

PROTEIN KINASE REGULATION OF
SARCOPLASMIC RETICULUM FUNCTION IN
ISOLATED ADULT RAT VENTRICULAR MYOCYTES

By

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ABSTRACT

The sarcoplasmic reticulum (SR) is one of the major regulators of the cytosolic Ca^{2+} concentration in cardiac ventricular muscle cells. In the myocardium, relaxation results from a decrease in cytoplasmic free Ca^{2+} levels mediated through an efflux of Ca^{2+} from the cell *via* the sarcolemmal $\text{Na}^+/\text{Ca}^{2+}$ -exchanger and by the sequestration of Ca^{2+} by the network SR membranes through the actions of a calcium pump (a Mg^{2+} -dependent, $\text{Ca}^{2+}/\text{K}^+$ -activated adenosine triphosphatase; $\text{Ca}^{2+}/\text{K}^+$ -ATPase). During an action potential, the Ca^{2+} stored in the SR is released to the cytoplasm *via* a Ca^{2+} -release channel present in the junctional SR and Ca^{2+} also enters the cell through voltage-controlled Ca^{2+} channels in the sarcolemmal membrane. These two processes result in an increase in cytoplasmic Ca^{2+} concentration which leads to contraction of the myocardium.

SR membrane function is regulated in part by the phosphorylation of proteins present in this membrane. Subsequent to β -adrenergic stimulation of the heart by catecholamines, levels of cAMP are increased leading to the activation of cAMP-dependent protein kinase (PK A). In isolated cardiac SR membrane vesicles and perfused hearts, phosphorylation of an indigenous SR protein, phospholamban, is mediated by PK A. Phosphorylated phospholamban acts as a modulator of the $\text{Ca}^{2+}/\text{K}^+$ -ATPase to stimulate active Ca^{2+} uptake by increasing the affinity of this enzyme for Ca^{2+} . This stimulation of Ca^{2+} -uptake is the main mechanism by which catecholamines accelerate relaxation in the heart. When phospholamban is in the dephosphorylated state, a cytoplasmic portion of the molecule interacts near the phosphorylation site of the Ca^{2+} -pump to inhibit Ca^{2+} -transport.

Although a number of studies have examined the phosphorylation of phospholamban in different experimental models, there appears to be an incongruity between the results obtained from isolated SR vesicles and perfused whole hearts. In isolated SR membranes, phospholamban can be phosphorylated by

PK A, Ca^{2+} /calmodulin-dependent protein kinase (CAM PK), cGMP-dependent protein kinase (PK G) and Ca^{2+} /phospholipid-dependent protein kinase (PK C). However, in isolated perfused hearts, only PK A and CAM PK (only after activation of PK A) are found to phosphorylate phospholamban. Thus, in a cardiac cell, there may be a functional basis for the phosphorylation of phospholamban by several different types of protein kinases. Further, the protein kinase pathways may not act independently of one another but may act synergistically or antagonistically to modulate SR function.

In this study, isolated adult rat ventricular myocytes were used to examine the phosphorylation of proteins in response to activators of PK A and PK C. As well, the activation of PK C was investigated in the isolated myocytes. Experiments were also carried out to analyze the oligomeric species of phosphorylated phospholamban obtained in the presence of activators of PK A, PK C and PK A and C, together.

In the present study, a method for the isolation of a high number of viable adult rat ventricular myocytes was established. The availability of these myocytes enabled the development of methods for myocyte homogenization and isolation of SR membranes from these homogenates. This study describes, for the first time, the isolation of sarcoplasmic reticulum (SR) membranes from adult rat ventricular myocytes obtained from a single rat heart. The myocyte SR preparation exhibits similar Ca^{2+} -transport and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity as well as a similar protein profile to SR membranes isolated from intact rat heart tissue. This SR preparation exhibited a $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity of 414 ± 62 nmol/min/mg protein (mean \pm S.E.M.; n=6) and an oxalate-stimulated Ca^{2+} -uptake activity of 107 ± 4 nmol/min/mg protein (mean \pm S.E.M.; n=6). Pretreatment of the SR vesicles with 5 μM ruthenium red increased the oxalate-stimulated Ca^{2+} -uptake to 208 ± 10 nmol/min/mg protein demonstrating the presence of junctional SR membranes. Sodium dodecylsulphate polyacrylamide gel electrophoresis shows that the isolated

SR membranes contained protein bands at 100 (Ca²⁺/K⁺-ATPase), 55 (calsequestrin and/or calreticulin) and 53 kDa (glycoprotein). Western blots of myocyte SR membranes stained with ruthenium red detected 2 major Ca²⁺-binding protein bands in this preparation at 53-55 kDa (calsequestrin and/or calreticulin) and 97-100 kDa (Ca²⁺/K⁺-ATPase). The presence of phospholamban was confirmed in the myocyte SR membranes on immunoblots probed with a monoclonal antibody to phospholamban.

The availability of purified SR membranes from adult rat ventricular myocytes provided a useful model for the study of the regulation of SR function by protein phosphorylation. For these studies, myocyte SR membranes were isolated and characterized in buffers developed to prevent the dephosphorylation of proteins. These SR membranes exhibited a protein profile similar to those isolated in control buffers, less contamination by enzymatic activities from other cellular membranes and lower recovery of Ca²⁺-uptake and Ca²⁺/K⁺-ATPase activities. Three distinct proteins (phospholamban, a 31 and a 152 kDa protein) were phosphorylated by PK A in homogenates and SR membranes from adult rat myocytes stimulated with isoproterenol or forskolin. The stimulation of protein phosphorylation in myocyte homogenates and SR membranes by isoproterenol was not affected by two different inhibitors of PK A. Also, an inhibitor of CAM PK did not affect the stimulation of protein phosphorylation in myocyte homogenates and SR membranes by isoproterenol. Treatment of isolated adult rat myocytes with DMSO (dimethylsulfoxide) or phorbol esters dissolved in DMSO resulted in the phosphorylation of phospholamban in myocyte homogenates and SR membranes. When OAG (1-oleoyl-2-acetyl-glycerol) and isoproterenol were used together to stimulate protein phosphorylation in isolated adult rat myocytes, the same proteins were phosphorylated as were found in homogenates and SR membranes treated with isoproterenol alone. In cytosolic fractions isolated from isoproterenol and OAG plus isoproterenol-treated myocytes, the phosphorylation of a 21, 31 and 152 kDa

protein was stimulated. The phosphorylation of a 24 kDa protein appeared to be decreased in myocytes treated with isoproterenol, OAG and isoproterenol plus OAG.

The separation of phosphorylated pentameric species of phospholamban from rat myocyte SR was found to be more difficult to achieve than from SR membranes prepared from canine heart. In control and OAG-treated myocytes, two species of phosphorylated pentameric phospholamban were obtained. In myocytes treated with isoproterenol or OAG plus isoproterenol, five species of phosphorylated pentameric phospholamban were obtained.

To assay the activity of PK C from myocyte cytosol and membrane fractions, FPLC fractionation to remove an inhibitory or interfering activity and the inclusion of the peptide inhibitor of PK A were required. The specific activity of PK C in myocyte cytosol was found to be much higher than that previously found from cytosolic fractions of canine, rat or guinea pig heart. Three peaks of Ca^{2+} and lipid-dependent PK C activity were found in cytosolic fractions isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes. The main peak of activity contained type II and type III isozymes of PK C, in perhaps in both autophosphorylated and nonphosphorylated states. The increase in molecular weight upon autophosphorylation of type III PK C has not been documented previously. The second major peak may contain only nonphosphorylated forms of type III PK C in control myocytes and type II and III PK C from OAG-treated myocytes. The third peak of PK C activity in the cytosol did not contain type III PK C protein. Two peaks of Ca^{2+} and lipid-dependent PK C activity were found in membranes isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes. The main peak of activity contained type III PK C, in perhaps both autophosphorylated and nonphosphorylated states. The second peak which contained a large Ca^{2+} and lipid-independent kinase activity contained type III PK C only, in perhaps the nonphosphorylated and proteolyzed PK M form. There were no differences in the number or types of PK C formed with respect to incubation

time. There was significantly less PK C activity in membrane fractions from myocytes that had been treated with ethanol. As well, this study demonstrates that in isolated adult rat ventricular myocytes, OAG was not able to activate PK C as determined by translocation, autophosphorylation and protein phosphorylation studies.

TABLE OF CONTENTS

ABSTRACT	ii
TABLE OF CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xv
ACKNOWLEDGEMENTS	xix
DEDICATION	xx
 1. INTRODUCTION	 1
1.1. Regulation of Myocardial Contractility and Relaxation	1
1.1.1. Primary Control	1
1.1.2. Excitation-Contraction Coupling	2
1.1.3. Relaxation	4
1.2. The Sarcoplasmic Reticulum	5
1.2.1. Structure of the Sarcoplasmic Reticulum	5
1.2.2. Function of Sarcoplasmic Reticulum Proteins	7
1.2.2.1. Storage of Ca^{2+}	7
1.2.2.2. Release of Ca^{2+}	7
1.2.2.3. Accumulation of Ca^{2+}	8
1.2.3. Regulation of Sarcoplasmic Reticulum Function	8
1.2.3.1. Regulation of Ca^{2+} Storage and Release	8
1.2.3.2. Regulation of Ca^{2+} Accumulation	9
1.3. Experimental Models of the Myocardium	12
1.4. Hypothesis, Rationale and Aims of This Study	17
1.4.1. Hypothesis and Rationale	17
1.4.2. Specific Aims	19
 2. MATERIALS AND METHODS	 20
2.1. Materials	20
2.1.1. Animals	20
2.1.2. Chemicals	20
2.1.2.1. Materials for Isolation of Adult Rat Ventricular Myocytes	20
2.1.2.2. Chemicals for the Treatment of Isolated Adult Rat Ventricular Myocytes	21

2.1.2.3.	Radioactivity and Autoradiography	21
2.1.2.4.	Materials for the Isolation and Characterization of Myocyte Homogenates and SR Membranes	22
2.1.2.5.	Materials for the Isolation and Quantitation of PK C	23
2.2.	Methods	23
2.2.1.	Isolation of Adult Rat Ventricular Myocytes	23
2.2.1.1.	Modified Method of Piper <i>et al.</i>	23
2.2.1.2.	Modified Method of Li <i>et al.</i> and Wimsatt <i>et al.</i>	24
2.2.2.	Characterization of Isolated Adult Rat Ventricular Myocytes	26
2.2.2.1.	Cell Counting, Morphology and Maintenance of Contractile Function	26
2.2.2.2.	Quantitation of cAMP	27
2.2.3.	Homogenization of Isolated Adult Rat Ventricular Myocytes	27
2.2.3.1.	Preliminary Homogenization Methods	27
2.2.3.2.	Successful Homogenization Methods	28
2.2.4.	Isolation of Sarcoplasmic Reticulum Membranes from Isolated Adult Rat Ventricular Myocytes	29
2.2.4.1.	Preliminary Methods for the Isolation of SR Membranes from Isolated Myocytes	29
2.2.4.2.	Final Method for the Isolation of SR Membranes from Isolated Myocytes	31
2.2.5.	Characterization of SR Membranes from Isolated Myocytes	32
2.2.5.1.	Biochemical Assays	32
2.2.5.1.1.	Determination of Ca^{2+} -Transport Activity	32
2.2.5.1.2.	Determination of $\text{Ca}^{2+}/\text{K}^{+}$ and $\text{Na}^{+}/\text{K}^{+}$ -ATPase Activities	33
2.2.5.1.3.	Determination of Cytochrome C Oxidase Activity	34
2.2.5.1.4.	Determination of Protein Concentration	35
2.2.5.1.5.	Determination of Free Calcium Concentrations	36
2.2.5.2.	Electrophoretic Methods	36
2.2.5.2.1.	Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis	36
2.2.5.2.1.1.	Polyacrylamide Gradient (5 -20% and 10 - 20%) Gels	36
2.2.5.2.1.2.	Polyacrylamide and Bis-acrylamide Gradient Gels	38
2.2.5.2.1.3.	Polyacrylamide Non-Gradient Gels	38
2.2.5.2.2.	Staining of Sodium Dodecylsulfate Polyacrylamide Gels	39
2.2.5.2.2.1.	Coomassie Brilliant Blue Stain	39
2.2.5.2.2.2.	Stainsall Stain	39
2.2.5.2.3.	Drying of SDS-PAGE Gels	40
2.2.5.2.4.	Western Blotting	40
2.2.5.2.4.1.	Antibody Localization	40
2.2.5.2.4.2.	Ruthenium Red Staining	42
2.2.5.2.5.	Analysis of SDS-PAGE Gels, Autoradiographs and Western Blots	42

	ix
2.2.5.2.6. Autoradiography	42
2.2.6. Isolation and Quantitation of Protein Kinase C	43
2.2.6.1. Preparation of Myocyte Cytosol and Membrane Fractions	43
2.2.6.2. Preparation of Cytosol from Rat Brain and Cytosol and Membrane Fractions from Bovine Trachea	44
2.2.6.3. DEAE-Cellulose Chromatography	45
2.2.6.4. Fast Protein Liquid Chromatography	46
2.2.6.5. Determination of Protein Kinase C Activity	46
2.2.6.6. Protein Concentration of PK C Activity Peaks	48
2.2.7. Phosphorylation of Proteins	49
2.2.7.1. Phosphorylation of Proteins in Isolated Myocytes	49
2.2.7.2. Phosphorylation and Dephosphorylation of Proteins in Isolated SR Vesicles	50
3. RESULTS	51
3.1. Characterization of Isolated Adult Rat Ventricular Myocytes	51
3.1.1. General Viability Studies	51
3.1.2. Maintenance of Hormone Responsiveness	54
3.2. Homogenization of Isolated Adult Rat Ventricular Myocytes	54
3.3. Characteristics of SR Membranes Isolated From Adult Rat Ventricular Myocytes	57
3.3.1. Preparation of SR Membranes from Isolated Myocytes	57
3.3.2. Marker Enzyme Activities	57
3.3.2.1. SR Membranes Prepared in Control Buffers	58
3.3.2.2. SR Membranes Prepared in Buffers That Prevent Dephosphorylation	64
3.3.3. Protein Profile of SR Membranes	67
3.3.3.1. SR Membranes Prepared in Control Buffers	67
3.3.3.2. SR Membranes Prepared in Buffers That Prevent Dephosphorylation	71
3.4. Phosphorylation of Proteins in Isolated Intact Adult Rat Ventricular Myocytes	75
3.4.1. Proteins Phosphorylated in Response to PK A Activation	75
3.4.1.1. Effect of PK A Inhibitors on PK A Stimulated Protein Phosphorylation	81
3.4.2. Proteins Phosphorylated in Response to PK C Activation	87
3.4.3. Concerted Kinase Regulation of Protein Phosphorylation	90
3.4.3.1. Possible Involvement of Calcium/Calmodulin-Dependent Protein Kinase	90
3.4.3.2. Possible Involvement of Protein Kinase C	94
3.5. Phosphorylated Species of Phospholamban	101

3.6.	Stimulation of Protein Kinase C in Adult Rat Ventricular Myocytes	103
3.6.1.	Activation of Protein Kinase C in Adult Rat Ventricular Myocytes	103
3.6.1.1.	Isolation and Quantitation of PK C After DEAE-Cellulose Chromatography	106
3.6.1.2.	Isolation and Quantitation of PK C After Fast Protein Liquid Chromatography	106
4.	DISCUSSION	123
4.1.	Isolated Adult Rat Ventricular Myocytes	123
4.2.	Sarcoplasmic Reticulum Membranes Isolated from Adult Rat Ventricular Myocytes	125
4.2.1.	Homogenization of Isolated Adult Rat Ventricular Myocytes	125
4.2.2.	Preparation of Sarcoplasmic Reticulum Membranes from Isolated Myocytes	125
4.2.3.	Enzymatic Activities and Protein Profile of Myocyte Sarcoplasmic Reticulum	127
4.2.4.	Comparison of Myocyte Sarcoplasmic Reticulum Membranes Prepared in Control Buffers Versus Buffers to Prevent Dephosphorylation	131
4.3.	Protein Phosphorylation in Isolated Adult Rat Ventricular Myocytes Stimulated with PK A and PK C	133
4.3.1.	Proteins Phosphorylated in Response to PK A Activation	134
4.3.2.	Proteins Phosphorylated in Response to PK C Activation	136
4.3.3.	Calcium/Calmodulin-dependent Protein Kinase Involvement	140
4.3.4.	Protein Phosphorylation in Response to PK A and PK C Activation	141
4.4.	Phosphorylated Species of Oligomeric Phospholamban	143
4.5.	Activation of PK C in Isolated Adult Ventricular Myocytes	146
4.5.1.	Translocation of PK C in Isolated Adult Rat Ventricular Myocytes	147
4.5.2.	Autophosphorylation of PK C in Isolated Adult Rat Ventricular Myocytes	152
5.	SUMMARY AND CONCLUSIONS	155
6.	REFERENCES	159

LIST OF TABLES

TABLE 1. cAMP accumulation in isolated adult rat ventricular myocytes.	55
TABLE 2. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities of several preparations obtained by different myocyte homogenization and SR centrifugation protocols.	56
TABLE 3. Ca^{2+} -uptake activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes.	59
TABLE 4. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes.	61
TABLE 5. Ca^{2+} -ATPase and Ca^{2+} -uptake activities of SR membranes isolated from whole adult rat hearts compared to those from isolated adult rat ventricular myocytes.	62
TABLE 6. Marker enzyme activities of homogenate and SR membranes isolated from adult rat ventricular myocytes.	63
TABLE 7. Ca^{2+} -uptake activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes using buffers to prevent dephosphorylation.	65
TABLE 8. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes using buffers to prevent dephosphorylation.	66
TABLE 9. Marker enzyme activities of homogenate and SR membranes isolated from adult rat ventricular myocytes using buffers to prevent dephosphorylation.	68
TABLE 10. Protein Kinase C Activities.	107

LIST OF FIGURES

Figure 1. Photograph of a crude preparation of adult rat ventricular myocytes.	52
Figure 2. Trypan blue staining of a crude preparation of adult rat ventricular myocytes.	53
Figure 3. Coomassie blue-stained SDS-PAGE gel of homogenates and SR membranes from whole rat heart and isolated rat ventricular myocytes.	69
Figure 4. Stainsall stained SDS-PAGE gel of homogenates and SR membrane preparations from whole rat heart and isolated rat ventricular myocytes.	70
Figure 5. Ca^{2+} -binding proteins of SR membranes purified from isolated rat ventricular myocytes and whole rat heart.	72
Figure 6. Identification of phospholamban in SR membranes purified from isolated rat ventricular myocytes and whole rat heart.	73
Figure 7. Coomassie blue-stained SDS-PAGE gel and western blot of myocyte homogenates and SR membranes prepared in buffers to prevent dephosphorylation.	74
Figure 8.A. Autoradiograph of homogenates and SR membranes from control and isoproterenol-stimulated rat ventricular myocytes.	76
Figure 8.B. SDS-PAGE gel of homogenates and SR membranes from control and isoproterenol-stimulated rat ventricular myocytes.	78
Figure 9. Western blot of homogenates and purified SR membranes from control and isoproterenol-stimulated rat ventricular myocytes.	79
Figure 10.A. Autoradiograph of crude membranes from control, isoproterenol and forskolin-stimulated myocytes.	80
Figure 10.B. SDS-PAGE gel of crude membranes from control, isoproterenol and forskolin-stimulated myocytes.	82
Figure 11.A. Autoradiograph of homogenates and SR membranes isolated from isoproterenol-stimulated myocytes in the absence and presence of HA1004.	83
Figure 11.B. SDS-PAGE gel of homogenates and SR membranes isolated from isoproterenol-stimulated myocytes in the absence and presence of HA1004.	84
Figure 12.A. Autoradiograph of homogenates and SR membranes isolated from isoproterenol-stimulated myocytes in the absence and presence of HA1004 and H-8.	85
Figure 12.B. SDS-PAGE gel of homogenates and SR membranes isolated from isoproterenol-stimulated myocytes in the absence and presence of HA1004 and H-8.	86

Figure 13. Autoradiograph of homogenates and SR membranes isolated from myocytes stimulated with isoproterenol, α -phorbol didecanoate and phorbol myristate acetate.	88
Figure 14. Autoradiograph of homogenates and SR membranes isolated from myocytes stimulated with isoproterenol, DMSO and OAG (in DMSO).	89
Figure 15.A. Autoradiograph of homogenates and SR membranes isolated from myocytes stimulated with isoproterenol, ethanol and OAG (in ethanol).	91
Figure 15.B. SDS-PAGE gel of homogenates and SR membranes isolated from myocytes stimulated with isoproterenol, ethanol and OAG (in ethanol)	92
Figure 16. Autoradiograph of homogenates and SR membranes isolated from control myocytes and myocytes stimulated with OAG.	93
Figure 17. Autoradiograph of homogenates and SR membranes isolated from DMSO and isoproterenol-stimulated myocytes in the absence and presence of CGS 9343 B.	95
Figure 18. Autoradiograph of homogenates and SR membranes isolated from myocytes stimulated with isoproterenol and with OAG and isoproterenol.	96
Figure 19. Incorporation of radioactivity into 27 and 8.5 kDa protein bands from SDS-PAGE gels of homogenates and SR membranes from control myocytes and myocytes stimulated with isoproterenol, OAG and OAG & isoproterenol.	97
Figure 20. Incorporation of radioactivity into 31 and 152 kDa protein bands from SDS-PAGE gels of homogenates and SR membranes from control myocytes and myocytes stimulated with isoproterenol, OAG and OAG & isoproterenol.	99
Figure 21. Autoradiograph of myocyte cytosolic fraction isolated from control, isoproterenol, isoproterenol & OAG and OAG-treated myocytes.	100
Figure 22. Western blot and SDS-PAGE gel of canine cardiac ventricular SR membranes probed with a monoclonal antibody to phospholamban.	102
Figure 23. Western blot and SDS-PAGE gel of rat myocyte and canine cardiac ventricle SR membranes probed with a monoclonal antibody to phospholamban.	104
Figure 24. Western blot of homogenates and SR membranes from control, isoproterenol, OAG and isoproterenol & OAG-stimulated myocytes.	105
Figure 25. FPLC profile of purified rat brain PK C.	109
Figure 26. Protein elution FPLC profile of control myocyte cytosol and membrane fractions.	110

Figure 27. PK C activity of FPLC profiles of control myocyte cytosolic fractions and membranes assayed in the absence and presence of cAMP-dependent protein kinase inhibitor.	111
Figure 28. PK C activities of FPLC profiles of cytosolic and membrane fractions from control myocytes incubated for 1, 5 and 10 min.	112
Figure 29. Area under the curve (AUC) of PK C activity from FPLC fractions 27 - 31 of myocyte cytosol and membrane fractions.	113
Figure 30. Western blots of PK C activity peaks from control (1 min) myocyte cytosol and membrane fractions, probed with antibodies to Type II and Type III PK C.	115
Figure 31. PK C activities of FPLC profiles of cytosol and membrane fractions from ethanol-treated myocytes incubated for 1, 5 and 10 min.	116
Figure 32. PK C activities of FPLC profiles of cytosol and membrane fractions from OAG-treated myocytes incubated for 1, 5 and 10 min.	118
Figure 33. Western blots of PK C activity peaks from OAG-treated (10 min) myocyte cytosol and membrane fractions, probed with antibodies to Type II and Type III PK C.	120
Figure 34. PK C activities of FPLC profiles of cytosol and membrane fractions from R59022 and OAG-treated myocytes incubated for 1, 5 and 10 min.	121

LIST OF ABBREVIATIONS

&	and
%	percent
³² P	phosphorus-32
³ H	tritium
⁴⁵ Ca	calcium-45
α-PDD	4α-phorbol didecanoate
ANOVA	analysis of variance
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
AUC	area under the curve
AV	atrioventricular
BIS	N,N'-methylene-bis-acrylamide
BCIP	5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt
BSA	bovine serum albumin
Ca ²⁺	free calcium ions
Ca ²⁺ /K ⁺ -ATPase	calcium-dependent/potassium-stimulated-ATPase
CAM	calmodulin
CAM PK	Ca ²⁺ /calmodulin-dependent protein kinase
cAMP	adenosine 3':5'-cyclic monophosphate
CGS 9343 B	1,3-dihydro-1-(1-((4-methyl-4H, 6H-pyrrolo(1,2-a) -(4,1)benzoxazepin-4-yl)methyl)-4-piperindinyl)-2H -benzimidazol-2-one maleate
Ci	Curie
cpm	counts <i>per</i> minute
DAG	diacylglycerol
ddH ₂ O	distilled, deionized water
DEAE	diethylaminoethyl

DMSO	dimethylsulfoxide
dpm	disintegrations <i>per</i> minute
DTT	dithiothreitol
E-P	phosphoenzyme intermediate of the SR $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase
E_1 , E_2	conformational states of the SR $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis(β -aminoethylether)-N,N,N'N'-tetraacetic acid
ETOH	ethanol
Forsk.	forskolin
FPLC	fast protein liquid chromatography
g	gravitational force
g	gram
g_{max}	gravitational force at maximum radius
H-8	N-(2-(methylamino) ethyl)-5-isoquinolinesulfonamide dihydrochloride
HA1004	N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride
HEPES	N-(2-hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)
HPL	high molecular weight form of phospholamban
IBMX	isobutylmethylxanthine
IP_3	inositol-1,4,5-trisphosphate
ISO.	isoproterenol
K-H	Krebs-Henseleit buffer
kDa	kilodaltons
K_i	dissociation constant for inhibitor
K_m	Michealis constant
l	litre

LDL	low density lipoprotein
LPL	low molecular weight form of phospholamban
M	molar
mA	milliamps
MEM	Joklik-Modified minimum essential medium
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
MgATP	magnesium adenosine triphosphate
min	minute
ml	millilitre
mol	mole
MOPS	3-(N-morpholino)propanesulfonic acid
n	number of separate experiments
Na ⁺ /K ⁺ -ATPase	sodium/potassium-dependent-ATPase
OAG	1-oleoyl-2-acetyl glycerol
PAGE	polyacrylamide gel electrophoresis
pH	negative logarithm of the hydrogen ion activity
P _i	inorganic phosphate (PO ₄ ³⁻)
PK A	cAMP-dependent protein kinase
PK C	Ca ²⁺ /phospholipid-dependent protein kinase
PK M	catalytic fragment of PK C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine
PTFE	Teflon
R59022	6-(2-(4-((p-fluorophenyl)-phenylmethylene)-1-piperidinyl)-ethyl)-7-methyl-5-H-thiazolo-3,2-a-pyrimidine-5-one
r _{av}	average radius

rpm	revolutions per minute
s	second
S.D.	standard deviation
S.E.M.	standard error of the mean
SA	sinoatrial
SAG	1-stearoyl-2-arachidonylglycerol
SDS	sodium dodecylsulfate
SL	sarcolemma
SR	sarcoplasmic reticulum
STI	soybean trypsin inhibitor
t-tubule	transverse tubule
TBS	TRIS-buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPCK	L-1-tosylamide-2-phenylethyl chloromethyl ketone
TRIS	tris(hydroxymethyl)aminomethane
TTBS	TRIS-buffered saline containing 0.2% Tween 20
μ	micro
V	volt

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DEDICATION

This thesis is dedicated to my husband, Dr. Matthew Rowland Wright, for his
patience, love, support and encouragement.

1. INTRODUCTION

1.1. *Regulation of Myocardial Contractility and Relaxation*

1.1.1. Primary Control

At rest, the heart can generate and conduct electrical impulses which allow the myocardial cells of the heart to contract and relax as a functional syncytium. There are three pacemaker regions in cardiac muscle, the SA (sinoatrial) node, the AV (atrioventricular) node and the His-Purkinje system. These specialized cells are capable of spontaneous depolarization to the threshold of initiation of an action potential. The SA node generates impulses at the fastest rate (70/min at rest in man) and dominates the other pacemakers to determine the heart rate. Normally, the electrical impulse from the SA node is transmitted throughout the cells of the atria, which are stimulated to contract, to the AV node. The AV node conducts the impulse from the atria to the ventricles, slowing the conduction from the SA node and by genesis of its own impulses at 50 - 60/min. From the AV node, the impulse travels down the bundle of His to the ventricles, causing them to contract. When the rate of impulse generation by the SA node is slowed, due to disease or intense vagal stimulation, the AV node becomes the dominant pacemaker of the heart. If there is failure of both the SA and AV nodes, the His-Purkinje system will generate impulses at 30 - 40/min which may be too low a rate to sustain life.

The heart is regulated by the autonomic nervous system and it has an intrinsic ability to regulate its own function by responding to changes in venous return. This matching of venous return to cardiac output is known as the Frank-Starling law of the heart [Frank (1895); Patterson *et al.* (1914)] and is the primary mechanism for the control of cardiac output in the resting state. Briefly, an increased amount of blood entering the heart causes an increased stretch of cardiac

muscle. The stretched muscle contracts with a greater force hence, pumping the extra blood out of the heart.

In addition, the contractile force of the heart and heart rate may also be controlled by the autonomic nervous system. Sympathetic nerves, releasing noradrenaline, increase both contractility and heart rate. There are a large number of sympathetic inputs into both the atria and ventricles. Cholinergic nerves, releasing acetylcholine, decrease both contractility and heart rate. The two atria are well supplied by parasympathetic nerves but the ventricles have few parasympathetic fibers. The SA and AV nodes are also innervated cholinergically, by branches of the vagus nerve.

1.1.2. Excitation-Contraction Coupling

When an electrical impulse reaches a contractile cell of the heart, the cell becomes excited and an action potential is generated. The action potential consists of depolarization by a fast inward Na^+ current followed by an inward Ca^{2+} current. These two depolarizing currents are balanced by a repolarizing current, the inwardly rectifying K^+ current. Repolarization then occurs due to a decay of the inward Ca^{2+} current and activation of an outward delayed rectifying K^+ current. The action potential travels along the sarcolemma (SL) and down the transverse tubules (t-tubules), causing Ca^{2+} to enter the cell through voltage-controlled Ca^{2+} -channels in the SL membrane. Ca^{2+} -induced Ca^{2+} release [Fabiato (1983); Fabiato and Fabiato (1975)] results from the increase of cytosolic Ca^{2+} which triggers a large release of Ca^{2+} from the sarcoplasmic reticulum (SR). Upon influx of Ca^{2+} from outside the cell and release of Ca^{2+} from the SR, the intracellular Ca^{2+} concentration rises from a resting level of 0.1 to a maximum of 5 μM [Blinks (1986)]. The Ca^{2+} in the cytosol binds to the Ca^{2+} binding site on troponin C causing this protein to interact more strongly with troponin I, thereby terminating the inhibition

of the interaction between actin with myosin. This conformational change displaces the troponin-tropomyosin complex from actin and allows actin to interact with myosin, consequently allowing force generation [Bers (1991)]. Contraction of the myofibril then occurs according to the sliding filament crossbridge attachment theory of force generation elaborated by Huxley and Simmons (1971).

Myocardial contractility can be regulated by both branches of the autonomic nervous system, the sympathetic and parasympathetic nervous system. Sympathetic stimulation *via* the interaction of noradrenaline and adrenaline with α - and β -adrenergic receptors on the surface of the SL membrane causes an increase in both the Ca^{2+} and the K^+ currents. β -receptors are linked to adenylate cyclase through a stimulatory G-protein and when activated, adenylate cyclase produces cAMP (cyclic adenosine-monophosphate) which is then available to activate the cAMP-dependent protein kinase (PK A). When active, PK A phosphorylates the voltage-dependent Ca^{2+} channel in the SL which results in an increase in the amount of Ca^{2+} entering the cell [Hartzell *et al.* (1991)]. Increased Ca^{2+} in the cytosol of the cell leads to a stimulation of the rate and force of contraction due to a greater activation of troponin C. The stimulation of α -receptors results in positive inotropy by increasing the sensitivity of the contractile proteins to Ca^{2+} [Endoh and Blinks (1988)] and by an increase in cytoplasmic Ca^{2+} caused, perhaps, by release of Ca^{2+} from the SR *via* an inositol(1,4,5)-trisphosphate-sensitive receptor [Scholz *et al.* (1988)]. Activation of α -receptors also prolongs the action potential duration by decreasing an outward K^+ current, which may also lead to an increase in contractility [Fedida *et al.* (1989)]. There are still many unresolved questions regarding the mechanism of positive inotropy caused by α -receptor activation [Bers (1991)]. Activation of muscarinic receptors by acetylcholine causes an inhibition of the stimulation by β -receptor agonists of adenylate cyclase *via* an inhibitory G-protein and thus, attenuation of the phosphorylation of proteins by decreasing

cAMP production [Fleming *et al.* (1987)]. Therefore, parasympathetic stimulation results in a decrease in contractility.

1.1.3. Relaxation

Relaxation of the myocardium occurs as the cell membrane repolarizes and by the removal of Ca^{2+} from the cytosol of the cell. The decrease in cytosolic Ca^{2+} concentration causes Ca^{2+} to dissociate from troponin C, which allows troponin I to inhibit actin and thus, disrupt its interaction with myosin. Ca^{2+} is removed mainly from the cytosol by sequestration into the SR by a Ca^{2+} -pump, the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase (a Mg^{2+} -dependent, $\text{Ca}^{2+}/\text{K}^{+}$ -activated adenosine triphosphatase) and through efflux from the cell *via* the $\text{Na}^{+}/\text{Ca}^{2+}$ -exchanger [Bers (1991)].

Cardiac relaxation may also be regulated by sympathetic and parasympathetic influences. Noradrenaline, *via* the activation of β -receptors and cAMP production, stimulates the PK A phosphorylation of several proteins involved in relaxation. The major cytosolic proteins phosphorylated include troponin I and C-protein (a protein associated with myofibrils) and from the SR, a protein named phospholamban. Phosphorylation of troponin I results in an increased rate of Ca^{2+} dissociation from troponin C thus, separating troponin I from C and allowing troponin I to once again inhibit the interaction of actin with myosin. When C-protein is phosphorylated it is not as effective in stimulating the myosin ATPase and thus, promotes relaxation [Hartzell (1985)]. The phosphorylation of phospholamban has been shown to activate the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase by increasing its affinity for Ca^{2+} and thus, stimulating Ca^{2+} uptake into the SR leading to an acceleration of relaxation [Tada *et al.* (1982)]. This stimulation of Ca^{2+} -uptake is the main mechanism by which sympathetic neurotransmitters accelerate relaxation in the heart. Parasympathetic stimulation results in a negative chronotropic effect due to hyperpolarization of SA node cells and a decrease in conduction velocity in

the AV node and Purkinje fibers [Hutter and Trautwein (1956); Taylor (1985)]. Further, acetylcholine interacts with muscarinic receptors on the surface of the SL membrane leading to modulation of a K^+ channel directly through a G protein [Pfaffinger *et al.* (1985)]. This results in an increase in the K^+ conductance through the channel and subsequently, faster hyperpolarization of the membrane.

1.2. *The Sarcoplasmic Reticulum*

1.2.1. Structure of the Sarcoplasmic Reticulum

The sarcoplasmic reticulum (SR) is an intracellular tubular membranous network analogous to the endoplasmic reticulum of noncontracting cells. The SR can be separated into two main types: a) network (longitudinal or free SR), a meshwork surrounding the myofilaments and b) junctional SR [Katz *et al.* (1986)]. Continuous with the network SR, the junctional cisternae are closely associated with sarcolemmal and t-tubule membranes. Between the junctional SR and the t-tubule membrane are regularly spaced, densely staining processes, called "feet" [Franzini-Armstrong (1970)]. These processes suggest a continuity between the junctional SR and the t-tubule membrane. Ultrastructurally, another specialized type of junctional SR can be recognized known as corbular SR [Dolber and Sommer (1981)]. Corbular SR does not associate with the SL or t-tubule membrane but appears to bud from the network SR and is associated with Z and I bands [Jorgensen *et al.* (1988)]. The lumen of junctional and corbular SR contain electron-dense granules, which are not present in network SR [Sommer and Jennings (1986)].

In the myocardium, proteins specific to the SR may be uniformly distributed or localized to certain types of SR. The Ca^{2+}/K^+ -ATPase is uniformly distributed in the membrane of network SR but in junctional SR is not present in areas associated

with SL or t-tubule membranes [Jorgensen *et al.* (1982)]. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase is also not found in corbular SR membranes. A 53 kDa glycoprotein has been demonstrated in both network and junctional SR but in network SR it appears to co-localize with the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase [Leberer *et al.* (1990)]. The SR also contains a 130 kDa glycoprotein, which is immunochemically similar to the 53 kDa glycoprotein [Campbell *et al.* (1983)]. Phospholamban, the Ca^{2+} -pump regulator protein, has been found to be uniformly distributed throughout the membranes of both network and corbular SR but in junctional SR is not present in areas associated with SL or t-tubule membranes [Jorgensen and Jones (1987)]. The electron-dense granules present in the lumen of both junctional and corbular SR have been identified as the Ca^{2+} -binding protein, calsequestrin [Jorgensen *et al.* (1988)]. Calreticulin, the 55 kDa high affinity Ca^{2+} -binding protein of SR and a 165 kDa Ca^{2+} -binding protein that also binds LDL were found to be distributed throughout the lumen of network and junctional SR [Fliegel *et al.* (1989); Hofmann *et al.* (1989)]. The "feet" structures between the junctional and t-tubule membranes have recently been identified as oligomers of the SR Ca^{2+} -release channel (also known as the cardiac ryanodine receptor) [Inui *et al.* (1987)]. There are several other ion channels in the membranes of the SR including a chloride channel that has been found to be randomly distributed throughout the SR [Rousseau *et al.* (1988)] and a potassium channel that has been isolated from network SR [Hill *et al.* (1989)]. A Ca^{2+} /calmodulin-dependent protein kinase [Molla and Demaille (1986)] and a phosphatase [Kranias and Di Salvo (1986)] are also associated with SR membranes but the localization of these proteins in the SR is unknown.

Recently, in human SR membranes, the following proteins have been identified: the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase, calsequestrin, Ca^{2+} -release channel, phospholamban and glycoproteins of 53, 155 and 165 kDa [Movsesian *et al.* (1990)].

1.2.2. Function of Sarcoplasmic Reticulum Proteins

Although cardiac SR performs some functions similar to the endoplasmic reticulum, such as lipid metabolism [Cornell and MacLennan (1985); Kasinathan and Kirchberger (1985)] and glycolysis [Entman *et al.* (1976)], the main functions are to store, release and accumulate Ca^{2+} .

1.2.2.1. Storage of Ca^{2+}

Ca^{2+} is stored in the lumen of the SR by binding mainly to two proteins: 1) a low affinity, high capacity Ca^{2+} -binding protein, calsequestrin and 2) calreticulin, which exhibits both high affinity, low capacity and low affinity, high capacity Ca^{2+} -binding sites. Calsequestrin is a 55 kDa protein that can bind 12 - 40 moles of Ca^{2+} /mole with an affinity (K_d) of 0.1 - 1.0 mM [Mitchell *et al.* (1988)]. As calsequestrin is localized to junctional and corbular SR, it is available to release Ca^{2+} near the Ca^{2+} -release channels of the junctional SR. Calreticulin can bind 25 moles of Ca^{2+} /mole at the high capacity site and 1 mole of Ca^{2+} /mole at the low capacity site [Ostwald and MacLennan (1974)]. The role of the 165 kDa Ca^{2+} -binding protein of the SR remains unknown.

1.2.2.2. Release of Ca^{2+}

Ca^{2+} is mainly released from cardiac SR *via* the Ca^{2+} release channel present in the junctional SR. This channel exhibits a molecular weight of 350 - 450 kDa and the homotetramer protein forms a quatrefoil structure. In electron micrographs, the quatrefoil structure was found to be identical to the "feet" structures observed between the junctional SR and the t-tubule membrane [Saito *et al.* (1988)]. A minor pathway of Ca^{2+} release from cardiac SR involves the

activation of an inositol(1,4,5)-trisphosphate-sensitive receptor [Hirata *et al.* (1984)]. This release of Ca^{2+} by IP_3 has been found to be too slow and too small to be a primary mechanism in cardiac excitation-contraction coupling [Feher and Fabiato (1990)].

1.2.2.3. Accumulation of Ca^{2+}

The accumulation of Ca^{2+} into the cardiac SR occurs *via* the action of a calcium pump, the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase. The ATPase has a molecular weight of 110 kDa and transports 2 moles of Ca^{2+} for every mole of MgATP hydrolyzed [Yamada *et al.* (1970)]. In the basic reaction sequence, the enzyme undergoes an E_1 to E_2 conversion with the formation of an E-P acylphosphate intermediate [Tada *et al.* (1988)]. For charge balance, during Ca^{2+} -uptake, the counter-transport of positive charges or co-transport of negative charges may be mediated by the anion and cation channels present in the SR membrane [Feher and Fabiato (1990)]. Recently, the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase has been shown to be able to operate as a Ca^{2+} channel for rapid Ca^{2+} efflux, under conditions where certain drugs have uncoupled the enzyme [de Meis (1991)].

1.2.3. Regulation of Sarcoplasmic Reticulum Function

1.2.3.1. Regulation of Ca^{2+} Storage and Release

The regulation of the storage of Ca^{2+} in the SR by its binding and release to Ca^{2+} -binding proteins is not well understood. It has been suggested that the potassium concentration within the SR modulates the Ca^{2+} -binding properties of calsequestrin, since the affinity of calsequestrin for Ca^{2+} is reduced by potassium [Slupsky *et al.* (1987)]. In junctional SR, there is a 26 kDa protein which binds to

calsequestrin. It is not known if this protein serves to anchor calsequestrin to the inside of the SR membrane or whether it is involved in the interaction between Ca^{2+} storage and release mechanisms [Mitchell *et al.* (1988)]. Calsequestrin can be phosphorylated *in vitro* by casein kinase II but this phosphorylation was found not to affect its function [Cala and Jones (1991)].

The regulation of Ca^{2+} -release from the SR is mainly a result of effects on the Ca^{2+} -release channel. The channel is activated to release Ca^{2+} at submicromolar concentrations of cytosolic Ca^{2+} [Meissner *et al.* (1988)], by mM concentrations of adenine nucleotides (only when Ca^{2+} had already partially activated the channel; Rousseau *et al.* (1986)) and caffeine [Rousseau *et al.* (1988)]. Ruthenium red and magnesium (both μM ; Rousseau and Meissner (1989)), calmodulin (1 - 4 μM ; Meissner and Henderson (1987)) and decreasing pH [Ma *et al.* (1988)] inhibit the activity of the channel. Recently, the Ca^{2+} -release channel has been shown to be a preferred substrate for Ca^{2+} /calmodulin-dependent protein kinase (CAM PK) and when phosphorylated, the channel became active [Witcher *et al.* (1991)].

1.2.3.2. Regulation of Ca^{2+} Accumulation

The regulation of Ca^{2+} -uptake into the SR is primarily effected through actions on the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase. Monovalent cations (10 - 20 mM) can stimulate the activity of the pump with the most effective being K^{+} [Jones *et al.* (1977)]. There is some controversy as to the function of the 53 kDa glycoprotein that co-localizes with the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase and whether it has any effect upon the function of the Ca^{2+} -pump [Leberer *et al.* (1989)]. As described previously, the activity of the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase is modulated by phospholamban. Phospholamban is a homopentamer with an apparent molecular mass of 25 - 27 kDa as determined by alkaline SDS-PAGE [Jones *et al.* (1985)]. Each monomer (obtained upon boiling in

SDS) has an approximate molecular mass of 8.5 kDa as determined by alkaline SDS-PAGE and 6 kDa as inferred from the amino acid sequence. Recently, Watanabe *et al.* (1991) have used low-angle laser light scattering photometry to more accurately determine the molecular mass of phospholamban. These investigators found that the phospholamban oligomer had a molecular mass of 30.4 kDa and confirmed that it was composed of 5 subunits.

Phospholamban stimulates the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase by lowering its K_m for Ca^{2+} , only when it is phosphorylated [Le Peuch *et al.* (1980)]. Recently, in human cardiac SR membranes, phospholamban was found to stimulate Ca^{2+} -uptake by increasing the apparent affinity of the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase for Ca^{2+} [Movsesian *et al.* (1990)]. When phospholamban is in the dephosphorylated state, a cytosolic portion of the monomer interacts near the phosphorylation site of the Ca^{2+} pump to inhibit Ca^{2+} transport [Kirchberger (1991)]. Upon phosphorylation, phospholamban undergoes a conformational change and no longer interacts with the Ca^{2+} pump. Removal of the cytoplasmic portion of phospholamban by proteolytic cleavage also leads to activation of Ca^{2+} -uptake [Kirchberger *et al.* (1986)]. Direct evidence for an interaction of phospholamban with the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase has come from SR reconstitution studies and other studies employing a monoclonal antibody to phospholamban or crosslinking reagents. In the reconstitution studies [Kim *et al.* (1990)], purified phospholamban and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase were reconstituted into phospholipid vesicles and the Ca^{2+} transport of these vesicles modulated by the state of phospholamban phosphorylation. Crosslinking of purified phospholamban and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase identified a region close to the phosphorylation site of this enzyme which bound to dephosphorylated, but not phosphorylated, phospholamban [James *et al.* (1989)]. By incubating SR vesicles with a monoclonal antibody to phospholamban, Suzuki and Wang (1986) found Ca^{2+} -uptake to be increased to a slightly higher level than that produced by PK A phosphorylation. More recently, these investigators have demonstrated that phospholamban amino acid residues 7

to 16 are involved in the regulation of the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase [Morris *et al.* (1991)]. Dialysis of isolated ventricular myocytes with a monoclonal antibody to phospholamban was shown to result in an increase in Ca^{2+} -uptake and release in response to depolarizing pulses and inhibited the ability of isoproterenol (a nonspecific β -receptor agonist) to stimulate Ca^{2+} -uptake and release [Sham *et al.* (1991)].

Phospholamban was found to be phosphorylated at serine 16 [Wegener *et al.* (1989)] by cAMP-dependent protein kinase in response to β -receptor agonists in intact perfused hearts [Kranias and Solaro (1982); Lindemann *et al.* (1983)]. Also, PK A was found to phosphorylate phospholamban in isolated adult rat ventricular myocytes [Blackshear *et al.* (1984)] and isolated SR vesicles [Kirchberger and Tada (1976)]. Ca^{2+} /calmodulin-dependent protein kinase (CAM PK) also phosphorylates phospholamban in the perfused heart and in isolated SR vesicles, on the threonine at position 17 [Simmerman *et al.* (1986)]. The physiological significance of CAM PK phosphorylation is not understood as, in the perfused heart, it only occurs after β -receptor activation [Lindemann and Watanabe (1985)]. In experiments in isolated SR vesicles, Ca^{2+} /phospholipid-dependent protein kinase (PK C) was also shown to phosphorylate phospholamban with a concurrent increase in Ca^{2+} -uptake [Moesian *et al.* (1984)]. However, in perfused adult rat hearts, activators of PK C have been found to decrease the rate and force of contraction and phospholamban was found not to be phosphorylated [Edes and Kranias (1990)]. A recent study cast doubt on the observation that activation of PK C causes a decrease in contractility [MacLeod and Harding (1991)]. These authors found that in isolated adult rat and guinea pig ventricular myocytes, activation of PK C caused a positive inotropic effect by increasing systolic Ca^{2+} . In isolated SR vesicles, cGMP-dependent protein kinase (PK G) was also found to phosphorylate phospholamban [Raeymaekers *et al.* (1988)], however, in intact perfused hearts, phospholamban was not phosphorylated in response to agents that increased intracellular cGMP [Huggins *et al.* (1989)].

The majority of phospholamban was shown to be dephosphorylated by protein phosphatase 1 activity in isolated SR vesicles [MacDougall *et al.* (1991)]. As well, both an inhibitor of protein phosphatase 1 and protein phosphatase 1 itself, have been shown to be phosphorylated by PK A in perfused guinea pig heart and isolated SR membranes [MacDougall *et al.* (1991); Neumann *et al.* (1991)]. These phosphorylations prevent protein phosphatase 1 from dephosphorylating phospholamban, thus, contributing to the positive inotropic effect of β -adrenergic stimulation.

In isolated SR vesicles, the rate of Ca^{2+} -uptake can be correlated to the phosphorylated and dephosphorylated levels of phospholamban [Kasinathan *et al.* (1988)]. Since each monomer of phospholamban contains two phosphorylation sites, a pentamer of phospholamban contains 10 sites in total. Phosphorylation of phospholamban reduces its electrophoretic mobility on SDS-PAGE gels, under certain conditions [Wegener and Jones (1984)]. Western immunoblots of these gels demonstrate multiple bands of the phospholamban pentamer as a result of filling one or both sites of phosphorylation in each of the monomers. Therefore, 6 bands are observed following phosphorylation by PK A, corresponding to the phosphorylation of monomers 0 to 5 at a single site [Li *et al.* (1990)]. Eleven bands are observed following phosphorylation by both PK A and CAM PK, indicating the phosphorylation of monomers 0 to 5 at both sites [Wegener *et al.* (1989)].

1.3. *Experimental Models of the Myocardium*

Commonly used experimental models of the myocardium include: the whole heart *in vivo*, isolated perfused hearts, tissue slices, organ culture, isolated ventricular myocytes, heart homogenates and intracellular organelles isolated from hearts. Each model has distinct properties that are advantageous for the

performance of certain types of experiments and disadvantageous for others [Jennings and Morgan (1986)].

From the whole heart *in vivo*, in closed-chest experiments, one can measure heart sounds and electrical activity by monitoring echo- and electrocardiograms [Shepherd and Vanhoutte (1979)]. It is also possible to measure arteriovenous differences across the myocardium of endogenous substrates, hormones, drugs or toxins [Jennings and Morgan (1986)]. In open-chested experiments, it is possible to obtain samples of tissue from specific areas of the heart through biopsy before, during and after the experiment, as well as sampling from coronary arteries and veins. Because of these features, the open-chested dog is often utilized as a model of regional ischemia. Disadvantages include stability of the test subject during the study (for example: heart failure, arrhythmia or ischemia that may develop upon application of hormones, drugs toxins, etc.), tissue heterogeneity which makes studies on metabolism difficult to interpret and finally, reactions can only be studied through flux of the entire pathway rather than the individual steps of the pathway or process.

Isolated perfused hearts can be prepared for perfusion in a Langendorff [Langendorff (1895)] or working heart mode [Neeley *et al.* (1967)]. The Langendorff mode maintains the intrinsic beating rate of the heart and the buffers are perfused through the coronary arteries *via* the aorta in a retrograde fashion. Langendorff perfusion is easy to perform and useful for studies involving the incorporation of radioactivity into phospholipids, proteins, *etc.* The working heart is a left heart preparation where the left atrium and subsequently, the left ventricle is perfused via the pulmonary vein and the myocardial tissue is perfused via the coronary arteries from outflow through the aorta. Contraction is stimulated electrically and both the preload and afterload pressures can be varied. Thus, the working heart is useful for studies where mechanical performance and metabolism are correlated following the addition of drugs, hormones or substrates. Advantages of the perfused

heart model include: i) compound delivery *via* the normal capillary bed, ii) intact cells, iii) stability of the preparation is easily measured, iv) mechanical function is stable for hours and v) the heart can be rapidly frozen after the study for the measurement of metabolites. Perfused hearts have several disadvantages including: i) the cellular heterogeneity of the heart, ii) coronary flow is increased greatly over the *in vivo* situation, iii) events that require 3 to 6 hrs or longer to develop *in vivo* can not be measured, iv) the identity of a single hormone or agent must be known in order to add it to the perfusate and v) in ischemic studies, the heart develops contracture-rigor and the no-flow phenomenon develops [Jennings and Morgan (1986)].

Handcut tissue slices of atrial, ventricular or papillary muscle are especially useful for studies on the regulation of cell volume of the adult heart [Grochowski *et al.* (1976)]. These slices are also useful in suspension for studying the effect of agents on integrated metabolism. Tissue slices have the following advantages: i) they maintain cell volume, ultrastructure and ion gradients, ii) there is no dependence on vascular integrity for substrate supply or loss of metabolic products and iii) the slice functions in an integrated way, therefore, studies on anoxia and osmolarity are possible. The major disadvantages of tissue slices include: i) the heterogeneity of the tissue, ii) the impossibility of assessing contractile function within the slice and iii) the slice is diffusion limited.

Fetal mouse hearts can be cultured intact for 24 to 48 hrs [Wildenthal (1971)]. An advantage over tissue slices is that all cells are intact and experiments can be performed over a much longer period of time. Disadvantages of fetal organ culture include: i) limitations by diffusion leading to necrosis at the centre if small hearts are not used, ii) cellular heterogeneity and iii) the fact that myocytes are fetal and not adult.

Fetal, neonatal and adult hearts can be used to isolate cardiac muscle cells (myocytes) by digestion of the hearts with proteolytic enzymes [Harary and Farley

(1960); Jacobson (1989)]. Fetal or neonatal myocytes can be grown in culture for extended periods of time making them useful for studies involving hypertrophy and differentiation. However, because the cells are growing and dividing they have the disadvantage of de-differentiation during their time in culture and overgrowth by fibroblasts interferes with the interpretation of data from metabolic studies. Myocytes from adult hearts do not divide and are usually utilized as fresh preparations. Isolated myocytes offer the advantage of being a homogeneous cell population as well as being free of neural and humoral influences. The ability to localize a metabolic or other activity to the cardiac muscle cell is the main advantage in the use of this model. They are an intact, viable cellular preparation that can be easily manipulated. The medium surrounding isolated myocytes can be controlled and quickly changed to facilitate treatment with different agents. As a result, myocytes (especially adult ventricular cells; Watanabe *et al.* (1986)) are widely used as a model for: i) receptor binding studies [*ex.* Martens *et al.* (1987)], ii) studies on hormone-receptor interaction [*ex.* Bode and Brunton (1989)], iii) metabolic studies [*ex.* Hee-Cheong and Severson (1989)], iv) studies on protein phosphorylation [*ex.* Robinson-Steiner and Corbin (1986)], v) ion flux studies [*ex.* Brierly *et al.* (1983)], vi) electrophysiological studies on ion channels and currents including patch-clamping and techniques for internal dialysis of the cell [*ex.* Harvey and Hume (1989); Sham *et al.* (1991)], vii) contractility studies utilizing single cell edge detection [*ex.* MacLeod and Harding (1991)], viii) drug uptake studies [*ex.* Cramb and Dow (1983)] and ix) in studies utilizing calcium imaging techniques to monitor spontaneous Ca^{2+} -oscillations and Ca^{2+} -induced Ca^{2+} -release from the SR [*ex.* Berridge and Galione (1988); Takamatsu *et al.* (1990)]. Disadvantages of isolated myocytes include: i) the viability of the preparation initially and during experimentation, ii) enzymes used for isolation may damage the glycocalyx and iii) intact quiescent adult myocytes do not spontaneously contract (*i.e.* they do not perform the work of contraction and relaxation). However, adult myocytes can be

electrically stimulated to contract at rates of perfused hearts and studies have shown that they will consume the same amount of oxygen as the perfused heart [Haworth *et al.* (1983)]. Further, it has been observed that isolated adult myocytes which spontaneously contract after isolation are electrochemically shunted and freely permeable to Ca^{2+} [Dani *et al.* (1979)]. Neonatal and fetal myocytes will spontaneously contract in culture and observations and measurements of contractile function can be made.

Heart homogenates can be prepared by grinding the tissue in various ways. Homogenates can be used to study metabolism but the results obtained from these studies are difficult to interpret since all cells are broken and cellular organelles are arranged randomly in solution. As well, it is not possible to localize an enzymatic activity or organelle to a specific cell type. Homogenates are most often used to prepare intracellular organelles such as nuclei, mitochondria, myofibrils, lysosomes, sarcolemma and sarcoplasmic reticulum membranes.

Isolated cellular organelles can be separated from homogenized heart tissue usually by differential centrifugation. Little work has been done on organelles prepared from isolated myocytes. In an isolated organelle, one can determine the origin of a function or enzymatic activity and can study the regulation of specific functions without interference from other cell components. Disadvantages in using isolated organelles include the yield and purity of the fraction obtained. As well, only the function of the isolated organelle itself can be investigated.

1.4. *Hypothesis, Rationale and Aims of This Study*

1.4.1. Hypothesis and Rationale

Although a number of studies have examined the phosphorylation of phospholamban in different experimental models (see section 1.2.3.2.), there appears to be some incongruity between the results obtained from isolated SR membrane vesicles and perfused whole hearts. In isolated SR vesicles, PK A, PK C, CAM PK and PK G all phosphorylate phospholamban. However, in isolated perfused hearts, only PK A and CAM PK (only after activation of PK A) are found to phosphorylate phospholamban. Thus, in the present study, it was hypothesized that in a cardiac cell, there may be a functional basis for the phosphorylation of phospholamban by several different types of protein kinases and further, that the protein kinase pathways may not act independently of one another but may synergize or antagonize each other to modulate SR function.

For the present studies, the isolated adult rat ventricular myocyte was chosen as the model system. Ventricular myocytes were chosen since these are the contractile cells of the heart and as such, regulation of their SR function is of prime interest. Adult myocytes were chosen since the SR of neonatal cells is poorly developed and t-tubules are almost nonexistent [Hirakow and Gotoh (1975)]. A recent study shows that in newborn myocardium the SR plays a negligible role in excitation-contraction coupling [Klitzner and Friedman (1989)]. Isolated myocytes are intact cells that possess the internal regulatory pathways used in the functional myocardium. In the intact heart, myocytes represent only 20% of the cellular population but 80% of the heart mass. The remaining cells (80%) are neuronal, fibroblastic, smooth muscle, endothelial and epithelial. Due to this cellular heterogeneity it is difficult to identify a population of cells responsible for a specific property of the myocardium. Isolated adult ventricular myocyte preparations offer

the advantage of being a more homogeneous cell population than is present in the isolated perfused heart thereby allowing a particular function to be localized to the muscle cell. As well, complex regulatory mechanisms may be studied in the myocyte without the additional complications brought about by the neuronal and hormonal effects that exist in intact myocardium. For our experiments, an added advantage was that the medium surrounding the isolated myocytes can be controlled and quickly changed to facilitate treatment with various agonists and antagonists.

Several studies on protein phosphorylation in adult cardiac ventricular myocytes have been published. In an early study utilizing adult rat ventricular cells, isoproterenol stimulated the phosphorylation of 5 proteins (150, 94, 33, 28, and 12 kDa), none of which were thought to be phospholamban [Onorato and Rudolph (1981)]. Another study utilized several methods of compromising the cellular integrity (colchicine and saponin treatment) of the adult myocytes and found that isoproterenol-stimulation increased the phosphorylation of 3 proteins (150, 28 and 26 kDa) and that phospholamban phosphorylation was a minor component of the overall phosphorylation pattern observed [MacKay and Sulakhe (1988)]. In saponin permeabilized, spontaneously beating adult rat myocytes, elevated cAMP levels were also found to increase the phosphorylation of 5 proteins (150, 28, 24, 15 and 12 kDa). The 24 kDa protein was identified as phospholamban [Miyakoda *et al.* (1987)]. Blackshear *et al.* (1984) demonstrated the phosphorylation of 13 proteins in response to isoproterenol treatment of isolated myocytes, one of which was identified as phospholamban. Recently, George *et al.* (1991) found 3 proteins (155, 31 and 6 kDa) phosphorylated in response to isoproterenol treatment of isolated myocytes.

In this study, isolated adult rat ventricular myocytes were used to study the phosphorylation of proteins in response to activators of PK A and PK C. SR

membranes were isolated from the myocytes to enable the localization of phosphorylated proteins to this organelle.

1.4.2. Specific Aims

- 1) To establish a method for the isolation of adult rat ventricular myocytes from a single rat heart that would result in a preparation with a high number of viable, rod-shaped cells.
- 2) To develop a method for the isolation of purified SR membranes from adult rat ventricular myocytes.
- 3) To characterize the SR membranes obtained in regards to Ca^{2+} -transport activity, $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity, purity and protein profile.
- 4) To determine the proteins phosphorylated in the intact myocyte subsequent to the stimulation of the cells with isoproterenol (a non-specific β -agonist).
- 5) To determine the proteins phosphorylated in the intact myocyte subsequent to the stimulation of the cells with PK C activators and to study the activation of this kinase in the myocytes.
- 6) To analyze the oligomeric species of phosphorylated phospholamban obtained in the presence of activators of PK A, PK C and PK A and C, together.

2. MATERIALS AND METHODS

2.1. *Materials*

2.1.1. Animals

Male Sprague-Dawley rats (300 - 600 g) were used throughout this study. The animals were obtained from Animal Care, U.B.C. or from Charles River, Montreal, Que. The animals were maintained on Purina Rat Chow and tap water, *ad libitum*, in a light and temperature controlled room.

2.1.2. Chemicals

2.1.2.1. Materials for Isolation of Adult Rat Ventricular Myocytes

CLS II collagenase (130 - 160 units/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ. Bovine serum albumin (Fraction V, fatty acid free) was obtained from Boehringer Mannheim, Dorval, Que. Joklik-modified minimum essential medium, Medium-199 with Earle's salts, minimum essential medium non-essential amino acid solution (10 mM) (100x), minimum essential medium amino acid solution without glutamine (50x), HEPES, pyruvic acid (Na⁺ salt), heparin (Na⁺ salt) (porcine intestinal mucosa, average molecular weight 4,000 - 6,000), EGTA, carnitine, creatine, citric acid, tissue culture-tested insulin (bovine pancreas), CaCl₂, glutamine, NaHCO₃ and taurine were obtained from Sigma Chemical Co., St. Louis, MO. Pentobarbital sodium (65 mg/ml) was obtained from Canada Packers Inc., Cambridge, Ont. Basal medium Eagle vitamin solution (100x) and trypan blue (0.4%) were obtained from Gibco Labs, Grand Island, NY. All other chemicals were at least of AnalaR^R grade from BDH Chemicals, Canada Ltd.

2.1.2.2. Chemicals for the Treatment of Isolated Adult Rat Ventricular Myocytes

Catalytic subunit of PK A (bovine heart), calmodulin, (\pm) isoproterenol-HCl, 4 α -phorbol 12, 13-didecanoate and phorbol 12, 13-myristate acetate were obtained from Sigma Chemical Co., St. Louis, MO. HA1004 (N-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride) and H-8 (N-(2-(methylamino) ethyl)-5-isoquinolinesulfonamide dihydrochloride) were obtained from Seikagaku America, Inc., St. Petersburg, FL. CGS 9343 B (1,3-dihydro-1-(1-((4-methyl-4H, 6H-pyrrolo(1,2-a)-(4,1)benzoxazepin-4-yl)methyl)-4-piperindinyl)-2H-benzimidazol-2-one maleate) was a kind gift from Ciba-Geigy Ltd., Basle, Switzerland. 1-Oleoyl-2-acetyl glycerol was obtained from Serdary Research Laboratories Inc., London, Ont. Ethanol and dimethylsulfoxide were AnalaR^R grade and obtained from BDH Chemicals Canada Inc. Forskolin (*Coleus forskohlii*) and diacylglycerol kinase inhibitor (6-(2-(4-((p-fluorophenyl)-phenylmethylene)-1-piperidinyl)-ethyl)-7-methyl-5-H-thiazolo-3,2-a-pyrimidine-5-one; R59022) were obtained from Calbiochem Corp., La Jolla, CA.

2.1.2.3. Radioactivity and Autoradiography

[γ -³²P]adenosine triphosphate in ethanol (30 Curies/mmol; 1 mCi/ml), cAMP protein binding [³H] kit and ³²P-orthophosphate in aqueous solution (HCl free) (10 mCi/ml) were obtained from Amersham, Oakville, Ont. ⁴⁵CaCl₂ (2 mCi/ml) was obtained from ICN Radiochemicals, Irvine, CA. Kodak X-OMAT film, Cronex intensifying screens and Kodak GBX developer and fixer (with replenisher) were obtained from Sigma Chemical Co., St. Louis, MO. Unisolve 1 (Terochem) and Ready Safe (Beckman Instruments Inc.) were used for liquid scintillation counting.

2.1.2.4. Materials for the Isolation and Characterization of Myocyte Homogenates and SR Membranes

Dithiothreitol, bovine serum albumin (Fraction V), phenylmethylsulfonylfluoride (PMSF), tosyl-L-phenylalanine chloromethyl ketone (TPCK) and alkaline phosphatase were obtained from Boehringer Mannheim Canada Ltd. NaHCO_3 , KCl, Na_2HPO_4 , methanol, acetic acid, MgCl_2 , sucrose, sodium molybdate, stainsall (1-ethyl-2-(3-(1-ethylnaphthol(1,2*d*)thiazolin-2-ylidene)-2-methylpropenyl)-naphthol-(1,2*d*)-thiazolium bromide, Kodak Organic Chemical, No. 2718), NaF and NaCl of AnalaR^R grade were obtained from BDH Chemicals Canada Ltd. L-Histidine, MOPS (3-(N-morpholino)propanesulfonic acid), Tris base (tris(hydroxy-methyl)aminomethane), Na_2EDTA , EGTA, adenosine 5'-triphosphate (Tris salt), ouabain, oxalate (Tris salt), alamethicin (antibiotic U-22324, from *Trichoderma viridae*), imidazole, leupeptin, soybean trypsin inhibitor, cytochrome c (horse heart), aprotonin, pepstatin A and NaN_3 were obtained from Sigma Chemical Co., St. Louis, MO. Ruthenium red was obtained from Fluka Chemical Corp., Ronkonkoma, NY. Acrylamide, bis-acrylamide, ammonium persulfate, sodium dodecylsulfate, Bradford protein reagent concentrate, bovine γ -globulin protein standard, Coomassie brilliant blue R250, glycine, TEMED, Zeta-probe membranes, SDS-PAGE high and low molecular weight standards, nitro blue tetrazolium, BCIP, alkaline phosphatase and horse radish peroxidase avidin conjugates, horse radish peroxidase color development reagent, prestained and biotinylated SDS-PAGE high and low molecular weight standards were obtained from Bio-Rad Labs., Mississauga, Ont. Goat F(ab')₂ anti-mouse (alkaline phosphatase and horse radish peroxidase-conjugated) and goat F(ab')₂ anti-rabbit IgG (G & L) (alkaline phosphatase and horse radish peroxidase-conjugated) were obtained from Tago Immunologicals, TAGO Inc., Burlingame, CA. BioGel wrap was obtained from BioDesign Inc., NY.

2.1.2.5. Materials for the Isolation and Quantitation of PK C

Histone Type III-S from calf thymus, protein kinase inhibitor (PKI, synthetic rabbit sequence), HEPES, leupeptin, EGTA, Na₂EDTA, MOPS, Na₃VO₄, DEAE-cellulose and β -glycerophosphate were obtained from Sigma Chemical Co., St. Louis, MO. Dithiothreitol was obtained from Boehringer Mannheim Canada Ltd. P-81 (cellulose-phosphate) ion exchange chromatography paper was obtained from Whatman International Ltd., Maidstone, England. NaCl, MgCl₂, KOH, CaCl₂ and orthophosphoric acid were obtained from BDH Chemicals Canada Ltd. Phosphatidylserine and 1-stearoyl-2-arachidonylglycerol were obtained from Serdary Research Laboratories Inc., London, Ont.

2.2. Methods

2.2.1. Isolation of Adult Rat Ventricular Myocytes

2.2.1.1. Modified Method of Piper *et al.*

The following method was modified from that of Piper *et al.* (1982). Hearts were rapidly removed from the anesthetized rats (70 mg/kg pentobarbital sodium and 1000 units/0.3 kg heparin), following attainment of surgical anaesthesia and rinsed with Joklik-Modified Minimum Essential Medium (MEM) containing 1 mM CaCl₂, at 4°C. Hearts were cannulated *via* the aorta and Langendorff perfusion at 37°C was started immediately with MEM (pH 7.4 at 20°C) containing the following additions (mM): HEPES, 21.1; NaHCO₃, 4.4; taurine, 60; MgCl₂, 2.42; glucose, 4; creatine, 20; glutamate, 8. and carnitine, 2. This buffer (HEPES-MEM) also contained 1% fatty acid free BSA and essential amino acids, however, no calcium was added. Subsequent to a 4.5 min non-recirculating perfusion at 37°C, hearts

were perfused in a recirculating manner for 35 min with HEPES-MEM containing 25 μ M CaCl_2 and 0.8 mg/ml collagenase. The buffers were aerated with 100% O_2 before use and throughout the perfusion. After perfusion, the ventricles were minced with a scalpel and the tissue pieces incubated in a 37°C water bath for 10 min in 10 ml of the recirculation medium. Following this incubation, the tissue pieces were drawn up and down very gently (no air bubbles) with plastic transfer pipettes of 3 diameters (2 to 4 mm), starting with the widest pipette, until the tissue pieces became small enough to easily pass through the narrowest diameter pipette. Subsequent to filtration through 200 μ m nitex mesh, the cells were washed three times with HEPES-MEM containing increasing concentrations of calcium (0.2, 0.5 and 1.0 mM CaCl_2). Rod-shaped myocytes were purified by settling through 4% BSA in Medium-199 with Earle's salts and then were resuspended in Medium-199. From each rat heart, 3 - 5 $\times 10^6$ rod shaped myocytes were isolated, of which 75 to 90% were viable.

2.2.1.2. Modified Method of Li *et al.* and Wimsatt *et al.*

The following method was modified from that of Li *et al.* (1988) and Wimsatt *et al.* (1990). Hearts were rapidly removed from anesthetized rats (70 mg/kg pentobarbital sodium and 1000 units/0.3 kg heparin), following attainment of surgical anaesthesia and rinsed with 1 ml of Krebs-Henseleit (K-H) buffer at 4°C. This buffer contained (mM): NaCl, 118.0; KCl, 4.8; CaCl_2 , 1.0; MgSO_4 , 1.2; KH_2PO_4 , 1.2; glutamine, 0.7; glucose, 11.0; Na-pyruvate, 5.0; HEPES, 25.0; pH 7.3 - 7.35 at 20°C. Hearts were cannulated *via* the aorta and Langendorff perfusion was started immediately in a non-recirculating mode at a flow rate of 10 ml/min for 5 min at 37°C with K-H containing vitamins and amino acids. Subsequently, hearts were perfused (in non-recirculating mode) with K-H containing no added CaCl_2 and 0.02 mM EGTA. Hearts were then perfused in a recirculating mode with K-H

containing 1 mg/ml BSA (fatty acid free) and 1 mg/ml collagenase. After 20 min, 125 μ l 0.1 M CaCl_2 was added to the perfusate to bring the Ca^{2+} concentration to 250 μ M. After 5 min, this was repeated to bring the total Ca^{2+} concentration to 500 μ M, then after another 5 min, 250 μ l of 0.1 M CaCl_2 was added to bring the total Ca^{2+} concentration of the perfusate to 1 mM. After 40 min, the flow rate of the perfusion was increased over 10 min to 16 ml/min. Perfusion was continued until hearts became soft or the aorta was digested through. Usually the hearts were perfused for a total time of approximately 50 min. The buffers were aerated with 100% O_2 before use and throughout the perfusion. After perfusion, the ventricles were minced with a scalpel and the tissue pieces incubated in a 37°C shaking (100 cycles/min) water bath under O_2 for 10 min in 10 ml of K-H containing 1 mM CaCl_2 , vitamins, amino acids, 0.6 μ g/ml insulin, 2% BSA and 2 mg/ml collagenase. After this incubation, the tissue pieces were drawn up and down very gently (no air bubbles) with plastic transfer pipettes of 3 diameters (2 to 4 mm), starting with the widest pipette, until the tissue pieces became small enough to pass through the narrowest diameter pipette. Subsequent to filtration through 200 μ m nitex mesh, the cells were centrifuged at 400 x g for 1 min. The pellet was gently resuspended in K-H containing 1 mM CaCl_2 , vitamins, amino acids, 0.6 μ g/ml insulin and 0.5% BSA, centrifuged as before and resuspended in the same K-H buffer with 0.5% BSA.

Myocytes were purified by layering 3 ml aliquots of the above suspension onto 10 ml of K-H containing 1 mM CaCl_2 , vitamins, amino acids, 0.6 μ g/ml insulin and 4.0% BSA. Myocytes were allowed to settle by gravity for 5 min, the supernatants removed and the cell pellets resuspended in K-H containing 1 mM CaCl_2 , vitamins, amino acids, 0.6 μ g/ml insulin and 2.0% BSA. Finally, after centrifugation (400 x g for 1 min), the myocytes were resuspended in K-H containing 1 mM CaCl_2 , vitamins, amino acids and 0.6 μ g/ml insulin. Using this method, from each rat heart, 3 - 8 x 10⁶ rod shaped myocytes were isolated, of which from 75 to 90% were viable.

2.2.2. Characterization of Isolated Adult Rat Ventricular Myocytes

2.2.2.1. Cell Counting, Morphology and Maintenance of Contractile Function

Isolated myocytes were quantitated using a Fuchs-Rosenthal Ultra Plane counting chamber of 2 mm depth. An aliquot (20 μ l) of the myocyte suspension was drawn under the coverslip of the counting chamber by capillary action. The counting chamber was placed under a microscope and myocytes were counted in each of the 1 mm² squares in the four corners of the grid. The number of myocytes in the preparation was calculated as follows:

$$\frac{\text{average cell number per 1 mm}^2 \text{ square}}{\text{volume counted (ml)}} \times \text{volume of the cell suspension (ml)}$$

The volume counted for this counting chamber is 2×10^{-4} ml (1 mm² x 0.2 mm).

The number of viable myocytes in a preparation was determined by using the cell viability stain, trypan blue. An aliquot of the myocyte cell suspension (250 μ l) was mixed with 50 μ l of 0.1% trypan blue. After 4 min, the viable myocytes (cells that did not take up the dye) were counted as described above.

Electrical stimulation was used to determine whether the isolated myocytes had retained the functional ability to contract, using the method of Haworth *et al.* (1980). An aliquot of the myocyte suspension (2 drops) was placed on a glass slide, covered with a coverslip and placed on the microscope stage. The two leads from a Grass stimulator (model SD9) were placed in the fluid on either side of the coverslip and attached with electrical tape. Square-wave stimulating pulses of 10 ms in duration and up to 100 V were used to elicit contractions from the myocytes.

2.2.2.2. Quantitation of cAMP

Isolated myocytes ($1.2 - 1.5 \times 10^4$ rod shaped cells per condition) were incubated at 37°C for 2 min in Medium-199 containing 0.5 mM IBMX and either 1 μ M isoproterenol or 100 μ M forskolin. The reaction was terminated by the addition of 35% TCA to the reaction mixture to bring the final concentration of TCA to 7%. The tubes were left on ice for 10 min and then centrifuged at 2500 x g for 15 min at 4°C. The supernatants were then extracted 4 times with a 5x volume of water-saturated diethyl ether. The ether phases were discarded and the residue was dried at 50°C for 30 min in an oven and, if necessary, was lyophilized to remove any remaining water. The amount of cAMP present was determined in a 50 μ l aliquot of each sample using the cAMP protein binding assay kit from Amersham.

2.2.3. Homogenization of Isolated Adult Rat Ventricular Myocytes

2.2.3.1. Preliminary Homogenization Methods

Several different methods of myocyte homogenization were attempted before suitable methods were found. Methods utilizing the French Press, sonication, grinding under liquid N₂, wiggle bug (dentistry amalgam mixer) and Polytron homogenizer were all tried and found to be unsuitable.

For homogenization by French Press, myocytes were resuspended in buffer containing 0.29 M sucrose, 3 mM NaN₃, 10 mM imidazole (pH 6.9 at 4°C), 0.1 M KCl, 10 mM DTT, 1.3 mM PMSF, 1 μ g/ml leupeptin, 25 μ g/ml STI, 1 μ g/ml aprotonin and 1 mM EGTA. Myocytes were passed once through the press, at a pressure of 1500 psi.

For sonication, the myocytes were resuspended in the same homogenization buffer as above but without 0.1 M KCl. Sonication was carried out with a Branson

Probe Sonicator at 4°C with four bursts of 30 s each at an output of 2, % duty of 80 and output control setting at 2.5.

Myocytes (frozen) were ground in liquid N₂ by hand with a porcelain mortar and pestle. Subsequently, the frozen cell slurry was resuspended in the same buffer (without KCl) as was used for sonication.

Homogenization with the wiggle bug (dentistry amalgam mixer) was carried out in the following buffer: 0.29 M sucrose, 3 mM NaN₃, 2.0 mM EGTA, 25 mM NaF, 50 mM Na₂HPO₄ (pH 6.9 at 4°C), 10 mM DTT, 0.1 mM PMSF, 1 µg/ml leupeptin, 25 µg/ml STI, 1 µg/ml aprotonin, 0.7 µg/ml pepstatin and 100 µg/ml TPCK. Myocyte pellets, frozen in liquid N₂, were placed into a precooled (with liquid N₂) wiggle bug. The wiggle bug was agitated for 15 s, 500 µl homogenization buffer added and the bug agitated for another 15 s. The resulting cell slurry was diluted with homogenization buffer under slow stirring.

Myocytes were homogenized by polytron at a setting of 5.5 for 9 x 10 s with a Brinkman PT10/35 Polytron Homogenizer, in the same buffer as was used for the wiggle bug.

2.2.3.2. Successful Homogenization Methods

Complete homogenization of all of the myocytes isolated was successfully carried out at 4°C in a hypotonic buffer using 1 pass (up and down) with a 'Zero'-clearance homogenizer (Kontes) on a motorized Potter-Elvehjem, set at 3.8 on reverse. The hypotonic buffer contained (mM): Tris-maleate, 10 (pH 7.4 at 4°C); HEPES, 20; NaF, 25; EGTA, 10; PMSF, 1; and DTT, 1. Homogenization was carried out immediately after the myocytes were isolated and after preparation, aliquots of the homogenate were immediately frozen in liquid N₂ and stored at -70°C.

When SR membranes were to be isolated (see section 2.2.4.2.) from the myocyte homogenates, the following homogenization procedure was used. Myocytes

(3 - 5 x 10⁶ cells) in a soft pellet (approximately 0.7 ml) at 4°C were gently resuspended in 15 ml of Buffer 1 (10 mM NaHCO₃ and 4 mM DTT, pH 7.4 at 4°C). The myocytes were then homogenized 3 times (total = 90 strokes) alternating 15 strokes of a 7 ml glass-glass douncer (Pestle B = small clearance) with 15 strokes of a motorized PTFE-glass Potter-Elvehjem (50 ml) set at approximately 800 rpm. The homogenate was then diluted to 25 ml with Buffer 1 and gentle stirring. It was necessary to dilute the homogenates (final 1:35 v/v; cells/buffer 1) to prevent aggregation of membranes and proteins during the SR isolation procedure. This procedure homogenized approximately 20-30% of the rod-shaped cells.

2.2.4. Isolation of Sarcoplasmic Reticulum Membranes from Isolated Adult Rat Ventricular Myocytes

2.2.4.1. Preliminary Methods for the Isolation of SR Membranes from Isolated Myocytes

Several different methods [Chamberlain and Fleischer (1988); Jones *et al.* (1979)] for the isolation of SR membranes from the isolated adult rat ventricular myocytes were attempted prior to the development of the final method utilized.

SR membrane vesicles were isolated by a modification of the method of Chamberlain and Fleischer (1988) as follows: Myocytes were homogenized in 0.29 M sucrose, 3 mM NaN₃, 20 mM imidazole (pH 6.9 at 4°C), 0.1 M KCl, 10 mM DTT, 0.1 mM PMSF, 1 µg/ml leupeptin, 25 µg/ml STI, 1 µg/ml aprotonin, 0.7 µg/ml pepstatin, 100 µg/ml TPCK and 1 mM EGTA. Homogenates were centrifuged for 15 min at 5,000 rpm (3,800 x g_{max}) in a Beckman high speed centrifuge at 4°C. Supernatants were then centrifuged for 15 min at 13,000 rpm (20,000 x g_{max}) in the same centrifuge. The resulting supernatant was centrifuged for 2 hrs at 55,000 rpm (120,000 x g) in a Beckman ultracentrifuge (Ty65 rotor) at 4°C. The supernatant

was discarded and the pellet was resuspended in 0.29 M sucrose, 0.65 M KCl, 3 mM NaN₃, 0.5 mM EGTA, 10 mM imidazole, pH 6.7 at 4°C, 5 mM DTT, 0.1 mM PMSF and the protease inhibitors (µg/ml): aprotonin, 1; leupeptin, 1; STI, 25; pepstatin, 0.7 and TPCK, 50. Resuspension of the pellet was carried out with 8 strokes of a PTFE-glass homogenizer. At this point, the resuspended pellet may have been frozen in liquid N₂ and stored at -70°C. Subsequently, the resuspended pellet was layered onto the top of a 20 - 45% continuous sucrose density gradient containing 10 mM imidazole (pH 6.7 at 4°C), 3 mM NaN₃, 0.65 M KCl, 5 mM DTT and the above listed protease inhibitors. The gradient was centrifuged for 6.5 hrs at 28,000 rpm in a Beckman ultracentrifuge (SW41 rotor). Following centrifugation, 1 ml fractions were collected from the top of the gradient with a Hamilton syringe.

When the original Chamberlain and Fleischer (1988) method was used the following procedure was followed: Myocytes were homogenized in the same buffer as above, except that the DTT concentration was 0.5 mM and no protease inhibitors were added. The first centrifugation was the same as above, the second centrifugation was omitted and the third centrifugation was also the same as above. The pellets were resuspended in the same buffer except that the EGTA concentration was 0.5 mM, the DTT concentration was 0.5 mM and no protease inhibitors were added. The resuspended pellet was kept on ice for 30 min and then centrifuged at 6,000 rpm (JA20 rotor) for 10 min at 4°C. The supernatant was then centrifuged for 100 min at 55,000 rpm (Ty65 rotor) at 4°C. Final pellets were resuspended in the same buffer, as the first resuspension, with 10 strokes of a PTFE-glass homogenizer.

SR membranes were isolated by the method of Jones *et al.* (1979) as follows: Myocytes were homogenized in 10 mM NaHCO₃, 10 mM DTT, 1.3 mM PMSF, 1 µg/ml leupeptin, 25 µg/ml STI, 1 µg/ml aprotonin, 0.7 µg/ml pepstatin and 1 mM EGTA. The homogenate was centrifuged at 14,000 × g_{max} (10,500 rpm in a Beckman JA20 rotor) for 20 min at 4°C. The pellet was discarded and the

supernatant was centrifuged in the same manner as the homogenate. Subsequent to discarding the pellet, the supernatant was centrifuged at $45,000 \times g_{\max}$ (18,500 rpm JA20 rotor) for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended in 30 mM histidine (pH 7.0 at 4°C) and 0.6 M KCl with 10 strokes of a small handheld Teflon-glass homogenizer. The resuspended pellet was centrifuged at $45,000 \times g_{\max}$ (18,500 rpm JA20 rotor) for 30 min at 4°C. The final pellet was resuspended in 0.25 M sucrose, 0.3 M KCl and 0.1 M Tris (pH 7.2 at 4°C) with 10 strokes of a small hand-held PTFE-glass homogenizer.

2.2.4.2. Final Method for the Isolation of SR Membranes from Isolated Myocytes

The basis for the development of a method to isolate purified SR membranes from myocytes was the SR preparation of Harigaya and Schwartz (1969) as modified by Jones *et al.* (1979). Intact adult rat ventricular myocytes were homogenized in Buffer 1 (10 mM NaHCO₃ and 4 mM DTT, pH 7.4 at 4°C) as described in section 2.2.3.2. All subsequent steps were carried out at 4°C and all centrifugations were done in Beckman centrifuges. Myocyte homogenates were centrifuged at $330 \times g$ ($r_{av} = 7.0$ cm; 2,000 rpm JA20 rotor) for 15 min to pellet unbroken cells. Supernatants were centrifuged at $5,000 \times g$ ($r_{av} = 7.0$ cm; 8,000 rpm JA20 rotor) for 15 min to pellet intact nuclei and mitochondria. The resultant supernatant was then centrifuged at $23,000 \times g$ ($r_{av} = 7.0$ cm; 17,000 rpm JA20 rotor) for 30 min to pellet SR membranes. The pellets were resuspended in Buffer 2 (30 mM histidine-Cl, 0.6 M KCl and 4 mM DTT, pH 7.0 at 4°C) with 10 strokes of a 7 ml glass-glass douncer. SR membranes were then collected by centrifuging at $23,000 \times g$ ($r_{av} = 7.0$ cm; 17,000 rpm JA20 rotor) for 30 min. SR membrane pellets were resuspended in Buffer 3 (0.25 M sucrose, 0.3 M KCl and 0.1 M Tris-Cl, pH 7.2 at 4°C), using 10 strokes of a 1 ml Potter-Elvehjem homogenizer, frozen in liquid N₂ and stored at -70°C.

When homogenates and SR membranes were prepared from ^{32}P -labeled myocytes subsequent to stimulation, alterations in protein-bound phosphate due to the action of kinase, phosphatase or protease activities were minimized by carrying out all procedures at 4°C and by homogenizing the myocytes in Buffer 4 (50 mM Na_2HPO_4 , 10 mM Na_2EDTA and 25 mM NaF). The SR pellets were washed in Buffer 4 with 0.6 M NaCl added (Buffer 5). The final SR preparation was resuspended in Buffer 3. Under these conditions, [^{32}P]phosphoproteins have been shown to be stable for several hours [Lindemann and Watanabe (1985); Lindemann *et al.* (1983)].

2.2.5. Characterization of SR Membranes from Isolated Myocytes

2.2.5.1. Biochemical Assays

2.2.5.1.1. Determination of Ca^{2+} -Transport Activity

Ca^{2+} -uptake activity was determined by a modification of the method by Tada *et al.* (1974). This assay was linear with respect to protein concentration (0 - 80 mg/ml) and incubation time (0 -15 min; Mahey (1986)). Fractions (10 μg protein in 50 μl) were added and pre-incubated for 140 s at 30°C in a reaction medium (400 μl) containing (final concentrations): 40 mM histidine/HCl buffer (pH 6.8 at 20°C), 110 mM KCl, 5 mM MgCl_2 , 5 mM NaN_3 , 0.25 M sucrose, 1 mM DTT, 5 mM ATP (Tris salt) in the presence and absence of 2.5 mM oxalate (Tris salt) and 5 μM ruthenium red. The reaction was initiated by the addition of 50 μl EGTA-buffered $^{45}\text{Ca}^{2+}$ (126 μM CaCl_2 and 175 μM EGTA resulting in 2 μM free Ca^{2+} ; 200,000 dpm/50 μl) and was terminated after 140 s by transferring an aliquot (400 μl) of the reaction mixture onto a Whatman GF/C filter. The filter was washed once with 15 ml of 40 mM Tris-Cl, pH 7.2 at 20°C to remove non-specifically bound calcium. After

drying, the radioactivity on the filters was quantitated by liquid scintillation counting in a Canberra Packard Tricarb scintillation spectrometer. All membrane fractions (fresh or frozen) were assayed for Ca^{2+} -uptake activity within 24 hrs of their isolation. The Ca^{2+} -transport activity of each fraction was calculated as follows:

$$\text{nmol Ca}^{2+}/\text{min}/\text{mg protein} = \frac{\text{average dpm}}{\text{specific activity of } ^{45}\text{Ca}^{2+} \text{ (dpm/nmol)}} (140 \text{ s})^{-1} (\text{mg protein})^{-1}$$

For the calculation of the total Ca^{2+} -transport activities of each SR or other membrane-containing fraction, the yield of protein (mg) has been normalized so that each was obtained from a starting myocyte population of 3×10^6 cells. These normalized protein values were used in the calculation of the total Ca^{2+} -transport activities in Tables 3 and 7 (sections 3.3.2.1. and 3.3.2.2.).

2.2.5.1.2. Determination of $\text{Ca}^{2+}/\text{K}^{+}$ and $\text{Na}^{+}/\text{K}^{+}$ ATPase Activities

Potassium-stimulated, calcium-dependent ATPase ($\text{Ca}^{2+}/\text{K}^{+}$ -ATPase) activity was measured under the conditions described by Jones and Besch (1984). Inorganic phosphate was determined by the method of Raess and Vincenzi (1980). The reaction buffer contained (400 μl): 45 mM histidine, 1 mM EGTA, 5 mM MgCl_2 , 100 mM KCl, 1.84 mM ouabain, 11 mM NaN_3 and 1.15 mM DTT in the absence or presence of 0.6 mM CaCl_2 (1.5 μM *free* Ca^{2+}). Tubes contained either 3 μg (SR) or 10 μg (other membrane fraction) of protein. Following a 10 min pre-incubation at 37°C, the reaction was initiated by the addition of 5 mM Tris-ATP and terminated after 30 min by the addition of 200 μl 10% (w/v) SDS solution with rapid mixing. Following this, 200 μl of 9% ascorbic acid (w/v) was added with rapid mixing. Subsequently, 200 μl of 1.25% ammonium molybdate in 6.5% H_2SO_4 was added to each tube every 30 s with rapid mixing. After a 30 min incubation at room

temperature, the absorbance at 660 nm was measured at 30 s intervals on a Hewlett Packard Diode Array Spectrophotometer (model 8452A). The P_i content of each sample was determined from a calibration curve ranging from 0 - 750 nmol P_i /ml. The Ca^{2+}/K^+ -ATPase activity was taken as the difference between ATP hydrolysis measured in the presence and absence of 0.6 mM $CaCl_2$ (1.5 μ M *free* Ca^{2+}) and was calculated as follows:

$$\text{nmol/min/mg protein} = (\text{nmol } P_i) (30 \text{ min})^{-1} (\text{mg protein})^{-1}$$

For the calculation of total Ca^{2+}/K^+ -ATPase activities of each SR or other membrane containing fraction, the yield of protein (mg) has been normalized so that each was obtained from a starting myocyte population of 3×10^6 cells. These normalized protein values were used in the calculation of the total Ca^{2+}/K^+ -ATPase and Na^+/K^+ -ATPase activities in Tables 4, 6, 8 and 9 (sections 3.3.2.1. and 3.3.2.2.).

Sodium and potassium-dependent ATPase (Na^+/K^+ -ATPase) activity was measured as described above, except that Ca^{2+} was omitted and the reaction buffer contained 10 mM KCl and 110 mM NaCl and was determined in the absence or presence of 1.5 mM ouabain. Tubes contained either 3 μ g (SR) or 10 μ g (other membrane fraction) of protein. To unmask latent activity [Jones and Besch (1984)], prior to the start of the reaction, protein samples were incubated for 10 min in the presence of alamethicin (0.1 mg/mg protein). Na^+/K^+ -ATPase activity was taken as the difference between ATP hydrolysis measured in the presence and absence of 1.5 mM ouabain and the activity of each sample and fraction was determined as above.

2.2.5.1.3. Determination of Cytochrome C Oxidase Activity

Mitochondrial membrane cytochrome c oxidase activity of myocyte homogenates and SR membranes was assayed according to the method of Smith (1955) as described by Wharton and Tzagoloff (1967). A cuvette containing 30 mM

potassium phosphate buffer (pH 7.4 at 37°C) and 50 µl of 1% (w/w) reduced cytochrome c was preincubated at 37°C. The reaction was initiated by the addition of 10 µl of membrane vesicles (10 µg protein) to the cuvette. Cytochrome c oxidase activity was determined by monitoring the decrease in absorbance at a wavelength of 550 nm over a period of one minute. The rate of cytochrome c oxidase was determined according to the following formula:

$$\text{Specific Activity} = k [\text{cytochrome c}] / [\text{protein}]$$

where:

$$\text{Specific Activity} = \text{nmoles cytochrome c/minute/mg protein}$$

$$k = \ln[A_0 / A_{0+1 \text{ min}}] \quad A_0 = A_{550} \text{ at time } 0 \text{ s}$$

$$A_{0+1 \text{ min}} = A_{550} \text{ at time } 60 \text{ s}$$

The absorbance of each sample in the cuvette in the presence of KCN was subtracted prior to the calculation.

2.2.5.1.4. Determination of Protein Concentration

Protein was determined by a modified Lowry procedure [Markwell *et al.* (1981)] which included SDS to solubilize the protein samples. BSA (Fraction V, Boehringer Mannheim) was used for the standard curve (0 - 25 µg protein). For protein samples in SDS-sample buffer, this Lowry method was modified by adding SDS-sample buffer to the standard curve. Protein content was also determined by the dye binding method of Bradford (1976) using the reagents and procedures for the microassay supplied by Bio-Rad Laboratories. Bovine γ-globulin was used to standardize each assay. For both methods, the protein concentration of the sample solution was then determined from linear regression of the standard curve samples.

2.2.5.1.5. Determination of Free Calcium Concentrations

Free calcium concentrations were calculated using the FORTRAN program "CATIONS" written by Goldstein (1979). Equilibrium constants for cations and ligands were obtained from Martell and Smith (1979-1982) except in the case of monoprotonated ligands which were calculated as described by Blinks *et al.* (1982). Constants were corrected for temperature using the BASIC program "LOGTEMP" based on the formula given by Tinoco *et al.* (1978) and using enthalpy values tabulated by Martell and Smith (1979-1982). The constants were then adjusted for pH and ionic strength [Blinks *et al.* (1982); Martell and Smith (1979-1982)].

2.2.5.2. Electrophoretic Methods

2.2.5.2.1. Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

2.2.5.2.1.1. Polyacrylamide Gradient (5 -20% and 10 - 20%) Gels

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), employing polyacrylamide gradients (5 - 20% and 10 - 20%), was performed using the discontinuous buffer system described by Laemmli (1970). Gloves were worn throughout the SDS-PAGE procedure including the preparation of all buffers and stock solutions. All stock solutions were filtered to 0.22 μ m. All components of the casting apparatus were cleaned using lint-free paper towels. Glass plates were soaked immediately prior to use and between uses in 0.1 M HCl. The 5% "resolving" gel solution consisted of 375 mM Tris/HCl buffer (pH 8.8 at 20°C) containing: 5% (w/v) acrylamide; 0.14% (w/v) N,N'-methylene-bis-acrylamide (BIS); 0.1% (w/v) sodium dodecylsulfate (SDS); 1 mM Na₂EDTA; 1.35% (v/v) glycerol; 0.025% tetraethylmethylene diamine (TEMED) and 0.3 mg/ml ammonium persulfate. The

10% "resolving" gel solution consisted of 375 mM Tris/HCl buffer (pH 8.8 at 20°C) containing: 10% (w/v) acrylamide; 0.27% (w/v) BIS; 0.1% (w/v) SDS; 1 mM Na₂EDTA; 1.35% (v/v) glycerol; 0.025% TEMED and 0.3 mg/ml ammonium persulfate. The 20% "resolving" gel solution consisted of 375 mM Tris/HCl buffer (pH 8.8 at 20°C) containing: 20% (w/v) acrylamide; 0.53% (w/v) BIS; 0.1% (w/v) SDS; 1 mM Na₂EDTA; 5.8% (v/v) glycerol; 0.05% TEMED and 0.23 mg/ml ammonium persulfate.

Once prepared, the resolving gel solutions were transferred to a gradient former and the gradient poured over a period of five minutes. The gel was then overlaid with 0.6 ml of isobutanol and allowed to polymerize for 12-18 hours at room temperature. Prior to use, the isobutanol was decanted and the resolving gel flushed with distilled and deionized water and then overlaid with a "stacking" gel solution.

The "stacking" gel consisted of the following: 126 mM Tris/HCl buffer (pH 6.8 at 20°C); 5% acrylamide; 0.14% BIS; 1 mM Na₂EDTA; 0.14% TEMED and 0.4 mg/ml ammonium persulfate. A PTFE "comb" was inserted to facilitate formation of sample wells. This solution was allowed to polymerize for 50 minutes at room temperature.

Electrophoresis of gels was performed in a Bio-Rad Protean II Apparatus with cooling core. The electrode buffers consisted of 25 mM Tris base, 192 mM glycine and 0.1% SDS in the upper reservoir and 25 mM Tris/HCl buffer (pH 8.3 at 20°C) in the lower reservoir. Gels were run at 25 mA (constant current) and 1000 V until the dye front entered the slab gel, after which, the current was increased to 35 mA. Gels were run until all dye fronts were off the bottom of the gels for at least 10 min or for a total of 1.5 volt-hours. The protein standards used for the estimation of molecular mass (in kilodaltons) were: myosin (200), β -galactosidase (116.25), phosphorylase *b* (92.5), bovine serum albumin (66.2), ovalbumin (45), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), lysozyme (14.4) and ubiquitin (8.3).

Samples were prepared for electrophoresis by solubilization in SDS-sample buffer containing: 5% SDS, 2.5% glycerol, 0.2 M Tris-Cl (pH 6.8 at 20°C), 0.5 M β -mercaptoethanol and 0.025% bromophenol blue. Samples were then either boiled for 3 min or incubated at 37°C for 10 min. Samples were allowed to cool, then centrifuged at 14,000 rpm for 2 minutes in an Eppendorf microcentrifuge at maximum speed and loaded into the sample wells.

2.2.5.2.1.2. Polyacrylamide and Bis-acrylamide Gradient Gels

SDS-PAGE was also carried out employing polyacrylamide and bis-acrylamide gradients (10 - 20% polyacrylamide with 0.27 - 1.0% BIS). The 10% “resolving” gel solution consisted of 375 mM Tris/HCl buffer (pH 8.8 at 20°C) containing: 10% (w/v) acrylamide; 0.27% (w/v) BIS; 0.1% (w/v) SDS; 1 mM Na_2EDTA ; 2.7% (v/v) glycerol; 0.025% TEMED and 0.3 mg/ml ammonium persulfate. The 20% “resolving” gel solution consisted of 375 mM Tris/HCl buffer (pH 8.8 at 20°C) containing: 20% (w/v) acrylamide; 1.0% (w/v) BIS; 0.1% (w/v) SDS; 1 mM Na_2EDTA ; 5.8% (v/v) glycerol; 0.05% TEMED and 0.23 mg/ml ammonium persulfate.

The procedures for the formation of the gradient gel, the stacking gel, the electrophoresis and the sample preparation for these gels was the same as in section 2.2.5.2.1.1.

2.2.5.2.1.3. Polyacrylamide Non-Gradient Gels

SDS-PAGE was also performed using polyacrylamide (15%) gels that did not contain a gradient according to the method of Li *et al.* (1990). The 15% “resolving” gel solution consisted of 375 mM Tris/HCl buffer (pH 8.8 at 20°C) containing: 15%

(w/v) acrylamide; 0.4% (w/v) BIS; 0.1% (w/v) SDS; 1 mM Na₂EDTA; 1.35% (v/v) glycerol; 0.019% TEMED and 0.3 mg/ml ammonium persulfate.

The procedures for the formation of the stacking gel, the electrophoresis run and the sample preparation for these gels was the same as in section 2.2.5.2.1.1.

2.2.5.2.2. Staining of Sodium Dodecylsulfate Polyacrylamide Gels

2.2.5.2.2.1. Coomassie Brilliant Blue Stain

The gels were allowed to stain for 1 hr at room temperature, with gentle agitation, in 500 ml of 0.25% (w/v) Coomassie Brilliant Blue R250 in methanol/ddH₂O/acetic acid (5:5:1). The first destaining step was for 1 hr in 500 ml (3 changes) of methanol/ddH₂O/acetic acid (5:5:1). This was followed by a second destaining step in ddH₂O/methanol/acetic acid (15:4:1) until the background became transparent.

2.2.5.2.2.2. Stainsall Stain

Glycoproteins and calcium binding proteins in myocyte SR membranes from whole rat heart and isolated ventricular myocytes were visualized using the cationic carbocyanine dye "Stainsall" according to the method of King and Morrison (1976) as modified by Campbell *et al.* (1983). SDS-PAGE gels were fixed overnight in 25% (v/v) isopropanol. As SDS will cause the dye to precipitate, fixed gels were then washed with 5 changes of (25%; v/v) isopropanol (200 ml) over an 8 hour period. The efficiency of the washing procedure was tested by adding a drop of the wash solution to 1 ml of the staining solution in a small (10 x 75 mm) test tube. If no precipitate was observed, the gel was transferred into a solution of 30 mM Tris/HCl buffer (pH 8.8 at 20°C) containing 0.0025% (w/v) "Stainsall", 25% (v/v) isopropanol

and 7.5% (v/v) formamide. Gels were maintained in the dark at room temperature with constant gentle agitation for 48 hours during color development. Due to the photosensitivity of this stain, gels were stored in staining solution in a dark place.

2.2.5.2.3. Drying of SDS-PAGE Gels

After staining with Coomassie blue, SDS-PAGE gels, were dried between two sheets of porous cellulose (BioGel Wrap, BioDesign Inc). Gels were clamped into a Plexiglass gel drying frame and were allowed to dry in a fume hood, at room temperature, for 12 hrs.

2.2.5.2.4. Western Blotting

2.2.5.2.4.1. Antibody Localization

Polypeptides from each sample were first separated by SDS-PAGE and then immunoblotted using the buffer system of Towbin *et al.* (1979). Gels were equilibrated for 30 minutes in buffer containing 25 mM Tris and 192 mM glycine and then electrophoretically transferred onto Zeta-Probe membranes. Transfer was for 60 minutes at 20 volts using a Bio-Rad Trans Blot Semi-Dry blotting apparatus. All of the following steps were carried out at room temperature with gentle rotation. Non-specific binding sites on the Zeta-Probe membrane were blocked by incubation overnight in 5% (w/v) skimmed milk powder (Carnation) in Tris-buffered saline (20 mM Tris/HCl buffer (pH 7.5 at 20°C) containing 0.5 M NaCl; TBS). Following a 20 min rinse with Tris-buffered saline containing 0.2% Tween-20 (TTBS), the membrane was incubated with the primary antibody in TTBS and 1% (w/v) skim milk powder for 3 hrs. Subsequent to two 10 min rinses with TTBS, membranes were incubated with secondary antibody (alkaline phosphatase or horse-radish

peroxidase conjugated Goat F(ab')₂ immunoglobulins; 1:2,000 v/v dilution) in TTBS and 1% (w/v) skim milk powder for 3 hrs. Membranes were then rinsed twice for 10 min in TTBS and then once for 15 min in TBS. Immunoreactive bands were then visualized by reaction with alkaline phosphatase development reagent (45 ml 100 mM Tris-HCl (pH 9.5 at 20°C) and 5 mM MgCl₂; 5 ml 0.1% (w/v) p-nitro blue tetrazolium chloride and 0.5 ml 0.5% 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) in 70% N,N-dimethylformamide) or horse-radish peroxidase development reagent (60 mg 4-chloro-1-naphthol in 20 ml methanol mixed into 100 ml TBS with 60 µl ice cold 30% H₂O₂). The molecular masses of the immunoreactive bands were determined relative to biotinylated SDS-PAGE standards, which had also been transferred to the membrane and visualized by including their avidin alkaline phosphatase- or horse radish peroxidase-conjugates in the secondary antibody incubation.

Primary antibodies were obtained from the following sources: A monoclonal antibody to phospholamban, antibody A1 [Suzuki and Wang (1986)], was a generous gift from Dr. Jerry. H. Wang, University of Calgary, Calgary, Alta., Canada. This antibody was used at a dilution of 1:2,735 v/v. Polyclonal antibodies to PKCβ₂ (Type II) and PKCα (Type III) were provided by Dr. Christopher Wilson (Department of Pediatrics, University of Washington, Seattle, WA) and were raised against synthetic peptides which appear in the amino acid sequences of the subspecies as deduced from their cDNA sequence [Kikkawa *et al.* (1987)]. These polyclonal antibodies against PKC Type II and Type III were prepared against peptides SFVNSEFLKPEVK (Type II sequence, amino acid residues 660-673) and AGNKVISPSEDRRQ (Type III sequence, amino acid residues 313-326), respectively. In the latter peptide, the conservative substitution of an arginine for lysine-325 was made [Makowski *et al.* (1988)]. These antibodies were used at a dilution of 1:250 v/v.

2.2.5.2.4.2. Ruthenium Red Staining

For the detection of Ca^{2+} -binding proteins, the method of Charuk *et al.* (1990) was utilized. SR membrane samples were separated by SDS-PAGE and transferred to 0.45 μm nitrocellulose using the semi-dry apparatus and the buffer system of Towbin *et al.* (1979) as described in the previous section (2.2.5.2.4.1.). After transfer, the nitrocellulose was stained in 60 mM KCl, 5 mM MgCl_2 , 10 mM Tris-HCl (pH 7.5 at 20°C) with 25 μM ruthenium red and in the presence and absence of 50 mM CaCl_2 .

2.2.5.2.5. Analysis of SDS-PAGE Gels, Autoradiographs and Western Blots

Electrophoretic gels, autoradiographs and western blots were analyzed using computer image analysis with a Visage 110 Bio Image Analyzer (Bio Image, Ann Arbor, MI) consisting of a high resolution camera (1024 x 1024 pixels) and a Sun Microsystems work station with Whole Band Analysis software to determine the molecular mass of the protein bands. This software calculates the molecular mass of each band by logarithmic interpolation using an average of the *per* pixel differences among all the defined standard molecular mass markers.

2.2.5.2.6. Autoradiography

Dried SDS-PAGE gels were exposed to X-ray film (Kodak X-Omat AR, X-Omat RP or MR-1) with an intensifying screen (Cronex Lightning Plus, Dupont) for 24 - 96 hours at -70°C. Following this, films were developed by incubating in Developer (Kodak) for 3 minutes, a 60 s rinse in 3% acetic acid, followed by a 3 minute incubation in Fixer (Kodak) and washing for 10 minutes under running water.

2.2.6. Isolation and Quantitation of Protein Kinase C

2.2.6.1. Preparation of Myocyte Cytosol and Membrane Fractions

Isolated adult rat ventricular myocytes were washed and resuspended in Krebs-Henseleit buffer containing (mM): NaCl, 134; KCl, 4.7; CaCl₂, 1.0; glucose, 10; ascorbic acid, 0.56; and HEPES, 15. Myocytes (1×10^6 rod shaped cells/ml) were incubated for 1, 5 or 10 min at 37°C in the presence and absence of 1% ethanol, 0.2 mM OAG (in 1% ethanol) or 30 μ M R59022 and 0.2 mM OAG (in 1% ethanol). Immediately after the incubation, the cells were quickly centrifuged at 300 rpm for 60 s, the supernatant removed and the cells were frozen in liquid N₂. Frozen myocyte pellets were homogenized in a wiggle bug apparatus in a buffer containing (mM): MOPS, 20; EGTA, 15; Na₂EDTA, 2; Na₃VO₄, 1; DTT, 1 and β -glycerophosphate, 75. The frozen pellet was placed into a precooled (with liquid N₂) capsule. The capsule was agitated for 15 s, 250 μ l homogenization buffer was added and the capsule was agitated for another 15 s. The homogenate was removed to a centrifuge tube, another 250 μ l homogenization buffer was added and the capsule agitated for 5 s. This rinse was also added to the centrifuge tube. The capsule was rinsed twice more with 250 μ l aliquots of homogenization buffer and these rinses were also added to the centrifuge tube.

The myocyte homogenates were centrifuged at 100,000 rpm for 11 min (240,000 x g) in a Beckman Optima TLA-100.2 Ultracentrifuge or at 50,000 rpm for 30 min (240,000 x g) in a Beckman L-8 Ultracentrifuge. The supernatant was reserved for the cytosolic fraction and the pellet was resuspended in 1 ml of the homogenization buffer with a 1 ml PTFE-glass douncer. The resuspended pellet was centrifuged at 100,000 rpm for 25 min (240,000 x g) in a Beckman Optima TLA-100.2 Ultracentrifuge or at 50,000 rpm for 30 min (240,000 x g) in a Beckman L-8 Ultracentrifuge. This supernatant was combined with the first supernatant and

constitutes the myocyte cytosolic fraction. This fraction was immediately frozen in liquid N₂ and stored at -70°C. The pellet was resuspended for 15 min on ice with a PTFE-glass douncer in 1 ml of homogenization buffer containing 1% Triton X-100. The resuspended pellet was centrifuged as before. The supernatant was reserved as the detergent-solubilized membrane fraction and immediately frozen in liquid N₂ and stored at -70°C.

2.2.6.2. Preparation of Cytosol from Rat Brain and Cytosol and Membrane Fractions from Bovine Trachea

Rat brain pieces, frozen in liquid N₂ immediately after dissection, were homogenized with 12 strokes of a motorized Potter-Elvehjem (50 ml) in 10 volumes of a buffer containing (mM): Tris-HCl, 20 (pH 7.5 at 20°C); EGTA, 10; Na₂EDTA, 5; sucrose, 330; PMSF, 2; DTT, 2; 25 µg/ml leupeptin and 0.5% Triton X-100. The brain homogenate was centrifuged at 60,000 rpm for 35 min (240,000 x g) in a Beckman L-8 Ultracentrifuge. The supernatant was reserved for the cytosol fraction and the pellet was resuspended in 10 ml of homogenization buffer with the motorized Potter-Elvehjem. The resuspended pellet was centrifuged as described for the homogenate. The resulting supernatant was combined with the first supernatant and referred to as the cytosolic fraction.

Bovine trachea sections, frozen in liquid N₂, were homogenized in a buffer containing (mM): Tris-HCl, 20 (pH 7.5 20°C); EGTA, 10; Na₂EDTA, 5; sucrose, 330; PMSF, 2; DTT, 2; and 25 µg/ml leupeptin. Homogenization was carried out with a wiggle bug apparatus as described in section 2.2.6.1. Cytosol and membrane fractions were isolated as described in section 2.2.6.1.

2.2.6.3. DEAE-Cellulose Chromatography

DEAE-cellulose chromatography was utilized to fractionate myocyte, rat brain and bovine trachea cytosol and detergent-solubilized membrane preparations to remove endogenous compounds and excess detergent which may interfere with the assay of PK C activity. DE-52 cellulose was prepared by washing with 0.1 N HCl, removing the fines 3 times by washing with ddH₂O, washing with 0.1 N NaOH and then ddH₂O before resuspending in 0.1 N HCl and bringing the pH to 7.4 (at 20°C) with 2.0 M Tris base. Columns were prepared as described by Thomas *et al.* (1987): DE-52 cellulose (2 ml) (1:1 v/v) was pipetted with a Pasteur pipette into disposable polypropylene columns (11 ml). The packed column (1 ml bed volume) was equilibrated with and stored in 20 mM Tris-HCl (pH 7.5) at 4°C. Before use, columns were washed with 10 ml of buffer containing (mM): Tris-HCl (pH 7.5 at 4°C), 20; EGTA, 10; Na₂EDTA, 5; sucrose, 330; PMSF, 2; DTT, 2; and 25 µg/ml leupeptin. Cytosol and detergent-solubilized membrane fractions (2 ml each) were slowly applied to the columns with pasteur pipettes and the columns washed twice with 3 ml of the above buffer. PK C was then eluted with 1 ml of buffer containing (mM): Tris-HCl (pH 7.5 at 4°C), 20; EGTA, 0.5; Na₂EDTA, 0.5; NaCl, 300; PMSF, 2; DTT, 2; and 25 µg/ml leupeptin. Leupeptin (25 µg/ml) was added immediately to the eluted fractions and PK C activity was assayed directly or the fractions frozen in liquid N₂ and stored at -70°C for assay the next day. On occasion, the protein in the eluted fractions was concentrated to a volume of 100 µl by centrifugation (5,000 x g) for 30 - 60 min in Centricon-10 microconcentrators (Amicon). Leupeptin (25 µg/ml) was added immediately to the concentrated fractions and PK C activity was assayed directly or the fractions frozen in liquid N₂ and stored at -70°C for assay the next day.

2.2.6.4. Fast Protein Liquid Chromatography

Fast protein liquid chromatography (FPLC) was utilized to fractionate myocyte cytosol and detergent-solubilized membrane preparations to remove endogenous compounds and excess detergent which interferes with the assay of PK C activity. FPLC was carried out using a 1 ml Mono-Q column (anion-exchange, Pharmacia) on a Pharmacia FPLC system according to the method of Pelech *et al.* (1991). Myocyte cytosol and detergent-solubilized membrane samples (4 mg in 2 ml) were loaded onto the Mono-Q column washed with (mM): MOPS (pH 7.2 at 20°C), 10; EGTA, 5; Na₂EDTA, 2; Na₃VO₄, 1; DTT, 1 and β -glycerophosphate, 25. PK C was eluted at a flow rate of 0.8 ml/min into 250 μ l fractions with a 15 ml linear gradient of 0 - 0.8 M NaCl in the above buffer. PK C activity was assayed either immediately after elution or the fractions were frozen in liquid N₂ and stored at -70°C until assayed.

2.2.6.5. Determination of Protein Kinase C Activity

Protein kinase C activity was determined by a modification of the method described by Kikkawa *et al.* (1982). The reaction mixture (55 μ l) contained (final concentration) 20 mM HEPES/KOH buffer (pH 7.5 at 20°C), 1 mg/ml histone Type III-S, 0.1 mM [γ -³²P]ATP (1 x 10⁶ dpm/tube; 0.08 Ci/mmol), 5 mM DTT, 10 mM MgCl₂, 500 nM protein kinase inhibitor peptide, 20 μ g/ml leupeptin, 1 mM EGTA, 0.875 mM CaCl₂ (1 μ M free Ca²⁺), 80 μ g/ml phosphatidylserine (PS), 8 μ g/ml 1-stearoyl-2-arachidonylglycerol (SAG) and 10 μ l of FPLC or other cytosol and membrane fraction. The phospholipid-independent protein kinase activity was measured under the same conditions without the addition of calcium or phospholipid and in the presence of 3 mM EGTA. All assays were performed in polypropylene tubes. Following a 200 s preincubation, phosphorylation was

initiated by adding [γ - ^{32}P]ATP and quenched after an additional 140 s by transferring a 45 μl aliquot of the mixture onto 2 cm^2 pieces of Whatman P-81 (cellulose-phosphate) ion exchange chromatography paper. The P-81 paper squares were then individually washed 3 times for 10 min with 10 ml of 70 mM phosphoric acid and then each was washed overnight with 10 ml of 70 mM phosphoric acid. The ^{32}P content of each square was determined by liquid scintillation counting. Lipids were stored at -20°C in either chloroform/methanol (95:5) (phospholipids) or hexane (neutral lipids, fatty acids) containing 0.05% (w/v) butylated hydroxytoluene to prevent oxidation of double bonds. Lipid vesicles were prepared immediately prior to use by evaporation to dryness under a gentle stream of nitrogen gas followed by sonication under nitrogen, until clear, in 20 mM HEPES/KOH (pH 7.5 at 20°C) using a bath-type sonicator (Branson 1200). The sonicating bath was maintained at a constant temperature by running cold water through a cooling coil.

PK C activity was calculated as phosphate incorporated into histone as follows:

$$\text{pmol Pi/min/ml of fraction} = \frac{(\text{cpm}/10 \mu\text{l sample} - \text{blank cpm}) (100)}{(140 \text{ s}) (\text{specific activity of ATP; cpm/pmol})}$$

The area under the curve (AUC) for each major PK C activity peak was calculated, from the FPLC profiles, using trapezoidal approximation. Prior to the calculation, the specific enzyme activity measured in the absence of Ca^{2+} and lipids was subtracted from the specific activity measured in the presence of Ca^{2+} and lipids.

$$\text{AUC} = \sum_{i=1}^n (\text{vol. fraction}_i) \frac{(c_i + c_{i+1}) (f_{i+1} - f_i)}{2}$$

where: vol. fraction_i = volume of fraction i

c_i = activity in fraction i

f_i = fraction # of fraction i

For these experiments, where the fraction volume was constant (0.25 ml) the above formula simplifies to:

$$\text{AUC (pmol/min)} = 0.25 \text{ ml } (C_1/2 + C_2 + C_3 + \dots + C_f/2)$$

where: C₁ was pmol/min/ml of the first fraction of the peak

C₂, C₃, etc. were pmol/min/ml of the fractions in the body of the peak

C_f was pmol/min/ml of the last fraction of the peak

Analysis of variance (Two-way ANOVA) was performed on the means of the AUC using the statistical computer program SYSTAT. The level of significance was $\alpha = 0.05$.

2.2.6.6. Protein Concentration of PK C Activity Peaks

The proteins in the peaks of PK C activity from the FPLC profile were concentrated by combining the FPLC fractions of the peaks and centrifuging each in Centricon-10 microconcentrators (Amicon). Subsequent to centrifugation at 8,000 rpm for 60 min, the samples were washed with 1 ml of 20 mM Tris-HCl (pH 7.5 at 20°C), 0.5 mM Na₂EDTA and 0.5 mM EGTA and centrifuged at 8,000 rpm for 90 min. After concentration, SDS-sample buffer (4-fold concentrate) was added to solubilize the proteins. Samples were then boiled for 90 s and centrifuged for 2 min in an Eppendorf microcentrifuge at maximum speed. Samples were separated by SDS-PAGE using 5 -20% gradient gels as described in section 2.2.5.2.1.1.

Subsequent to electrophoresis, gels were western blotted as described in section 2.2.5.2.4.1.

2.2.7. Phosphorylation of Proteins

2.2.7.1. Phosphorylation of Proteins in Isolated Myocytes

Isolated adult rat ventricular myocytes ($3 - 6 \times 10^6$ rod shaped myocytes) were incubated in a Krebs-Henseleit buffer (pH 7.4 at 20°C) containing (mM): NaCl, 134; KCl, 4.7; CaCl_2 , 1.0; glucose, 10; ascorbic acid, 0.56; and HEPES, 15; with and without [^{32}P]orthophosphate (0.5 mCi/ml) for 60 min at 37°C. After incubation, myocytes (1×10^6 rod shaped myocytes) were stimulated (from 1 to 30 min at 37°C) with one or more of the following: 0.01 or 1 μM isoproterenol, 0.1 or 1 mM HA1004, 1 mM H-8, 0.1 mM forskolin, 120 μM CGS 9343 B, 5% DMSO, 4 μM α -PDD or PMA, 10% ethanol, 0.2, 0.25 or 0.4 μM OAG. The reaction was terminated by rapid centrifugation at 300 rpm for 60 s and the supernatant discarded. Myocytes were homogenized to prepare homogenates and purified SR fractions in buffers to prevent the dephosphorylation of proteins as described in section 2.2.3.2. and 2.2.4.2. The proteins in the homogenate samples and final SR pellets were precipitated by the addition of cold 10% TCA. Samples were kept on ice for 15 min and then centrifuged at maximum speed in an Eppendorf microcentrifuge for 30 min. The TCA was removed and the pellets resuspended in SDS-sample buffer, vigorously mixed and neutralized with 2 M Tris-base. Aliquots of each sample were removed for the determination of protein. Subsequently, one-half of each sample was boiled and gel electrophoresis carried out using 5 - 20% polyacrylamide gradient gels as described in section 2.2.5.2.1.1. Gels were stained as described in section 2.2.5.2.2.1. and dried as described in section 2.2.5.2.3. Autoradiography was performed as described in section 2.2.5.2.6. For the non-radioactive phosphorylation

of proteins in isolated myocytes, SDS-PAGE was carried out as described in section 2.2.5.2.1.2. and 2.2.5.2.1.3., followed by western blotting as described in section 2.2.5.2.4.1.

To quantitate the radioactivity incorporated into specific protein bands, phosphorylated bands were localized on the dried gel by comparison with the autoradiograph. The protein bands were then excised from the gel with scissors and the gel pieces soaked for 12 hrs in liquid scintillation fluid in the dark, prior to quantitation in the scintillation spectrometer.

2.2.7.2. Phosphorylation and Dephosphorylation of Proteins in Isolated SR Vesicles

When isolated canine cardiac ventricular (a kind gift from Dr. B. Allen; Allen (1992)) or rat myocyte SR membrane vesicles were phosphorylated, the reaction medium (100 μ l) consisted of 20 mM Histidine/HCl buffer (pH 7.0 at 30°C), SR vesicles (75 μ g), 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4 , 5 mM NaN_3 , 5 mM NaF and 15 μ g catalytic subunit of PK A. Phosphorylation was initiated by the addition of 5 μ l of 0.15 mM ATP (Tris salt). After 5 min at 30°C, the reaction was terminated by the addition of 100 μ l of cold 10% TCA to the reaction medium. The sample was mixed and kept on ice for 15 min prior to centrifugation in an Eppendorf microcentrifuge for 30 min at maximum speed. The TCA was removed and 90 μ l of SDS-sample buffer was added. The pellet was resuspended by vigorous mixing and then neutralized with 2 M Tris base.

When isolated canine cardiac ventricular or rat myocyte SR membrane vesicles were dephosphorylated after isolation, the reaction medium (100 μ l) consisted of 40 mM Histidine/HCl buffer (pH 6.8 at 30°C), SR vesicles (75 μ g), 10 mM EGTA, 5 mM NaN_3 and 120 mM KCl. Dephosphorylation was initiated by the addition of 1 μ l (25 units) of alkaline phosphatase. After 15 - 30 min at 30°C, the reaction was terminated and the sample processed as described previously.

3. RESULTS

3.1. *Characterization of Isolated Adult Rat Ventricular Myocytes*

3.1.1. General Viability Studies

Two methods for the isolation of myocytes were used throughout these studies (section 2.2.1.1. and 2.2.1.2.). Both methods yielded $3 - 5 \times 10^6$ rod-shaped, Ca^{2+} tolerant (1.0 mM), quiescent (non-beating) myocytes/rat heart. The major criteria utilized in the demonstration of myocyte viability were an elongated striated morphology, the exclusion from the cell of trypan blue and the ability of the cells to contract upon electrical stimulation. The elongated and striated morphology of these viable myocytes was maintained for at least 4 hours (Figure 1). Utilizing trypan blue exclusion staining (Figure 2), it was demonstrated that the rod-shaped cells in the preparation did not take up the dye and thus, had intact sarcolemmal membranes. However, the round, blebbed cells (non-viable myocytes) present, were stained intracellularly with the dye, indicating breaks in the sarcolemmal membrane.

The current literature [Jacobson and Piper (1986)] indicates that adult ventricular myocytes which are physically and biochemically intact and functional do not contract spontaneously in culture. Previous studies [Dani *et al.* (1979)] have shown that adult myocytes which contract spontaneously after isolation are electrochemically shunted and undergo the Ca^{2+} paradox. Intact myocytes retain the morphological and functional ability to contract in response to electrical stimulation [Pelzer *et al.* (1984)]. When myocytes were isolated and then stimulated as described by Haworth *et al.* (1980), all of the rod-shaped cells contracted synchronously. The non-viable myocytes (round cells) did not respond to electrical stimulation.



Figure 1. Photograph of a crude preparation of adult rat ventricular myocytes.
(magnification: x 400)



Figure 2. Trypan blue staining of a crude preparation of adult rat ventricular myocytes. (magnification: x 100)

3.1.2. Maintenance of Hormone Responsiveness

The retention of cell surface receptors on the isolated myocytes was assessed by probing the integrity and coupling of the β -adrenergic system. This criterion was utilized since the ability of the myocytes to produce cAMP intracellularly requires intact β -receptors on the cell surface and the coupled sarcolemmal intramembrane components of the adenylate cyclase system (G proteins and adenylate cyclase). Isolated myocytes in suspension were incubated with isoproterenol (a nonspecific β -receptor agonist) and forskolin (direct activator of adenylate cyclase) to stimulate the production of cAMP. As can be seen from Table 1, isoproterenol and forskolin elevate the intracellular level of cAMP in the myocytes 3.7- and 11.7-fold, respectively, over the resting basal level.

3.2. *Homogenization of Isolated Adult Rat Ventricular Myocytes*

Two different myocyte homogenization methods were developed in order to accommodate the different types of experiments performed. Complete homogenization of all of the myocytes isolated was carried out using a 'Zero'-clearance Potter-Elvehjem homogenizer. This method was suitable for experiments where thorough homogenization was necessary. It was found that SR membranes isolated from myocytes homogenized with the 'Zero'-clearance Potter-Elvehjem or with other harsh methods of homogenization (sonication, French press, Polytron homogenizer and wiggle bug) resulted in preparations that had very low Ca^{2+} -uptake and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities (Table 2). Also, homogenization with the French Press, wiggle bug and sonication effectively broke the cells open but the resulting SR cell membranes isolated were highly contaminated with mitochondrial and sarcolemmal membranes. Polytron homogenization did not break very many of the cells as well as resulting in isolated cell membranes that had very little

TABLE 1. cAMP accumulation in isolated adult rat ventricular myocytes.

Isolated myocytes were incubated at 37°C with 0.5 mM IBMX and were stimulated with 1 μ M isoproterenol or 100 μ M forskolin for 2 min. Results are the mean \pm S.E.M. (n) denotes the # of experiments.

cAMP (pmol/10 ⁴ rod-shaped cells)	
Basal	16.2 \pm 1.4 (6)
Isoproterenol	60.4 \pm 4.2 (5)
Forskolin	190.1 \pm 52.5 (3)

TABLE 2. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities of several preparations obtained by different myocyte homogenization and SR centrifugation protocols.

$\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity was determined as described in section 2.2.5.1.2. Only the calcium-stimulated values are shown. Ca^{2+} -uptake activity was determined as described in 2.2.5.1.1. and only the oxalate-stimulated values are shown. Specific enzyme activities are expressed as nmol/min/mg protein. Each result represents the data from a single experiment. N.D. - not determined.

Fraction	Method	Specific Activity	
		Ca^{2+} -ATPase	Ca^{2+} -Uptake
Homogenate	French Press	N.D.	N.D.
SR	Modified C & F ^a	369.61	N.D.
Homogenate	Sonication	16.19	N.D.
SR	Modified C & F	34.13	N.D.
Homogenate	Wiggle Bug	30.32	N.D.
SR	Modified C & F	36.82	N.D.
Homogenate	Polytron	N.D.	N.D.
SR	Modified C & F	14.36	N.D.
Homogenate	Zero-clearance	18.79	N.D.
SR	Modified C & F	40.11	N.D.
Homogenate	Zero-clearance	19.59	N.D.
SR	Jones ^b	86.94	N.D.
Homogenate	Zero-clearance	36.24	5.50
SR	Original C & F ^c	50.77	3.30
Homogenate	Dounce	46.32	3.01
SR	Original C & F	85.01	18.57
Homogenate	Dounce	76.82	8.59
SR	Original C & F	95.48	13.64

a - modified from Chamberlain and Fleischer (1988)

b - from Jones *et al.* (1979)

c - from Chamberlain and Fleischer (1988)

enzymatic activity.

Thus, when SR membranes were to be isolated from the myocytes a different homogenization procedure was required. In previous attempts to homogenize these cells with a close fitting (20 μ) glass douncer and a loose-fitting teflon-glass Potter-Elvehjem homogenizer it was noted that only approximately 1/3 of the myocytes were broken. However, when SR membranes were isolated from such a myocyte homogenate they contained high Ca^{2+} -uptake and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities. This procedure was therefore used whenever SR membranes were isolated from the myocytes (see section 2.2.3.2. and 2.2.4.2.).

3.3. Characteristics of SR Membranes Isolated From Adult Rat Ventricular Myocytes

3.3.1. Preparation of SR Membranes from Isolated Myocytes

A method was developed for the isolation of SR membrane vesicles from adult rat ventricular myocytes prepared from a single rat heart (see section 2.2.4.2.). Utilizing this method, SR membranes were prepared from myocytes within 3 h. When using the control buffers, this SR isolation method yielded 542.7 ± 50.1 μg of SR protein (mean \pm S.D.) from 3×10^6 rod-shaped myocytes ($n = 5$). The yield of SR protein was less when buffers were used which prevented the dephosphorylation of proteins to prepare SR membranes (194.5 ± 45.8 μg SR protein; mean \pm S.D., from 3×10^6 rod-shaped myocytes; $n = 5$).

3.3.2. Marker Enzyme Activities

3.3.2.1. SR Membranes Prepared in Control Buffers

The SR membranes isolated were characterized for the presence of SR marker enzymes and other enzymatic activities indicative of contamination with membranes from other cellular organelles. Oxalate-stimulated Ca^{2+} -uptake activity was used as a specific marker for the SR since it had previously been shown that mitochondrial and sarcolemmal membranes do not support oxalate-facilitated Ca^{2+} -transport [Jones *et al.* (1979); Solaro and Briggs (1974)]. Table 3 shows Ca^{2+} -uptake activities in the presence and absence of oxalate in homogenates and SR membrane vesicles from isolated adult rat ventricular myocytes. Ca^{2+} -uptake activity was found to be 98% ATP-dependent in the myocyte SR membranes. The final SR fraction transported Ca^{2+} in the presence of oxalate at a rate of 107.3 nmol/min/mg protein. When compared to the specific activity of the homogenate, this result suggests that the SR was enriched in this activity approximately 18-fold (Table 3). In the absence of oxalate, the specific activity of Ca^{2+} -uptake in the SR and homogenate preparations were 4.0 and 1.7 nmol/min/mg protein, respectively. This indicates only a 2.4-fold enrichment of oxalate-independent Ca^{2+} -uptake activity and suggests that the preparation of SR membranes did not result in the co-purification of oxalate-independent Ca^{2+} -transporting membranes. Upon comparing the total Ca^{2+} -uptake activities in the SR and homogenates prepared from myocytes, approximately 60% of the oxalate-stimulated Ca^{2+} -uptake activity of the homogenate was recovered in the SR membrane preparation (Table 3). As can be seen from Table 3, when the Ca^{2+} -release channels in the junctional SR were blocked by ruthenium red (5 μM ; Nagasaki and Fleischer (1988)) the Ca^{2+} -uptake activity in the presence of oxalate increased by 93% and the oxalate-stimulated Ca^{2+} -uptake increased by 97%. Ruthenium red had no effect on the specific activity of oxalate-independent Ca^{2+} -uptake in myocyte SR membranes.

TABLE 3. Ca^{2+} -uptake activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes.

Ca^{2+} -transport activity was determined as described in section 2.2.5.1.1. Results are reported as mean \pm S.E.M. Specific activities are expressed as nmol/min/mg protein. Total activities were calculated by multiplying the specific activity measured by the total amount of protein in the specific fraction and are expressed as nmol/min. The number in brackets () indicates the number of fractions assayed. Pellet 1 and 2 - from 330 x g and 5000 x g centrifugations, respectively; RR - Ruthenium red.

	Ca^{2+} -Uptake (+oxalate)		Ca^{2+} -Uptake (-oxalate)		Ca^{2+} -Uptake Oxalate-Stimulated	
	Specific Activity	Total Activity	Specific Activity	Total Activity	Specific Activity	Total Activity
Homogenate	5.9 \pm 2.4 (6)	135.0 \pm 28.8 (5)	1.7 \pm 0.4 (6)	55.1 \pm 3.9 (5)	5.1 \pm 2.8 (5)	83.0 \pm 38.0 (4)
Pellet 1	2.4 \pm 0.4 (6)	59.9 \pm 12.5 (6)	1.1 \pm 0.2 (6)	25.1 \pm 5.4 (5)	1.5 \pm 0.3 (5)	39.8 \pm 10.1 (5)
Pellet 2	9.9 \pm 4.6 (6)	38.3 \pm 15.5 (6)	1.3 \pm 0.1 (6)	5.6 \pm 0.7 (6)	8.6 \pm 4.6 (6)	32.7 \pm 15.6 (6)
SR	107.3 \pm 3.8 (6)	52.1 \pm 5.6 (6)	4.0 \pm 0.3 (6)	2.0 \pm 0.3 (6)	103.3 \pm 3.8 (6)	50.1 \pm 5.4 (6)
SR + RR	207.5 \pm 9.7 (4)	110.0 \pm 8.8 (4)	3.8 \pm 0.3 (4)	2.0 \pm 0.2 (4)	203.7 \pm 11.8 (4)	108.0 \pm 8.7 (4)

The specific and total activities of the SR $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase were also measured in the SR membranes isolated from adult ventricular myocytes. As shown in Table 4, during the isolation procedure the specific activity of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase in the SR fraction increased 5.6-fold over the activity found in the homogenate. However, only 9% of the total $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity in the homogenate was recovered in the final SR fraction and 91% of the total activity was lost during the isolation. After the first centrifugation, 76% of the activity was in the first pellet and after the second centrifugation, 9% was in the second pellet.

In each of the tables (Table 3 and 4), the yield of protein (mg) for each fraction has been normalized so that each was obtained from a starting myocyte population of 3×10^6 cells. These normalized protein values were used in the calculation of the total activities in Tables 3 and 4.

Comparing the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase and Ca^{2+} -uptake activities of the SR membrane vesicles obtained from isolated adult rat ventricular myocytes to the activities from SR preparations isolated from whole rat heart(s) (Table 5) shows that the activities of the myocyte SR preparation obtained to be similar to or higher than the activities of SR preparations from whole rat heart.

Since the most likely membranes to contaminate the SR membrane preparation were sarcolemmal and mitochondrial in origin, the following markers were chosen as a measure of contamination: ouabain-sensitive $\text{Na}^{+}/\text{K}^{+}$ -ATPase (sarcolemma) and cytochrome c oxidase (inner mitochondrial membrane). The latent activity of the $\text{Na}^{+}/\text{K}^{+}$ -ATPase was exposed by pre-treating the membrane samples with alamethicin. In Table 6, the specific ouabain-sensitive $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity in the myocyte SR preparation was increased 6.5-fold over that found in the homogenate. However, the total ouabain-sensitive $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity was decreased by 93% during the purification of the SR membranes from the homogenate and only 7% of the total activity was recovered in the SR membranes. The specific activity of cytochrome c oxidase in the myocyte SR

TABLE 4. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes.

$\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity was determined as described in section 2.2.5.1.2. Only the calcium-stimulated values are shown. Total activities (nmol/min) were calculated by multiplying the specific activity (nmol/min/mg protein) measured by the total amount of protein (mg) in the fraction. Results are reported as mean \pm S.E.M. The number in brackets () indicates the number of fractions assayed. Pellet 1 and Pellet 2 are the same as defined in Table 3.

	Specific Activity	Total Activity
Homogenate	74.1 ± 19.6 (6)	$2,248. \pm 345.$ (5)
Pellet 1	68.1 ± 14.9 (6)	$1,699. \pm 378.$ (6)
Pellet 2	50.0 ± 13.4 (6)	210.0 ± 60.5 (6)
SR	414.0 ± 61.8 (6)	201.7 ± 37.3 (6)

TABLE 5. Ca^{2+} -ATPase and Ca^{2+} -uptake activities of SR membranes isolated from whole adult rat hearts compared to those from isolated adult ventricular myocytes.

Ca^{2+} -ATPase activities are the Ca^{2+} -stimulated data only. Ca^{2+} -uptake activities are the oxalate-stimulated uptake data only. N.A. - not available.

<u>nmol/min/mg protein</u>		
Ca^{2+} -ATPase	Ca^{2+} -Uptake	Reference
414 ± 62^a	107 ± 4^a	Wientzek and Katz (1991) ^d
38 ± 1.4^a	74 ± 2.6^a	Limas (1978) ^e
$80^{a,c}$	166 ± 13^a	Penpargkul <i>et al.</i> (1980) ^e
$153 \pm 1^{a,c}$	$31 \pm 3.5^{a,c}$	Wei <i>et al.</i> (1976) ^e
$213^{a,c}$	8.1 ± 0.6^a	Lamers and Stinis (1980) ^e
$274 \pm 16^{a,c}$	$23.8 \pm 1.6^{a,c}$	Ganguly <i>et al.</i> (1983) ^e
$360 \pm 50^{a,c}$	68 ± 5^a	Narayanan (1983) ^e
437 ± 48.8^b	13^c	Lopaschuk <i>et al.</i> (1983) ^e
510 ± 70^b	263^c	Barker <i>et al.</i> (1988) ^e
N.A.	130^c	DeFoor <i>et al.</i> (1980) ^e
N.A.	$131.6 \pm 13^{a,c}$	Naylor <i>et al.</i> (1975) ^e

^a Mean \pm S.E.M.

^b Mean \pm S.D.

^c Value was recalculated from reference

^d Data from isolated myocytes

^e Data from whole rat hearts

TABLE 6. Marker enzyme activities of homogenate and SR membranes isolated from adult rat ventricular myocytes.

Na⁺/K⁺-ATPase activity (latent and patent) was determined by pre-incubating the membrane fractions with alamethicin for 10 min at 37°C. Only the ouabain-sensitive values are shown. Total marker activities (μmol/min) were calculated by multiplying the specific activity (μmol/min/mg protein) measured by the total amount of protein (mg) in the rat myocyte homogenate or SR membrane vesicle preparation. Results are reported as mean ± S.E.M. The number in brackets () indicates the number of myocyte homogenates or SR preparations assayed.

	Na ⁺ /K ⁺ -ATPase		Cytochrome c Oxidase	
	Specific Activity	Total Activity	Specific Activity	Total Activity
Homogenate	0.04 ± 0.01 (6)	1.70 ± 0.18 (5)	0.21 ± 0.05 (5)	12.28 ± 3.18 (6)
SR	0.26 ± 0.05 (6)	0.12 ± 0.02 (6)	2.42 ± 0.31 (5)	0.95 ± 0.12 (4)

membranes increased 11.5-fold from the homogenate values. Again, as with the Na^+/K^+ -ATPase activity, the total cytochrome c oxidase activity was decreased by 92% during the isolation so that only 8% of the total mitochondrial activity of the homogenate was recovered in the SR fraction.

3.3.2.2. SR Membranes Prepared in Buffers That Prevent Dephosphorylation

Table 7 shows Ca^{2+} -uptake activities from isolated adult rat ventricular myocytes, in the presence and absence of oxalate, in homogenates and SR membrane vesicles prepared using methods to prevent dephosphorylation. The final SR fraction transported Ca^{2+} in the presence of oxalate at a rate of 116.1 nmol/min/mg protein. When compared to the specific activity of the homogenate, this result suggests that the SR was enriched in this activity approximately 33-fold over the homogenate. In the absence of oxalate, the specific activity of Ca^{2+} -uptake in the SR and homogenate preparations were 6.3 and 1.2 nmol/min/mg protein, respectively. This indicates only a 5.3-fold enrichment of oxalate-independent Ca^{2+} -uptake activity in the final SR membranes and suggests that the preparation of SR membranes did not result in the co-purification of oxalate-independent Ca^{2+} -transporting membranes. Upon comparison of the total Ca^{2+} -uptake activities of the SR to that in homogenates prepared from myocytes, 22% of the oxalate-stimulated Ca^{2+} -uptake activity of the homogenate was recovered in the SR membrane preparation. As can be seen from Table 7, when the Ca^{2+} -release channels were blocked by ruthenium red (5 μM) the Ca^{2+} -uptake activity of the myocyte SR preparation, in the presence of oxalate, increased by 34% and when corrected for oxalate-independent Ca^{2+} -uptake, increased by 35%. Ruthenium red increased the oxalate-independent Ca^{2+} -uptake in myocyte SR membranes by 11%.

Table 8 shows that the SR $\text{Ca}^{2+}/\text{K}^+$ -ATPase specific activity increased 2.0-fold during the purification of SR membranes from the myocyte homogenate.

TABLE 7. Ca^{2+} -uptake activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes using buffers to prevent dephosphorylation.

Ca^{2+} -transport activity was determined as described in section 2.2.5.1.1. Results are reported as mean \pm S.E.M. Specific activities are expressed as nmol/min/mg protein. Total activities were calculated by multiplying the specific activity measured by the total amount of protein in the specific fraction and are expressed as nmol/min. The number in brackets () indicates the number of fractions assayed. Pellet 1 and 2 - from 330 x g and 5000 x g centrifugations, respectively; RR - Ruthenium red.

	Ca^{2+} -Uptake (+oxalate)		Ca^{2+} -Uptake (-oxalate)		Ca^{2+} -Uptake Oxalate-Stimulated	
	Specific Activity	Total Activity	Specific Activity	Total Activity	Specific Activity	Total Activity
Homogenate	3.5 \pm 0.6 (5)	148.2 \pm 27.0 (5)	1.2 \pm 0.1 (5)	49.8 \pm 7.4 (5)	2.3 \pm 0.4 (5)	98.4 \pm 20.0 (5)
Pellet 1	2.3 \pm 0.2 (5)	70.0 \pm 11.1 (5)	1.0 \pm 0.1 (5)	30.4 \pm 2.3 (5)	1.2 \pm 0.3 (5)	39.6 \pm 10.3 (5)
Pellet 2	11.0 \pm 0.9 (5)	36.0 \pm 4.0 (5)	1.4 \pm 0.3 (5)	4.8 \pm 1.3 (5)	9.6 \pm 0.8 (5)	31.2 \pm 2.8 (5)
SR	116.1 \pm 20.8 (5)	22.6 \pm 3.8 (5)	6.3 \pm 0.5 (5)	1.2 \pm 0.2 (5)	109.9 \pm 20.4 (5)	21.4 \pm 3.7 (5)
SR + RR	155.3 \pm 34.4 (5)	30.0 \pm 6.7 (5)	7.0 \pm 0.9 (5)	1.4 \pm 0.2 (5)	148.3 \pm 33.6 (5)	28.5 \pm 5.7 (5)

TABLE 8. $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities of fractions obtained during the isolation of SR membranes from adult rat ventricular myocytes using buffers to prevent dephosphorylation.

$\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity was determined as described in section 2.2.5.1.2. Only the calcium-stimulated values are shown. Total activities (nmol/min) were calculated by multiplying the specific activity (nmol/min/mg protein) measured by the total amount of protein (mg) in the fraction. Results are reported as mean \pm S.E.M. The number in brackets () indicates the number of fractions assayed. Pellet 1 and Pellet 2 are the same as defined in Table 7.

	Specific Activity	Total Activity
Homogenate	122.2 ± 20.2 (5)	$5,227. \pm 147.$ (3)
Pellet 1	63.9 ± 14.1 (5)	$1,993. \pm 528.$ (5)
Pellet 2	36.5 ± 10.3 (5)	131.9 ± 45.9 (5)
SR	250.8 ± 72.9 (3)	74.6 ± 26.0 (4)

However, the total $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity decreased by 98.6% during the purification so that only 1.4% of the activity in the homogenate was recovered in the final SR membrane fraction.

The sarcolemmal and mitochondrial marker enzyme activities were also measured in this SR preparation. Table 9 shows that although the specific activity of $\text{Na}^{+}/\text{K}^{+}$ -ATPase and cytochrome c oxidase increased by 3.8- and 9.7-fold respectively, the total $\text{Na}^{+}/\text{K}^{+}$ -ATPase and cytochrome c oxidase activity decreased by 97.8 and 95.3%, respectively. Therefore, only 2.2% of the total $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity and 4.7% of the total cytochrome c oxidase activity in the homogenate was recovered in the SR membrane fraction.

3.3.3. Protein Profile of SR Membranes

3.3.3.1. SR Membranes Prepared in Control Buffers

One-dimensional SDS-PAGE analysis using Coomassie blue staining demonstrated that myocyte and rat heart homogenate and SR preparations had proteins of similar molecular mass and staining intensity (Figure 3). The characteristic SR protein band at 110 kDa has been shown to be the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase [Brandl *et al.* (1986)]. Several protein bands in close proximity to each other at 53 to 55 kDa were also detected. This molecular mass region of the gel was expected to contain at least three known SR proteins; calsequestrin, calreticulin, and the 53 kDa glycoprotein. A Stainsall stained gel (Figure 4) of myocyte and rat heart SR preparations demonstrated several pink stained bands as glycoproteins and a blue stained band at 55 kDa, which may be calsequestrin.

A new method for the detection of various types of Ca^{2+} -binding proteins following electrophoresis is staining with ruthenium red. In a previous study [Charuk *et al.* (1990)], this inorganic dye was found to bind to the same proteins as

TABLE 9. Marker enzyme activities of homogenate and SR membranes isolated from adult rat ventricular myocytes using buffers to prevent dephosphorylation.

Na⁺/K⁺-ATPase activity (latent and patent) was determined by pre-incubating the membrane fractions with alamethicin for 10 min at 37°C. Only the ouabain-sensitive values are shown. Total marker enzyme activities (μmol/min) were calculated by multiplying the specific enzyme activity (μmol/min/mg protein) measured by the total amount of protein (mg) in the rat myocyte homogenate or SR membrane vesicle preparation. Results are reported as mean ± S.E.M. The number in brackets () indicates the number of myocyte homogenate or SR preparations assayed.

	Na ⁺ /K ⁺ -ATPase		Cytochrome c Oxidase	
	Specific Activity	Total Activity	Specific Activity	Total Activity
Homogenate	0.09 ± 0.01 (4)	3.61 ± 0.47 (4)	0.48 ± 0.05 (5)	20.18 ± 1.67 (5)
SR	0.34 ± 0.01 (3)	0.08 ± 0.01 (3)	4.67 ± 0.85 (3)	0.95 ± 0.05 (3)

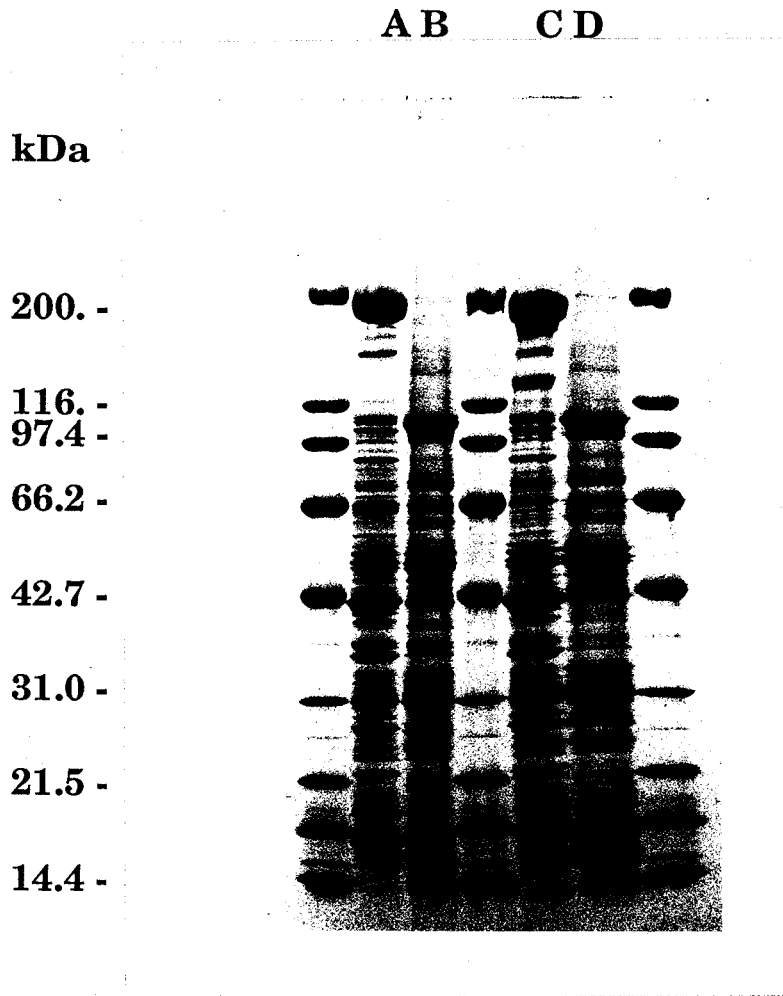


Figure 3. Coomassie blue-stained SDS-PAGE gel of homogenates and SR membranes from whole rat heart (A & B) and isolated rat ventricular myocytes (C & D). Lanes A and C contain homogenates and B and D contain SR membranes. Lanes contain 20 μ g protein each and samples were heated at 37°C for 10 min prior to electrophoresis. Molecular mass markers are in the left, right and center lane of the gel. This gel was a representative result from at least 10 separate experiments. kDa -kilodaltons.

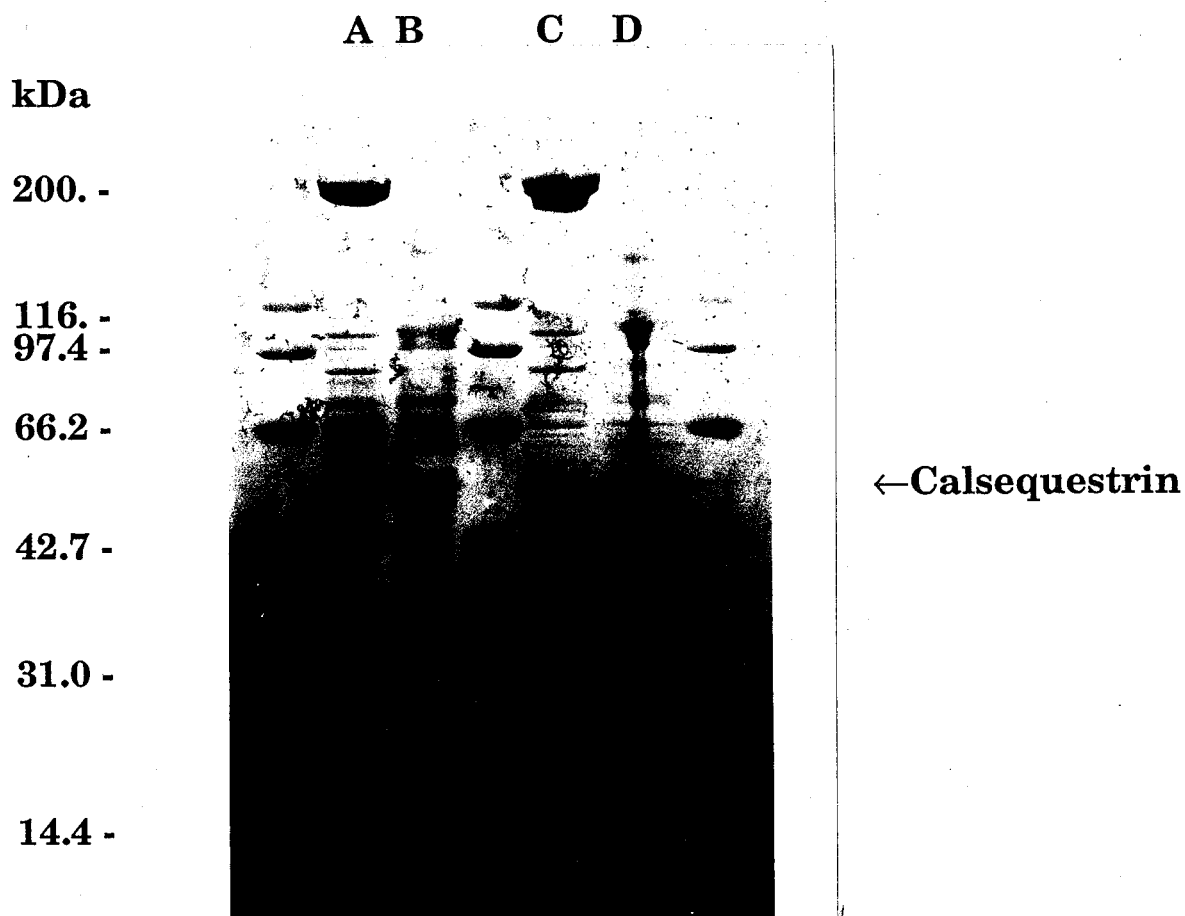


Figure 4. Stainsall stained SDS-PAGE gel of whole rat heart (A & B) and isolated rat ventricular myocytes (C & D). Lanes A and C contain homogenate and B and D contain SR membrane samples. Lanes contain 20 μ g protein each and samples were heated at 37°C for 10 min prior to electrophoresis. Molecular mass markers are in the left, right and center lane of the gel and are indicated in kilodaltons (kDa). This gel was a representative result from 3 separate experiments.

detected by the $^{45}\text{Ca}^{2+}$ -overlay technique. Figure 5 shows that in both myocyte and rat heart SR preparations, ruthenium red in the absence of CaCl_2 (lanes A & B), bound mainly to 2 wide bands of protein of molecular mass 53-55 kDa (calsequestrin and/or calreticulin and glycoprotein) and 97-100 kDa ($\text{Ca}^{2+}/\text{K}^{+}$ -ATPase). Another protein band which became stained, but not as intensely, was detected at 16 kDa in both SR preparations. The presence of 50 mM CaCl_2 in the staining buffer greatly reduced the binding of ruthenium red to all of these proteins (Figure 5, lanes C & D).

Using SDS-PAGE of isolated SR membrane vesicles, it was generally not possible to visualize phospholamban with either Coomassie blue or silver staining techniques. Therefore, visualization of phospholamban in SR membrane preparations was usually accomplished by autoradiography (after radioactive labelling) or immunoblotting techniques. Figure 6 illustrates a Western blot of myocyte and rat heart SR preparations separated by SDS-PAGE and probed with the monoclonal antibody A1, which is specific for phospholamban [Suzuki and Wang (1986)]. This blot shows that in unboiled samples there were 2 major mobility forms of phospholamban, visualized at 8 and 25 kDa. A third fainter band at approximately 27 kDa was also visualized. This band may represent a phosphorylated species of oligomeric phospholamban [Wegener and Jones (1984)]. When the sample was boiled prior to SDS-PAGE electrophoresis, the majority of phospholamban dissociated into the 8 kDa form with a small amount dissociating to a 15 kDa form.

3.3.3.2. SR Membranes Prepared in Buffers That Prevent Dephosphorylation

The protein profile of myocyte homogenate and SR membranes prepared in buffers that prevent the dephosphorylation of proteins was carried out using one-dimensional SDS-PAGE analysis with Coomassie blue staining. Figure 7

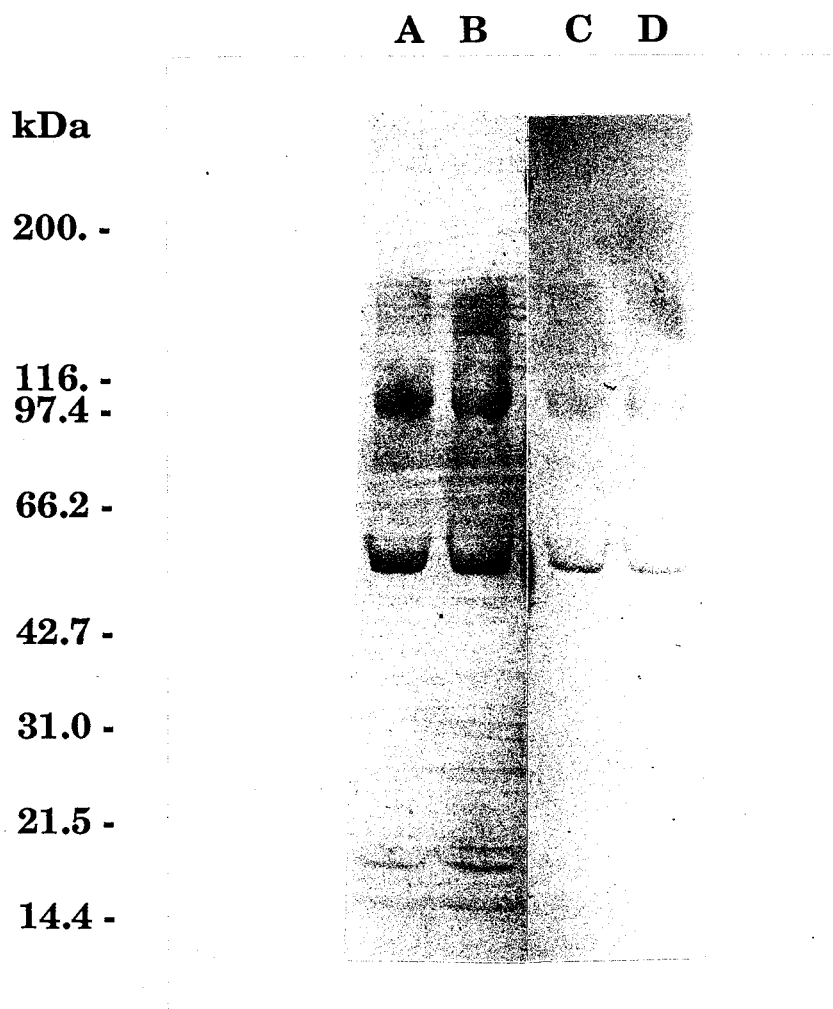


Figure 5. Ca^{2+} -binding proteins of SR membranes purified from isolated rat ventricular myocytes (A & C) and whole rat heart (B & D), separated by SDS-PAGE and transferred to nitrocellulose. The blot was stained with ruthenium red in the absence (A & B) and presence (C & D) of 50 mM CaCl_2 . Lanes contain 50 μg protein each and samples were boiled for 90 s prior to electrophoresis. Molecular mass is indicated in kilodaltons (kDa). This blot was a representative result from 3 separate experiments.

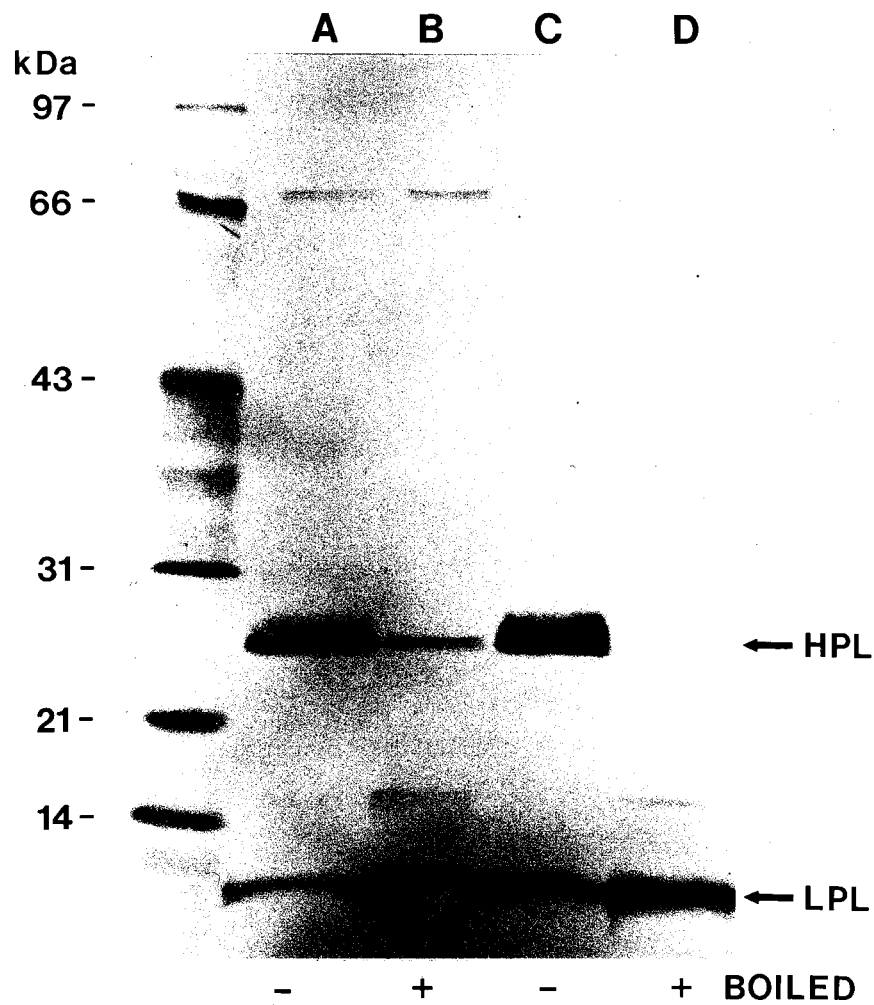


Figure 6. Identification of phospholamban in SR membranes purified from isolated rat ventricular myocytes (A & B) and whole rat heart (C & D), separated by SDS-PAGE and transferred to Zeta-Probe membranes. The blot was probed with a monoclonal antibody to phospholamban. Each lane contains 50 μ g protein. Lanes B & D were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass markers are in the left lane of the blot. kDa - kilodaltons. This blot was a representative result from at least 3 separate experiments.

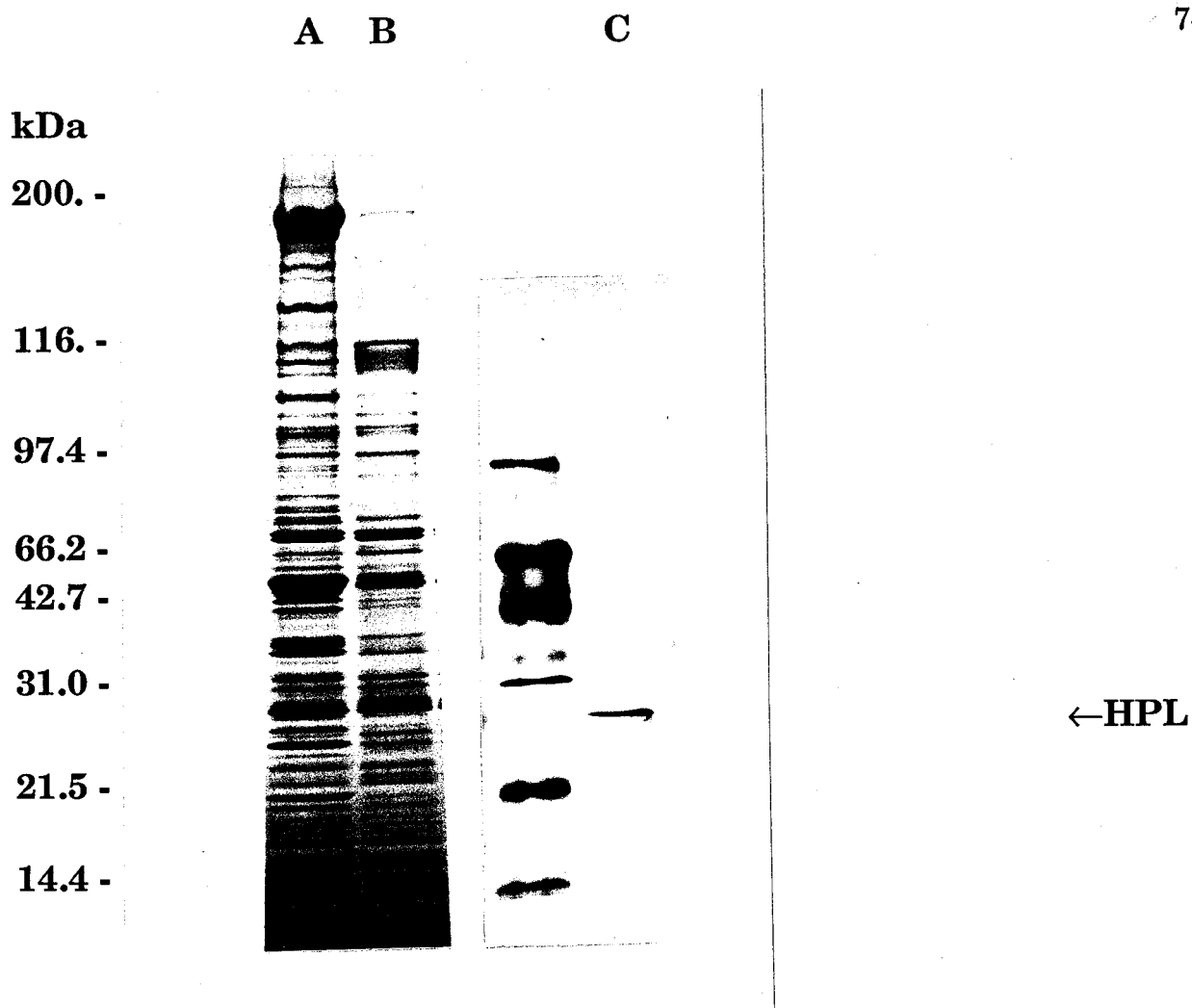


Figure 7. Coomassie blue-stained SDS-PAGE gel (A & B) and western blot (C) of myocyte homogenates and SR membranes prepared in buffers to prevent dephosphorylation. Lane A contains myocyte homogenate and B and C contain SR membranes. Lane C contains 25 μ g protein and all samples were heated at 37°C for 10 min prior to electrophoresis. The blot was probed with a monoclonal antibody to phospholamban. HPL is the high molecular mass form of phospholamban, respectively. The gel and blot are a representative result from at least 10 separate experiments for the gel and 2 separate experiments for the blot. Molecular mass markers are in the left lane of the blot. kDa - kilodaltons.

demonstrates that the protein profile of this preparation was very similar to the myocyte and rat heart homogenate and SR (isolated in control buffers) preparations shown in Figure 3, containing proteins of similar molecular mass and staining intensity. A Western blot of unboiled myocyte SR membranes prepared in buffers that prevent dephosphorylation (Figure 7) demonstrates a single band at 26 kDa, which is the high mobility form of phospholamban.

3.4. *Phosphorylation of Proteins in Isolated Intact Adult Rat Ventricular Myocytes*

3.4.1. Proteins Phosphorylated in Response to PK A Activation

Protein kinase A (cAMP-dependent protein kinase; PK A) was stimulated in intact myocytes by treatment of the cells with isoproterenol, a non-specific β -receptor agonist. Treatment of intact myocytes with isoproterenol leads to the intracellular accumulation of cAMP as shown previously in Table 1. A rise in myocyte intracellular cAMP has previously been shown to activate PK A [Tsien (1977); Tada *et al.* (1976)]. In the present phosphorylation studies, SR membrane vesicles were prepared from intact isolated adult rat ventricular myocytes which were radioactively labelled with [^{32}P]orthophosphate and then stimulated with isoproterenol. After which the SR membranes were isolated using buffers which minimized the dephosphorylation of proteins (see section 2.2.4.2.).

Incubation of [^{32}P]-labelled myocytes with 1 μM isoproterenol for 1 min specifically increased the phosphorylation of 4 protein bands (8.5, 27, 31 and 152 kDa) in myocyte homogenates (Figure 8.A.). Upon boiling this sample, the phosphorylated band at 27 kDa was lost and the phosphorylated band at 8.5 kDa became more intense, suggesting the dissociation of phospholamban from the oligomeric to the monomeric form. In the SR fraction isolated from myocytes stimulated for 1 min with 1 μM isoproterenol, the phosphorylation of 3 protein

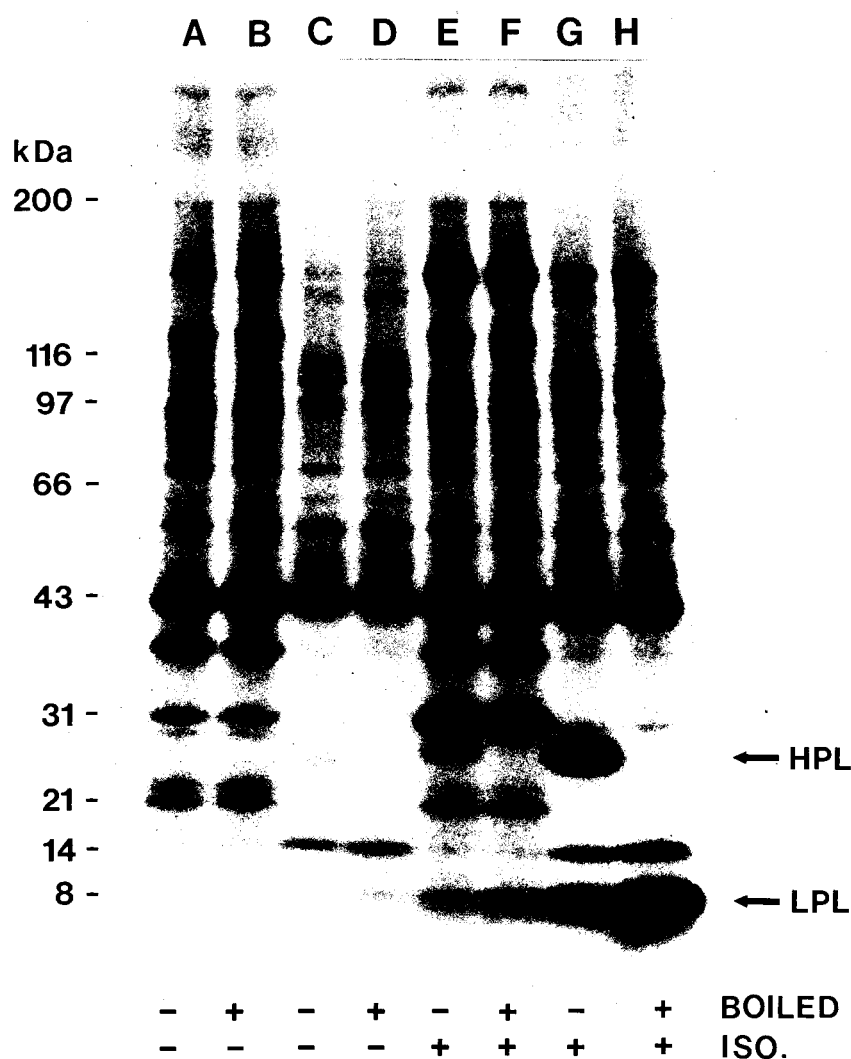


Figure 8.A. Autoradiograph of homogenates (A, B, E, & F) and SR membranes (C, D, G, & H) from control and isoproterenol-stimulated rat ventricular myocytes. Myocytes were labelled with ^{32}P -orthophosphate and then incubated as control (A, B, C & D) or stimulated with isoproterenol (E, F, G & H). Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from at least 10 separate experiments. Iso. - isoproterenol.

bands was increased (8.5, 15 and 27 kDa). Boiling of the SR preparation prior to electrophoresis also caused the loss of the phosphorylated band at 27 kDa and an increase in phosphorylation of the bands at 8.5 and 15 kDa. Boiling did not affect the phosphorylation profile of any of the other proteins in the homogenate or SR preparation in either control or isoproterenol-stimulated myocytes. Figure 8.B. shows the SDS-PAGE gel of the experiment in Figure 8.A. Each corresponding sample lane of the gel has a similar protein profile. In the SR samples from myocytes treated with isoproterenol, more protein may have been recovered during the isolation procedure as these lanes seem to contain more protein, as judged by the intensity of the Coomassie blue staining.

Figure 9 illustrates the Western blot of homogenate and SR fractions isolated from myocytes stimulated with 1 μ M isoproterenol for 1 min and probed with an antibody to phospholamban. In SDS-PAGE gels of myocyte homogenates, it was not possible to detect phospholamban by Coomassie blue staining since this protein does not stain well with this dye. However, as shown in Figure 9, the monoclonal antibody A1 was able to detect phospholamban in the homogenate fraction. Both the low (8.5 kDa) and high (27 kDa) molecular mass forms of phospholamban, in homogenate and SR membrane preparations from control and isoproterenol-stimulated myocytes, bound the antibody. Together with the boiling-induced shift in molecular weight, antibody studies confirm that the phosphorylated bands stimulated by isoproterenol were, in fact, phospholamban.

Myocytes pre-labelled with [32 P]orthophosphate were treated with 0.1 mM forskolin in DMSO (final DMSO was 5% v/v) to activate PK A *via* the direct activation of adenylate cyclase (Figure 10.A.). Forskolin increased the phosphorylation of 4 protein bands (8.5, 27, 31 and 152 kDa) in a myocyte crude particulate fraction. In this figure, the protein phosphorylation pattern obtained using forskolin can be compared to that found when the myocytes were stimulated with 1 μ M isoproterenol. The number and types of protein bands

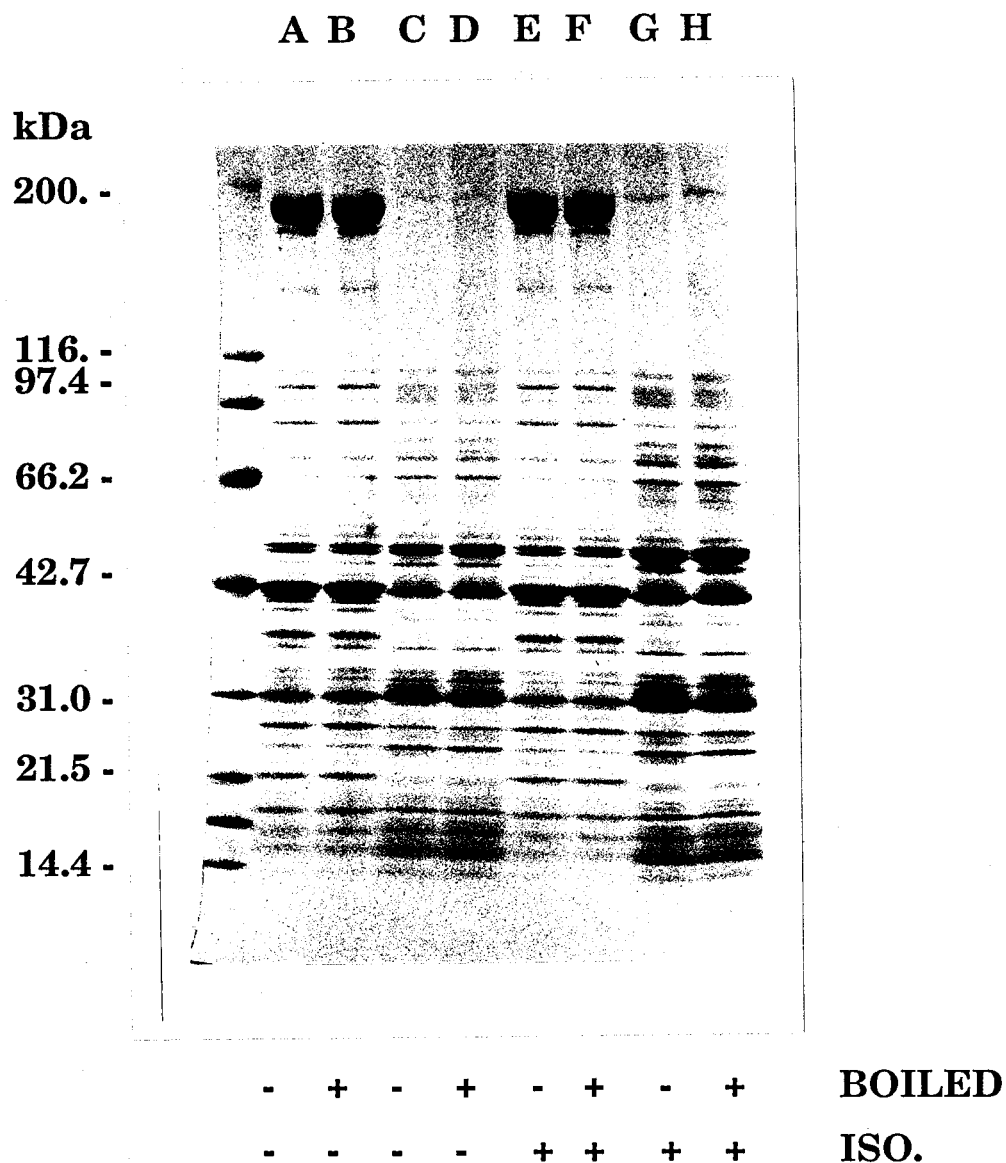


Figure 8.B. SDS-PAGE gel of homogenate (A, B, E, & F) and SR membranes (C, D, G, & H) from control and isoproterenol-stimulated rat ventricular myocytes. Myocytes were labelled with ^{32}P -orthophosphate and then incubated as control (A, B, C & D) or stimulated with isoproterenol (E, F, G & H). Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. Molecular mass is indicated in kilodaltons (kDa). This gel was a representative result from at least 10 separate experiments. Iso. - isoproterenol.

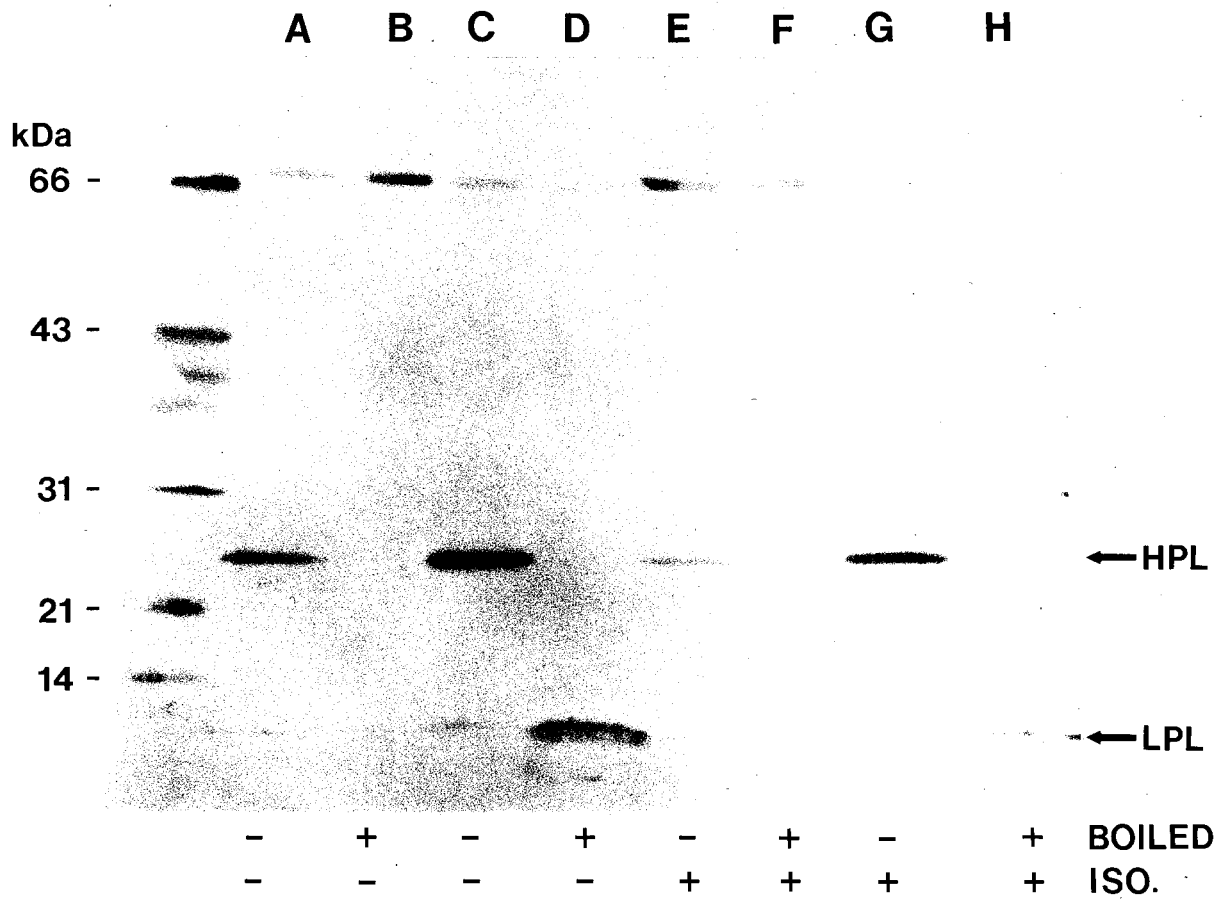


Figure 9. Western blot of homogenates (A, B, E & F) and purified SR membranes (C, D, G & H) from control (A, B, C & D) and isoproterenol-stimulated (E, F, G & H) rat ventricular myocytes. The blot was probed with the monoclonal antibody to phospholamban, A1. Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. This blot was a representative result from 3 separate experiments. Molecular mass markers are in the left lane of the blot. kDa - kilodaltons. Iso. -isoproterenol.

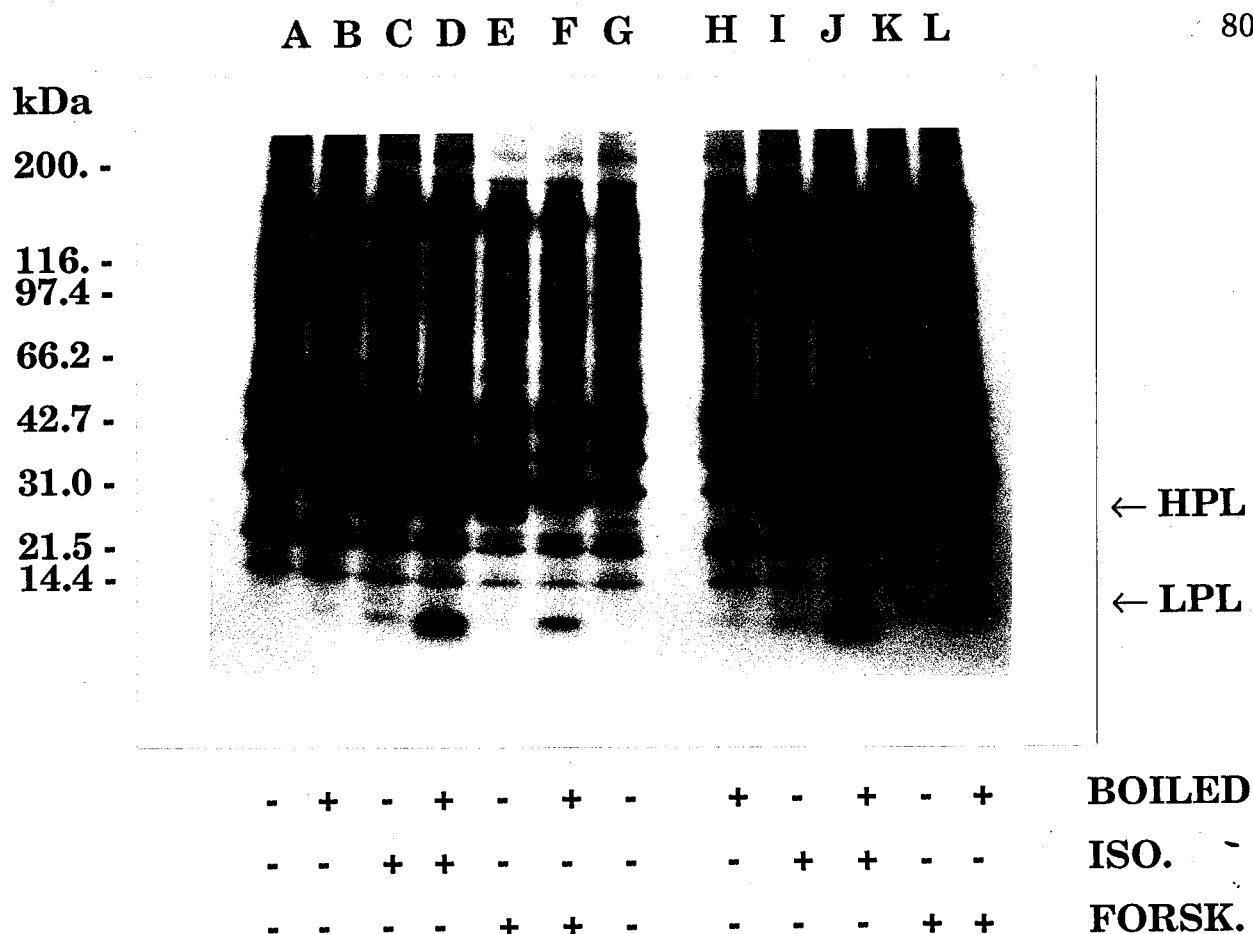


Figure 10.A. Autoradiograph of crude membrane fractions from control (A, B, G & H), isoproterenol (C, D, I & J) and forskolin-stimulated (E, F, K & L) myocytes. Lanes A to F were stimulated for 1 min and lanes G to L for 2 min. Lanes B, D, F, H, J & L were boiled for 90 sec prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 3 separate experiments. ISO - isoproterenol, FORSK - forskolin.

phosphorylated by PK A in these crude membrane fractions isolated from the treated myocytes were the same whether PK A was activated by either forskolin or isoproterenol. Figure 10.B. shows the SDS-PAGE gel of the experiment illustrated in Figure 10.A. This gel shows that the corresponding sample lanes all contained a similar protein profile and that the proteins were stained to the same intensity.

3.4.1.1. Effect of PK A Inhibitors on PK A Stimulated Protein Phosphorylation

To block the phosphorylation of proteins by PK A the intact ^{32}P -labelled myocytes were pretreated with inhibitors of PK A, prior to stimulation with isoproterenol. Two isoquinolinesulfonamide derivatives were used as specific inhibitors of PK A, HA1004 and H-8. As can be seen from Figure 11.A., pretreatment of the myocytes for 30 min with 100 μM HA1004 did not inhibit the ability of isoproterenol (1 μM) to increase the phosphorylation of 4 protein bands (8.5, 27, 31 and 152 kDa) in myocyte homogenates and of 3 protein bands (8.5, 15 and 27 kDa) in myocyte SR membranes. Figure 11.B. illustrates the Coomassie blue-stained gel of the experiment shown in Figure 11.A. This gel shows that the corresponding sample lanes all contained a similar protein profile. However, on this gel there appears to be more protein in lanes containing SR samples from HA1004 treated myocytes judging by the intensity of the Coomassie blue staining; this may be due to increased protein recovery during the SR isolation procedure. In Figure 12.A., myocytes were pretreated for 15 min with 1 mM HA1004 or H-8 and then stimulated with 0.01 μM isoproterenol. Isoproterenol increased the phosphorylation of 4 protein bands (8.5, 27, 31 and 152 kDa) in myocyte homogenates and 2 protein bands (8.5 and 27 kDa) in myocyte SR membranes. It appears that HA1004 and H-8 did not inhibit the activation of PK A. Figure 12.B. shows the SDS-PAGE gel of the experiment in Figure 12.A. This gel shows that the corresponding sample lanes all contained a similar protein profile.

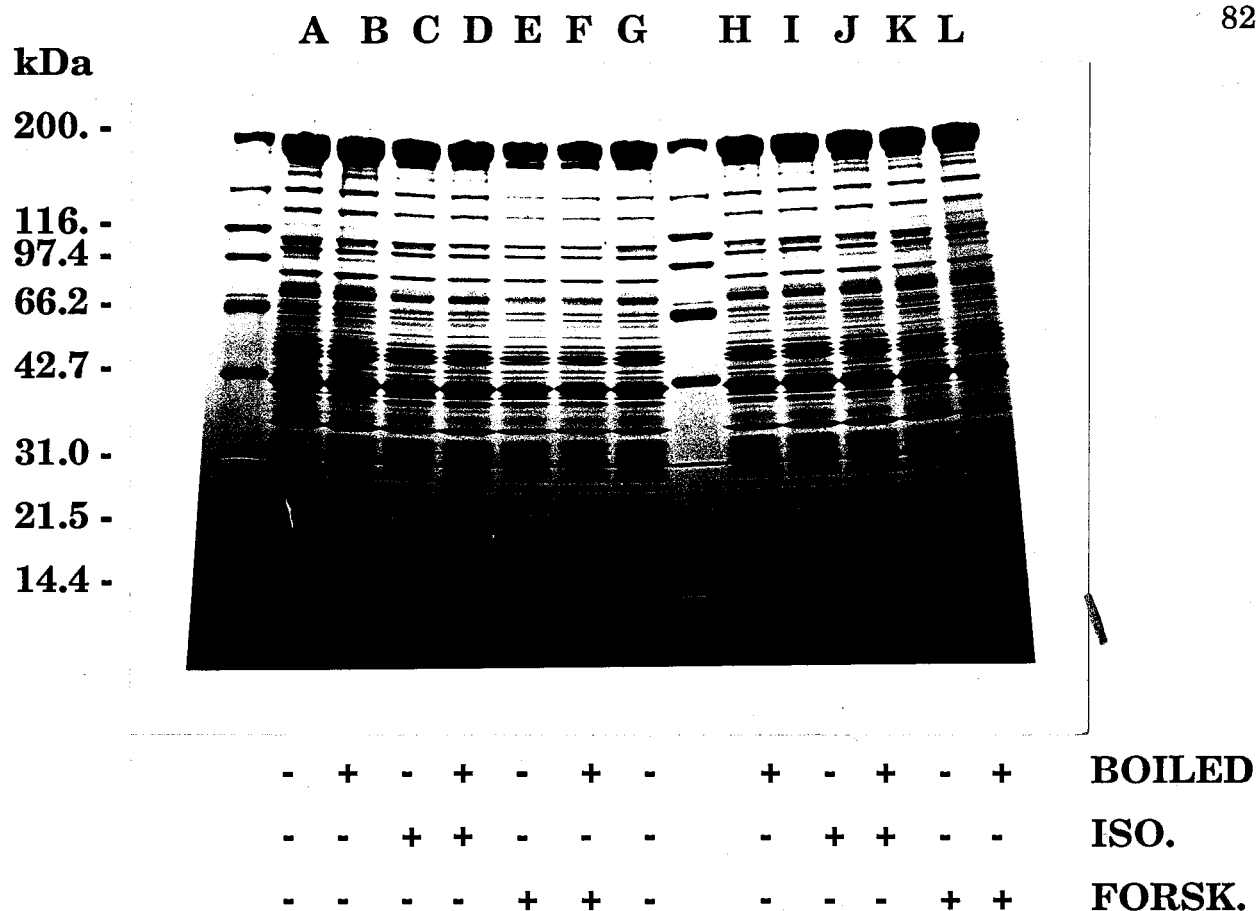


Figure 10.B. SDS-PAGE gel of crude membrane fractions from control (A, B, G & H), isoproterenol (C, D, I & J) and forskolin-stimulated (E, F, K & L) myocytes. Lanes A to F were stimulated for 1 min and lanes G to L for 2 min. Lanes B, D, F, H, J & L were boiled for 90 sec prior to electrophoresis. Molecular mass markers are in the left lane and the sixth lane from the right and are indicated in kilodaltons (kDa). This gel was a representative result from 3 separate experiments. ISO - isoproterenol, FORSK - forskolin.

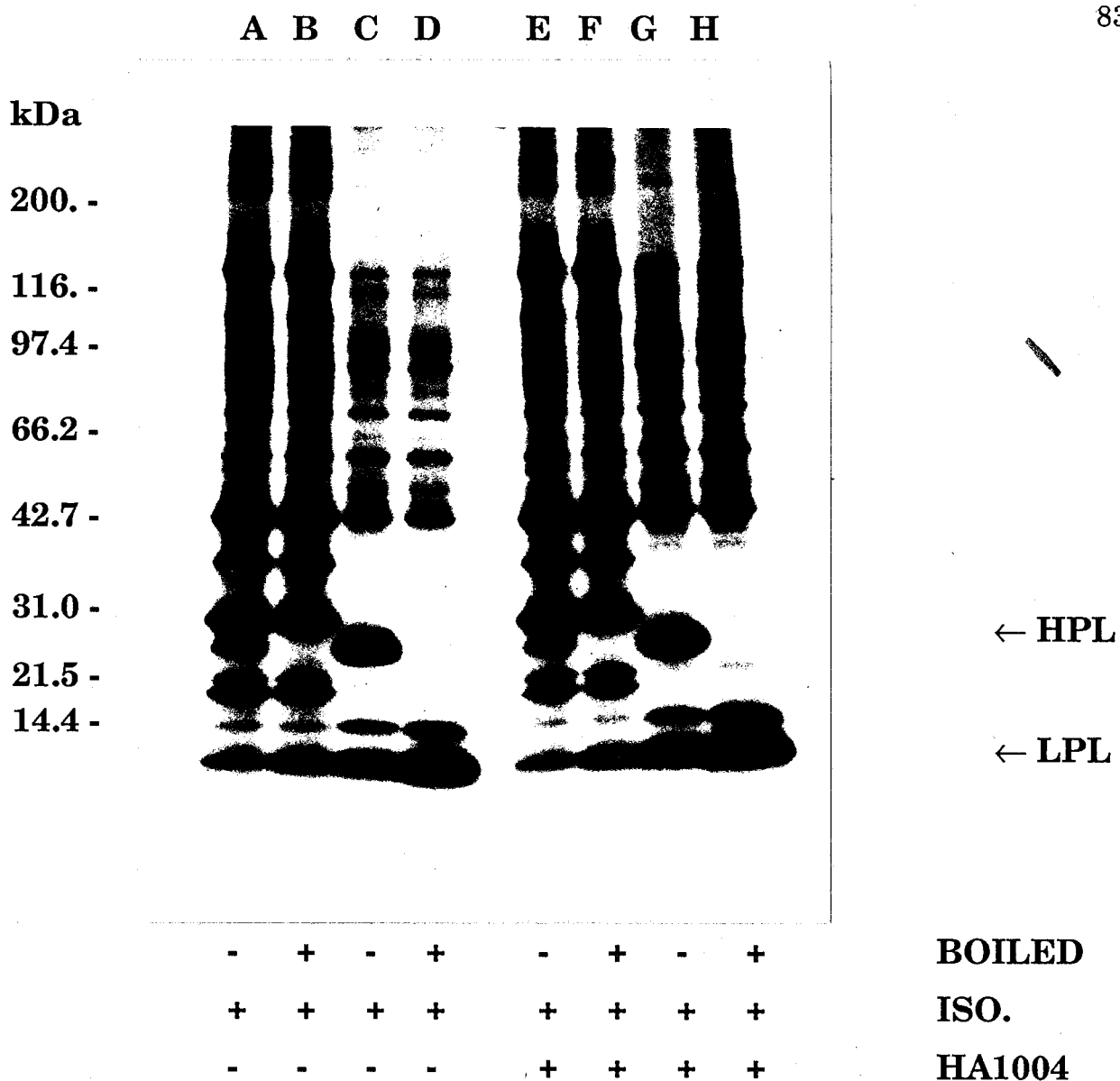


Figure 11.A. Autoradiograph of homogenates (A, B, E & F) and SR membranes (C, D, G & H) isolated from isoproterenol-stimulated myocytes in the absence (A, B, C & D) and presence (E, F, G & H) of HA1004. Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 3 separate experiments. ISO - isoproterenol.

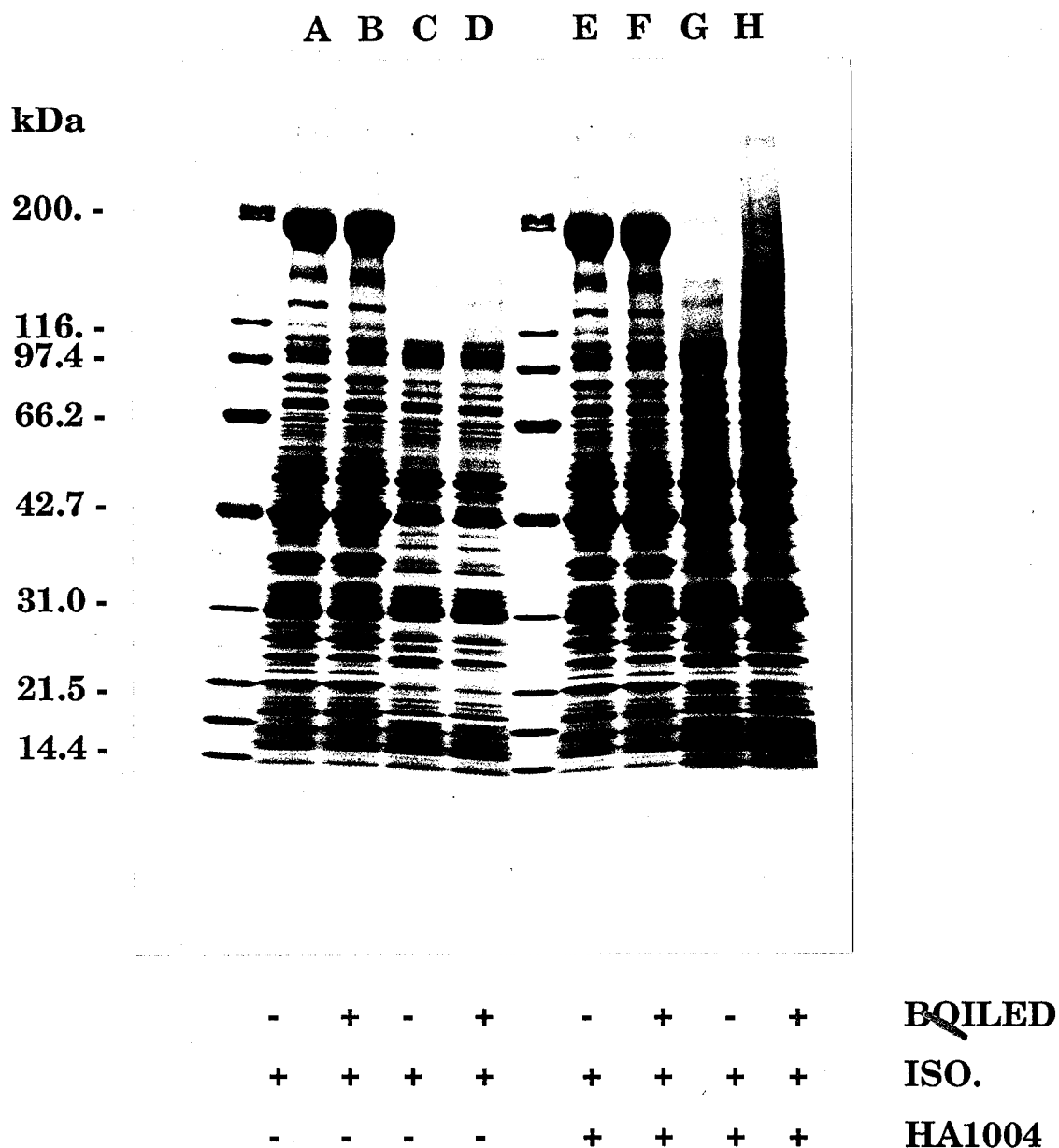


Figure 11.B. SDS-PAGE gel of homogenates (A, B, E & F) and SR membranes (C, D, G & H) isolated from isoproterenol-stimulated myocytes in the absence (A, B, C & D) and presence (E, F, G & H) of HA1004. Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. Molecular mass markers are in the left lane and the fifth lane from the right and are indicated in kilodaltons (kDa). This gel was a representative result from 3 separate experiments. ISO - isoproterenol.

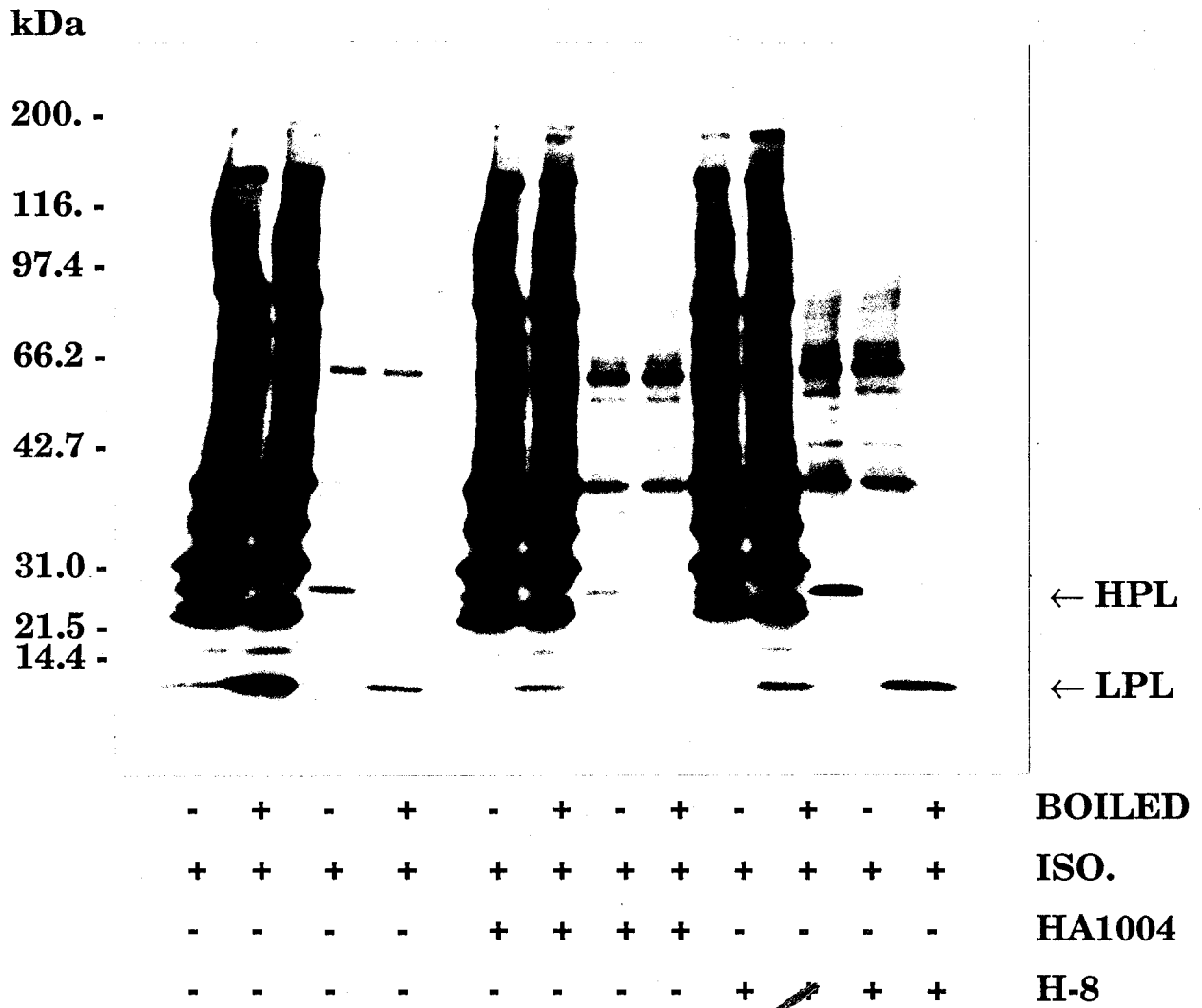


Figure 12.A. Autoradiograph of homogenates (A, B, E, F, I & J) and SR membranes (C, D, G, H, K & L) isolated from isoproterenol-stimulated myocytes in the absence (A, B, C, D, I, J, K & L) and presence (E, F, G & H) of HA1004 and in the absence (A - H) and presence (I, J, K & L) of H-8. Lanes B, D, F, H, J & L were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result of 3 separate experiments. ISO - isoproterenol.

kDa

200. -

116. -

97.4 -

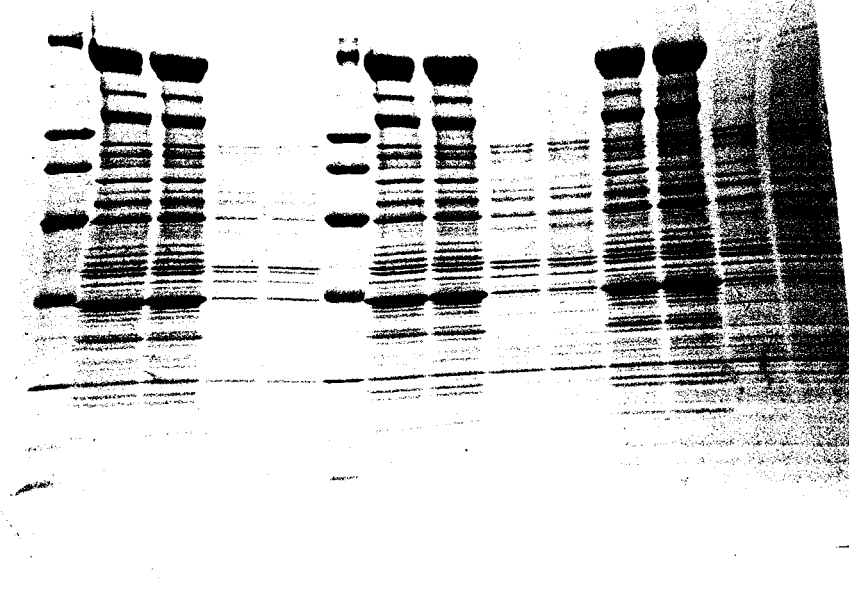
66.2 -

42.7 -

31.0 -

21.5 -

14.4 -



-	+	-	+	-	+	-	+	-	+	-	+	BOILED
+	+	+	+	+	+	+	+	+	+	+	+	ISO.
-	-	-	-	+	+	+	+	-	-	-	-	HA1004
-	-	-	-	-	-	-	-	+	+	+	+	H-8

Figure 12.B. SDS-PAGE gel of homogenates (A, B, E, F, I & J) and SR membranes (C, D, G, H, K & L) isolated from isoproterenol-stimulated myocytes in the absence (A, B, C, D, I, J, K & L) and presence (E, F, G & H) of HA1004 and in the absence (A - H) and presence (I, J, K & L) of H-8. Lanes B, D, F, H, J & L were boiled for 3 min prior to electrophoresis. Molecular mass markers are in the left lane and the sixth lane from the left and are indicated in kilodaltons (kDa). This gel was a representative result from 3 separate experiments. ISO - isoproterenol.

More protein appears to have been recovered in the SR membranes isolated from H-8 and HA1004 treated myocytes, since the lanes on the gel seem to contain more protein, as judged by the intensity of the Coomassie blue staining.

3.4.2. Proteins Phosphorylated in Response to PK C Activation

In preliminary studies, intact ^{32}P -labelled myocytes were treated with an active phorbol ester, PMA (phorbol 12-myristate, 13-acetate) or an inactive phorbol ester, α -PDD (4 α -phorbol 12, 13-didecanoate), to modulate the activity of Ca^{2+} /phospholipid-dependent protein kinase (PK C). Myocytes were treated for 3 min either with 4 μM PMA or 4 μM α -PDD (in 0.4% DMSO). As shown in Figure 13 and previously, isoproterenol increased the phosphorylation of 4 protein bands (8.5, 27, 31 and 152 kDa) in myocyte homogenates and 2 protein bands (8.5 and 27 kDa) in myocyte SR membranes. Both phorbol esters stimulated the phosphorylation of the 2 protein bands of phospholamban (8.5 and 27 kDa) in myocyte homogenates and SR membranes isolated from phorbol ester-treated myocytes. In this particular experiment, the amount of SR membranes isolated from isoproterenol-treated myocytes was lower than that isolated from cells treated with the phorbol esters, resulting in a faint signal on the autoradiograph.

Myocytes were also stimulated with OAG (1-oleoyl-2-acetyl-glycerol), a cell permeant, synthetic, diacylglycerol analog known to activate PK C in other cells and tissues [Witters and Blackshear (1987)]. As shown in Figure 14, when ^{32}P -labelled myocytes were treated with isoproterenol (1 μM for 1 min), 5% DMSO or OAG (0.25 mM in 5% DMSO for 5 min), 2 protein bands of phospholamban (8.5 and 27 kDa) in myocyte homogenates and SR membranes were phosphorylated. Also, with isoproterenol treatment a 31 kDa protein band was phosphorylated.

Stimulation of intact ^{32}P -labelled myocytes with ethanol (10%) and 0.2 mM OAG (in 10% ethanol) for 10 min to activate PK C, did not result in the stimulation

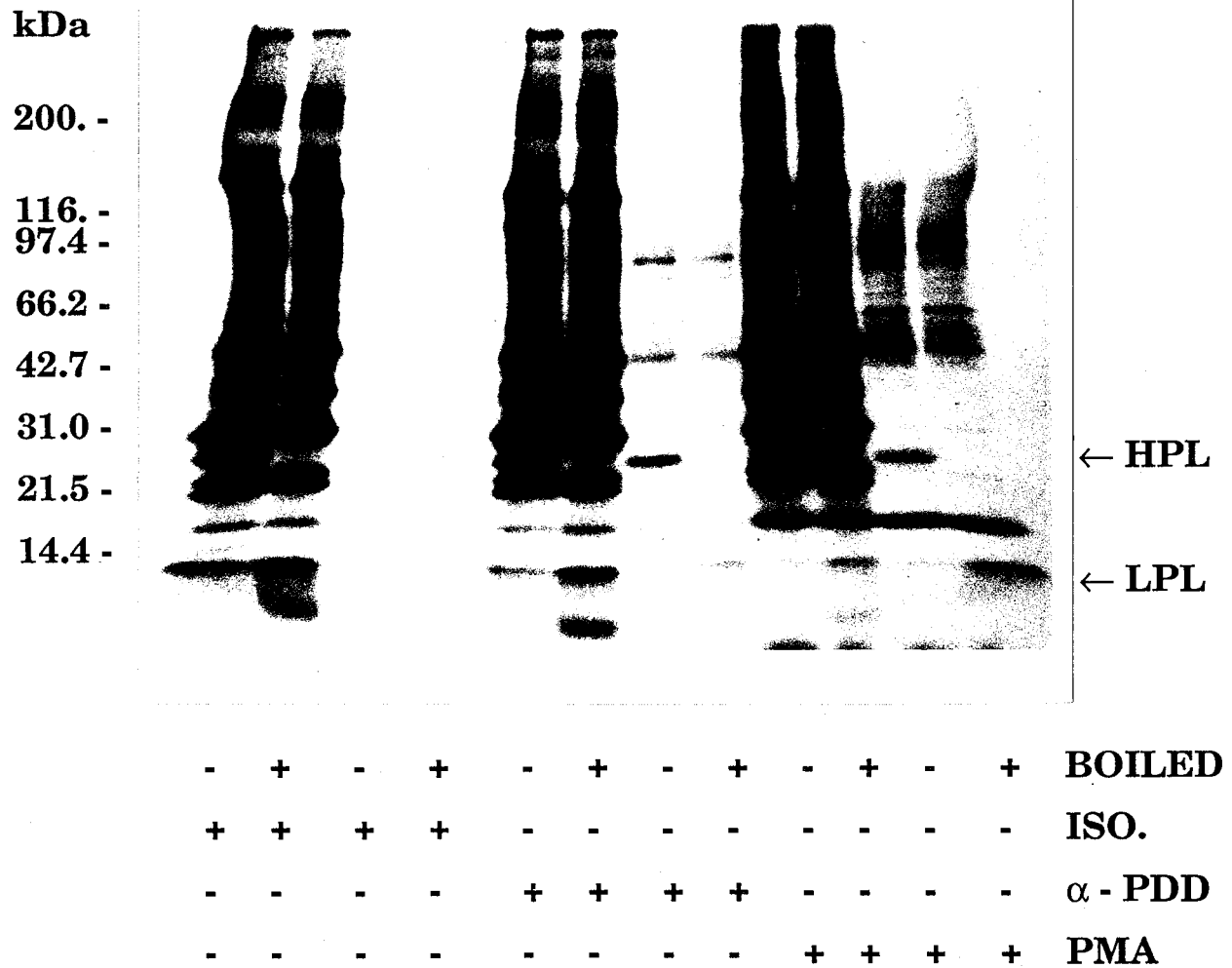


Figure 13. Autoradiograph of homogenates (A, B, E, F, I & J) and SR membranes (C, D, G, H, K & L) isolated from myocytes stimulated with isoproterenol (A, B, C & D), α -phorbol didecanoate (E, F, G & H) and phorbol myristate acetate (I, J, K & L). Lanes B, D, F, H, J & L were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 2 separate experiments. ISO - isoproterenol, α -PDD - α -phorbol didecanoate, PMA - phorbol myristate acetate.



Figure 14. Autoradiograph of homogenates (A, B, E, F, I & J) and SR membranes (C, D, G, H, K & L) isolated from myocytes stimulated with isoproterenol (A, B, C & D), DMSO (E, F, G & H) and OAG (in DMSO) (I, J, K & L). Lanes B, D, F, H, J & L were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 2 separate experiments. ISO - isoproterenol, DMSO - dimethyl sulfoxide, OAG - 1-oleoyl-2-acetyl glycerol.

of phosphorylation of any proteins in either case (Figure 15.A.). For comparison, the stimulation of protein phosphorylation (bands at 8.5, 27, 31 and 152 kDa in myocyte homogenates and 8.5 and 27 kDa in myocyte SR membranes) by isoproterenol treatment of the myocytes (1 μ M for 1 min) is also shown. The SDS-PAGE gel of this experiment (Figure 15.B.) shows that each corresponding sample lane has a similar protein profile. However, as illustrated on the gel by the intensity of the Coomassie blue staining, the protein recovered in the homogenate samples from ethanol and OAG in ethanol-treated myocytes appears to be less than that recovered in the homogenate from isoproterenol-treated cells. Less protein also appeared to be recovered in the SR membrane samples from OAG in ethanol-treated myocytes than from isoproterenol or ethanol-treated myocytes.

Figure 16 shows an autoradiograph of control myocytes and myocytes stimulated with 0.4 mM OAG (in 2% ethanol) for 10 min. Treatment of myocytes with OAG resulted in the phosphorylation of bands at approximately 18 kDa in SR membranes and 300 kDa in the homogenate fraction however, the stimulation of phosphorylation of these proteins was not a consistent result. Therefore, it appears that OAG treatment of myocytes did not result in the stimulation of phosphorylation of protein bands above the control level.

3.4.3. Concerted Kinase Regulation of Protein Phosphorylation

3.4.3.1. Possible Involvement of Calcium/Calmodulin-Dependent Protein Kinase

The possible contribution of calcium/calmodulin-dependent protein kinase (CAM PK) to the stimulation of protein phosphorylation upon the activation of PK A was also examined by treating the myocytes with an inhibitor of CAM PK. Myocytes were pretreated with 120 μ M CGS 9343 B (in 1.2% DMSO) for 5 min and then stimulated with 1 μ M isoproterenol (in 1.2% DMSO) for 1 min. As shown in

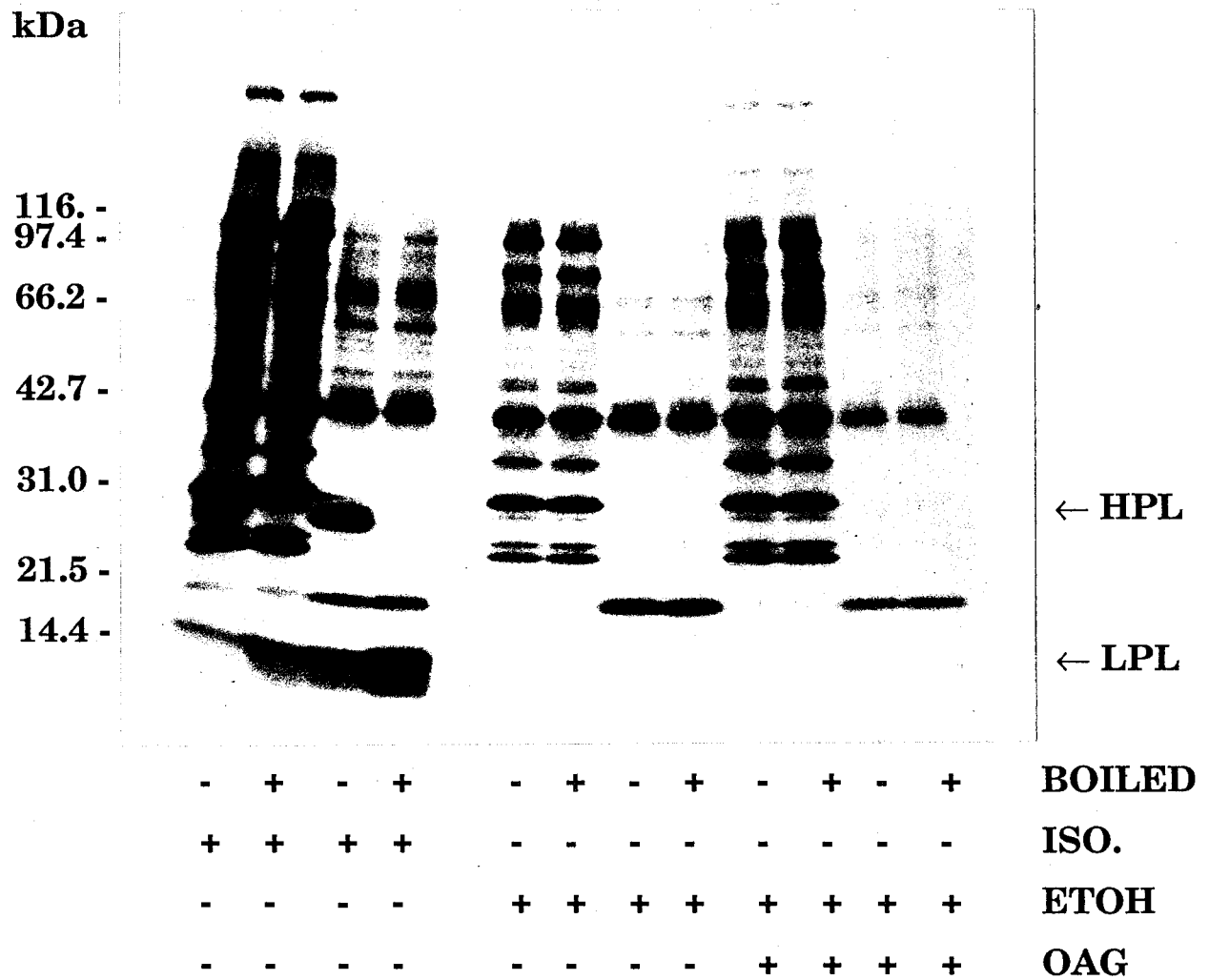
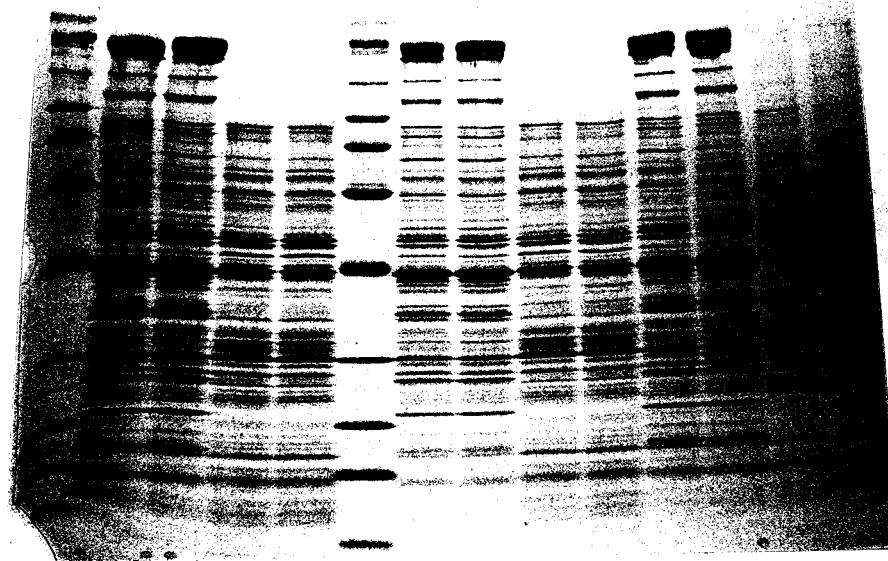


Figure 15.A. Autoradiograph of homogenates (A, B, E, F, I & J) and SR membranes (C, D, G, H, K & L) isolated from myocytes stimulated with isoproterenol (A, B, C & D), ethanol (E, F, G & H) and OAG (in ethanol) (I, J, K & L). Lanes B, D, F, H, J & L were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 2 separate experiments. ISO - isoproterenol, ETOH - ethanol, OAG - 1-oleoyl-2-acetyl glycerol.

kDa
 200. -
 116. -
 97.4 -
 66.2 -
 42.7 -
 31.0 -
 21.5 -
 14.4 -



-	+	-	+		-	+	-	+	-	+	-	+	BOILED
+	+	+	+		-	-	-	-	-	-	-	-	ISO.
-	-	-	-		+	+	+	+	+	+	+	+	ETOH
-	-	-	-		-	-	-	-	+	+	+	+	OAG

Figure 15.B. SDS-PAGE gel of homogenates (A, B, E, F, I & J) and SR membranes (C, D, G, H, K & L) isolated from myocytes stimulated with isoproterenol (A, B, C & D), ethanol (E, F, G & H) and OAG (in ethanol) (I, J, K & L). Lanes B, D, F, H, J & L were boiled for 3 min prior to electrophoresis. Molecular mass markers are in the left lane and in the sixth lane from the left and are indicated in kilodaltons (kDa). This gel was a representative result from 2 separate experiments. ISO - isoproterenol, ETOH -ethanol, OAG - 1-oleoyl-2-acetyl-glycerol.

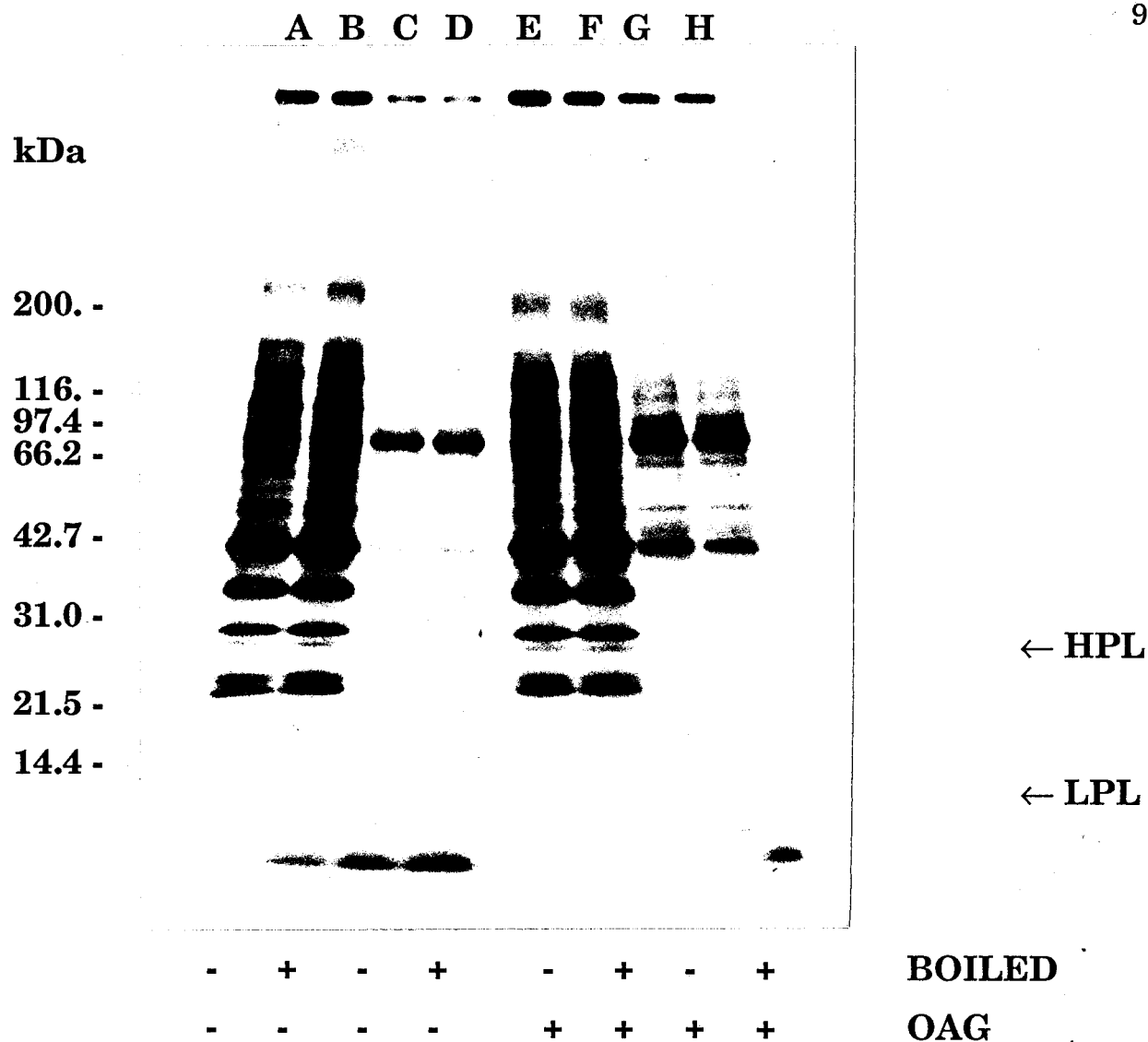


Figure 16. Autoradiograph of homogenates (A, B, E & F) and SR membranes (C, D, G & H) isolated from control (A, B, C & D) myocytes and myocytes stimulated with OAG (E, F, G & H). Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 3 separate experiments. OAG - 1-oleoyl-2-acetyl-glycerol.

Figure 17, isoproterenol in the presence or absence of CGS 9343B, increased the phosphorylation of 4 protein bands (8.5, 27, 31 and 152 kDa) in myocyte homogenates and 2 protein bands (8.5 and 27 kDa) in myocyte SR membranes. Inhibition of CAM PK did not alter the pattern of stimulated protein phosphorylation upon activation of PK A in intact myocytes.

3.4.3.2. Possible Involvement of Protein Kinase C

Phosphorylation of proteins by PK C was further investigated by treating the myocytes simultaneously with 1 μ M isoproterenol and 0.4 mM OAG (in 2% ethanol). Intact 32 P-labelled myocytes were pretreated for 10 min with OAG and then stimulated with isoproterenol for 1 min. Figure 18 shows that treatment of myocytes with isoproterenol alone resulted in the stimulation of phosphorylation of 5 protein bands (8.5, 15, 27, 31 and 152 kDa) in myocyte homogenates and 3 protein bands (8.5, 15 and 27 kDa) in myocyte SR membranes. Myocytes treated simultaneously with isoproterenol and OAG show the same phosphorylated bands as with isoproterenol alone. Upon comparison of the samples from myocytes stimulated with isoproterenol, in the absence and presence of OAG, there do not appear to be any differences in the number of protein bands phosphorylated.

The protein bands whose phosphorylation were stimulated by isoproterenol (8.5, 27, 31 and 152 kDa) were excised from the gels and the radioactivity incorporated into these bands quantitated using liquid scintillation counting. Figure 19 shows the degree of incorporation of radioactivity into the 8.5 and 27 kDa bands of phospholamban from homogenates and SR membranes isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes. There are no apparent differences in the incorporation of radioactivity into the 8.5 and 27 kDa bands (unboiled and boiled) of the homogenate samples between the four different myocyte treatments. In contrast, there appears to be an increase in the

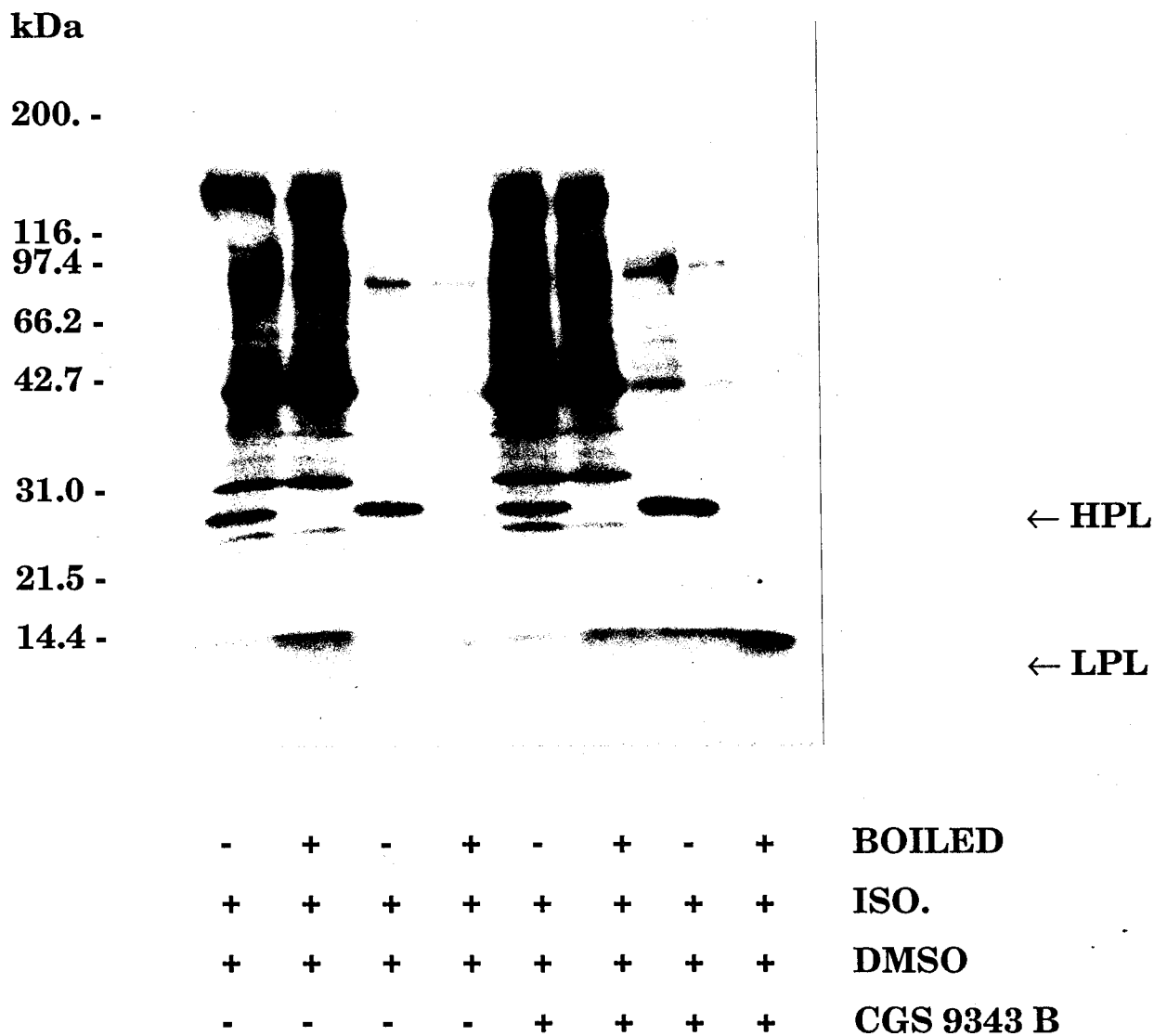


Figure 17. Autoradiograph of homogenates (A, B, E & F) and SR membranes (C, D, G & H) isolated from DMSO and isoproterenol-stimulated myocytes in the absence (A, B, C & D) and presence (E, F, G & H) of CGS 9343 B. Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 2 separate experiments. ISO - isoproterenol, DMSO - dimethyl sulfoxide.

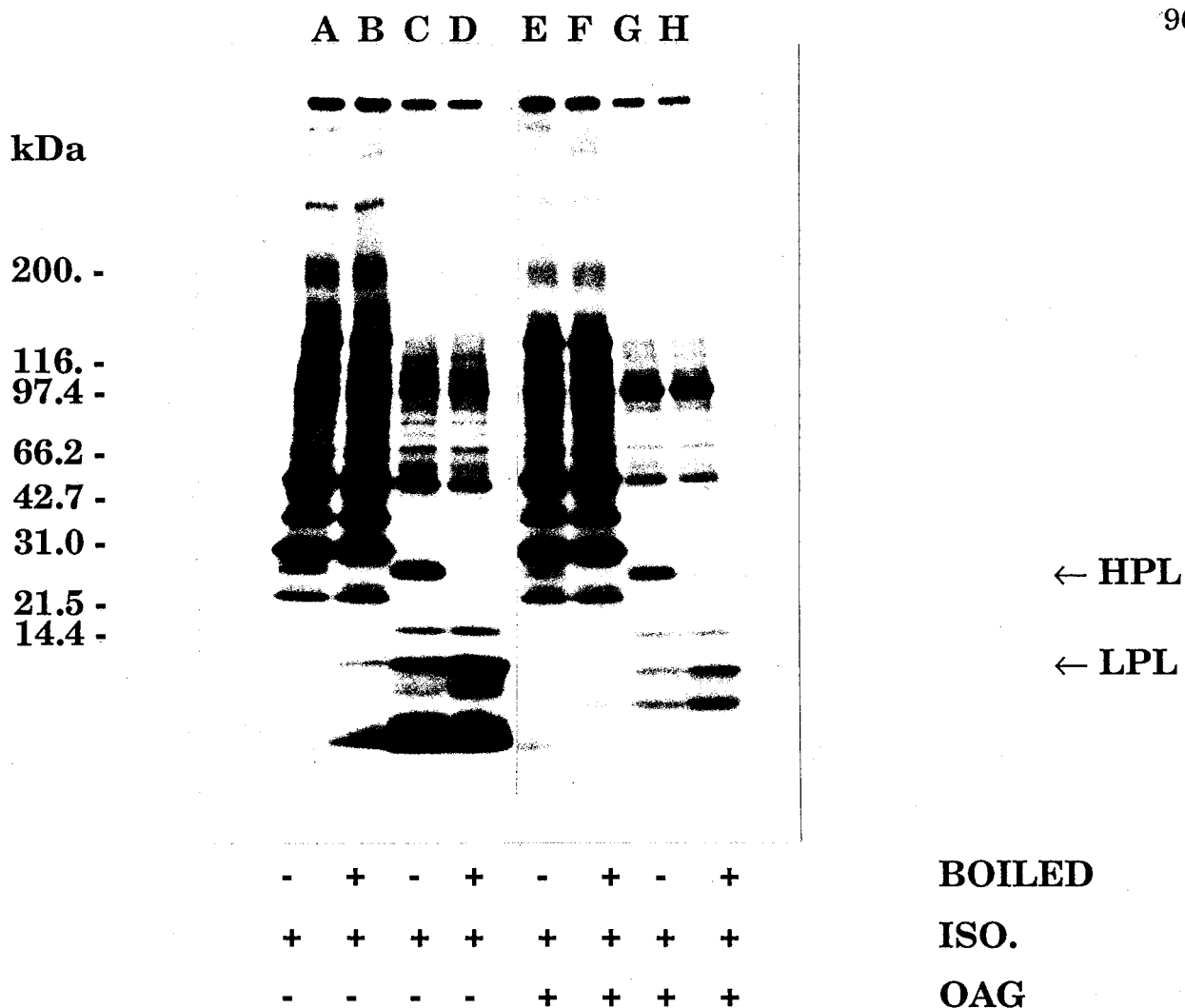


Figure 18. Autoradiograph of homogenates (A, B, E & F) and SR membranes (C, D, G & H) isolated from myocytes stimulated with isoproterenol (A, B, C & D) and with OAG and isoproterenol (E, F, G & H). Lanes B, D, F & H were boiled for 3 min prior to electrophoresis. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph was a representative result from 3 separate experiments. ISO - isoproterenol, OAG - 1-oleoyl-2-acetyl glycerol.

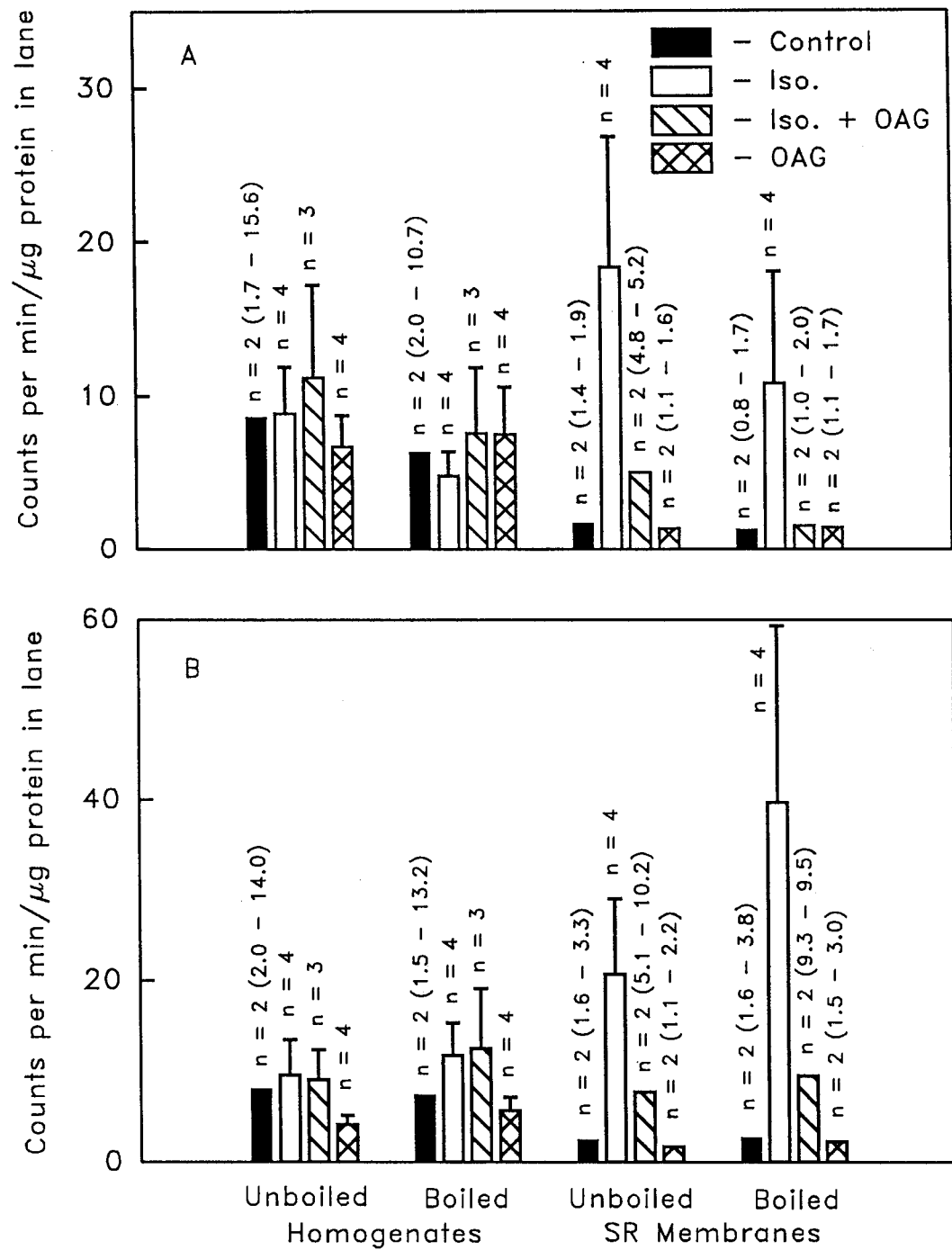


Figure 19. Incorporation of radioactivity into 27 (A) and 8.5 (B) kDa protein bands from SDS-PAGE gels. Results are reported as mean \pm S.E.M., except where noted in the figure. Numbers in brackets () represent ranges. ISO - isoproterenol, OAG - 1-oleoyl-2-acetylgllycerol.

incorporation of radioactivity into the 8.5 and 27 kDa bands (unboiled and boiled) in the SR membranes from isoproterenol-treated myocytes as compared to the control, OAG and isoproterenol plus OAG-treated myocytes. There seems to be no difference in the incorporation of radioactivity into the 8.5 and 27 kDa bands (unboiled and boiled) between the control, OAG and isoproterenol plus OAG-treated myocytes. In Figure 20, the incorporation of radioactivity into the 31 and 152 kDa protein bands in homogenates from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes is shown. There are apparently no differences in the incorporation of radioactivity into these two bands between unboiled and boiled or between the four different myocyte treatments. However, these results (Figure 19 and 20) must be viewed with caution since the data varies over a wide range and were obtained from a small number of experiments.

Figure 21 shows an autoradiograph of cytosolic fractions isolated from control myocytes and myocytes stimulated with isoproterenol (1 μ M), OAG (0.4 mM in 2% ethanol) and isoproterenol (1 μ M) plus OAG (0.4 mM in 2% ethanol). Cytosolic fractions isolated from isoproterenol and OAG plus isoproterenol-treated myocytes show an increase in the phosphorylation of the 21, 31 and 152 kDa proteins over the phosphorylation levels of these proteins noted in cytosolic fractions isolated from control and OAG-treated myocytes. The phosphorylation of a 24 kDa protein appears to decrease in the cytosolic fractions isolated from isoproterenol or OAG plus isoproterenol-treated myocytes as compared to the phosphorylation of this protein in the cytosolic fractions from control or OAG-treated myocytes. There appears to be no apparent difference in the proteins phosphorylated in the cytosolic fraction from isoproterenol or OAG plus isoproterenol-treated myocytes.

Several protein bands from the myocyte cytosolic fractions (152, 31, 24 and 21 kDa), whose state of phosphorylation seemed to have changed with the four different myocyte treatments, were excised from the gels and the radioactivity incorporated into these bands quantitated using liquid scintillation counting. The

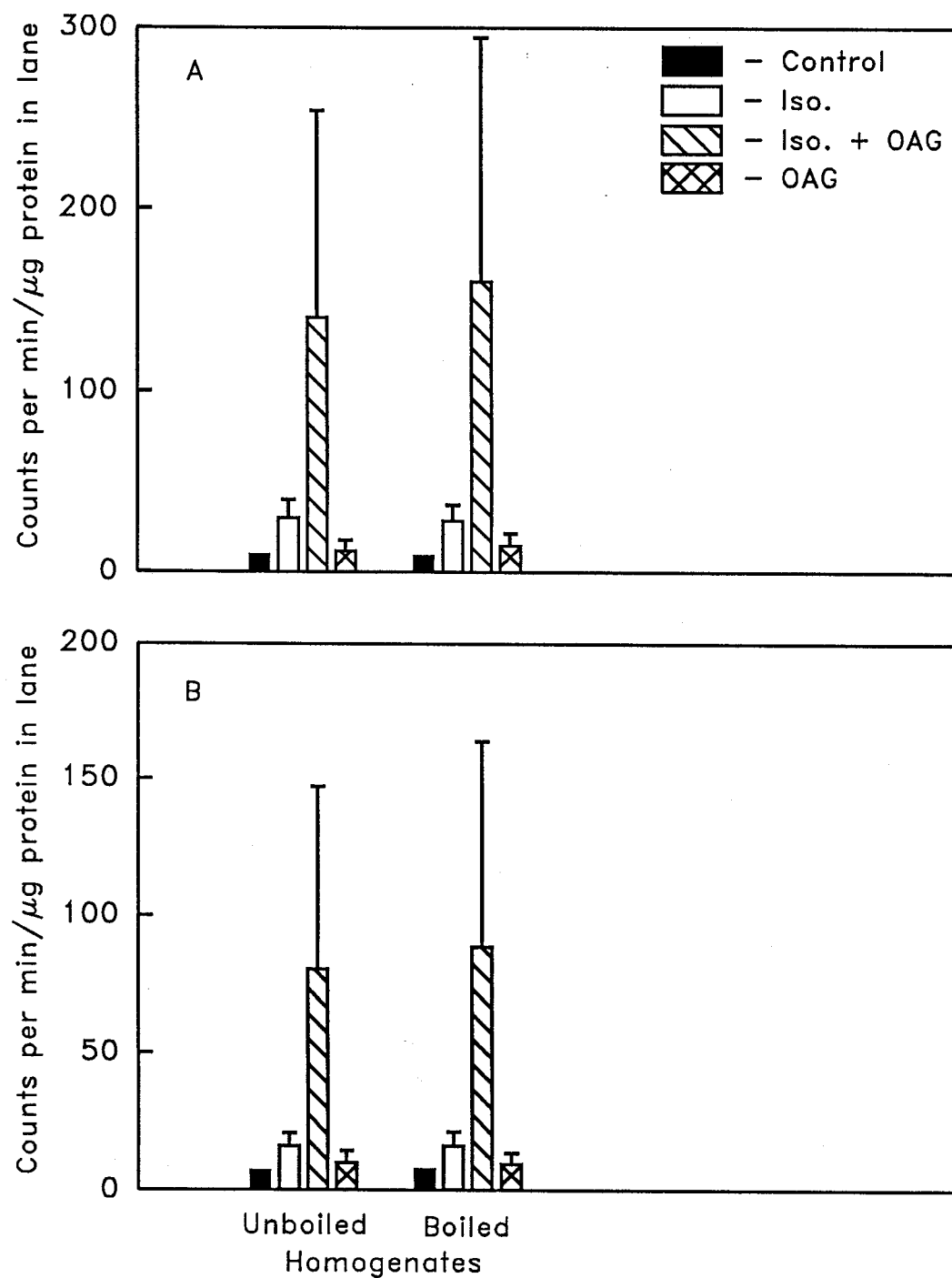


Figure 20. Incorporation of radioactivity into 31 (A) and 152 (B) kDa protein bands from SDS-PAGE gels. Results are reported as mean \pm S.E.M. ($n = 3$), except for the controls ($n = 2$). Ranges for the controls are: A) unboiled (4.2 - 14.8) and boiled (3.8 - 14.2); B) unboiled (4.7 - 9.6) and boiled (4.8 - 11.0). ISO - isoproterenol, OAG - 1-oleoyl-2-acetylglcerol.

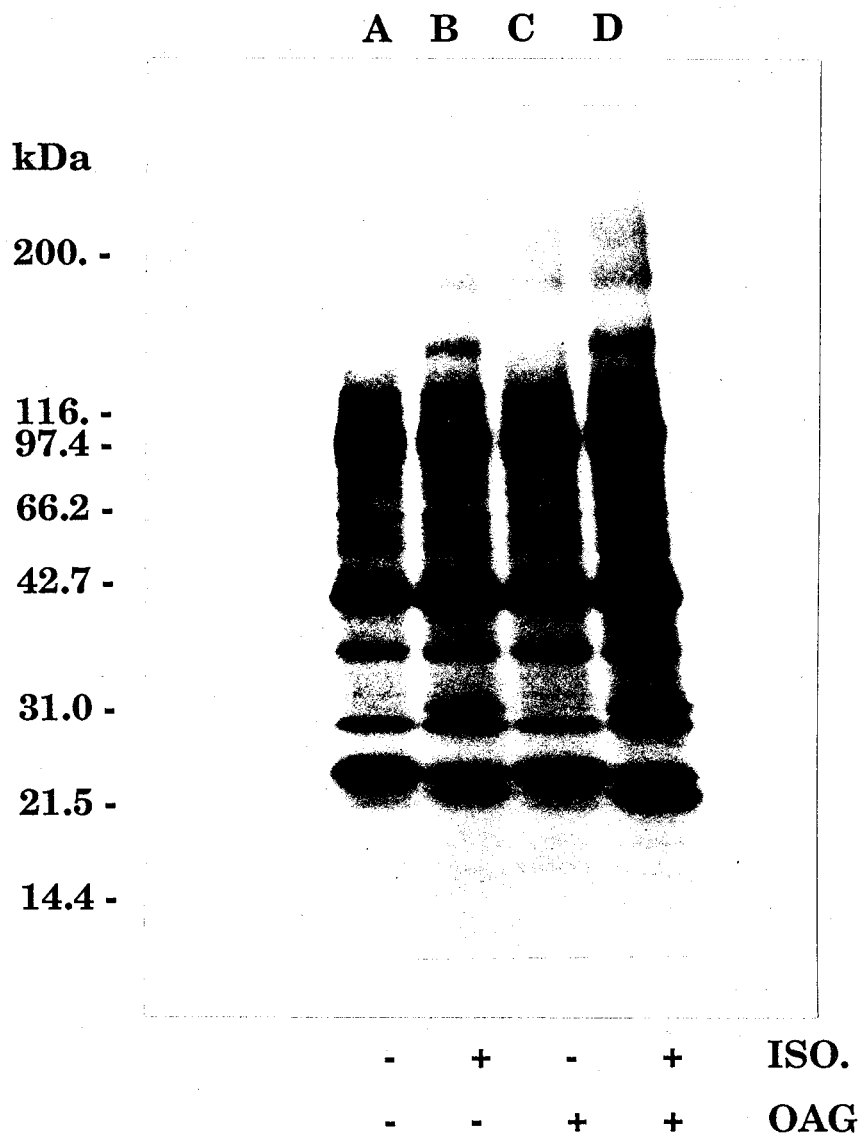


Figure 21. Autoradiograph of myocyte cytosolic fraction isolated from control (A), isoproterenol (B), OAG (C) and isoproterenol and OAG-treated (D) myocytes. Samples were heated at 37°C for 10 min prior to electrophoresis. Molecular mass is indicated in kilodaltons (kDa). This autoradiograph is a representative result from 2 separate experiments. ISO - isoproterenol, OAG - 1-oleoyl-2-acetyl glycerol.

incorporation of radioactivity into the 31 kDa band appeared to be increased in the cytosolic fraction from isoproterenol and OAG plus isoproterenol-treated myocytes as compared to control and OAG-treated myocytes [control (n = 2): 3.41 (1.25 - 5.57), isoproterenol (n = 2): 6.72 (1.07 - 12.36), OAG (n = 2): 2.28 (0.96 - 3.59), isoproterenol plus OAG (n = 2): 4.30 (1.61 - 6.98) cpm/ μ g protein in the lane; mean (range)]. Also, the incorporation of radioactivity into the 152 kDa protein band appeared to be increased in the cytosolic fraction from isoproterenol and OAG plus isoproterenol-treated myocytes as compared to control and OAG-treated myocytes [control (n = 2): 2.68 (1.12 - 4.24), isoproterenol (n = 2): 5.32 (1.24 - 9.40), OAG (n = 2): 1.60 (0.78 - 2.41), isoproterenol plus OAG (n = 2): 3.61 (1.88 - 5.34) cpm/ μ g protein in the lane; mean (range)]. The radioactivity incorporated into the 24 kDa band appeared to be the highest from the cytosolic fractions from control myocytes with incorporation decreasing to the same level in myocytes treated with isoproterenol, OAG and isoproterenol plus OAG [control (n = 2): 9.76 (3.51 - 16.01), isoproterenol (n = 2): 5.45 (1.49 - 9.41), OAG (n = 2): 4.3 (1.91 - 6.69), isoproterenol plus OAG (n = 2): 2.81 (1.31 - 4.31) cpm/ μ g protein in the lane; mean (range)]. The radioactivity incorporated into the 21 kDa band appeared to be similar between control, isoproterenol and OAG plus isoproterenol-treated myocytes but appeared to be decreased in OAG-treated myocytes. Since the above results were obtained from a small number of experiments they must be viewed with caution.

3.5. Phosphorylated Species of Phospholamban

Using 15% (non-gradient) SDS-PAGE gels and immunoblotting it was possible to separate distinct species of oligomeric phospholamban from control, dephosphorylated and phosphorylated canine cardiac ventricle SR membranes (Figure 22) as described by Li *et al.* (1990). However, using this methodology it was

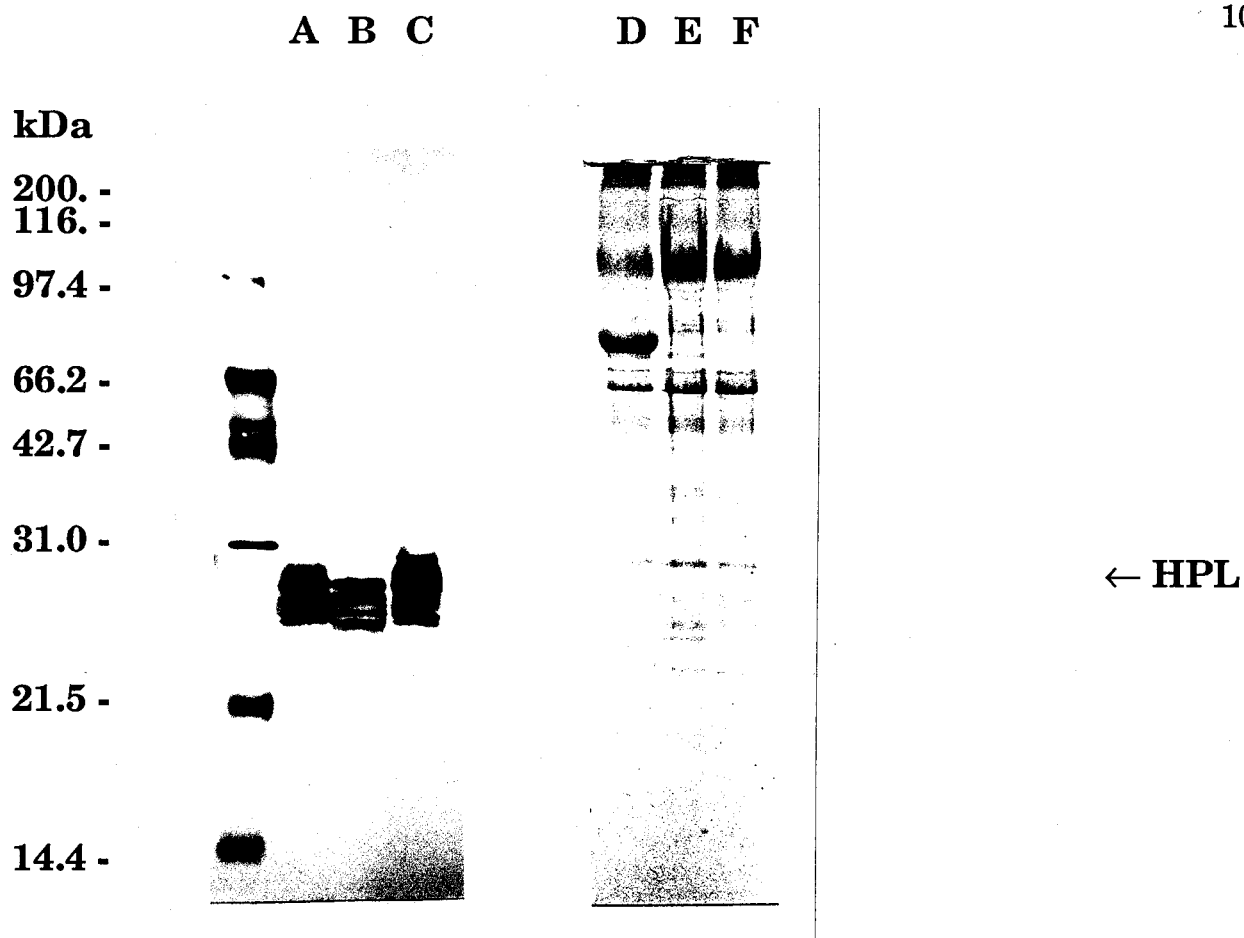


Figure 22. Western blot (A - C) and SDS-PAGE gel (D - F) of canine cardiac ventricular SR membranes probed with a monoclonal antibody to phospholamban. Lanes A & D contain SR membranes dephosphorylated with alkaline phosphatase, lanes B & E contain control SR and lanes C & F contain SR phosphorylated by catalytic subunit of PK A. Samples were heated at 37°C for 10 min prior to electrophoresis and each lane of the gel contained 25 μ g protein. HPL are the high molecular mass forms of phospholamban. The gel was stained subsequent to transfer. Molecular mass markers are in the left lane of the blot and are indicated in kilodaltons (kDa). The blot and gel are a representative result from 2 separate experiments.

not possible to separate distinct oligomeric species of phospholamban from rat myocyte SR membranes. Utilizing 10 - 20% double gradient (10 - 20% polyacrylamide and 0.27 - 1.0% bisacrylamide) SDS-PAGE gels and immunoblotting, however, it was possible to achieve some separation of the different oligomeric species of phospholamban from control, dephosphorylated and phosphorylated myocyte SR membranes (Figure 23). A sample of phosphorylated canine SR membranes separated by this system is also shown for comparison.

Homogenate and SR membrane samples from control myocytes and myocytes stimulated with isoproterenol (1 μ M) alone, OAG (0.4 mM in 2% ethanol) alone and isoproterenol (1 μ M) and OAG (0.4 mM in 2% ethanol), together, were separated with the 10 - 20% double gradient gel and then western blotted and probed with a monoclonal antibody to phospholamban (Figure 24). This blot shows that in homogenates and SR membranes from myocytes treated with isoproterenol or isoproterenol plus OAG, phospholamban was phosphorylated to a similar, higher degree (as evidenced by the increase in number of the species of oligomeric phospholamban separated) than in the presence of OAG alone or from control myocytes. There seem to be no differences between the number of oligomeric species of phospholamban formed in the homogenates and SR membranes of myocytes treated with isoproterenol or isoproterenol plus OAG. The number of oligomeric species of phospholamban formed was not different between homogenates and SR membranes from control and OAG-treated myocytes.

3.6. *Stimulation of Protein Kinase C in Adult Rat Ventricular Myocytes*

3.6.1. Activation of Protein Kinase C in Adult Rat Ventricular Myocytes

From these foregoing studies, it was not evident that PK C had been activated since results show that there was no protein whose phosphorylation was

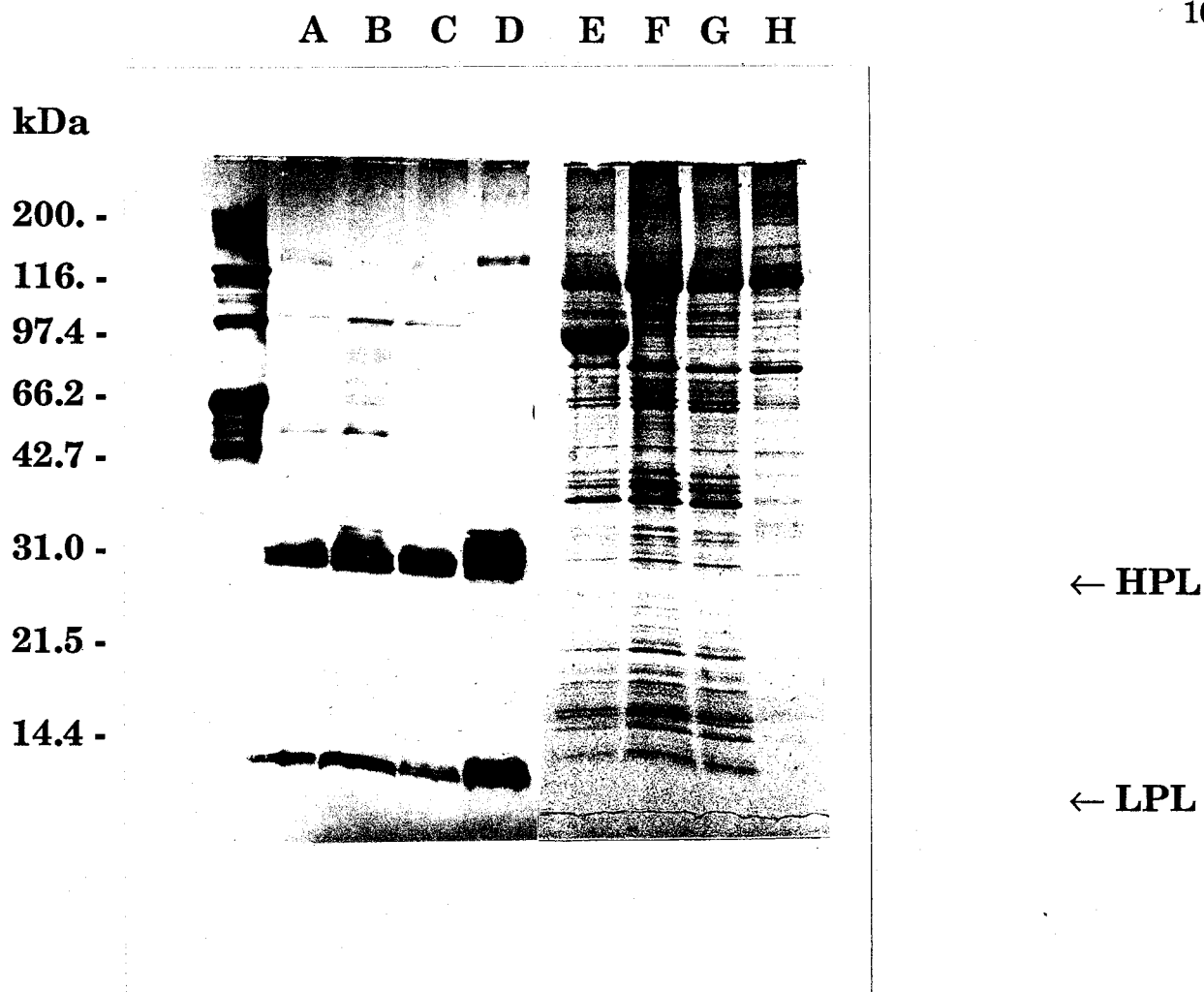


Figure 23. Western blot (A - D) and SDS-PAGE gel (E - H) of rat myocyte (A - C & E - G) and canine cardiac ventricle (D & H) SR membranes probed with a monoclonal antibody to phospholamban. Lanes A & E contain SR membranes dephosphorylated with alkaline phosphatase, lanes B & F contain control SR and lanes C, D, G & H contain SR phosphorylated by catalytic subunit of PK A. Samples were heated at 37°C for 10 min prior to electrophoresis and each lane of the gel contained 25 μ g protein. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. The gel was stained subsequent to transfer. Molecular mass markers are in the left lane of the blot and are indicated in kilodaltons (kDa). The blot and gel are a representative result from 4 separate experiments.

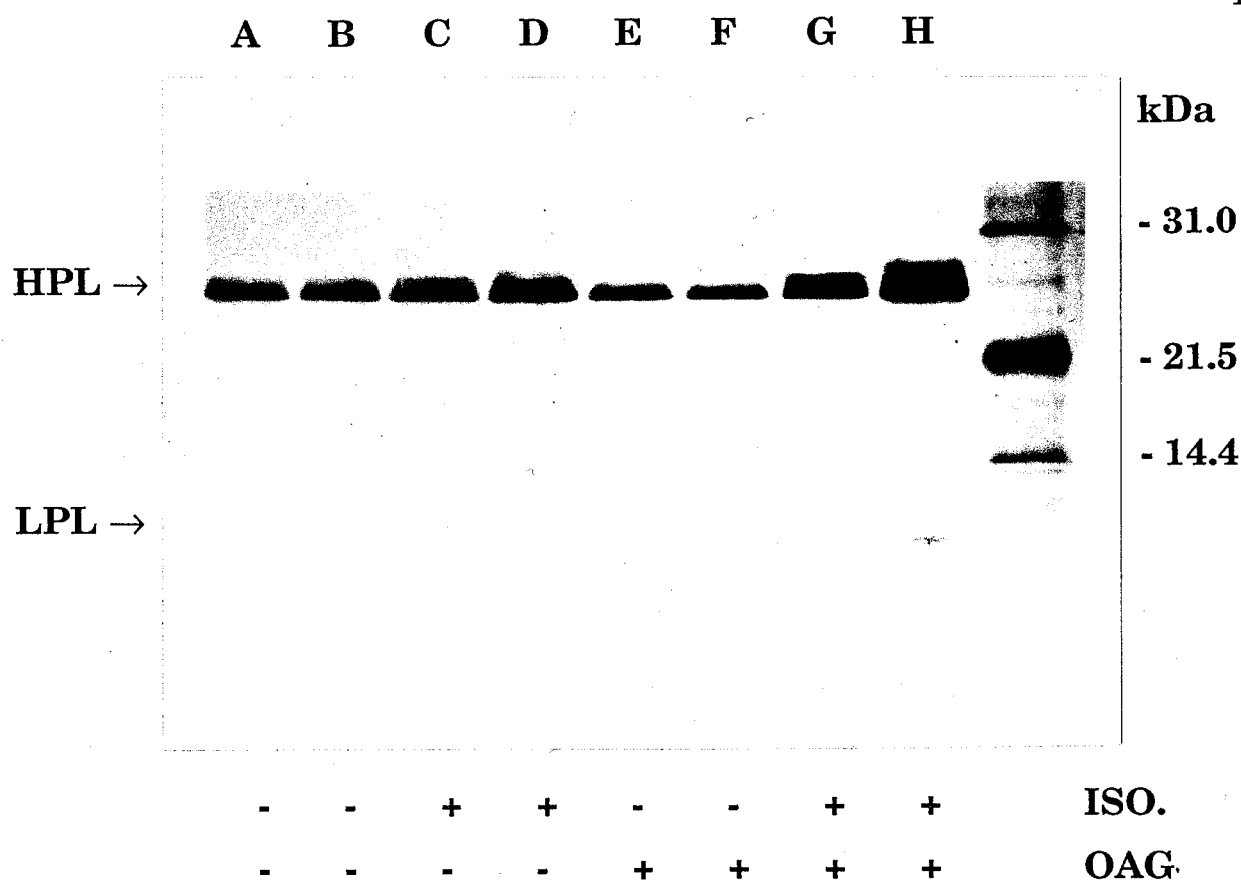


Figure 24. Western blot of homogenates (A, C, E & G) and SR membranes (B, D, F & H) from control (A & B), isoproterenol (C & D), OAG (E & F) and isoproterenol and OAG-stimulated (G & H) myocytes. The blot was probed with a monoclonal antibody to phospholamban. Samples were heated at 37°C for 10 min prior to electrophoresis and each lane of the gel contained approximately 4 µg protein. HPL and LPL are the high and low molecular mass forms of phospholamban, respectively. Molecular mass is indicated in kilodaltons (kDa). This blot was a representative result from 3 separate experiments. ISO - isoproterenol, OAG - 1-oleoyl-2-acetyl glycerol.

stimulated by PK C activators. Therefore, we undertook a closer examination of the activation of PK C in isolated myocytes.

3.6.1.1. Isolation and Quantitation of PK C After DEAE-Cellulose Chromatography

In a series of preliminary experiments, it was attempted to isolate PK C from cytosolic fractions and membranes prepared from control and OAG-treated myocytes by DEAE-cellulose chromatography. Table 10 shows that in the presence of the PK C activators, Ca^{2+} and lipids (phosphatidylserine and diacylglycerol), the activity of PK C from the rat brain and bovine trachea cytosolic preparations was much higher than in the absence of the activators. The PK C activity from the rat myocyte preparations was similar to that found in the rat brain and bovine trachea in the absence of the activators, Ca^{2+} and lipids. However, in the presence of Ca^{2+} and lipids, the PK C activity from the rat myocyte cytosol was not increased. The DEAE separation of rat myocyte cytosol was repeated in six separate experiments and all had similar results to those shown here. When the myocyte cytosol was separated by FPLC, a large increase in the activity of PK C in the presence of activators was demonstrated. Therefore, future studies on the isolation and quantitation of PK C from myocyte cytosolic fractions and membranes utilized FPLC to partially purify these preparations.

3.6.1.2. Isolation and Quantitation of PK C After Fast Protein Liquid Chromatography

To investigate the possible movement of PK C from the cytosol to the crude membrane fraction in intact ventricular myocytes, FPLC (fast protein liquid chromatography) was used. Fractions obtained using FPLC were assayed for PK C activity with a standard PK C assay which utilized histone as the substrate. To

Protein kinase C activity was determined for all preparations as described in section 2.2.7. Only the values from the cytosolic fraction are shown from each preparation. Ca^{2+} /Lipid stimulated activities were calculated by subtracting the specific enzyme activity (pmol/min/mg protein) measured in the absence of Ca^{2+} /Lipids from that measured in the presence of Ca^{2+} /Lipids. Results shown are of a representative experiment in each case. (DEAE) indicates that the cytosols were separated by DEAE-cellulose column chromatography. (FPLC) indicates that the cytosol was separated by fast protein liquid chromatography.

	pmol/min/mg protein		Ca^{2+} /Lipid Stimulated Activity
	– Ca^{2+} /Lipids	+ Ca^{2+} /Lipids	
Rat Brain PK C (DEAE)	186.0	2,030.0	1,844.0
Bovine Trachea (DEAE)	254.0	1,361.0	1,107.0
Rat Myocyte (DEAE)	188.9	190.5	1.6
Rat Myocyte (FPLC)	42.1	768.3	726.2

determine the approximate fractions where PK C would elute from the FPLC column, a sample of pure rat brain PK C was fractionated on the column. Figure 25 shows that the activity of rat brain PK C eluted from the FPLC column from fractions 27-35. Cytosolic and membrane fractions obtained from control myocytes and separated by FPLC (Figure 26) show that a large part of the protein in these fractions does not bind very tightly to the Mono Q column and eluted in the initial fractions collected. From the cytosol, the next largest amount of protein eluted from fractions 27-35 and from the membrane fraction the next largest amount of protein eluted from fractions 47-50. Figure 27 shows the PK C activity profile of control myocyte cytosolic and membrane fractions assayed in the absence and presence of the peptide inhibitor of cAMP-dependent protein kinase. The inhibition of PK A during the assay of PK C activity greatly decreased the variability of the phosphorylating activity. Subsequently, all assays for PK C activity contained the PK A inhibitor.

PK C activity was measured in cytosolic and membrane fractions isolated from control myocytes incubated for 1, 5 and 10 min (Figure 28) at 37°C. In the cytosol, three peaks of Ca^{2+} and lipid-dependent PK C activity were eluted. Fraction 27-31 contained the peak with the highest activity. Two smaller peaks of activity occurred at fractions 32-34 and 39-42. In the membrane fraction, two major peaks of PK C activity were eluted. Eluting in the initial fractions, from fractions 6-15 was a peak of PK C activity that contained a large proportion of Ca^{2+} and lipid-independent activity. Fractions 27-31 contained the major peak of Ca^{2+} and lipid-dependent PK C activity from the membrane fraction. Calculation of the area under the curve (AUC) of the major peaks of Ca^{2+} and lipid-dependent PK C activity (fractions 27-31) for the cytosolic and membrane preparations (Figure 29) show that there were no significant differences (Two-way ANOVA, $p > 0.05$) between the peaks obtained from control myocytes that had been incubated for 1, 5 or 10 min. The PK C activities in the presence of 3 mM EGTA (without Ca^{2+} or

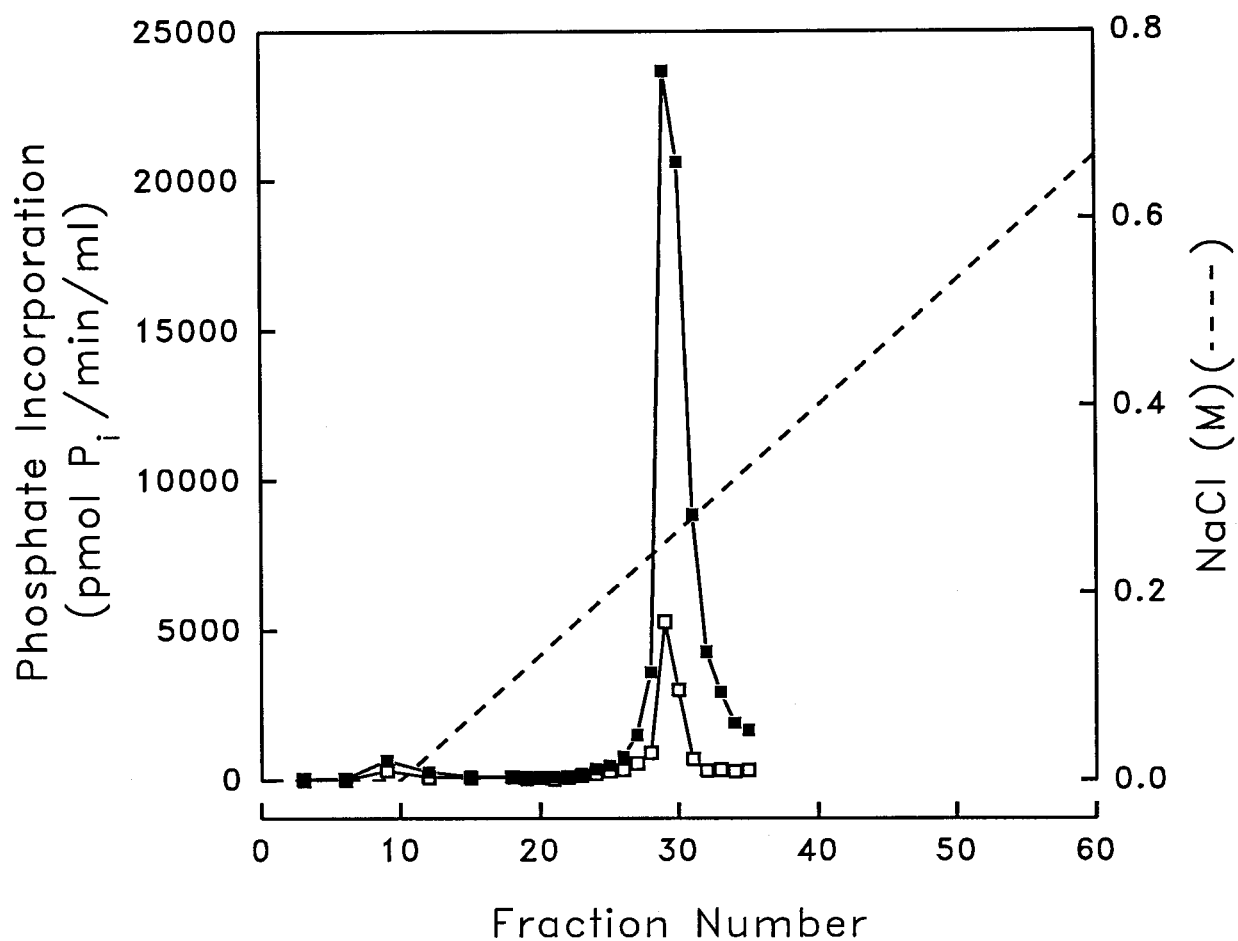


Figure 25. FPLC profile of purified rat brain PK C. PK C was assayed either in the presence of 1 μM Ca^{2+} (free), 80 $\mu\text{g/ml}$ phosphatidylserine and 8 $\mu\text{g/ml}$ 1-stearoyl-2-arachidonylglycerol (■) or in the presence of no added Ca^{2+} or lipids and 3 mM EGTA (□). The data presented here was from a single experiment.

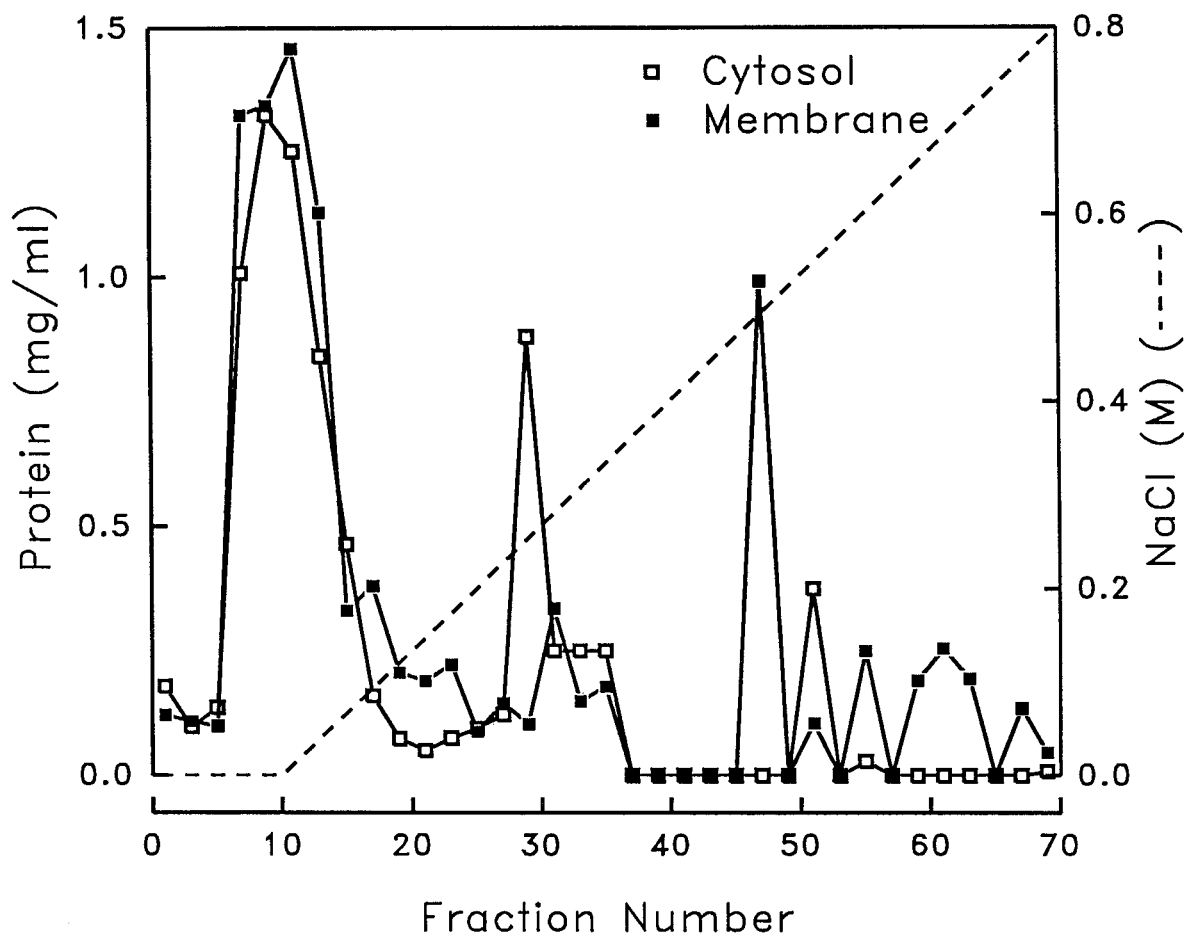


Figure 26. Protein elution FPLC profile of control myocyte cytosol (□) and membrane (■) fractions. The data presented here was from a single experiment.

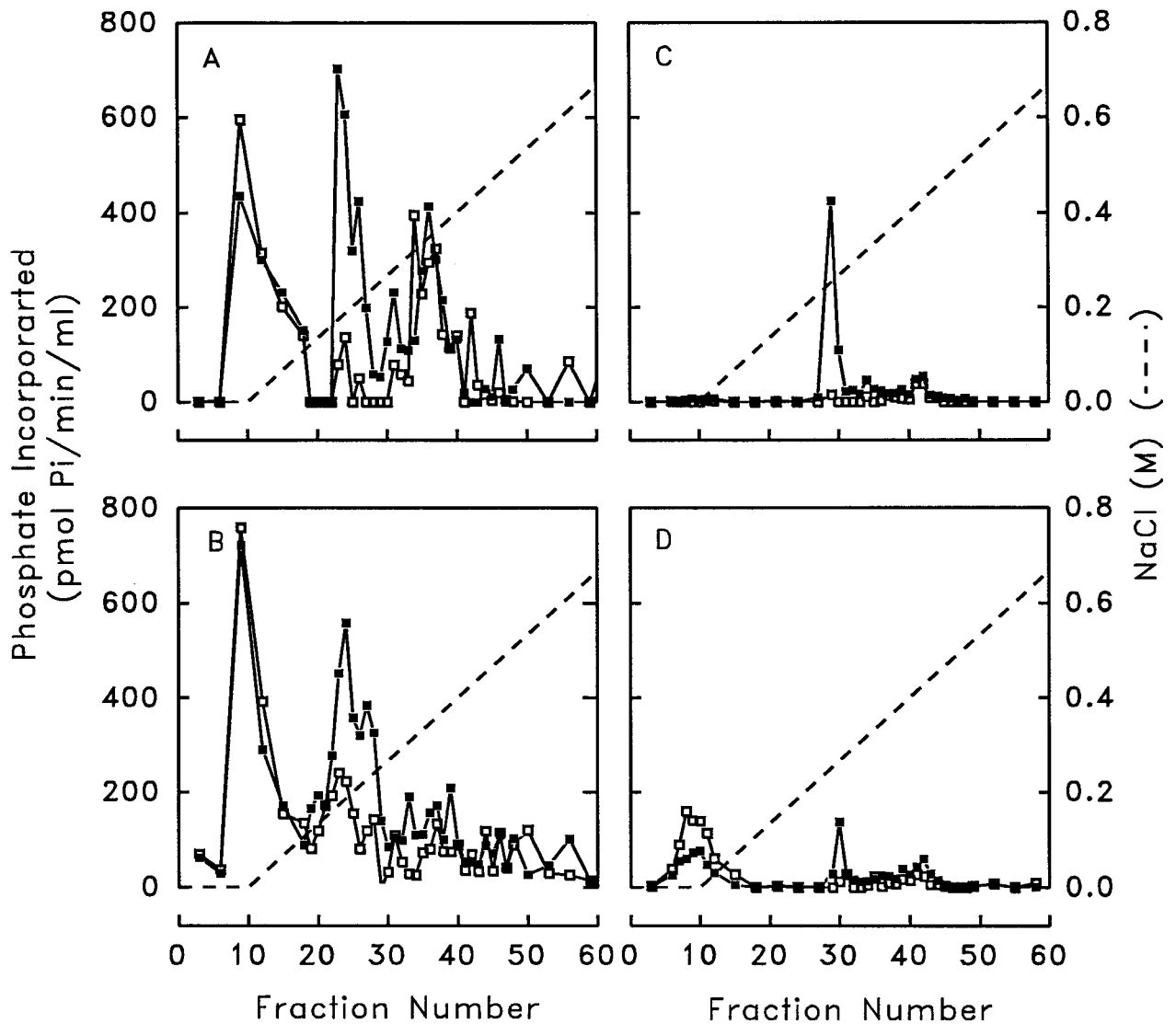


Figure 27. PK C activity of FPLC profiles of control myocyte cytosols (A & C) and membranes (B & D) assayed in the absence (A & B) and presence (C & D) of cAMP-dependent protein kinase inhibitor. PK C was assayed either in the presence of $1 \mu\text{M}$ Ca^{2+} (free), $80 \mu\text{g/ml}$ phosphatidylserine and $8 \mu\text{g/ml}$ 1-stearoyl-2-arachidonylglycerol (■) or no added Ca^{2+} or lipids and 3 mM EGTA (□). The data presented are from two separate experiments. The experiment shown in C & D was repeated for $n = 3$.

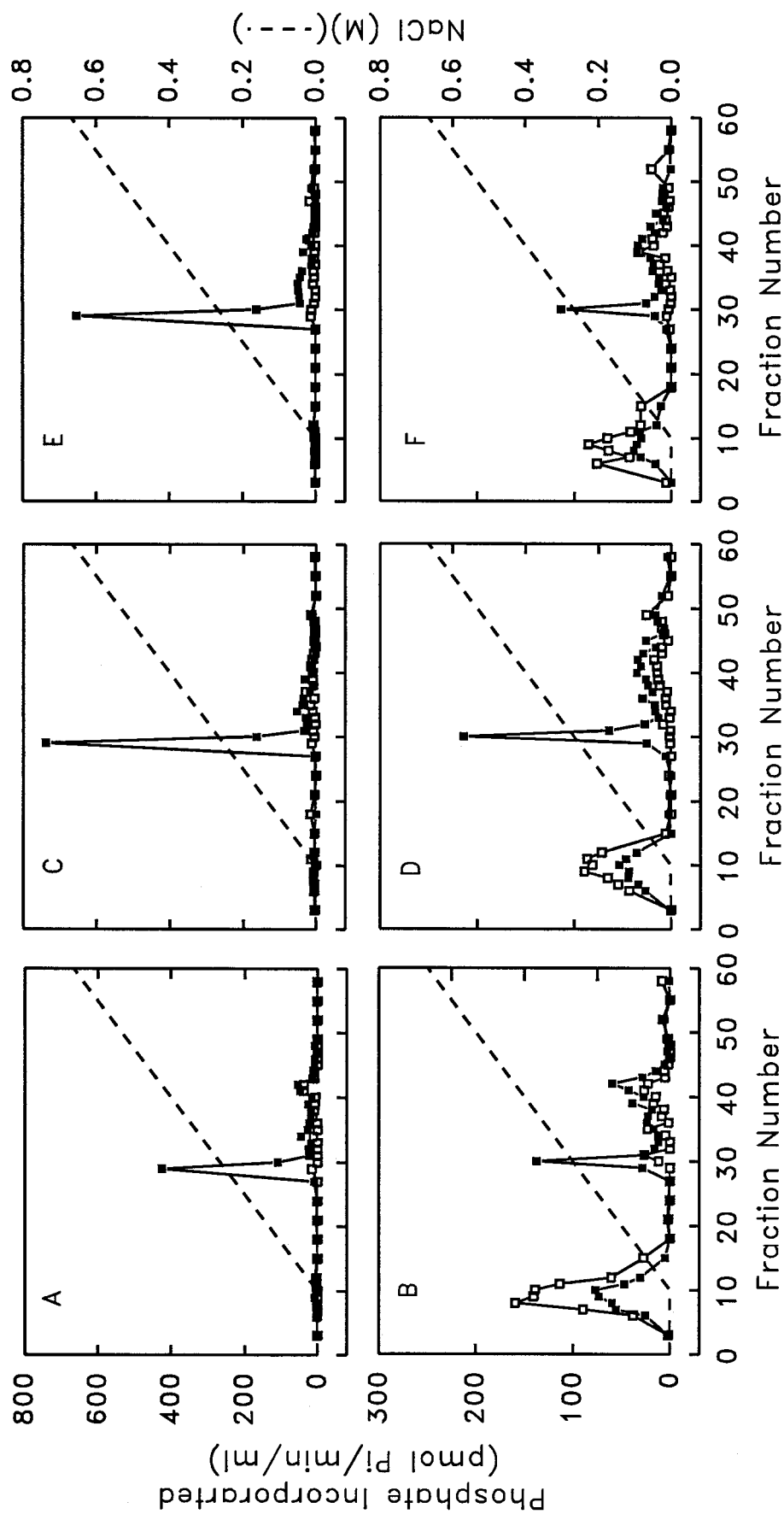


Figure 28. PK C activities of FPLC profiles of control myocyte cytosols (A, C & E) and membranes (B, D & F) incubated for 1 (A & B), 5 (C & D) and 10 min (E & F). PK C was assayed either in the presence of $1 \mu\text{M Ca}^{2+}$ (free), $80 \mu\text{g/ml}$ phosphatidylserine and $8 \mu\text{g/ml}$ 1-stearoyl-2-arachidonylglycerol (■) or no added Ca^{2+} or lipids and 3 mM EGTA (□). These data are each a representative plot from experiments that have each been repeated three times.

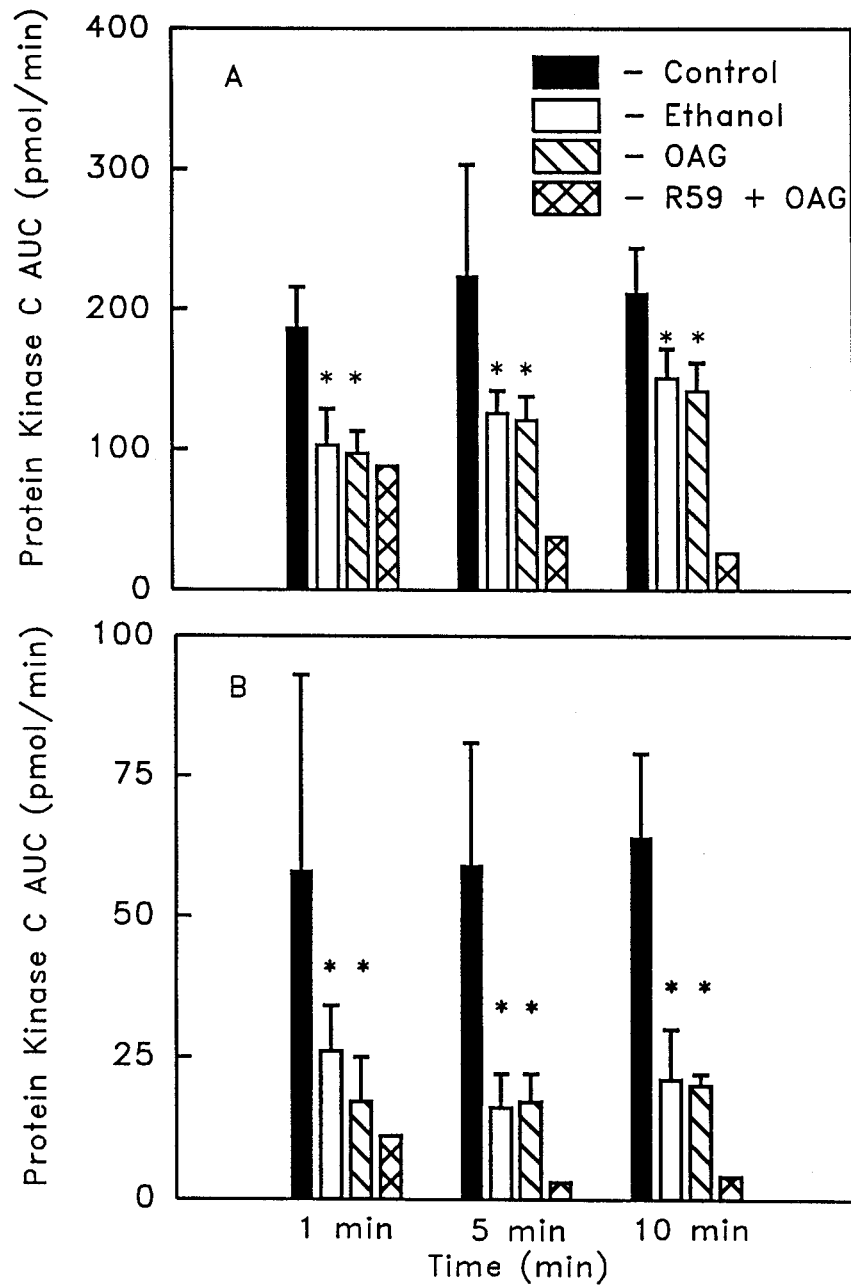


Figure 29. Area under the curve (AUC) of PK C activity from FPLC fractions 27 - 31 of myocyte cytosols (A) and membranes (B). Results are expressed as mean \pm S.E.M. ($n = 3$), except for ethanol cytosol (1 & 5 min), ethanol membrane (5 min) and OAG (1 min) where $n = 4$ and ethanol cytosol (5 min) where $n = 5$. Results for myocytes treated with R59 and OAG are mean ($n = 2$), ranges are: cytosols - 1 min (45 - 131); 5 min (17 - 59); 10 min (16 - 38) and membranes - 1 min (4 - 18); 5 min (0.4 - 5.2); 10 min (0 - 7.4). * - denotes significantly different from control (Two-way ANOVA, $p < 0.05$).

lipid) were subtracted from the activity in the presence of Ca^{2+} and lipids, prior to the AUC calculation.

The protein from the PK C activity peak fractions obtained from the control cytosol and membrane fractions were concentrated, separated by SDS-PAGE and immunoblotted. These blots were then probed with antibodies to the PK C isozymes type III and type II (Figure 30). The cytosolic peak at fractions 27-31 demonstrated two immunoreactive bands of PK C; the band which demonstrated a higher molecular weight may be an autophosphorylated species of PK C. These fractions also contained both type II and type III PK C. The second cytosolic peak at fractions 32-34 contained type III PK C and the third cytosolic peak at fractions 39-42 did not contain type III PK C. The membrane peak that contained a large proportion of Ca^{2+} and lipid-independent PK C activity (fractions 6-15) contained 2 bands of type III but not type II PK C. The band of type III PK C at approximately 50 kDa may be the proteolytically active form of PK C, PK M. Fractions 27-31 from the membrane preparation contained two bands of type III PK C; the band demonstrating a higher molecular weight may be an autophosphorylated species of PK C. The kinase peaks at fractions 39 and 42 from the membrane preparation did not contain type III PK C.

To stimulate PK C in intact myocytes, the cells were treated with OAG. Since OAG was dissolved in ethanol, the effect of ethanol alone on the PK C activity of myocytes was determined. Cytosolic and membrane fractions were prepared from myocytes treated with 1% ethanol for 1, 5 and 10 min (Figure 31). As compared to the PK C activity profiles from control cytosol and membrane fractions, ethanol did not affect the number or position of the peaks found in the cytosol or membrane preparations from ethanol-treated myocytes. The PK C activity in the membrane peak that contained a large proportion of Ca^{2+} and lipid-independent PK C activity (fractions 6-15) was also of the same magnitude as that found in the control. The major peak of Ca^{2+} and lipid-dependent PK C activity (fractions 27

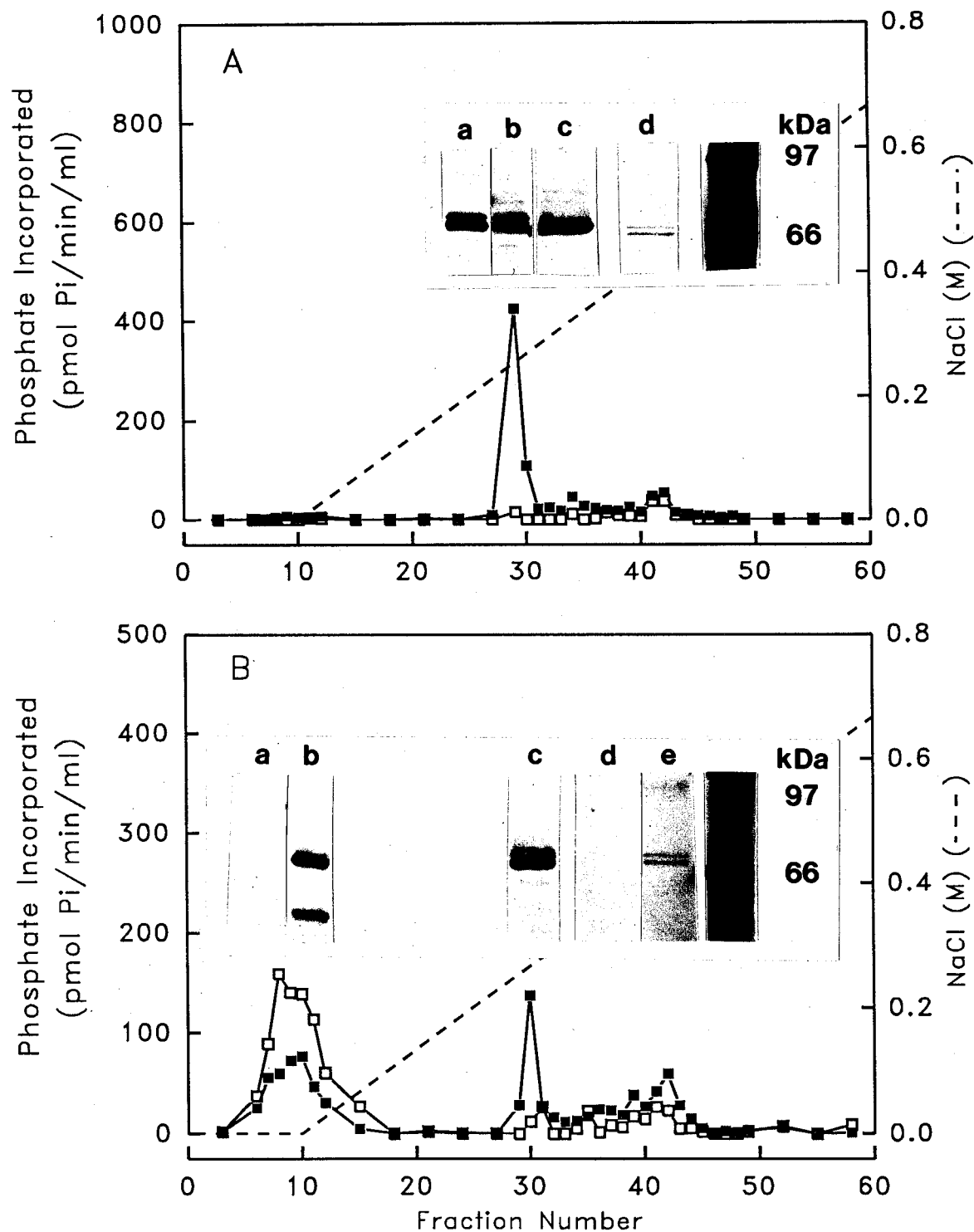


Figure 30. Western blots of PK C activity peaks from control (1 min) myocyte cytosol (A) and membrane (B) fractions, probed with antibodies to Type II (a) and Type III PK C (b - e). The right lane of the blot contains molecular mass markers expressed in kilodaltons (kDa). This blot was the result of 1 experiment.

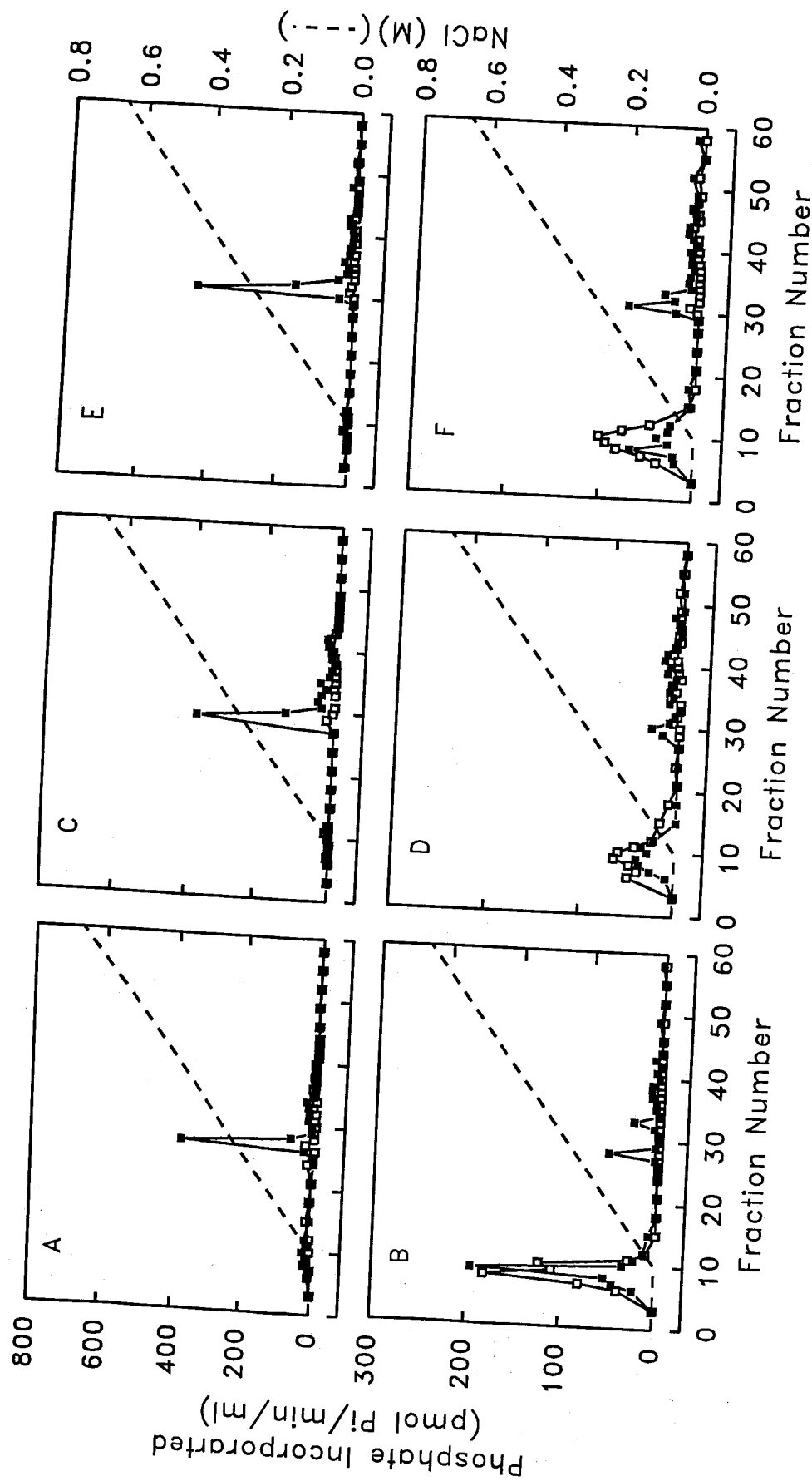


Figure 31. PK C activities of FPLC profiles of ethanol-treated myocyte cytosols (A, C & E) and membranes (B, D & F) incubated for 1 (A & B), 5 (C & D) and 10 min (E & F). PK C was assayed either in the presence of $1 \mu\text{M Ca}^{2+}$ (free), $80 \mu\text{g/ml}$ phosphatidylserine and $8 \mu\text{g/ml}$ 1-stearoyl-2-arachidonylglycerol (■) or no added Ca^{2+} or lipids and 3 mM EGTA (□). These data are each a representative plot from experiments that have each been repeated at least three times.

-31) from the membrane fraction appeared to contain less PK C activity than the same peak from the control profiles. Calculation of the AUC (Figure 29) of these peaks from both the cytosol and membrane fractions isolated from ethanol-treated myocytes revealed that, when compared to the AUC from control peaks there was significantly less PK C activity (Two-way ANOVA, $p < 0.05$). As shown in the control profiles, there were no significant differences (Two-way ANOVA, $p > 0.05$) found in the AUC of the major Ca^{2+} and lipid-dependent PK C activity peaks obtained from either the cytosol or membrane fractions prepared from ethanol-treated myocytes that had been incubated for 1, 5 or 10 min.

PK C activity profiles from cytosolic and membrane fractions isolated from myocytes treated with 0.2 mM OAG (in 1% ethanol) for 1, 5 and 10 min are shown in Figure 32. As compared to the control and ethanol PK C activity profiles, OAG has not affected the number or position of the peaks found in the cytosol or membrane preparations from OAG-treated myocytes. The PK C activity in the membrane peak that contained a large proportion of Ca^{2+} and lipid-independent PK C activity (fractions 6-15) was also of the same magnitude as that found in the control and ethanol profiles. Just as in the ethanol profile, the major peak of Ca^{2+} and lipid-dependent PK C activity (fractions 27-31) from the membrane fraction appeared to contain less PK C activity than the same peak from the control profiles. As before, calculation of the AUC of these peaks from both cytosol and membrane fractions isolated from OAG-treated myocytes was carried out to determine whether this reduction of PK C activity was of statistical significance. Figure 29 shows that when compared to the AUC from control peaks there was significantly less PK C activity in these peaks (Two-way ANOVA, $p < 0.05$). However, when the AUC of these OAG-treated peaks was compared to the AUC of the ethanol-treated peaks, from both cytosol and membrane fractions, there was no significant difference found (Two-way ANOVA, $p > 0.05$). As shown in the control and ethanol profiles, there were no significant differences found (Two-way ANOVA, $p > 0.05$) in the AUC of the

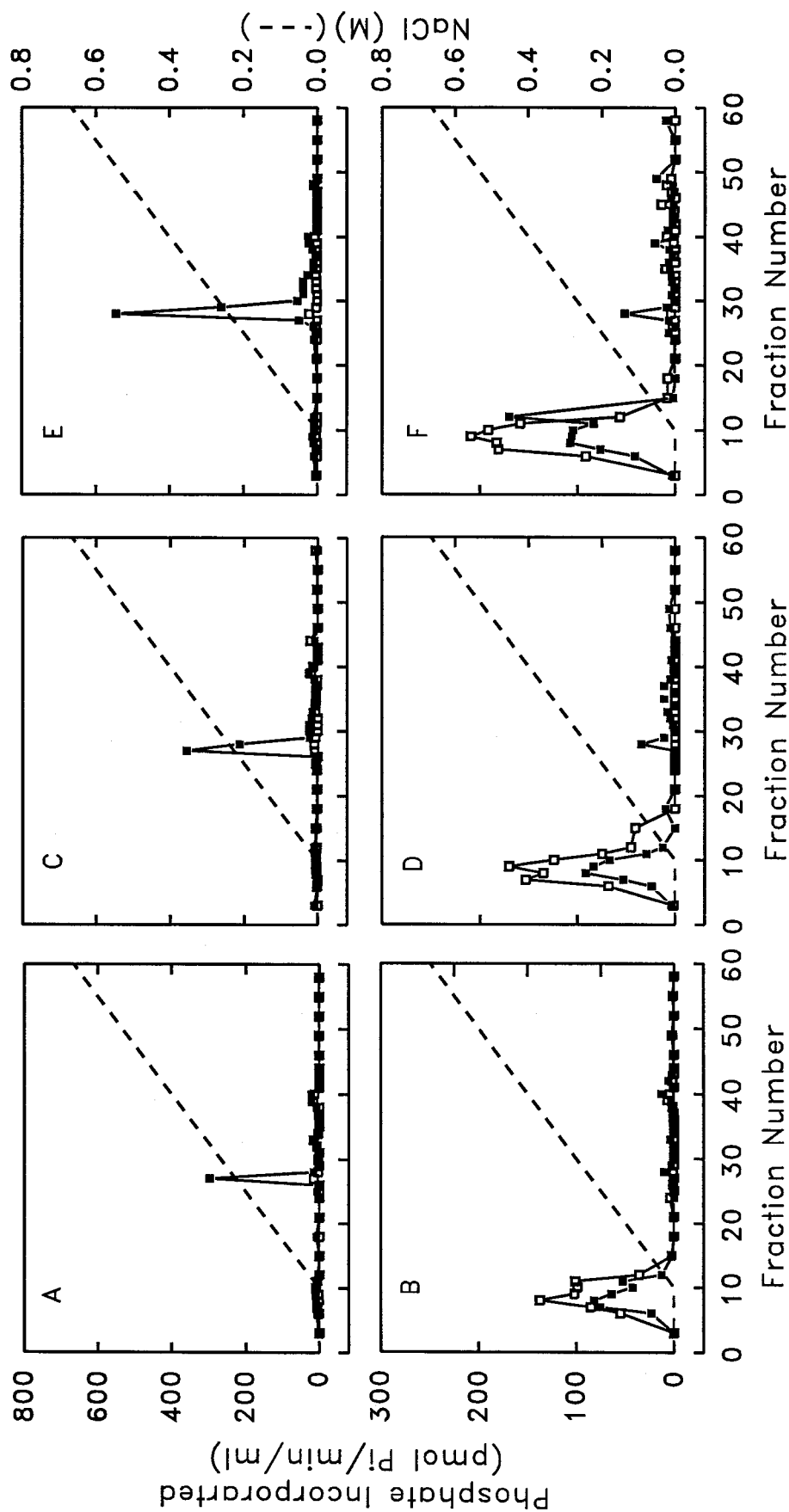


Figure 32. PK C activities of FPLC profiles of OAG-treated myocyte cytosols (A, C & E) and membranes (B, D & F) incubated for 1 (A & B), 5 (C & D) and 10 min (E & F). PK C was assayed either in the presence of $1 \mu\text{M Ca}^{2+}$ (free), $80 \mu\text{g/ml}$ phosphatidylserine and $8 \mu\text{g/ml}$ 1-stearoyl-2-arachidonylglycerol (□) or no added Ca^{2+} or lipids and 3 mM EGTA (■). These data are each a representative plot from experiments that have each been repeated at least three times.

major Ca^{2+} and lipid-dependent PK C activity peaks obtained from either the cytosol or membrane fractions prepared from OAG-treated myocytes that had been incubated for 1, 5 or 10 min. Taken together, the above results suggest that there was no movement of PK C from the cytosolic to the crude membrane fraction upon treatment of the myocytes with OAG to indicate the activation of PK C.

The protein from the PK C activity peak fractions obtained from the FPLC of the OAG-treated cytosol and membrane fractions were concentrated, separated by SDS-PAGE and western blotted. These blots were then probed with antibodies to the PK C isozymes type III and type II (Figure 33). The results obtained were similar to those obtained from the blots of peaks from control myocytes. The cytosolic peak at fractions 27-31 demonstrated two immunoreactive bands of PK C; the band demonstrating a higher molecular weight may be an autophosphorylated species of PK C. These fractions also contained both type II and type III PK C. The second cytosolic peak at fractions 32-34 contained both type III and type II PK C and the third cytosolic peak at fractions 39-42 did not contain type III PK C. The membrane peak that contained a large proportion of Ca^{2+} and lipid-independent PK C activity (fractions 6-15) contained 2 bands of type III but not type II PK C. Again, the PK C type III band at 50 kDa may be the proteolytically activated form, PK M. Fractions 27-31 from the membrane preparation contained type III PK C.

To investigate further the activation of PK C by OAG, intact myocytes were pretreated for 10 min with 30 μM R59022, a compound known to inhibit diacylglycerol kinase in intact cells and tissues [de Chaffoy de Courcelles *et al.* (1985)]. Diacylglycerol kinase metabolizes diacylglycerols in the cell to phosphatidic acid, which does not activate PK C. Inhibition of DAG kinase, therefore, would lengthen the amount of time that OAG remained unmetabolized by the kinase or decrease the amount of OAG that was metabolized by the kinase. After pretreatment, the myocytes were again stimulated by OAG (0.2 mM in 1% ethanol) for 1, 5 and 10 min (Figure 34). These profiles show results similar to those

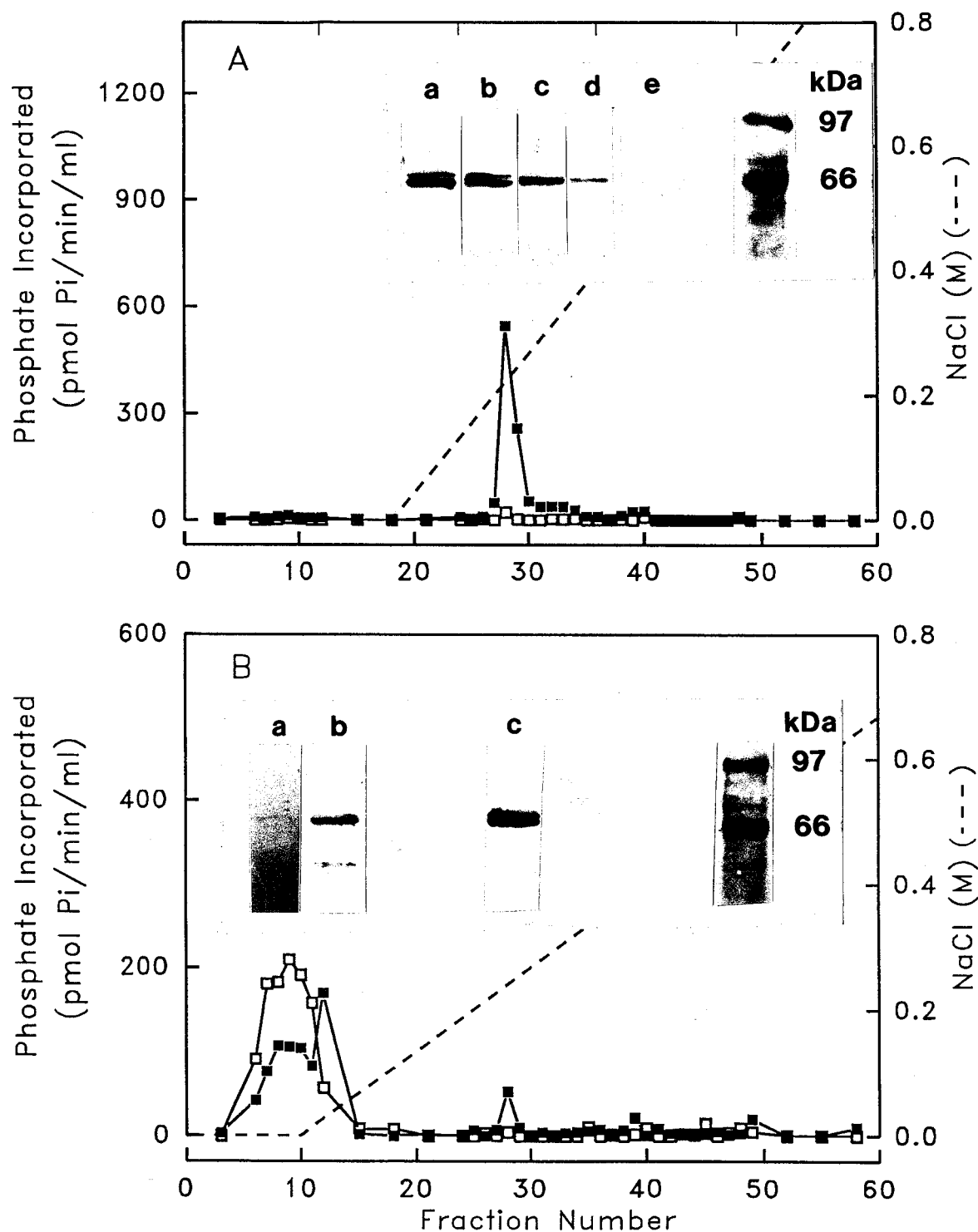


Figure 33. Western blots of PK C activity peaks from OAG-treated (10 min) myocyte cytosol (A) and membrane (B) fractions, probed with antibodies to Type II (in A: a & c; in B: a) and Type III PK C (in A: b, d & e; in B: b & c). The right lane of the blot contains molecular mass markers expressed in kilodaltons (kDa). This blot was the result of 1 experiment.

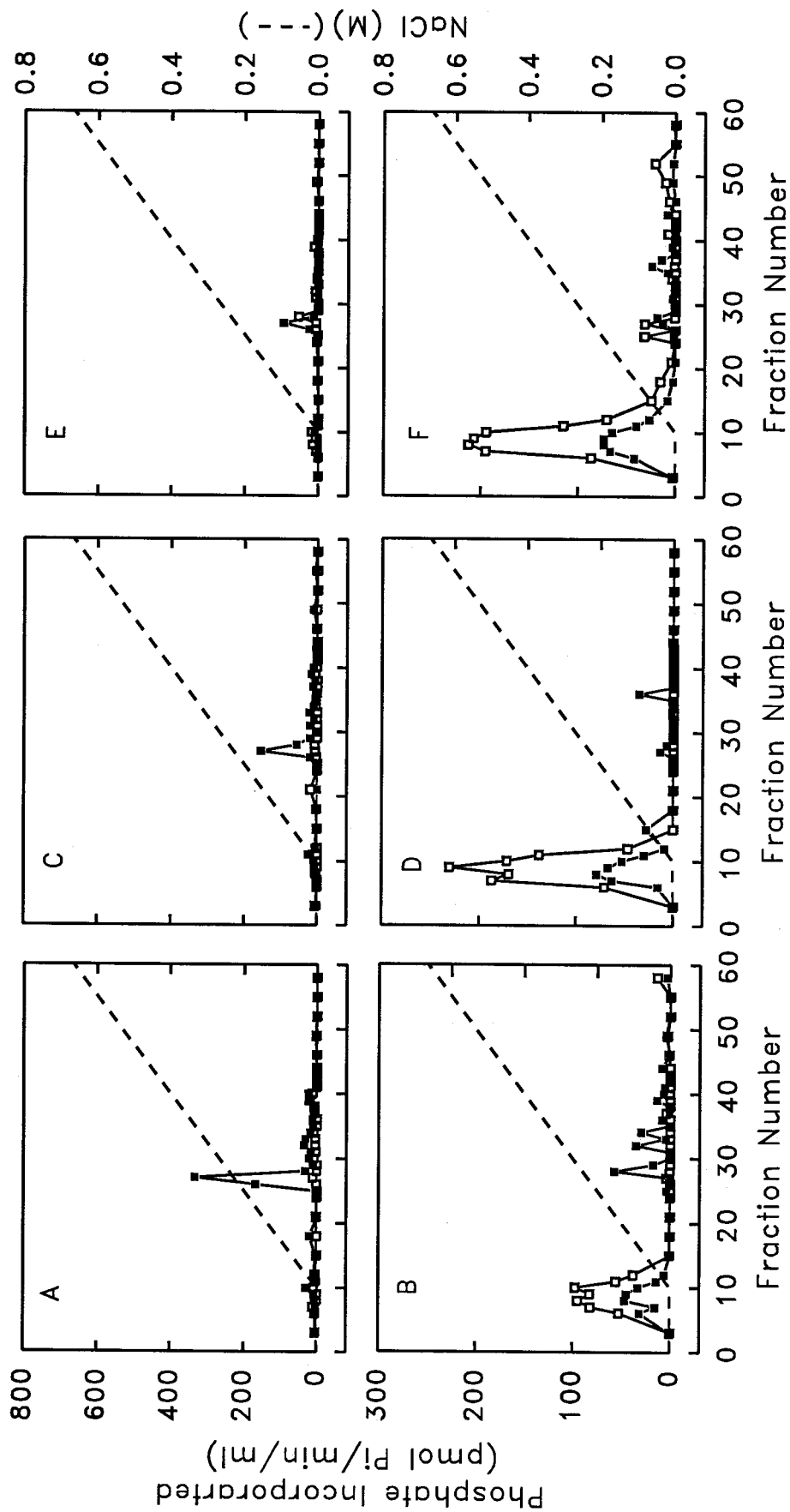


Figure 34. PK C activities of FPLC profiles of R59022 and OAG-treated myocyte cytosols (A, C & E) and membranes (B, D & F) incubated for 1 (A & B), 5 (C & D) and 10 min (E & F). PK C was assayed either in the presence of 1 μM Ca^{2+} (free), 80 $\mu\text{g/ml}$ phosphatidylserine and 8 $\mu\text{g/ml}$ 1-stearoyl-2-arachidonylglycerol (\blacksquare) or no added Ca^{2+} or lipids and 3 mM EGTA (\square). These data are each a representative plot from experiments that have each been repeated twice.

obtained with OAG treatment alone. As above, calculation of the AUC of the major peak of Ca^{2+} and lipid-dependent PK C activity (fractions 27-31) from both cytosol and membrane fractions was carried out to determine whether there were any differences between these PK C activity peaks and the AUC of the PK C activity peaks of the control, ethanol or OAG-treated myocytes. Figure 29 shows that the AUC of the peaks from both cytosol and membrane fractions isolated from R59022 and OAG-treated myocytes were similar to those obtained from ethanol or OAG-treated myocytes at 1 min. However, at 5 and 10 min the AUC was apparently lower than from either the ethanol or OAG-treated myocytes. These differences are reflected in both cytosol and membrane fractions. These results were not tested statistically due to the low number of experiments performed.

4. DISCUSSION

4.1. *Isolated Adult Rat Ventricular Myocytes*

Intact adult rat ventricular cardiac muscle cells (myocytes) were utilized for this study since they have several distinct advantages over isolated SR membrane vesicles and intact heart muscle preparations (see section 1.3.). Unlike isolated SR vesicles, myocytes contain the various regulatory mechanisms and structures necessary for contraction. Intact heart muscle preparations suffer from complications such as a heterogeneous cell population, intercellular interactions and multicompartimentation [Jacobson and Piper (1986)]. Isolated myocytes lack diffusional barriers and have the advantage of being a homogeneous cell preparation. As well, regulatory mechanisms can be studied without the complex syncytial, neuronal and hormonal effects that exist in the intact myocardium [Jacobson and Piper (1986)]. Finally, results obtained from isolated myocytes allow the specific localization of a particular activity to the muscle cell. In this study, the advantages of the isolated myocyte preparation experimentally, enabled investigations into protein kinase activities inside these cells. As well, by utilizing subcellular fractions from the myocytes, phosphorylated proteins and kinase activities were localized intracellularly. However, a disadvantage of the isolated myocyte preparation used in the present studies was unlike working myocytes or whole heart tissue these cells were in the quiescent state. The results of these studies therefore may not reflect the situation found in working myocytes during the excitation-contraction-relaxation cycle.

In order to successfully carry out the present studies, the isolated adult rat ventricular myocyte preparation was required to have a high yield/rat heart, a high proportion of viable cells and inter-preparation reproducibility. As well, the viable myocytes obtained were to possess the following qualities: Tolerance to

physiological temperature (37°C) and calcium concentrations (0.5-1.0 mM), quiescence (*i.e.* myocytes do not contract spontaneously), the ability to contract upon electrical stimulation and maintenance of characteristics such as rod-shaped morphology and SL membrane integrity for the duration of the experimental procedure. Several different methods of myocyte isolation were tried [Powell *et al.* (1980); Wittenberg and Robinson (1981); Farmer *et al.* (1983); Lundgren *et al.* (1984); Vander Heide *et al.* (1986); Langer *et al.* (1987); Haworth *et al.* (1989)] in an attempt to find a reproducible preparation, with the aforementioned cellular properties. However, many of these methods did not yield myocyte preparations with the above characteristics. The most difficult parameter to satisfy was the procurement of a high viable myocyte yield. It was found that this was greatly dependent on the type and lot of collagenase enzyme used for the cell dissociation steps as had been noted by other investigators [Jacobson (1989)]. The isolation of adult rat ventricular myocytes with the above characteristics was established using the methods outlined in section 2.2.1. The modified method of myocyte isolation by Li *et al.* (1988) and Wimsatt *et al.* (1990) (see section 2.2.1.2.) was found to produce preparations of viable myocytes on a more consistent basis than the modified method of Piper *et al.* (1982).

In addition to morphology, viability and contractility in response to electrical stimulation, isolated myocytes were characterized for their ability to produce cAMP. The results presented (see section 3.2.1. and Table 1) showed that the isolated myocytes retained the β -adrenergic receptor on the cell surface and the sarcolemmal intramembrane components of the adenylate cyclase system (G proteins and adenylate cyclase). As well, these results demonstrated that these components were still functionally coupled. In the isolated myocytes, the basal and stimulated levels of cAMP were higher than cAMP levels found in myocytes by other investigators [Buxton and Brunton (1983); Claycomb *et al.* (1984)]. There may be two reasons for this: 1) the incubation buffer contained 0.5 mM IBMX (a cAMP-

dependent phosphodiesterase inhibitor) which would have increased the amount of cAMP produced by inhibiting cAMP breakdown and 2) in Table 1, cAMP results were reported as pmol/ 10^4 viable rod-shaped cells. Many other studies [West *et al.* (1986); Millar *et al.* (1988); Bode and Brunton (1988); Pauwels *et al.* (1989)] report cAMP levels using units of pmol/mg protein, which are difficult to interpret, since these investigators do not state how many viable myocytes their preparation contains. Therefore, pmol/mg protein represents non-viable as well as viable myocytes.

4.2. *Sarcoplasmic Reticulum Membranes Isolated from Adult Rat Ventricular Myocytes*

4.2.1. Homogenization of Isolated Adult Rat Ventricular Myocytes

During these studies, it was noted that isolated myocytes were relatively robust to disruption by homogenization as compared to other cell types. Two different procedures were developed (see section 2.2.3.2.): One method (utilizing the 'Zero'-clearance homogenizer) succeeded in breaking 100% of the cells and releasing their cellular contents but the SR membranes isolated by this procedure had low Ca^{2+} -uptake and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities (Table 2) and were found to be highly contaminated with enzymatic activities from other cellular membranes (SL and mitochondrial). The second procedure (see section 2.2.3.2., using a glass douncer and a motorized Potter-Elvehjem) only broke 30% of the cells, but the SR membranes subsequently isolated retained enzymatic activities associated with the SR and contamination was held to a reasonably low level (< 10%). This method was used for the preparation of SR membranes for the studies involving protein phosphorylation.

4.2.2. Preparation of Sarcoplasmic Reticulum Membranes from Isolated Myocytes

There have been no previous reports of the isolation of SR membranes from isolated adult rat ventricular myocytes. In preliminary studies, an attempt was made to prepare SR membranes from frozen myocyte homogenates. However, it was not possible to obtain any SR membranes from these homogenates. Consequently, only fresh myocyte homogenates from freshly isolated cells were used to prepare SR membranes. Several methods for the preparation of SR membranes were used (see Table 2). With many of the methods, it was found that most of the cellular material (nuclei, myofibrils, mitochondria, SL, SR, *etc.*) was sedimented after the first centrifugation, leaving no material from which to purify SR membranes. It was concluded that upon homogenization, large complexes of cellular material formed, entrapping the membranes. Thus, attempts at further purification were unsuccessful. It was found that it was necessary to dilute homogenates (1 : 35 v/v, cells : Buffer) to prevent aggregation of membranes and proteins during the SR isolation procedure. In contrast, methods used for the homogenization of whole rat hearts prior to preparation of SR membranes only dilute the tissue 1 : 5 with homogenization buffer [Barker *et al.* (1988); Lamers and Stinis (1980); Feher and LeBolt (1990)].

Many studies of SR function and regulation utilize SR membrane vesicles prepared from canine heart [Wegener and Jones (1984); Chamberlain and Fleischer (1988)]. This tissue provides abundant starting material for the isolation of SR membranes and pure preparations of either network or junctional SR [Chamberlain and Fleischer (1988)]. However, due to the limited access to canine tissue and the unavailability of canine disease models, many investigators have preferred to use rat or guinea pig models. Utilizing whole rat heart(s), SR isolation methods now available yield preparations which contain both network and junctional SR membranes [Barker *et al.* (1988); Lopaschuk *et al.* (1983); Veleema and Zaagsma

(1981)]. An attempt was made to isolate SR membranes from myocytes by the method of Chamberlain and Fleischer (1988) since, with this method, purified network and junctional membranes can be separated. However, the Chamberlain and Fleischer method was optimized for canine heart and it was not possible to reproduce this preparation using isolated rat heart myocytes. Even when the buffers from this procedure were used together with the centrifugation protocol devised, the recovery of SR membranes was very low. This may be due to the presence of sucrose in the buffers which may have reduced the pelleting efficiency of the membranes to the point that SR vesicles could not be recovered from the small amount of starting material typically obtained from a myocyte isolation.

Subsequently, a method to isolate purified SR membranes from isolated adult rat ventricular myocytes was developed (see section 2.2.4.2.) based on the SR preparation of Harigaya and Schwartz (1969) as modified by Jones *et al.* (1979). For the isolation of SR membranes from myocytes during the phosphorylation studies, it was also necessary to use methods which prevented the dephosphorylation of proteins. Alterations in protein-bound phosphate due to the action of kinase, phosphatase or protease activities were minimized during membrane isolation through the use of a phosphate buffer system combined with the presence of NaF (a phosphatase inhibitor) and EDTA (to chelate Mg^{2+} and Ca^{2+} , thereby, inactivating Ca^{2+} -dependent proteases and phosphatases) [Lindemann *et al.* (1983)].

4.2.3. Enzymatic Activities and Protein Profile of Myocyte Sarcoplasmic Reticulum

The myocyte SR preparation developed contained the two major structural types of SR, network and junctional SR. The presence of phospholamban and the Ca^{2+}/K^{+} -ATPase (Figures 3, 5 and 6) was evidence for the existence of network SR. The presence of junctional SR in our preparation was illustrated by the occurrence of calsequestrin (Figure 4) and the presence of the Ca^{2+} -release channel as

indicated by an increase in Ca^{2+} -uptake activity upon the addition of ruthenium red (Table 3), a known blocker of the channel [Nagasaki and Fleischer (1988)].

Oxalate-stimulated Ca^{2+} -uptake activity was used as a specific marker for SR since it had previously been shown that mitochondrial and sarcolemmal membranes did not support oxalate-facilitated Ca^{2+} -transport [Jones *et al.* (1979); Solaro and Briggs (1974)]. In the presence of oxalate, the specific Ca^{2+} -uptake activity of the myocyte SR preparation prepared in control buffers increased 18-fold upon isolation from the myocyte homogenate (Table 3). Approximately 60% of the oxalate-stimulated Ca^{2+} -uptake activity present in the homogenate was recovered in the final SR preparation. In addition, oxalate-stimulated Ca^{2+} -uptake activity was increased in the SR preparation by 97% in the presence of ruthenium red. The specific activity of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase of the myocyte SR preparation prepared in control buffers increased 5.6-fold upon isolation from the myocyte homogenate and only 9% of the activity in the homogenate was recovered in the final SR preparation (Table 4). It is unclear why, during the purification of SR membranes, the specific activity of Ca^{2+} -uptake increased by 18-fold and 60% of this activity was recovered, while the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase specific activity only increased 5.6-fold and only 9% of this activity was recovered. Since these activities are functions of the same enzyme, the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase, it would be reasonable to expect that the same percentage of these activities would have been recovered. Further, it would seem unlikely that the ATPase activity of the enzyme could have deteriorated during the SR isolation procedure while the uptake activity remained intact, since the ATPase activity is required for uptake activity. Several different factors may contribute to this apparently paradoxical result: There are slight differences in the buffers and methods used for the determination of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase from those utilized for Ca^{2+} -uptake activity. As well, $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity was determined at steady state and Ca^{2+} -uptake activity was determined at initial rates. The measurement of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity is not as specific an activity of the SR as is the Ca^{2+} -

uptake activity, since there are other Ca^{2+} -stimulated ATPases and processes in the cell that utilize ATP while only the SR membrane can support oxalate-stimulated Ca^{2+} -uptake activity [Jones *et al.* (1979); Solaro and Briggs (1974)]. In the assay for $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity, ouabain and NaN_3 were included to inhibit the other two major ATPases in heart tissue, the $\text{Na}^{+}/\text{K}^{+}$ -ATPase and the mitochondrial ATPase, respectively. However, it is not unlikely that there may be another Ca^{2+} -stimulated ATP-utilizing activity that may have interfered with the measurement of SR $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity, especially in the homogenate. If the value obtained for SR $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity in the myocyte homogenate was artifactually increased by other ATP-utilizing activities, this may account for the low recovery of specific activity calculated in the final SR fraction.

The "coupling ratio" refers to the stoichiometry of Ca^{2+} -transport relative to ATP hydrolysis and is defined as the net Ca^{2+} -flux divided by the net ATPase rate mediated by the $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase [Feher and Fabiato (1990)]. The stoichiometry of the Ca^{2+} -pump reaction is 2 Ca^{2+} ions transported for each ATP molecule hydrolyzed [Yamada *et al.* (1970)]. Typical "coupling ratio" values found in canine SR by other authors range from 0.3 to 0.8 [Chamberlain *et al.* (1983); Jones and Cala (1981); Feher and Lipford (1985)]. Values for rat SR show a greater variation, ranging from 0.03 to 2.0 (calculated from Table 5). In the myocyte SR preparation used, the coupling ratio found (in the presence of ruthenium red), 0.5, is within these two ranges. Most often, the coupling ratio is approximated by simultaneously measuring the rate of oxalate-stimulated Ca^{2+} -uptake and ATP hydrolysis. However, most of the above authors (including the present studies) did not measure Ca^{2+} -uptake and ATPase activity simultaneously. Nevertheless, this calculation demonstrates that the specific activities of Ca^{2+} -uptake and ATPase obtained in the myocyte SR membranes used, appear to be coupled in a similar manner to previously published SR preparations.

The Ca^{2+} -uptake activity of the myocyte SR preparation developed was similar to SR preparations from whole rat heart reported by DeFoor *et al.* (1980) and Naylor *et al.* (1975) but higher than that found in other studies in whole rat heart SR preparations [Lopaschuk *et al.* (1983); Ganguly *et al.* (1983); Limas (1978); Wei *et al.* (1976); Lamers and Stinis (1980); Narayanan (1983); Table 5]. Two SR preparations from whole rat heart had higher Ca^{2+} -uptake activity [Penpargkul *et al.* (1980); Barker *et al.* (1988)]. Recently, Wimsatt *et al.* (1990), using digitonin-lysed adult rat ventricular myocytes, measured Ca^{2+} -uptake into the SR of myocytes at a rate of 160 nmol/min/mg protein in the presence of ruthenium red. This compares favorably with the Ca^{2+} -uptake activity found in this study in the presence of ruthenium red (208 nmol/min/mg protein). The myocyte SR $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity was similar to that found by Lopaschuk *et al.* (1983) and Narayanan (1983) and was higher than that obtained using the SR preparations from whole rat heart [Ganguly *et al.* (1983); Limas (1978); Wei *et al.* (1976); Penpargkul *et al.* (1980); Lamers and Stinis (1980)]. Only one SR preparation from whole rat heart [Barker *et al.* (1988)] possessed $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase and Ca^{2+} -uptake activities which were both higher than the SR preparation from myocytes reported here. However, this preparation [Barker *et al.* (1988)] consisted mainly of network SR and required a minimum of 5 rat hearts and 18 hours to prepare. Recently, Feher and LeBolt (1990) have developed an SR preparation from whole rat heart, stabilized by sodium metabisulfite, which exhibited very high $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase and Ca^{2+} -uptake activities (1,070 and 360 nmol/min/mg protein, respectively). It remains to be determined whether these high values truly reflect the activities present in the intact rat myocardium.

To measure contamination of the isolated SR membranes with enzyme activities from other organelles, determinations of ouabain-sensitive $\text{Na}^{+}/\text{K}^{+}$ -ATPase (SL marker) and cytochrome c oxidase (inner mitochondrial membrane) activity were carried out. Only 7% of the total sarcolemmal ouabain-sensitive

Na⁺/K⁺-ATPase activity and 8% of the total mitochondrial cytochrome c oxidase activity of the homogenate were recovered in the final myocyte SR preparation (Table 6). There are relatively few reports in the literature on the contamination of isolated SR membranes with enzyme activities from other organelles of the rat heart. Some reports present the specific activity of SL marker enzymes, but this activity is not a true quantitation of how much of the contaminating enzyme activity appears in the SR preparation. The specific activity is rather a reflection of the ability to isolate this enzymatic activity in an intact form. Values of contamination of isolated canine SR by sarcolemmal and mitochondrial enzyme activities, expressed as total activity, have been published by Chamberlain and Fleischer (1988). These authors found 7 - 10% contamination by SL enzymatic activity and 2% contamination by mitochondrial enzyme activity. Therefore, the contamination by SL and mitochondrial enzyme activity, by 7 and 8% respectively, of the myocyte SR preparation in this current study is comparable to that found in the SR membrane prepared from canine heart by other investigators.

4.2.4. Comparison of Myocyte Sarcoplasmic Reticulum Membranes Prepared in Control Buffers Versus Buffers Used to Prevent Dephosphorylation

For the isolation of SR membranes from myocytes during the phosphorylation studies, it was necessary to use methods which prevented the dephosphorylation of proteins during the procedure. These methods employed the same centrifugation protocol as was utilized during the isolation of SR in control buffers, but used a different buffer system. This change in buffer resulted in the isolation of SR membranes with a similar protein profile (Figure 7) but with slightly different enzymatic properties (Figures 7, 8 and 9) than SR isolated in control buffers. When buffers to prevent dephosphorylation were used to prepare SR membranes the yield of SR protein obtained was less (194 ± 45 μ g SR protein; mean \pm S.D., from 3×10^6

rod-shaped myocytes; n=5) when compared to that obtained using control buffers (542 ± 50 μ g SR protein; mean \pm S.D., from 3×10^6 rod-shaped myocytes; n=5).

There was no difference found between the oxalate-stimulated Ca^{2+} -uptake specific activities (Tables 3 and 7) in these two SR preparations (107.3 ± 3.8 , mean \pm S.E.M., n = 6 (control buffers) *vs* 116.1 ± 20.8 , mean \pm S.E.M., n = 5 (dephosphorylation buffers) unpaired t-test $p > 0.05$). However, only 22% of the total oxalate-stimulated Ca^{2+} -uptake activity was recovered in the SR membranes prepared in buffers to prevent dephosphorylation (Table 7), as compared to 60% in control buffers (Table 3). As well, while using buffers to prevent dephosphorylation, there was a 33-fold increase in the Ca^{2+} -uptake specific activity in the presence of oxalate during the isolation of the SR fraction from the homogenate, whereas in control buffers this activity had increased only 18-fold in the SR fraction as compared to the homogenate. Using buffers to prevent dephosphorylation (Table 7), oxalate-independent Ca^{2+} -uptake activity increased 5.3-fold in the SR membranes when compared with the activity found in the homogenate. In SR membranes prepared in control buffers, this activity was only increased by 2.4-fold over that found in the homogenate. Finally, ruthenium red increased oxalate-stimulated Ca^{2+} -uptake specific activity in the SR fraction prepared in control buffers by 97% (Table 3), whereas ruthenium red only increased this activity by 35% in SR preparations prepared in buffers to prevent dephosphorylation (Table 7).

The specific activity of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase of SR membranes prepared in buffers to prevent dephosphorylation (Table 8) was decreased by 40% as compared to the activity from SR membranes prepared in control buffers (Table 4 and 8; 414 ± 61 , mean \pm S.E.M., n = 6 (control buffers) *vs* 250 ± 72 , mean \pm S.E.M., n = 3 (dephosphorylation buffers)). The specific activity of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase (250 ± 72 nmol/min/mg protein; mean \pm S.E.M.; n = 3) found in the presence of buffers to prevent dephosphorylation in this study compares well to that found in another study where SR membranes were also isolated in the same buffers from whole rat

heart (333 ± 5 nmol/min/mg protein; mean \pm S.E.; $n = 148$; Lindemann *et al.* (1983)). During the isolation procedure in buffers to prevent dephosphorylation, the specific activity of $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase in the SR membranes increased only 2-fold over the activity found in the homogenate, whereas in control buffers there was a 5.6-fold increase; only 1.4% of the total $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activity in the homogenate was recovered as compared to 9% recovery in SR membranes prepared in control buffers.

SR membranes prepared in buffers to prevent dephosphorylation also demonstrated less contamination by SL and mitochondrial enzymatic activities (Table 9): Only 2.2% of the total sarcolemmal ouabain-sensitive $\text{Na}^{+}/\text{K}^{+}$ -ATPase activity and 4.7% of the total mitochondrial cytochrome c oxidase activity in the homogenate was recovered in the SR membranes, whereas 7% and 8% of these activities, respectively, were recovered in the SR membranes prepared in control buffers (Table 6). Ouabain-sensitive $\text{Na}^{+}/\text{K}^{+}$ -ATPase specific activity in SR membranes increased 3.8-fold over the activity in the myocyte homogenate in buffers to prevent dephosphorylation as compared to an increase of 6.5-fold in the SR membranes prepared in control buffers. In SR membranes isolated in buffers to prevent dephosphorylation, cytochrome c oxidase specific activity increased 9.7-fold during the purification of SR membrane vesicles from myocyte homogenates, whereas in SR membranes isolated in control buffers this activity was increased 11.5-fold.

These differences in enzymatic activities between SR membranes isolated in control buffers and in buffers to prevent the dephosphorylation of proteins may be due to effects on the extraction and sedimentation of SR membrane vesicles during the isolation procedure by components of the dephosphorylation buffer. However, even though there were some enzymatic activity differences between these two SR preparations, since their protein profiles were similar, these differences did not appear to affect the results obtained on the phosphorylation of proteins in intact ventricular myocytes.

4.3. *Protein Phosphorylation in Isolated Adult Rat Ventricular Myocytes Stimulated with PK A and PK C*

In early studies on the nucleotide, adenosine triphosphate (ATP), it was noted that the terminal phosphate group participated in transfer reactions as reviewed by Barany and Barany (1981). Subsequently, the hydroxyl groups of serine, threonine and tyrosine were shown to be acceptors for the γ -phosphate group of ATP. Now it is possible to phosphorylate all nucleophilic amino acid residues in proteins and that the phosphate transferred can originate from a variety of sources [Vener (1990)]. There are two major types of protein phosphorylation, catalytic and regulatory. Catalytic protein phosphorylation was found to include the formation of phosphorylated intermediates in the active sites of enzymes catalyzing the phosphoryl group transfer [Vener (1990); Knowles (1980)]. Physiologically, the first interest in protein phosphorylation occurred when cAMP was found to activate a protein kinase (PK A) isolated from rabbit skeletal muscle [Walsh *et al.* (1968)]. Since that time, many other kinases and functions of protein phosphorylation have been and are still being elucidated. Regulatory protein phosphorylation is the most common type of reversible post-translational modification of proteins. It controls almost all cellular processes by altering the catalytic activity or functional properties of other proteins [Cohen (1985); Krebs (1986)].

4.3.1. *Proteins Phosphorylated in Response to PK A Activation*

Results presented in this study (see section 3.4.1.) indicate that stimulation of PK A by the β -adrenergic receptor agonist, isoproterenol, specifically increased the phosphorylation of 5 protein bands (8.5, 15, 27, 31 and 152 kDa; Figure 8.A.). The 152 kDa protein was tentatively identified as C-protein by its molecular mass. C-protein is a myofibril protein and a major substrate of PK A in the heart

[Robinson-Steiner and Corbin (1986)]. C-protein was previously found to be phosphorylated in isoproterenol-stimulated saponin-permeabilized [Miyakoda *et al.* (1987)] and intact adult rat myocytes [Onorato and Rudolph (1981); Blackshear *et al.* (1984); George *et al.* (1991)]. The phosphorylated protein band at 31 kDa was tentatively identified as troponin I by its molecular mass. Troponin I usually displays a molecular mass of 28 kDa, however, in isolated perfused rat hearts, troponin I has been found to be phosphorylated in response to β -adrenergic stimulation and have a molecular mass ranging from 28 to 30 kDa [Karczewski *et al.* (1990); Le Peuch *et al.* (1980)]. Several other studies on the phosphorylation of proteins in isolated adult rat myocytes [Onorato and Rudolph (1981); Blackshear *et al.* (1984); Miyakoda *et al.* (1987)] have noted the phosphorylation of a 28 kDa protein, which they tentatively identify as troponin I. Recently, George *et al.* (1991) have found a 31 kDa protein phosphorylated in response to isoproterenol-treatment of isolated adult rat ventricular myocytes and have identified this protein as troponin I using a monoclonal antibody to cardiac troponin I. The 8.5, 15 and 27 kDa phosphorylated protein bands in this study were identified as the SR protein, phospholamban, on the basis of mobility on SDS-PAGE gels and by immunoblotting (Figures 6 and 8.A.). Phosphorylated phospholamban has also been identified following isoproterenol-stimulation of both saponin-permeabilized [Miyakoda *et al.* (1987)] and intact isolated adult rat myocytes [Blackshear *et al.* (1984); George *et al.* (1991)]. Previously, a 15 kDa protein from the SL membrane has been found to be phosphorylated in response to isoproterenol in the perfused guinea pig heart [Presti *et al.* (1985)]. In the present study, there was a phosphorylated band in the SR membranes from control myocytes at 15 kDa, whose phosphorylation appeared to increase in SR membranes isolated from isoproterenol-stimulated myocytes. The 15 kDa protein band in the present study was probably not the result of a contaminant from the SL since this band was recognized by the phospholamban monoclonal antibody (Figure 6). In addition, boiling the SR membranes isolated from

isoproterenol-stimulated myocytes prior to SDS-PAGE appeared to increase the intensity of this band (Figure 8.A.) whereas the SL 15 kDa band was not affected by boiling [Presti *et al.* (1985)]. These results suggest that the 15 kDa band in myocyte homogenates and SR membranes was a form of phospholamban (di- or trimeric).

Isolated myocytes were also treated with forskolin to activate PK A by direct action on adenylate cyclase (Figure 10.A.). Forskolin treatment was found to stimulate the phosphorylation of 4 proteins (152, 31, 27 and 8.5 kDa) in a crude membrane fraction isolated from the myocytes. Using isolated adult myocytes [George *et al.* (1991)] and in isolated perfused rat heart [England and Shahid (1987)], forskolin was found to stimulate the phosphorylation of troponin I and C-protein. Also, Fliegel and Drummond (1985) using perfused guinea pig heart, found that forskolin stimulated the phosphorylation of a 25 kDa protein, which was tentatively identified as phospholamban. Thus, regardless of whether the myocytes were treated with isoproterenol or forskolin, PK A increased the phosphorylation of the same proteins: C-protein, troponin I and phospholamban.

Several authors [Miyakoda *et al.* (1987); Blackshear *et al.* (1984); George *et al.* (1991); Onorato and Rudolph (1981)] have shown antagonism of the effects of isoproterenol on protein phosphorylation in isolated ventricular myocytes by propranolol (a β -receptor antagonist). This competition would, however, occur at the level of the receptor and not at the level of the kinase. In this study, an attempt was made to inhibit the actions of PK A directly by utilizing two isoquinolinesulfonamides, HA1004 and H-8. Both of these inhibitors display selectivity for PK A (K_i (μ M): 2.3 and 1.2, respectively) and inhibit PK A by competing with ATP for the free enzyme at the active site [Hidaka *et al.* (1984); Hagiwara *et al.* (1987)]. In the present study, neither of the two compounds (100 μ M (HA1004) or 1 mM (HA1004 or H-8)) were able to inhibit the stimulation of protein phosphorylation in intact myocytes treated with isoproterenol (0.01 or 1.0 μ M; Figures 11.A. and 12.A.). This lack of an inhibitory effect on PK A catalyzed

protein phosphorylation by these inhibitors may reflect an inability of these compounds to permeate the myocyte and to inhibit PK A in the compartments where it is localized [Buxton and Brunton (1983)].

4.3.2. Proteins Phosphorylated in Response to PK C Activation

In experiments using isolated SR membranes Ca^{2+} /phospholipid-dependent protein kinase (PK C) was shown to phosphorylate phospholamban with a concurrent increase in Ca^{2+} -uptake [Movsesian *et al.* (1984)]. PK C was also found to phosphorylate isolated cardiac troponin I and troponin T, either in the free form or in the troponin-tropomyosin complex [Kato *et al.* (1983); Risnik *et al.* (1987)]. PK C has also been implicated in the decreased Ca^{2+} -sensitivity of the myofilaments due to the phosphorylation of troponin I and T [Gwathmey and Hajjar (1990)]. In SL membranes isolated from canine heart, PK C was found to phosphorylate 7 different proteins [Yuan and Sen (1986)]. In perfused adult rat hearts, activators of PK C have been found to decrease the rate and force of contraction [Yuan *et al.* (1987)]. However, recently MacLeod and Harding (1991) found that treatment of isolated adult rat and guinea pig ventricular myocytes with phorbol esters results in a positive inotropic effect, which may be due to a PK C-induced increase in systolic Ca^{2+} . In addition, phospholamban, troponin I and C-protein were found not to be phosphorylated in response to PK C stimulation of the perfused heart but a 28 kDa cytosolic protein was phosphorylated [Edes and Kranias (1990)]. No studies on the phosphorylation of proteins by PK C have been carried out in isolated adult ventricular myocytes.

Phorbol esters are tetracyclic diterpenes isolated from croton oil [Bohm *et al.* (1935)] which were found to be tumor promoting on mouse skin [Berenblum (1941)]. Tumor-promoting phorbol esters have been shown to directly activate PK C [Castagna *et al.* (1982)] by substituting for naturally occurring diacylglycerols.

Non-tumor promoting phorbol esters have been shown not to affect PK C activity [Castagna (1987)]. In addition, PK C was found to act as a receptor for phorbol esters [Castagna *et al.* (1982); Driedger and Blumberg (1980)].

Although phorbol esters can be used to activate PK C in cells and tissues, the actual physiological activator of PK C is diacylglycerol (DAG). Because phorbol esters bypass the normal membrane signal transduction pathway, results with these compounds may be affected by the nonparticipation of some regulatory signals. It has also been observed that certain phorbol esters will only activate certain PK C isozymes [Ryves *et al.* (1991); Robles-Flores *et al.* (1991)]. Some studies have concluded that the effects of phorbol esters were independent of PK C activation [Murphy *et al.* (1991); Kraft *et al.* (1986); Motasim Billah *et al.* (1989); Tao *et al.* (1989); Pollock *et al.* (1986); Matsumoto *et al.* (1988)]. It has also been found that phorbol esters are not specific for PK C but can bind to another protein, neuronal chimaerin [Ahmed *et al.* (1990)]. Phorbol esters and cell-permeant diacylglycerols have frequently been used to activate PK C in intact tissues. However, there are some differences with respect to their effects on cellular functions and in their activation of PK C. In GH₃ pituitary cells, diacylglycerol activates a potential inhibitory pathway for PK C but phorbol esters did not [Kolesnik and Clegg (1988)]. In addition, in these cells phorbol ester inhibited K⁺-induced Ca²⁺ influx while diacylglycerol did not [MacEwan and Mitchell (1991)]. Further, diacylglycerol inhibits cAMP production in granulosa cells but phorbol esters do not [Shinohara *et al.* (1985)]. In HL-60 cells, tumor promoting phorbol ester caused 95% of the cells to adhere to culture dishes [Yamamoto *et al.* (1985)] and a 40% decrease in cellular ATP [Dawson *et al.* (1987)], whereas diacylglycerol had no effect on either of these parameters. In GH₄C₅ pituitary cells, phorbol esters and diacylglycerols both activate PK C but their actions on PK C-mediated functions such as prolactin release and synthesis and cell stretching differ [Ramsdell *et al.* (1986)]. In islet cells, diacylglycerol induced an increase in cytosolic

Ca^{2+} but phorbol ester did not [Thomas *et al.* (1991)]. In liver plasma membranes, phorbol esters were found to stimulate the phosphorylation of 10 proteins but DAG only increased the phosphorylation of 6 proteins [Kiss and Luo (1986)]. In rat pancreatic acini, unlike phorbol ester, DAG had no effect on basal or stimulated amylase release [Brockenbrough and Korc (1987)]. Finally, it must be kept in mind that addition of DAG or phorbol esters to activate PK C are an attempt to mimic an intracellular function and that their activation or inhibition of cellular processes may not reflect the actual situation inside the cell. An example of this is a study which suggests that OAG and phorbol esters depress the Ca^{2+} current in sensory neurons independent of their effects as activators of PK C [Hockberger *et al.* (1989)].

Since there are many reported differences between phorbol esters and diacylglycerols in the activation of PK C, studies were carried out with both phorbol esters and a synthetic diacylglycerol. In this study, isolated adult rat ventricular myocytes were treated with a tumor promoting and a non-tumor promoting phorbol ester (Figure 13). Both phorbol esters (in 0.4% DMSO) stimulated the phosphorylation of two protein bands of phospholamban (8.5 and 27 kDa). These results are interesting in that the active and inactive phorbol esters were apparently able to phosphorylate phospholamban, in contrast to a previous study, in perfused guinea pig heart, where tumor promoting phorbol esters did not stimulate the phosphorylation of phospholamban [Edes and Kranias (1990)]. Diacylglycerols such as the cell permeant synthetic diacylglycerol, OAG, have detergent-like properties which allow them to activate PK C without damaging intact cell membranes. In this study, OAG (in 5% DMSO) treatment of isolated adult myocytes was found to phosphorylate the two protein bands of phospholamban at 8.5 and 27 kDa (Figure 14). In this experiment, it was also found that 5% DMSO, by itself, could phosphorylate these two bands of phospholamban. There appeared to be no differences between DMSO, with and without OAG in the phosphorylation of phospholamban. It therefore seems

reasonable to conclude that OAG may not have contributed to the phosphorylation of phospholamban and that DMSO was solely responsible for this phosphorylation. This result shed a different light on the results obtained with tumor promoting and non-tumor promoting phorbol esters which were also dissolved in DMSO (0.4%). It now appears highly likely that the phosphorylation of phospholamban obtained with both active and inactive phorbol esters may have been due to the DMSO and not to the actions of the phorbol esters. It appears that the DMSO effect on protein phosphorylation may also be concentration-independent since, at both 0.4 and 5% DMSO, phospholamban became phosphorylated. To further investigate this solvent effect, isolated myocytes were treated with OAG dissolved in ethanol (10%; Figure 15.A.). These results show that neither ethanol, by itself or with OAG, was able to stimulate the phosphorylation of proteins in intact myocytes. Treatment of myocytes with a higher concentration of OAG and a lower concentration of ethanol (Figure 16) also did not consistently result in the stimulation of protein phosphorylation in isolated adult rat ventricular myocytes.

4.3.3. Calcium/Calmodulin-dependent Protein Kinase Involvement

Previous studies have shown that Ca^{2+} /calmodulin-dependent protein kinase (CAM PK) phosphorylates phospholamban in the perfused heart [Le Peuch *et al.* (1980); Wegener *et al.* (1989); Lindemann and Watanabe (1985); Karczewski *et al.* (1987); Vittone *et al.* (1990)] and in isolated SR vesicles [Davis *et al.* (1983); Molla *et al.* (1985); Plank *et al.* (1983); Katz and Remtulla (1978); Le Peuch *et al.* (1979)] with an associated increase in the rate of Ca^{2+} -uptake and in the affinity of the Ca^{2+} -pump for Ca^{2+} [Katz and Remtulla (1978); Davis *et al.* (1983); Plank *et al.* (1983)]. The physiological significance of CAM PK phosphorylation is not understood as, in the perfused heart, it does not occur in response to elevated extracellular Ca^{2+} [Lindemann and Watanabe (1985); Vittone *et al.* (1990)] but only

after β -receptor activation and PK A phosphorylation of phospholamban [Wegener *et al.* (1989); Karczewski *et al.* (1987)]. Recently, Colyer and Wang (1991) have found that the additional phosphorylation of phospholamban by CAM PK subsequent to phosphorylation by PK A, did not increase SR Ca^{2+} -pump activity above the level achieved by PK A phosphorylation. It has also been suggested that in perfused rat hearts, CAM PK may only be able to phosphorylate phospholamban when intracellular cAMP levels are high [1 pmol/mg wet wt.; Vittone *et al.* (1990)]. In addition, Gasser *et al.* (1988) have shown that CAM PK may preferentially phosphorylate phospholamban in junctional SR and PK A may preferentially phosphorylate phospholamban in network SR.

To investigate the possible effect of CAM PK on the protein phosphorylation observed with PK A in isolated myocytes, CGS 9343 B was used to specifically inhibit the Ca^{2+} /calmodulin protein kinase (CAM PK). CGS 9343 B is four times more potent than trifluoperazine as an inhibitor of calmodulin activity and does not inhibit the activity of PK C at concentrations over 200 μM [Norman *et al.* (1987); Hill *et al.* (1988)]. This compound has been found to be effective in inhibiting IP_3 -stimulated Ca^{2+} -release in rat liver epithelial cells at a concentration of 110 μM [Hill *et al.* (1988)] and at 10 μM , protected perfused pig hearts from reperfusion injury subsequent to ischemia [Das *et al.* (1989)]. In the present study, pre-treatment of isolated myocytes with CGS 9343 B had no effect on the stimulation of protein phosphorylation by isoproterenol (Figure 17). This result indicates that in isolated adult myocytes, CAM PK was probably not involved in the stimulation of protein phosphorylation by PK A.

4.3.4. Protein Phosphorylation in Response to PK A and PK C Activation

To date, there have been no studies on protein phosphorylation in any experimental model of the heart subsequent to activation of both PK A and PK C.

As described in section 4.3.3., in perfused hearts treated with isoproterenol, a synergistic phosphorylation of phospholamban occurs due to the activation of both PK A and CAM PK. However, these kinase pathways appear to operate under different conditions and, perhaps, in different areas of the SR (see section 4.3.3.). Since PK A and PK C have both been shown to phosphorylate phospholamban in SR vesicles and purified preparations of troponin I, the effect of the simultaneous activation of both of these kinases on protein phosphorylation in isolated adult ventricular myocytes was investigated. The results presented here show that in the presence of OAG and isoproterenol, together, the same proteins were phosphorylated as when myocytes were treated with isoproterenol alone (Figure 18). To further investigate the incorporation of radioactivity, the protein bands whose phosphorylation was stimulated by isoproterenol (8.5 and 27 kDa forms of phospholamban, troponin I and C-protein) were quantitated by liquid scintillation counting (Figure 19 and 20). In myocyte homogenate samples, there were no apparent differences in the incorporation of radioactivity into phospholamban, troponin I or C-protein between control, isoproterenol, OAG or isoproterenol plus OAG-treated myocytes. However, in myocyte SR membranes, there was an apparent increase in the incorporation of radioactivity into the two bands of phospholamban from isoproterenol-treated myocytes as compared with SR membranes from control, OAG and isoproterenol plus OAG-treated myocytes. There appeared to be no apparent difference in the incorporation of radioactivity into the two bands of phospholamban between SR membranes prepared from control, OAG or isoproterenol plus OAG-treated myocytes. The interpretation of these results is limited due to the wide variation of the data and by the small number of experiments performed.

Since Edes and Kranias (1990) have shown that PK C phosphorylated a 28 kDa cytosolic protein and Katoh *et al.* (1981) have found that PK C phosphorylated a 38 and a 49 kDa cytosolic protein in guinea pig heart, the phosphorylation of

cytosolic proteins was examined in isolated adult rat ventricular myocytes. The incorporation of radioactivity into cytosolic fractions isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes shows that the phosphorylation state of proteins at 21, 24, 31 and 152 kDa appeared to change with the different treatments (Figure 21). The phosphorylation of the 21, 31 (troponin I) and 152 (C-protein) kDa proteins were apparently increased in the cytosolic fractions from isoproterenol and OAG plus isoproterenol-treated myocytes as compared to that found in cytosolic fractions from control and OAG-treated myocytes. The results for troponin I and C-protein are in agreement with the results obtained earlier with isoproterenol treatment of myocytes. The phosphorylation of the 24 kDa protein appears to decrease in the cytosolic fractions from isoproterenol and OAG plus isoproterenol-treated myocytes as compared to that found in cytosolic fractions from control and OAG-treated myocytes. The identity of the 21 and 24 kDa proteins is unknown at this time. Quantitation of the radioactivity incorporated into these bands by liquid scintillation counting confirms the above result for troponin I and C-protein. The incorporation of radioactivity into the 24 kDa protein was the highest in the cytosol from control myocytes with decreased incorporation of radioactivity into this protein from cytosolic fractions prepared from isoproterenol, OAG and OAG plus isoproterenol-treated myocytes. Regarding the 21 kDa protein, the incorporation of radioactivity was similar between the cytosolic fractions isolated from control, isoproterenol and OAG plus isoproterenol-treated myocytes but decreased in cytosolic fractions from OAG-treated myocytes. However, interpretation of these results is limited due to the wide variation in the data and the small number of experiments performed. Unlike the previously reported studies [Edes and Kranias (1990); Katoh *et al.* (1981)], in the present study, neither a 28, 38 or 49 kDa cytosolic protein was found to be phosphorylated in either OAG or OAG plus isoproterenol-treated myocytes.

4.4. *Phosphorylated Species of Oligomeric Phospholamban*

A pentamer of phospholamban contains a total of 10 possible phosphorylation sites [Simmerman *et al.* (1986)]. Since the phosphorylation of phospholamban reduces its electrophoretic mobility on SDS-PAGE gels under certain conditions [Wegener and Jones (1984)] western immunoblots of these gels demonstrate multiple bands of the phospholamban pentamer as a result of filling one or both phosphorylation sites in each monomer. Following phosphorylation by PK A, 6 bands of phosphorylated pentameric phospholamban were observed, corresponding to the phosphorylation of monomers 0 to 5 at a single site [Li *et al.* (1990); Gasser *et al.* (1986); Imagawa *et al.* (1986); Wegener and Jones (1984); Wegener *et al.* (1989)]. Following phosphorylation by both PK A and CAM PK, eleven bands of phosphorylated pentameric phospholamban are observed, indicating the phosphorylation of monomers 0 to 5 at both phosphorylation sites.

Several authors have described the separation of oligomeric species of phosphorylated phospholamban from isolated canine SR [Li *et al.* (1990); Kasinathan *et al.* (1988); Gasser *et al.* (1988); Imagawa *et al.* (1986); Wegener and Jones (1984)] and from perfused guinea pig heart [Wegener *et al.* (1989)]. There have been no reports on the separation of oligomeric species of phosphorylated phospholamban from whole rat heart, rat SR preparations or from isolated adult rat ventricular myocytes.

In this study, four bands of phosphorylated pentameric phospholamban were obtained from control and dephosphorylated (with alkaline phosphatase) canine cardiac ventricular SR vesicles as described by Li *et al.* (1990) (Figure 22). Six bands of phosphorylated pentameric phospholamban were obtained from canine SR vesicles phosphorylated by the catalytic subunit of PK A (Figure 22). Using rat myocyte SR membranes, this method was not able to separate the phosphorylated pentameric species of phospholamban which all migrated as a single large band.

Another SDS-PAGE gel system was therefore developed (see section 2.2.5.2.1.2.) and on the immunoblot, four bands of phosphorylated pentameric phospholamban were separated from dephosphorylated (with alkaline phosphatase), seven bands from control and five bands from phosphorylated (with catalytic subunit of PK A) rat myocyte SR vesicles (Figure 23). A sample of canine SR vesicles phosphorylated by the catalytic subunit of PK A was separated into nine bands using this SDS-PAGE system (Figure 23). This was unusual since only six bands of phosphorylated pentameric phospholamban were expected. Perhaps, as seen in previous studies, an endogenous kinase activity was associated with the SR [Molla and Demaille (1986)] and during the phosphorylation reaction it was able to add to the species of phospholamban phosphorylated by the catalytic subunit of PK A. On inspection of these western blots (Figure 22 & 23), it is not clear why the phosphorylated phospholamban bands from canine SR vesicles appear to separate much more distinctly from one another than do the phosphorylated phospholamban bands from myocyte SR vesicles. However, this was a consistent result and may be due to some inherent differences between these two types of SR. In both the canine and myocyte SR vesicles, the control and dephosphorylated samples contain bands of phosphorylated phospholamban instead of only the dephosphorylated form. Again, it may be that under the control and dephosphorylation incubation conditions, an endogenous kinase activity associated with the SR was able to phosphorylate phospholamban into these phosphorylated forms or it may indicate that myocyte SR is in some way resistant to dephosphorylation.

The SDS-PAGE system developed to separate the phosphorylated forms of phospholamban in myocyte SR was used, together with immunoblotting, to determine the phosphorylated forms of pentameric phospholamban in SR membranes isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes (Figure 24). In homogenate and SR membranes from control and OAG-treated myocytes only two bands of phosphorylated pentameric

phospholamban were obtained. In homogenate and SR membranes from isoproterenol and OAG plus isoproterenol-treated myocytes, 5 bands of phosphorylated pentameric phospholamban were separated. Thus, the treatment of myocytes with OAG did not result in the phosphorylation of phospholamban above that found in control myocytes and did not affect the number of species of phosphorylated phospholamban formed in the presence of isoproterenol.

In addition to its importance as a regulatory mechanism in myocardial relaxation, the phosphorylation of phospholamban is modified in several disease states. In ischemic pig heart, with increasing duration of ischemia, phospholamban phosphorylation became increasingly reduced with a concurrent reduction in Ca^{2+} -pump activity [Schoutsen *et al.* (1989)]. In hyperthyroid hypertrophic rat hearts [Beekman *et al.* (1989)], there may be a decrease in the ratio of phospholamban to $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase in SR membranes. Endotoxin treatment of canine SR membranes resulted in an inhibition of phospholamban phosphorylation by CAM PK but not PK A and a stimulation in the rate of phospholamban dephosphorylation [Mohammed and Liu (1990)]. This last result may be significant to the mechanism of myocardial dysfunction in endotoxic shock. In idiopathic dilated cardiomyopathy studied in human failing hearts [Movsesian *et al.* (1990)], phospholamban mediated stimulation of Ca^{2+} -uptake was found to be normal. It remains to be determined what other roles phospholamban phosphorylation may play in other pathophysiological conditions of the heart. As well, the species of phosphorylated pentameric phospholamban formed in the functioning myocardium under normal and diseased conditions remains to be determined.

4.5. Activation of PK C in Isolated Adult Ventricular Myocytes

In the results presented in this study on the stimulation of protein phosphorylation by PK C in isolated adult rat ventricular myocytes (see section

3.4.2.), no proteins were specifically found to be phosphorylated by PK C, as had been found previously in perfused hearts [Edes and Kranias (1990); Katoh *et al.* (1981)]. As well, a PK C activator (OAG) had no significant effect on the phosphorylation of myocyte proteins by PK A stimulation. These results can be explained if PK C was found not to have been activated in the isolated myocytes. Thus, an examination into the activation of PK C in the intact adult ventricular myocyte was undertaken. There are three methods used to determine whether PK C has become activated in a cellular system [Mitchell *et al.* (1989)]: 1) to document the movement (*i.e.* translocation) of the kinase from the cytosol of the cell to the plasma membrane (or particulate fraction), 2) to isolate the kinase and determine the state of autophosphorylation and 3) to define possible protein targets for the enzyme and to determine whether they have been phosphorylated in response to treatments known to activate PK C in other cellular systems. Since results have already been presented to show that PK C was not able to phosphorylate specific substrate(s) in isolated adult myocytes in response to OAG-treatment, method 3) will not be discussed further. Instead, the autophosphorylation and translocation of PK C from myocyte cytosol to the membranes was investigated as a measure of PK C activation.

4.5.1. Translocation of PK C in Isolated Adult Rat Ventricular Myocytes

Due to the activation of PK C by phospholipids, diacylglycerols and other lipids, it was reasoned that the movement of the enzyme from its reservoir in the cytosol to the membrane must occur in order for the kinase to become activated [Gopalakrishna *et al.* (1986)]. Although, the mechanisms involved in this process are not fully known or understood, the following is a description of what is at present thought to occur [Nelsestuen and Bazzi (1991)]: PK C (inactive) in the cytosol, perhaps associated with microtubules [Ito *et al.* (1989)], becomes stimulated

(mechanism unknown) to travel to a membrane (for example: the plasma membrane) whereupon it can form two active membrane-associated states [Nelsetuen and Bazzi (1991)]. One state is dependent on low Ca^{2+} concentrations to bind to membrane phospholipids and is reversible [Nelsetuen and Bazzi (1991)]. This form of PK C can be removed from the membrane with Ca^{2+} chelators [Kikkawa *et al.* (1982)] and can bind phorbol esters; this binding reaction is also reversible [Bazzi and Nelsetuen (1989)]. The formation of the second membrane state, which is an irreversible complex, is promoted by increased Ca^{2+} concentrations and DAG or tumor-promoting phorbol esters. This irreversible complex behaves as an integral membrane protein and no longer requires Ca^{2+} , lipid or phorbol esters as cofactors for activity. In order to form this irreversible complex, it is thought that PK C undergoes a conformational change which allows a portion of the molecule to become inserted into the membrane [Bazzi and Nelsetuen (1988a)]. The irreversible membrane-bound form can be released by solubilizing the membrane in detergent [Bazzi and Nelsetuen (1988b)]. In addition, the catalytic fragment of PK C (PK M) can be generated from intact PK C by Ca^{2+} -dependent protease and is active in the absence of phospholipid and Ca^{2+} in the cytosol [Kishimoto *et al.* (1983)]. The possibility that the distribution of PK C between the cytosol and the membrane is in dynamic equilibrium controlled by levels of free intracellular Ca^{2+} has recently been proposed [Phillips *et al.* (1989)].

Unlike samples from rat brain and bovine trachea, chromatography of myocyte cytosolic fractions and detergent-solubilized membranes using DEAE-cellulose was found to be inadequate and did not allow detection of PK C activity (Table 10). In the present study, it was necessary to fractionate myocyte cytosolic fractions and detergent-solubilized membranes by FPLC prior to the assay of PK C activity, to partially purify the kinase and to remove the detergent from the membrane fraction. Fractionation of the myocyte cytosol and membrane samples by FPLC allowed the PK C activity to be subsequently stimulated by Ca^{2+} and lipids.

The specific activity of PK C in myocyte cytosol was found to be 726 pmol/min/mg protein. This value is approximately 12-fold higher than that found previously in cytosol from canine ventricles [Yuan and Sen (1986)], 17-fold higher than that previously found in cytosolic fractions from whole rat heart [Tanaka *et al.* (1991)] and 3-fold higher than that previously found in cytosolic fractions from perfused guinea pig heart [Edes and Kranias (1990)]. The use of FPLC fractionation may have removed an inhibitory or other conflicting endogenous activity in the myocyte cytosol and membrane fractions which interfered with the assay of PK C activity, since a large proportion of the protein in these samples did not bind very tightly to the Mono Q column (Figure 26). This inhibitory or other conflicting activity was apparently not removed by chromatography on DEAE-cellulose. In relation to this, Pearson *et al.* (1990) and Mozier *et al.* (1990) have purified two protein inhibitors (PKC inhibitors 1 and 2) of PK C from bovine brain. As well, there appeared to be a very large amount of PK A activity in the myocyte cytosol and membrane fractions (Figure 27). When the peptide inhibitor of PK A was included in the assay for PK C activity there was a substantial reduction in the number of interfering peaks, allowing the unmasking of kinase activity solely due to PK C.

PK C activity was measured in cytosol and membrane fractions isolated from control, ethanol, OAG (in ethanol) and OAG plus R59022-treated myocytes incubated for 1, 5 and 10 min. In each of the cytosol fractions isolated from myocytes from these 12 treatment groups, three peaks of Ca^{2+} and lipid-dependent PK C activity were eluted. The peak with the highest activity was always found in fractions 27-31 (Figure 28, 31, 32 and 34). In the control membrane fractions, two peaks of Ca^{2+} and lipid-dependent PK C activity were eluted and again the peak with the highest activity was usually found in fractions 27-31 (Figure 28, 31, 32 and 34). In the initial FPLC fractions (6 - 15) from each membrane sample, a large portion of the PK C activity peak contained Ca^{2+} and lipid-independent activity. It is not known at this time what other kinase activities may be included in this peak.

The major peak of Ca^{2+} and lipid-dependent PK C activity (fractions 27-31) from membrane fractions of ethanol and OAG-treated myocytes appeared to contain less PK C activity than the same peak from the control profiles.

Calculation of the area under the curve (AUC) of the major peaks of Ca^{2+} and lipid-dependent PK C activity (fractions 27-31) for the cytosolic and membrane preparations (Figure 29) show that in regards to incubation time, there were no significant differences (Two-way ANOVA, $p > 0.05$) between the peaks obtained from control, ethanol, OAG and OAG plus R59022-treated myocytes that had been incubated for 1, 5 or 10 min. When compared to the AUC from control peaks, there was significantly less PK C activity (Two-way ANOVA, $p < 0.05$) in the peaks from both the cytosol and membrane fractions isolated from ethanol and OAG-treated myocytes. When the AUC of the OAG-treated peaks was compared to the AUC of the ethanol-treated peaks, from both cytosol and membrane fractions, no significant differences were found (Two-way ANOVA, $p > 0.05$). The AUC of the peaks from both cytosol and membrane fractions isolated from R59022 plus OAG-treated myocytes were similar to those obtained from ethanol or OAG-treated myocytes at 1 min. However, at 5 and 10 min, the AUC was apparently lower than from either ethanol or OAG-treated myocytes, these differences are reflected in both cytosol and membrane fractions. Therefore, it appears that ethanol has decreased PK C activity in both cytosol and membrane fractions, including those treated with OAG, which was dissolved in ethanol. In addition to possible physical effects on the sarcolemmal membrane [Polimeni *et al.* (1983)], ethanol has several other effects on the myocardium. Ethanol (2.5 - 5%) has been shown to protect rat heart from calcium paradox injury [Auffermann *et al.* (1990)] and to increase the levels of cAMP in the heart through multiple effects on adenylate cyclase, including an enhancement of the interaction between G_s and the catalytic unit in cardiac membranes [Feldman *et al.* (1989)]. Ethanol has also been found to inhibit $\text{Na}^+/\text{Ca}^{2+}$ exchange and to arrest hamster heart during systole [Auffermann *et al.*

(1988)]. In isolated adult rat ventricular myocytes, ethanol (1%) has been found to cause contractile depression, to reduce the magnitude of the Ca^{2+}_i transient and to cause concentration-dependent depletion of SR Ca^{2+} [Danziger *et al.* (1991)]. At this time, the effect(s) of ethanol on PK C and its activity are not known. It is not known, as well, which of the above effects on the heart, if any, may have contributed to the decrease in PK C activity observed in this study in ethanol-treated isolated myocytes.

In the present study, treatment of myocytes with OAG did not produce activation of PK C as determined by the lack of movement from the cytosol to the membrane fraction. Previously, PK C was found to be translocated from the cytosol to the membrane fraction in perfused rat and guinea pig hearts in response to tumor-promoting phorbol esters [Edes and Kranias (1990); Yuan *et al.* (1987)]. However, in a perfused guinea pig heart study [Edes and Kranias (1990)], there was an incongruity between the decrease of PK C activity in the cytosol (from 232 to 180 pmol/min/mg protein; a decrease of 22%) and the increase in activity associated with the membrane (from 119 to 392 pmol/min/mg protein; an increase of 230%). In isolated adult rat ventricular myocytes, both tumor-promoting phorbol esters and 1,2-dioctanoylglycerol (a synthetic cell-permeant diacylglycerol; DiC8) increased the membrane association of PK C [Capogrossi *et al.* (1990)].

As discussed previously (see section 4.3.2.), there are many studies which indicate that phorbol esters do not act in the same way as diacylglycerols and this may be due to their binding to proteins other than PK C or to their sustained activation of PK C. Movement of PK C from the cytosol to the membrane fraction in experimental cardiac models has not been shown with the diacylglycerol, OAG. There has been a report which demonstrates that synthetic lipids, OAG and DiC8, do not cause the same biological effects in platelets indicating that they are not equal activators of PK C [Krishnamurthi *et al.* (1987)]. It has been suggested that the lack of effects with OAG could be the result of its weak ability to cross

membranes, other studies [Ebeling *et al.* (1985); Lapetina *et al.* (1985)] have found that it is a much less potent activator of PK C than DiC8. Since Capogrossi *et al.* (1990) have shown that DiC8 causes PK C translocation in isolated adult rat ventricular myocytes and in the present study, OAG did not cause translocation of PK C, there is some evidence in these cells that OAG and DiC8 are not equal activators of PK C. In the present study, an attempt was made to increase the concentration and persistence of OAG in the myocytes by inhibiting one of the enzymes responsible for diacylglycerol metabolism, diacylglycerol kinase, with R59022 [de Chaffoy de Courcelles *et al.* (1985)]. Elevated levels of OAG resulted in the augmentation of PK C activity in intact platelets [de Chaffoy de Courcelles *et al.* (1985)]. However, there are several limitations to the use of R59022. There are two isozymes of DAG kinase in mammalian tissues and R59022 only inhibits one of the isoforms [Sakane *et al.* (1989)]. As well, several studies [Mahadevappa and Sicilia (1988); Nasmith and Grinstein (1989)] have shown that R59022 exerts an effect on cellular functions that are independent of its action on DAG kinase. The most significant limitation to the use of R59022 is that in isolated adult rat ventricular myocytes, DAG kinase is not the major pathway for the metabolism of diacylglycerols, rather diacylglycerols are metabolized through a pathway involving DAG lipase [Hee-Cheong and Severson (1989)]. Taking into account these limitations of R59022, and the possibility that OAG may not be a potent activator of PK C in myocytes, it is perhaps not surprising that in this study, neither OAG nor OAG plus R59022 was seen to cause the translocation of PK C from the cytosol to the membrane fraction.

In some studies, the concept that translocation of PK C indicates activation of the phosphorylating activity of this enzyme has come into question [see review by Woodgett *et al.* (1987); Bosca *et al.* (1989)]. In astrocytoma cells, the redistribution of PK C was found not to correlate with either the extent or duration of the phosphorylation of PK C substrates [Trilivas *et al.* (1991)]. Another recent study

has found intracellular receptor proteins, in the cytoskeletal components of the membrane fraction of neonatal rat heart, that bind PK C (RACKS: receptors for activated C-kinase; Mochly-Rosen *et al.* (1991)). Binding of PK C to these proteins was specific, saturable and concentration-dependent. At this time, it is difficult to ascertain the ramifications of these results on the pathway of activation of PK C by translocation to membranes in the adult rat ventricular myocyte.

4.5.2. Autophosphorylation of PK C in Isolated Adult Rat Ventricular Myocytes

Another method used to determine the activation of PK C in cellular systems is evidence for the autophosphorylation of the enzyme [Mitchell *et al.* (1989)]. The autophosphorylation reaction of PK C involves an intrapeptide mechanism whereby a single polypeptide chain phosphorylates itself [Newton and Koshland (1987)]. This reaction occurs on both the catalytic and regulatory domains of PK C and a total of 6 autophosphorylation sites have been found in the sequence of type II PK C [Flint *et al.* (1990)]. The autophosphorylation of PK C can be monitored by two methods: 1) the incorporation and quantitation of radioactive phosphate [Mitchell *et al.* (1989)] and 2) the separation of PK C on SDS-PAGE gels and visualization of the increase in molecular weight upon autophosphorylation [Huang *et al.* (1986a & b)]. In this study, the protein in the PK C activity peak fractions obtained from cytosolic and membrane fractions isolated from control and OAG-treated myocytes was concentrated, separated by SDS-PAGE and western blotted. These blots were then probed with antibodies to the PK C isozymes type III and type II (Figure 30 and 33). The major cytosolic peak at fractions 27-31 from both control and OAG-treated myocytes demonstrated two immunoreactive bands of PK C for both type II and type III at approximately 80 kDa. The less intense band, demonstrating a higher molecular weight, may represent an autophosphorylated species of PK C and the lower, more intense band may represent the nonphosphorylated form. The second

cytosolic peak at fractions 32-34 from control myocytes contains a single band of type III PK C at approximately 80 kDa and this peak from OAG-treated myocytes contains a single band of type III and also a single band of type II PK C. Both of these single bands migrate at the position of what may represent nonphosphorylated PK C. The third cytosolic peak at fractions 39-42 from both control and OAG-treated myocytes was found not to contain type III PK C. The membrane peak that contains a large proportion of Ca^{2+} and lipid-independent PK C activity (fractions 6-15) from both control and OAG-treated myocytes contains 2 bands of type III but not type II PK C. The band of type III PK C at approximately 80 kDa may represent the nonphosphorylated form of PK C and the band at 50 kDa may represent the proteolytically active form of PK C, PK M. Fractions 27-31 of the membrane preparations from both control and OAG-treated myocytes contains 2 bands of type III PK C, which may represent the autophosphorylated and nonphosphorylated forms.

Since these western blots may have indicated that a portion of the PK C in cytosol and membrane fractions from control myocytes may already be in the autophosphorylated state, and since autophosphorylation of PK C is a prerequisite to its phosphorylation of substrate proteins, this may indicate that PK C was activated, prior to the control incubation. During the isolation of myocytes, insulin and several other amino acids and vitamins are used in the perfusion and washing buffers. Perhaps one of these buffer components caused the partial activation of PK C, as reflected in the production of the autophosphorylated form. However, these blots also show a similar large proportion of PK C from control and OAG-treated myocytes which may be in the nonphosphorylated form. Thus, using the increase in molecular mass upon autophosphorylation as an indication of PK C activation demonstrates that OAG was not able to activate a substantial portion of this kinase in isolated adult rat ventricular myocytes.

Previous studies have shown that autophosphorylation occurs with a rise in molecular weight for type II of PK C [Huang *et al.* (1986a & b); Flint *et al.* (1990); Pelech *et al.* (1991)]. However, type III PK C is known to become autophosphorylated but an upshift in the molecular weight upon the autophosphorylation of type III PK C has not been documented before.

In addition to type II and type III PK C, the heart also contains a large amount of the PK C isozyme ϵ [Schaap *et al.* (1989)]. The activity of this isozyme is Ca^{2+} -independent but it is activated by phospholipids, DAG and phorbol esters. Unlike PK C types II and III, PK ϵ does not phosphorylate the substrate histone H1 very well [Schaap and Parker (1990)]. Therefore, in the present studies, the activity of PK ϵ was not measured since histone H1 was used as the substrate. Insulin has been shown to activate the activity of PK ϵ in cytosol and membrane fractions from fetal chick neurons but did not cause translocation. As well, insulin and tumor-promoting phorbol ester were found to upshift the molecular weight of PK ϵ on SDS gels [Heidenreich *et al.* (1990)]. Although no studies have been carried out as yet on PK ϵ in experimental models of the myocardium, it will be of great interest in the future to determine whether this isozyme contributes functionally to the effects of PK C activation in the heart.

5. SUMMARY AND CONCLUSIONS

In summary, a method for the isolation of a high number of viable adult rat ventricular myocytes was established. The availability of these myocytes enabled the development of methods for myocyte homogenization and isolation of SR membranes from these homogenates. The myocyte SR membrane preparation exhibited similar Ca^{2+} -transport and Ca^{2+} -ATPase activity as well as a similar protein profile to SR membranes isolated from intact rat heart tissue. Also, these

SR membranes exhibited low levels of contamination by enzymatic activities from other cellular membranes.

The availability of purified SR membranes from adult rat ventricular myocytes provided a useful model for the study of the regulation of SR function by protein phosphorylation. For these studies, myocyte SR membranes were isolated and characterized in buffers developed to prevent the dephosphorylation of proteins. These SR membranes exhibited a protein profile similar to those isolated in control buffers, less contamination by enzymatic activities from other cellular membranes and lower recovery of Ca^{2+} -uptake and $\text{Ca}^{2+}/\text{K}^{+}$ -ATPase activities. Three distinct proteins (phospholamban, a 31 and a 152 kDa protein) were phosphorylated by PK A in homogenates and SR membranes from adult rat myocytes stimulated with isoproterenol and forskolin. The stimulation of protein phosphorylation in myocyte homogenates and SR membranes by isoproterenol could not be inhibited by two different inhibitors of PK A. Also, an inhibitor of CAM PK did not affect the stimulation of protein phosphorylation in myocyte homogenates and SR membranes by isoproterenol. Treatment of isolated adult rat myocytes with DMSO or phorbol esters dissolved in DMSO resulted in the phosphorylation of phospholamban in myocyte homogenates and SR membranes. When OAG and isoproterenol were used together to stimulate protein phosphorylation in isolated adult rat myocytes, the same proteins were phosphorylated to similar degrees as observed in homogenates and SR membranes treated with isoproterenol alone. In cytosolic fractions isolated from isoproterenol and OAG plus isoproterenol-treated myocytes, the phosphorylation of protein bands at 21, 31 and 152 kDa was stimulated. The phosphorylation of a 24 kDa protein appeared to be decreased in myocytes treated with isoproterenol, OAG and isoproterenol plus OAG.

The separation of phosphorylated pentameric species of phospholamban from rat myocyte SR was found to be more difficult to achieve than from SR membranes prepared from canine heart. In control and OAG-treated myocytes, two species of

phosphorylated pentameric phospholamban were obtained. In myocytes treated with isoproterenol or OAG plus isoproterenol, 5 species of phosphorylated pentameric phospholamban were obtained.

To assay the activity of PK C from myocyte cytosol and membrane fractions, FPLC fractionation and the inclusion of the peptide inhibitor of PK A were required in order to remove an inhibitory or interfering activity and to inhibit the large amount of PK A activity found in these fractions. The specific activity of PK C in myocyte cytosol was found to be much higher than that previously found present in cytosolic fractions from canine, rat or guinea pig heart. Three peaks of Ca^{2+} and lipid-dependent PK C activity were found in cytosolic fractions isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes. The main peak of activity contained type II and type III isozymes of PK C, in perhaps autophosphorylated and nonphosphorylated states. The second major peak contained perhaps only nonphosphorylated forms of type III PK C in control myocytes and type II and III PK C from OAG-treated myocytes. The third peak of PK C activity in the cytosol did not contain type III PK C protein. Two peaks of Ca^{2+} and lipid-dependent PK C activity were found in membranes isolated from control, isoproterenol, OAG and isoproterenol plus OAG-treated myocytes. The main peak of activity contained type III PK C, which may be present in both autophosphorylated and nonphosphorylated states. The second peak, which contained a large Ca^{2+} and lipid-independent kinase activity contained type III PK C perhaps only in the nonphosphorylated and proteolyzed PK M form. There were no differences seen in the number or types of peaks of PK C activity formed with respect to incubation time. There was significantly less PK C activity in membrane fractions from myocytes that had been treated with ethanol.

In conclusion, the results presented in this work have demonstrated:

- 1) For the first time, the isolation of SR membranes from isolated adult rat ventricular myocytes.

2) Stimulation of PK A in adult ventricular myocytes results in the phosphorylation of phospholamban, a 31 and a 152 kDa protein. These phosphorylations were not inhibited by specific inhibitors of PK A and did not seem to involve CAM PK.

3) Treatment of isolated adult myocytes with OAG resulted in the formation of the same species of phosphorylated pentameric phospholamban as found in control myocytes. As well, OAG in combination with isoproterenol had no effect on the number of species of phosphorylated pentameric phospholamban over that obtained with isoproterenol treatment alone.

4) Myocyte cytosolic fractions and membranes contain both type II and type III PK C, perhaps in both nonphosphorylated and autophosphorylated forms. The increase in molecular weight upon autophosphorylation of type III PK C has not been documented previously.

5) In isolated adult rat ventricular myocytes, OAG was not able to activate PK C as determined by translocation, autophosphorylation and protein phosphorylation studies.

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