MECHANISM OF INHIBITION OF ADENYLYL CYCLASE BY MUSCARINIC RECEPTOR STIMULATION OF INHIBITORY G-PROTEINS IN CARDIAC MYOCYTES

A Biochemical Approach

by

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ABSTRACT

In the mammalian myocardium, adenylyl cyclase is under dual regulation by β adrenoceptors and muscarinic receptors via stimulatory (G_s) and inhibitory (G_i) G proteins. It is well known that activation of G_s upon stimulation of β adrenoceptors cause increase in adenylyl cyclase activity. However, the mechanism of inhibition of adenylyl cyclase upon activation of G_i is not clear and two mechanisms have been proposed (Gilman, 1987) : an indirect inhibition due to decrease in the concentration of $G_{s\alpha.GTP}$ by βγ subunits generated upon activation of G_i and a direct inhibition by $G_{i\alpha.GTP}$.

In this study we investigated the mechanism of muscarinic receptor-mediated inhibition of adenylyl cyclase in sarcolemmal membranes isolated from rabbit atrial and ventricular myocyte. High affinity guanosine triphosphatase and adenylyl cyclase activity in the presence of the muscarinic agonists carbachol and oxotremorine alone and in combination with the β-adrenergic agonist isoproterenol were measured, and the different inhibitory G-protein subunits were identified in these membranes by immunoblotting.

In ventricular myocyte membranes, isoproterenol, carbachol and oxotremorine alone produced significant increases in guanosine triphosphatase activity above the basal value, whereas an additive effect was observed in the presence of isoproterenol and carbachol or isoproterenol and oxotremorine together. Adenylyl cyclase activity was increased by isoproterenol alone, whereas carbachol inhibited basal cyclase activity. However, the adenylyl cyclase activity measured in the presence of isoproterenol and carbachol together was very close to the value

predicted if the opposing effects of the two agonists were independent of each other. These observations suggest that in ventricular myocyte membranes, there is no interaction between β adrenoceptor and muscarinic receptor-stimulated G-proteins and inhibition of adenylyl cyclase occurs by a direct mechanism.

In atrial myocyte membranes, isoproterenol, carbachol and oxotremorine alone produced significant increases in guanosine triphosphatase activity above the basal value, whereas the activity measured in the presence of isoproterenol and carbachol or isoproterenol and oxotremorine together was not different from that in the presence of carbachol or oxotremorine alone. Isoproterenol increased adenylyl cyclase above the basal level, while carbachol did not affect basal cyclase activity. In the presence of both agonists together, the isoproterenol - stimulated cyclase activity was inhibited. Although statistically not significant, our results on cyclase activity are qualitatively consistent with earlier observations from our laboratory on rabbit atrial homogenates (Ray, 1992). These observations suggest that in atrial myocyte membranes, there is an interaction between β adrenoceptor and muscarinic receptor-stimulated G-proteins and inhibition of adenylyl cyclase occurs by an indirect mechanism. Western blotting of ventricular myocyte membranes identified $G_{i\alpha1}$ and $G_{i\alpha3}$ but not $G_{i\alpha2}$ subtypes, while the presence of $G_{o\alpha}$ could not be conclusively confirmed. In atrial myocyte membranes, Western blotting identified $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{o\alpha}$ subtypes. In conclusion, the results from this study suggest that there are differences in the mechanism of muscarinic receptor regulation of adenylyl cyclase in atria and ventricles, which is associated with the presence of different isoforms of G_i in these two tissues.

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AppNHp	Adenosine -5- [β,γ - imino] triphosphate
β AR	Beta adrenergic receptor
AS/7	Anti G-protein antibody with relative selectivity for $G_{i\alpha1}$ and $G_{i\alpha2}$
ATP	Adenosine triphosphate
(α-^{32}P) - ATP	ATP where the α-phosphate group is radioactive
(γ-^{32}P) - ATP	ATP where the γ-phosphate group is radioactive
BSA	Bovine serum albumin
C	Centigrade
cAMP	Adenosine 3',5' cyclic monophosphate
(^{3}H) - cAMP	Tritium - labeled cAMP
Cch	Carbachol
cGMP	Guanosine 3',5' cyclic monophosphate
cpm	Counts per minute
CO_2	Carbon dioxide
CTX	Cholera toxin
dpm	Disintegrations per minute
DAG	Diacylglycerol
DTT	Dithiothreitol
EC/2	Anti G-protein antibody with

	relative selectivity for $G_{i\alpha 3}$ and $G_{o\alpha}$
EDTA	Ethylene diamine tetra acetic acid
EGTA	Ethylene glycol -bis- (β amino ethyl ether)-, N, N', N'-tetra acetic acid
g	Gram(s)
G-protein	Guanine nucleotide binding protein
$G_{\beta\gamma}$	Beta and gamma subunits of G-protein
G_i	Inhibitory G-protein
$G_{i\alpha}$	α-subunit of the inhibitory G-protein
$G_{i\alpha 1}$, $G_{i\alpha 2}$, $G_{i\alpha 3}$	α-subunits of the isoforms of inhibitory G-protein
G_o	Other G-protein
$G_{o\alpha}$	α-subunit of the other G-protein
G_s	Stimulatory G-protein
$G_{s\alpha}$	α-subunit of stimulatory G-protein
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
$(\gamma\text{-}^{32}P)$ - GTP	GTP where the γ-phosphate group is radioactive
GTPase	Guanosine triphosphatase
HCl	Hydrochloric acid
IBMX	Isobutyl methylxanthine
IP_3	Inositol trisphosphate
Iso	Isoproterenol

KCl	Potassium chloride
KDa	Kilodalton
Kg	Kilogram
l	Litre
M	Molar
$MgCl_2$	Magnesium chloride
MEM	Minimum Essential Medium
$MgSO_4$	Magnesium sulphate
mg	Milligram
ml	Millilitre
mM	Millimolar
µCi	Microcurie
µg	Microgram
µl	Microlitre
µM	Micromolar
min	Minute
N	Normal
NaCl	Sodium chloride
nM	Nanomolar
O_2	Oxygen
Oxo	Oxotremorine
PAGE	Polyacrylamide gel electrophoresis
PLC	Phospholipase C

PKA	cAMP - dependent protein kinase
PMSF	Phenyl methyl sulfonyl fluoride
PTX	Pertussis toxin
Pi	Inorganic phosphate
pmol	Picomole
RIA	Radioimmunoassay
Rsp	Specific radioactivity (dpm/pmol)
SDS	Sodium dodecyl sulphate
SNP	Sodium nitroprusside
TEMED	N,N,N',N'- tetramethyl ethylene diamine

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Dedicated to

My parents

my beloved wife Manisha

and my brothers

1.0 INTRODUCTION

1.1 GENERAL OVERVIEW

The heart has the intrinsic capability of generating its own impulses without any interference from the central nervous system. However, the functioning of mammalian heart is under dual regulation of input from the sympathetic and parasympathetic branches of autonomic nervous system. The effects of sympathetic stimulation in the heart are mediated via α and β ARs, whereas parasympathetic influences are mediated by muscarinic cholinergic receptors. Input from the sympathetic nervous system stimulates inotropic, electrophysiological and metabolic states of the myocardium whereas parasympathetic input antagonizes the effects of sympathetic influences (Fleming et al., 1987). Physiologically in the normal heart, sympathetic stimulation of β AR causes an increase in heart rate (positive chronotropic effect), force of contraction (positive inotropic effect) and conduction velocity (positive dromotropic effect), while parasympathetic stimulation of muscarinic cholinergic receptors produces a decrease in heart rate (negative chronotropic effect), force of contraction (negative inotropic effect) and conduction velocity (negative dromotropic effect) (Lefkowitz et al., 1996). It appears that the β AR-mediated positive inotropic effect upon sympathetic stimulation is the predominant mechanism for regulation of myocardial contractility (Bruckner et al., 1985).

1.2. BETA ADRENOCEPTORS IN THE HEART

Interaction of β AR with the catecholamines, epinephrine or norepinephrine leads to significant increases in the intracellular levels of the second messenger

cAMP, (Hausdorff et al., 1990), an effect mediated by the stimulatory G-protein G_s and the intrinsic enzyme adenylyl cyclase.

β ARs belong to the G-protein-coupled superfamily of receptors that have seven hydrophobic membrane-spanning domains and consist of 402-560 amino acids (Dohlman et al., 1991). The hydrophilic N-terminus extends into the extracellular space whereas the hydrophilic C-terminus is located intracellularly and contains substrates for phosphorylation by β AR kinase (Frielle et al., 1987 ; Benovic et al., 1986) and PKA (Frielle et al., 1987 ; Benovic et al., 1985). In humans, molecular biological techniques have clearly demonstrated the presence of three distinct genes encoding β AR subtypes, namely β_1, β_2 and β_3 (Chung et al., 1987 ; Dixon et al., 1987a,b ; Frielle et al., 1987 ; Kobilka et al., 1987a,b ; Emorine et al., 1989).

Radioligand binding studies using high affinity β AR antagonists have provided evidence for the existence of both β_1 and β_2 AR subtypes in the mammalian myocardium (Hedberg et al., 1980). The authors used (^{125}I) hydroxybenzyl pindolol to demonstrate that in cat right atrium 78% of β ARs are of the β_1 subtype and 22% of the β_2 subtype, while in left ventricle the proportion of β_1 and β_2 ARs is 98% and 2% respectively. Similarly, in guinea pig right atrium 77% of β ARs were of the β_1 subtype and 23% were of the β_2 subtype. In human right atrium 74% of β ARs are of β_1 subtype and 26% of β_2 subtype while in left ventricle β_1 and β_2 AR constitute 86% and 14% respectively as determined by radioligand binding assays using (^{125}I) iodocyanopindolol (Stiles et al., 1983). Structurally, human β_1 AR cDNA is 2.4kb long and shares 54% sequence homology with β_2 AR and 69% with avian β_1 AR (Minneman et al., 1980) whereas β_3 AR exhibits 51% and 45% sequence homology

with β_1 and β_2 subtypes respectively. β_2 AR cDNA is 2.0 kb long and expressed in human ventricular myocardium. Several lines of evidence have indicated that in the human heart, adenylyl cyclase is preferentially activated by β_2 AR-stimulation, although β_1 ARs predominate (Bristow et al., 1989 ; Kauman et al.,1989 ; Brodde, 1991).

Biochemically, stimulation of β_1 and β_2 AR by catecholamines causes activation of a stimulatory G-protein (G_s) which leads to an increase in the production of cAMP via stimulation of adenylyl cyclase. cAMP in turn activates PKA which can lead to the following associated cellular responses: phosphorylation of phosphorylase kinase for mobilization of glycogen stores (Insel & Ransnas, 1988); phosphorylation of troponin I, which decreases the affinity of the protein for Ca^{2+} (Adelstein and Eisenberg, 1980); phosphorylation of myofibrillar C protein (Hartzell & Titus, 1982); phosphorylation and activation of inhibitor-1 of type 1 phosphatase (Ahmad et al., 1989); phosphorylation of phospholamban which regulates Ca^{2+}-ATPase activity (Lindemann et al., 1983) ; phosphorylation of a 15kDa sarcolemmal protein (Presti et al., 1985) ; inhibition of sarcolemmal Na^+ channels when the membranes are depolarized (Schubert et al., 1989) and activation of voltage-sensitive Ca^{2+} channels to cause increased influx of Ca^{2+} across the sarcolemma (Yatani & Brown, 1989).

1.3 MUSCARINIC RECEPTORS IN THE HEART

Parasympathetic stimulation causes release of the neurotransmitter Ach which binds to cell-surface muscarinic receptors in the heart (Felder, 1995). The functional effects of muscarinic receptor stimulation in the myocardium vary from one part to the

other (Loffelholz and Pappano, 1985). Thus in the atria, muscarinic agonists exert a direct negative inotropic and chronotropic effect and also antagonize α and β AR-stimulated positive inotropic responses. However, in ventricles the direct negative inotropic effect due to muscarinic receptor stimulation is minimal, although muscarinic agonists inhibit β AR-stimulation. At very high concentrations muscarinic agonists exert positive inotropic effects in both atria and ventricles (Loffelholz and Pappano, 1985; Pappano, 1990).

The realization that muscarinic receptor-stimulated effects of Ach involve more than one subtype of receptor was evident by the beginning of 1980's when radioligand binding studies demonstrated that tissue-specific differences existed in the affinity of pirenzepine, an antiulcer agent, for blocking muscarinic receptors in rat autonomic ganglia and atria. (Hammer & Giachetti, 1982; Hammer et al., 1980). Hammer and Giachetti (1982) demonstrated that pirenzepine exhibited 20 fold greater selectivity in blocking ganglionic than atrial muscarinic receptors, whereas atropine was equipotent in muscarinic receptor-blockade in both tissues. Earlier, Barlow et al. (1976) showed that the compound 4-diphenylacetoxy-N-methyl methiodide (4-DAMP) was 20-fold more selective in blocking muscarinic responses in the ileum compared to the heart. In the same year, Clark and Mitchelson (1976) also reported that gallamine was 10-100 fold more potent on guinea pig atrial muscarinic receptors than ileum. All these observations led to the suggestion that two subtypes of muscarinic receptors, designated as M_1 and M_2 existed (Caulfield et al., 1983). Subsequently, Mitchelson (1988) used more selective antagonists and differentiated three pharmacologically distinct muscarinic receptor subtypes namely M_1, M_2 and M_3.

Pharmacologically, an M_4 subtype of muscarinic receptor has also been identified in human (Dorje et al., 1991), rabbit (Lazareno et al., 1990), rat (Bernheim et al., 1992), mouse (Lazareno et al., 1990) and neuroblastoma x glioma hybrid (NG 108-15) cells (Caulfield & Brown, 1991). Molecular cloning studies have so far identified five genes (m_1-m_5) that code for muscarinic receptors (Kubo et al., 1986a,b ; Peralta et al., 1987a,b ; Bonner et al., 1987; Braun et al., 1988 ; Bonner et al., 1988). The major subtype of muscarinic receptor in the heart is m_2 as shown by studies on tissue distribution of muscarinic receptor mRNAs (Maeda et al., 1988) and also by pharmacological studies using specific muscarinic receptor antagonists (Yang et al., 1992). The use of receptor-specific antibody has enabled the identification of m_2 muscarinic receptor protein in mammalian myocardium (Caulfield, 1993). There is also evidence for the presence of the M_1 muscarinic receptor subtype in guinea pig ventricular myocytes (Gallo et al., 1993) and the M_3 subtype in canine left ventricles (Yang et al., 1992).

It is well established that in mammalian myocardium stimulation of muscarinic receptors cause activation of at least three pathways of signal transduction which can lead to inhibition of adenylyl cyclase, opening of muscarinic K^+ channels and/or activation of metabolism of polyphosphoinositides (Jakobs et al., 1979 ; Brown and Brown, 1983 ; Pfaffinger et al., 1985). It is also known that muscarinic receptor-stimulated effects are mediated primarily through inhibitory G-proteins. Binding of a muscarinic agonist such as Ach to its receptor in the heart causes activation of PTX-sensitive inhibitory G-proteins (G_i) which inhibit adenylyl cyclase and reduce the intracellular cAMP levels. This in turn inhibits phosphorylation of intracellular

proteins. The agonist-stimulated G_i may also act directly on atrial muscarinic K^+ channels and activate them or the stimulated G-protein may directly activate Na^+ channels. In another pathway, muscarinic receptor-stimulation may activate a PTX-insensitive G-protein, G_q which, in turn activates phospholipase C causing release of two intracellular second messengers, IP_3 and DAG. IP_3 causes release of intracellular Ca^{2+} while DAG activates PKC which phosphorylates and activates sarcoplasmic Ca^{2+} channels. Activation of the receptor may also result in stimulation of soluble guanylate cyclase which would cause an increase in the levels of cyclic GMP (fig. 1) (reviewed by Fleming et al., 1992).

1.3.1. Muscarinic receptor stimulation and regulation of atrial and ventricular ion channels

In the mammalian myocardium, differences in time and voltage-dependent properties and pharmacological sensitivities have identified two broad types of voltage-gated K^+ currents, namely a 4-AP-sensitive rapidly activating and inactivating transient outward current (I_{to}) and a delayed, slowly activating and deactivating tetraethylammonium-sensitive rectifier current (I_k). I_{to} contributes to the early phase of repolarization, whereas I_k contributes to the later phase of repolarization (reviewed by Barry and Nerbonne, 1996). I_{to} has been subdivided into a Ca^{2+} -independent, 4-AP-sensitive I_{to1} and a Ca^{2+} -dependent, 4-AP-insensitive I_{to2} (Coraboeuf and Carmeliet, 1982 ; Kenyon and Gibbons, 1979b). Several studies in atrial and ventricular myocardium have demonstrated that I_{to2} is a chloride current that is activated by intracellular Ca^{2+} and is exhibited even in the absence of potassium (Kenyon and Gibbons, 1979; Zygmunt and Gibbons, 1991, 1992; Zygmunt, 1994).

This current has since been referred to as $I_{Cl(Ca)}$ (Zygmunt and Gibbons, 1991). Three types of delayed rectifier currents have been characterized in mammalian myocardium, namely I_{k1} (strongly rectifying), $I_{K(ACh)}$ (Ach-activated K^+ current) and $I_{K(ATP)}$ (ATP-sensitive K^+ Current) (see Barry and Nerbonne, 1996 for a review). I_{K1} is K^+-selective, is blocked by Cs^+ and Ba^{2+} and is strongly rectifying (Vandenberg, 1994). It has been well characterized in atrial and ventricular myocytes and Purkinje fibres (Giles and Imaizumi, 1988 ; Hume and Uehara, 1985 ; Josephson and Brown, 1986 ; Shimoni et al., 1992). Several lines of evidence have indicated the presence of inwardly rectifying K^+ channels in ventricular myocytes that are inhibited by intracellular ATP and are referred as $I_{k(ATP)}$ (Noma, 1983 ; Trube and Hescheler, 1983 ; Trube and Hescheler, 1984). Activation of $I_{k(ATP)}$ during metabolic stress causes shortening of action potential (Lederer et al., 1989) which is blocked by tolbutamide, an $I_{k(ATP)}$ inhibitor (Faivre and Findlay, 1990). A third type of K^+ current which is sensitive to Ach ($I_{K(ACh)}$) has been identified in atrial and ventricular myocytes, pacemaker cells and Purkinje fibres (Heidbuchel et al., 1990 ; Koumi and Wasserstrom, 1994 ; Noma et al., 1979 ; Sakmann et al., 1983).

In an early study on atrial myocardium Ten Eick et al. (1976) observed that at a low concentration, Ach activates an outward K^+ current (now referred to as $I_{K(ACh)}$) and shortens the duration of action potential without affecting the slow inward Ca^{2+} current, whereas at high concentrations of Ach activation of K^+ current is also accompanied by inhibition of slow inward Ca^{2+} current. In both cases a negative inotropic effect was observed. Similar observations have been reported in other studies (DiFrancesco et al., 1980 ; Inoue et al., 1983; Bellardinelli and Isenberg,

1983; Soejima and Noma, 1984; Iijima et al., 1985). A negative chronotropic effect and a reduction in atrioventricular conduction due to increased K^+ conductance upon stimulation of muscarinic Ach receptors has also been demonstrated in the heart (Noma and Trautwein, 1978).

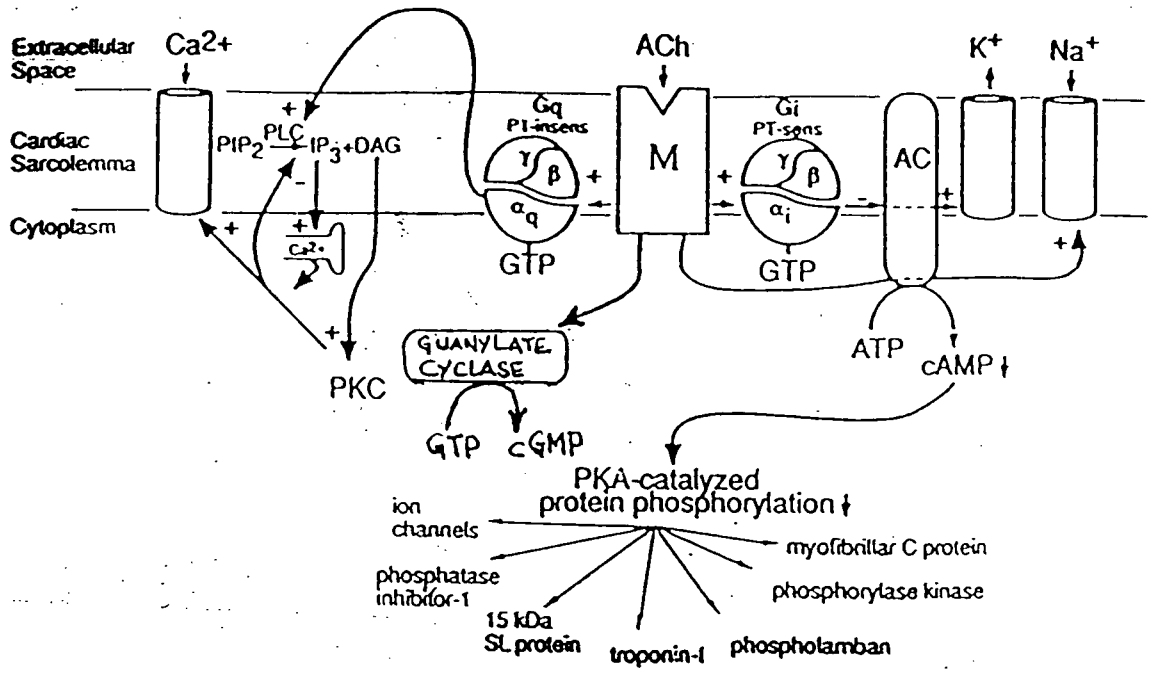

Fig. 1 Schematic representation of possible consequences of muscarinic receptor - effector coupling in the heart (modified from Fleming et al., 1992). See text for details.

Activation of $I_{K(ACh)}$ requires intracellular GTP (Breitweiser and Szabo, 1985 ; Kurachi et al., 1986b ; Kurachi et al., 1992) but does not require soluble intracellular

second messengers (cAMP or cGMP) or Ca^{2+} (Kurachi et al., 1986a). Ventricular $I_{k(Ach)}$ appears to be less sensitive to Ach than atrial currents which accounts for the relatively low current density of $I_{k(Ach)}$ in ventricular myocytes (Koumi and Wasserstrom, 1994).

It is well established that in atria the muscarinic receptors are coupled to K^+ channels by pertussis toxin-sensitive G-proteins (Pfaffinger et al., 1985 ; Breitweiser and Szabo, 1985 ; Endoh et al., 1985 ; Sorota et al., 1985 ; Kurachi et al., 1986 a,b,c ; Ito et al., 1991 ; Ito et al., 1995). The atrial muscarinic K^+ channels are activated by G-proteins directly without involving any intermediate events (Yatani et al., 1987). Kurachi et al. (1986c) showed that intracellular Mg^{2+} is essential for GTP-induced activation of atrial muscarinic K^+ channels. In contrast, however activation of muscarinic K^+ channels by purified G-protein $\beta\gamma$ subunits in chick embryonic atrial cells does not require intracellular Mg^{2+} (Logothetis et al., 1987). The activated α subunit of heterotrimeric G-proteins was initially suggested to be involved in the activation of muscarinic K^+ channels in guinea pig atrial myocytes (Codina et al., 1987), chick embryonic, neonatal and adult guinea pig atria (Kirsch et al., 1988). However other studies have shown that the $\beta\gamma$ subunit directly activates atrial muscarinic K^+ channels (Logothetis et al., 1987; Wickman et al., 1994).

The carboxyl (C)-terminus of a receptor-gated inwardly rectifying muscarinic K^+ channel cloned from mouse brain (MB-$GIRK_1$) has been shown to directly bind $G\beta\gamma$ subunits purified from bovine brain membranes (Inanobe et al., 1995). Similarly, Krapivinsky et al. (1995) reported that $G\beta\gamma$ subunits purified from bovine brain membranes bind directly to immunoprecipitated atrial muscarinic K^+ channels ($I_{K(Ach)}$)

as well as to its recombinant $GIRK_1$ and CIR subunits with equal affinity and cause activation of the channel. Huang et al. (1995) further demonstrated that although both N and C-terminal hydrophilic domains of $GIRK_1$ bind $G\beta\gamma$ subunit, the C-terminal domain has higher affinity for $G\beta\gamma$ subunit. In summary these observations suggest that in atrial myocardium, PTX-sensitive G-proteins activate muscarinic K^+ channels which may or may not require intracellular Mg^{2+}. Further, it appears to be the $\beta\gamma$ subunits of these G-proteins that activate the channels by directly binding to C-terminus of the channel protein.

Several studies have demonstrated that in ventricular myocardium, a delayed outward K^+ current (I_k) is increased by β adrenergic stimulation (Quadbeck and Reiter, 1975; Bennett and Begenisich, 1987; Matsuura et al., 1987; Walsh et al., 1988; Yazawa and Kameyama, 1990). In contrast, however Iso suppresses the inwardly rectifying K^+ current (I_{k1}) in guinea pig ventricular myocytes, an action that is blocked by Ach. Pretreatment of myocytes with PTX blocked the inhibitory effects of Ach on Iso-induced suppression of I_{k1} (Koumi et al., 1995a,b). Ach (10μM) has also been shown to antagonize the increase in action potential duration induced by Iso (33nM and 50nM) and forskolin (3μM), an adenylyl cyclase activator in isolated rabbit ventricular myocytes (Watson et al., 1988). In a more recent study on rat atrial and ventricular myocytes, McMorn et al. (1993) observed that Ach activates $I_{k(Ach)}$ directly even in the absence of β AR stimulation. Although activation of ventricular $I_{k(Ach)}$ was smaller than atrial $I_{k(Ach)}$, a transient direct negative inotropic response was observed in ventricular myocytes. However the decrease in contractile response was more pronounced in atrial cells.

Activation of L-type Ca^{2+} current (I_{Ca}) by Iso, forskolin and a non-specific phosphodiesterase inhibitor, IBMX has been demonstrated in guinea pig ventricular myocytes (Mubagwa et al., 1993; Levi et al., 1994). The IBMX-stimulated I_{Ca} was blocked by muscarinic agonists such as Cch and cGMP-elevating agents such as sodium nitroprusside (SNP) (Levi et al., 1994). The inhibitory effects of Cch and SNP on IBMX-induced increase in I_{Ca} were blocked by methylene blue, a soluble guanylate cyclase inhibitor.

In summary muscarinic agonists reverse the Iso-induced inhibition of inwardly rectifying K^+ conductance in ventricular myocardium and this inhibitory effect is PTX-sensitive. Further the increases in I_{Ca} produced by β adrenergic agonists (Iso), activators of adenylyl cyclase (forskolin) and phosphodiesterase inhibitors (IBMX) are also inhibited by muscarinic agonists and this effect is blocked by soluble guanylate cyclase inhibitors such as methylene blue.

1.3.2 Modulation of cGMP and cAMP levels by muscarinic receptor stimulation in atria and ventricles

The cause-effect relationship between muscarinic receptor-mediated alterations in cyclic nucleotide (cGMP or cAMP) levels and negative inotropic effects in atria and ventricles has been a subject of controversy for a long time and most of the reported studies provide conflicting observations. Early studies on different atrial preparations demonstrated a positive correlation between elevated cGMP levels and decrease in contractility (George et al., 1975 ; Goldberg et al., 1975) without any effect on cAMP levels. However in subsequent years other studies demonstrated a lack of relationship between muscarinic agonist-stimulated elevation in cGMP levels

and negative inotropic effects in atrial myocardium (reviewed by Linden and Brooker, 1979). In cat atrial strips a 17 - fold increase in cGMP levels with no effect on cAMP levels from the control value was accompanied by a marginal increase in contractile force with SNP (100μM), whereas Ach (5×10^{-7} M for 60 sec) did not alter cGMP or cAMP levels from the control but significantly decreased atrial contractility (Diamond et al., 1977). In another study on guinea pig left atria, Brooker (1977) showed that Cch (0.3μM, 5-60 sec) did not increase cGMP levels but decreased the contractility significantly to a maximum of 80% in 60 sec. Interestingly, the same concentration of Cch (0.3μM) alone produced significant increases in cGMP levels accompanied by a small decrease in contractile response without affecting cAMP levels in rabbit left atrial strips (MacLeod, 1986). But the same concentration of Cch (0.3 μM) significantly inhibited 30nM Iso-stimulated cAMP levels and increase in contractility, while the cGMP levels were increased. In an earlier study on rabbit atrial strips, Diamond and Chu (1985) demonstrated that the cGMP-lowering agent, LY83583 blocked Ach-induced elevation in cGMP levels without affecting its negative inotropic effect. In a more recent study on rabbit left atrial strips Ray and MacLeod (1992) observed that Cch (3μM) alone did not alter basal cAMP levels but significantly decreased Iso (100nM)-stimulated cAMP levels accompanied by a significant inhibition of contractile response. Pretreatment of animals with PTX (1.75 μg/kg) abolished the inhibitory effects of Cch on Iso-stimulated cAMP levels and contractile response. In the same study Cch inhibited forskolin and IBMX-stimulated inotropic response without affecting the cAMP levels. Despite the conflicting observations, it is becoming increasingly evident that there is a clear dissociation between muscarinic

agonist-stimulated increases in cGMP levels and negative inotropic effects in atrial myocardium. Further, most of these studies indicate that muscarinic agonists do not affect cAMP levels in atrial myocardium when present alone, but inhibit β agonist-stimulated cAMP levels.

In contrast to atrial myocardium, the relationship between cyclic nucleotide levels and muscarinic receptor-stimulated negative inotropic effect is still not clearly established in ventricular myocardium. In an early study on guinea pig ventricular myocardium, Ach (10^{-8} - 10^{-6} M) produced a concentration-dependent increase in cGMP levels with no significant effect on cAMP levels and contractility (Watanabe and Besch, 1975). With increasing concentrations of Iso (1nM - 100nM), 100 nM Ach produced a profound inhibition of contractile response without altering cAMP levels. The cGMP levels were, however still elevated above the basal value. Subsequently, Inui et al. (1982) observed that in rabbit right ventricular papillary muscles Ach (1nM - 100 μM) and SNP (1 μM - 1mM) produced concentration-dependent inhibition of positive inotropic effects evoked by 10 nM Iso accompanied by a significant decrease in cAMP levels and increase in cGMP levels. In a later study on rabbit right ventricular papillary muscles, MacLeod and Diamond (1986) observed that 10 μM LY83583 significantly decreased cGMP levels in the presence of 3 μM Cch alone or in combination with 3 μM forskolin. However Cch was unable to overcome the positive inotropic effect of forskolin in LY83583 - treated tissues. Further, the cAMP levels in the presence of forskolin and Cch together in LY83583 treated tissues were higher than control tissues exposed to both the agonists. A recent study from our laboratory on rabbit right ventricular papillary muscles (Hui et al., 1995)

demonstrated that L-N^G-monomethyl arginine (L-NMMA), a nitric oxide synthase inhibitor, significantly inhibited Cch-stimulated increases in cGMP levels without altering its inhibitory effects on forskolin-stimulated positive inotropic response. In the same study it was also shown that SNP significantly elevated cGMP levels while the positive inotropic effects of forskolin were not affected. At about the same time another study on rat ventricular myocytes (MacDonell et al., 1995) demonstrated that Cch (1 μM and 10 μM) and Ach (10μM) significantly increased cGMP levels and also antagonized 1μM Iso-stimulated positive inotropic effect without altering cAMP levels. It was also shown that SNP (10 μM and 100 μM) produced significant elevation in cGMP levels from the basal value, while the Iso-stimulated positive inotropic effects and cAMP levels were not affected. The authors (MacDonell et al., 1995) suggest that cGMP and cAMP may not be involved in the muscarinic receptor-mediated negative inotropic effects in rat ventricular myocytes. A lack of effect of 2 μM Ach on cAMP levels or cAMP-dependent protein kinase A activity stimulated by Iso has also been reported in a recent study on guinea pig ventricular myocytes (Gupta et al., 1994). Another recent study from our laboratory on rat ventricular myocardium (Zhang and MacLeod, 1996) indicated that perfusion of hearts concurrently with 0.1 μM Iso and 3 μM Cch abolished the Iso-stimulated increase in left ventricular pressure (LVP) without altering cAMP levels or PKA activity. On the other hand, preperfusion with 3 μM Cch initially for 1 min followed by perfusion with Iso and Cch for an additional 1.5 min not only reversed Iso-stimulated LVP but also inhibited total and particulate cAMP levels and partially decreased PKA activity.

In summary many of the observations described above have demonstrated that in ventricle, muscarinic agonists do not affect basal cAMP levels but inhibit β agonist-stimulated cAMP levels. However, it is still not clear whether cGMP plays a role in mediating muscarinic receptor-stimulated negative inotropic effects.

1.4 G-PROTEINS IN THE HEART

Guanine nucleotide binding proteins or G-proteins belong to a large superfamily of proteins that act as transducers of information across the cell membrane (see Eschenhagen, 1993 for a review). Structurally, G-proteins are heterotrimers, consisting of a large α subunit and two smaller tightly coupled β and γ subunits. The α subunit binds guanine nucleotides, possesses intrinsic GTPase activity and couples receptors to effectors (reviewed by Emala et al.,1994). The molecular weights of α subunits range from 39-52 kDa. In the inactive state, the α subunit is bound to GDP and associated with βγ subunit to form the heterotrimer (reviewed by Neer,1995). To date, 23 distinct α subunits encoded by 17 different genes have been identified and are divided into four major families depending on primary sequence homology: $G\alpha_s$ (stimulatory) including α_s and α_{olf} (olfactory), $G\alpha_i$ (inhibitory) including α_1, α_2, α_3, α_o (other) (α_{oA} & α_{oB}), α_{t1} and α_{t2} (transducins), α_{gust} (gustatory) and α_z; $G\alpha_q$, including α_q, α_{11}, α_{14}, α_{15} and α_{16} and $G\alpha_{12}$ including α_{12} and α_{13} (reviewed by Neer,1995).

G-protein α subunits often contain a cysteine residue on the fourth position from the carboxy terminus which is sensitive to ADP-ribosylation by bacterial toxins. G_α subunits are classified on the basis of their sensitivity to ADP-ribosylation by the bacterial toxins, CTX or PTX (reviewed by Robishaw and Hansen, 1994). CTX ADP-

ribosylates the α subunit of G_s, thereby inhibiting its GTPase activity which irreversibly activates G_s and causes persistent stimulation of the effector enzyme, adenylyl cyclase (Kahn and Gilman, 1984). The CTX-sensitive family of G-proteins include G_s and G_{olf} (reviewed by Eschenhagen, 1993). PTX catalyzes ADP-ribosylation of a cysteine residue fourth from the carboxy terminus which uncouples the G-protein from its receptor and thereby inhibits guanine nucleotide exchange (Spiegel et al., 1992). The PTX-sensitive G-proteins include G_i (G_{i1}, G_{i2} and G_{i3}), G_o (G_{o1} and G_{o2} or G_{oa} and G_{ob}) and transducins (G_{t1} and G_{t2}) whereas G-proteins belonging to G_q family (G_q, G_{11}, G_{14}, G_{15} and G_{16}) and G_{12} family (G_{12} and G_{13}) are PTX-insensitive. The use of bacterial toxins to ADP-ribosylate α subunits of G-proteins has provided a very useful tool to identify and distinguish different subtypes of G-proteins.

Molecular cloning studies have identified to date five β subunits (M.W. range 35 - 36 kDa) and ten γ subunits (M.W. range 8-9 kDa) (Ray et al., 1995). Mammalian β subunits display a high degree of amino acid sequence homology (Simon et al., 1991 ; Watson et al., 1994). Structurally, β subunits are composed of eight repetitive segments of approximately 40 amino acids each that contain the characteristic "tryptophan-aspartic acid pair" called "WD-40" motif, the functional significance of which is not yet clear (Simon et al., 1991). Despite the high sequence similarity, β subunits cannot form dimers with each kind of γ subunit. For example, $G_{\beta1}$ can associate with $G_{\gamma1}$ or $G_{\gamma2}$ while $G_{\beta2}$ can form a dimer with $G_{\gamma2}$ but not with $G_{\gamma1}$ and $G_{\beta3}$ cannot associate with $G_{\gamma1}$ or $G_{\gamma3}$ (Schmidt et al., 1992 ; Pronin and Gautam, 1992).

Each γ subunit consists of 70 amino acids and a "CAAX" (CysAlaAlaX) motif at the carboxy terminus which serves as a site for post translational modifications (Fung et al., 1990 ; Backlund et al., 1990 ; Fukada et al., 1990 ; Lai et al., 1990) such as polyisoprenylation (Mumby et al., 1990 ; Simonds et al., 1991 ; Sanford et al., 1991). It has been suggested that although polyisoprenylation is not necessary for association of β with γ subunit, it is essential for activation of adenylyl cyclase (Iniguez-Lluhi et al., 1992). Further, this post-translational modification also appears to increase the affinity of βγ dimer for the α.GDP complex which leads to inactivation of the G-protein by forming the heterotrimer (reviewed by Birnbaumer and Birnbaumer, 1995). The γ subunits are crucial for determining the function of βγ subunits (reviewed by Clapham and Neer, 1993).

Some of the functions of βγ subunits include : binding to an inactive G_{α}.GDP complex and reassociating it back to the membrane to form an inactive heterotrimer (see Emala et al., 1994 for a review), anchoring the inactive heterotrimer to the membrane due to isoprenylated γ subunit (Spiegel et al., 1991), regulation of cardiac K^+ channels (Logothetis et al., 1987), activation of retinal and cardiac phospholipase A_2 to produce arachidonic acid (Axelrod et al., 1988 ; Kim et al., 1989) and regulation of adenylyl cyclase (Katada et al., 1987 ; Gao and Gilman, 1991 ; Tang and Gilman, 1991).

Using G-protein α subunit-specific antibodies, Foster et al. (1990) demonstrated the presence of 41 KDa $G_{i\alpha1,3}$, 40 KDa $G_{i\alpha2}$ and 39 KDa $G_{oA,B}$ in adult rat hearts. Foster et al. observed that in neonatal rat ventricles, expression of $G_{i\alpha1,3}$,

$G_{o\alpha}$ and $G_{z\alpha}$ was greater than in adult ventricles whereas $G_{i\alpha 2}$, $G_{q\alpha}$ and $G_{\alpha 11}$ were expressed in equal amounts. Earlier, Luetje et al. (1988) found that $G_{o\alpha}$ is expressed at very low levels in rat atria and even lesser in ventricles. Similar observations have been reported in human heart (Eschenhagen et al., 1992). In a recent study on washed particulate membranes from rabbit whole ventricles and ventricular myocyte membranes, Kumar et al. (1994) used anti G-protein antibodies and identified $G_{i\alpha 1}$ and very low levels of immunodetectable $G_{i\alpha 2}$ but no $G_{i\alpha 3}$ subtypes in adult ventricular myocyte membranes. However, washed particulate membranes from whole ventricles contained $G_{i\alpha 1}$, $G_{i\alpha 2}$ and low levels of $G_{i\alpha 3}$ subtypes. In canine ventricles although the $G_{i\alpha 2}$ protein levels are several fold fold higher than $G_{s\alpha}$ (Longabaugh et al., 1988; Vatner et al., 1988) the $G_{s\alpha}$ mRNA levels are at least four fold greater than $G_{i\alpha 2}$ (Holmer et al., 1989).

It is well known that in the heart, the muscarinic receptor-stimulated effects on effectors such as adenylyl cyclase and ion channels are mediated via inhibitory G-proteins. Matesic et al. (1991) provided evidence for selective interaction of m_2 muscarinic receptors with inhibitory G-proteins in rat cardiac membranes by co-purification of the receptors and G_α subunits after incubation of the membranes with 1mM Cch and 31-ID1 (a monoclonal antibody that immunoprecipitates m_2 receptors but not other muscarinic receptors). The $G_{i\alpha}$ and $G_{o\alpha}$ subtypes of G-proteins were resolved in 1mM Cch-treated atrial and ventricular membranes by SDS-PAGE followed by Western blotting using anti-G-protein antibodies. In atrial membranes the m_2 receptors co-purified exclusively with $G_{o\alpha}$, whereas in ventricular membranes both $G_{i\alpha}$ and $G_{o\alpha}$ copurified with muscarinic receptors .

The $G_{i\alpha 2}$ subtype appears to be primarily involved in muscarinic receptor-stimulated inhibition of adenylyl cyclase, as demonstrated in an in vitro experiment on transgenic mice heart deficient in α_{i2} gene (α_{i2} -/-) [Rudolph et al., 1996] where the inhibitory effects of Cch (0.01 - 100 μM) on 10 μM Iso-stimulated adenylyl cyclase activity were attenuated. In cardiac homogenates from control (α_{i2} +/+) animals Cch produced a 31% inhibition of Iso-stimulated cyclase activity at the maximum concentration of the agonist (100 μM) which was inhibited by 60-70% in homogenates from α_{i2} -/- animals. $G_{i\alpha 2}$ was shown to be the predominant PTX substrate by (^{32}P) ADP-ribosylation of cardiac homogenates. Since the lack of α_{i2} only produced a partial inhibition of adenylyl cyclase, the authors (Rudolph et al., 1996) suggested that other PTX-sensitive G-proteins such as $G_{i\alpha 1}$ and/or $G_{i\alpha 3}$ may also be involved in the inhibitory regulation of adenylyl cyclase.

1.4.1. Activation - deactivation cycle of G-proteins (GTPase cycle)

In the inactive state of a G-protein, the α subunit of the heterotrimer is associated with GDP and βγ subunit. During the process of signal transduction, G-proteins constantly undergo an activation-deactivation cycle. Upon stimulation of a G-protein-coupled receptor by an agonist, the α subunit of the heterotrimer exchanges GDP for GTP, which causes activation of the α subunit and dissociation from βγ dimer. The liberated α.GTP complex and βγ dimer interact independently with numerous effectors (reviewed by Clapham and Neer, 1993) leading to a physiological response. The response is terminated when GTP bound to α subunit is hydrolyzed to GDP, a reaction catalyzed by an intrinsic enzyme, GTPase present in the α subunit (reviewed by Eschenhagen, 1993) (fig. 2). Agonist-stimulated high

affinity GTPase activity has been successfully employed as a measure of receptor-G-protein interaction in mammalian cardiac membranes (Fleming et al., 1987 ; Fleming and Watanabe, 1988) and in atrial and ventricular myocyte membranes (Braun and Walsh, 1993). The release of GDP and binding of GTP to the α subunit appears to be the rate limiting step in G-protein activation (see Holmer and Homcy, 1991 for a review). Codina et al. (1984) demonstrated that GTP-binding is a Mg^{2+} - dependent process and the concentration of Mg^{2+} required for maximum activation of G-proteins is different for different G-proteins.

In the absence of Mg^{2+}, $\beta\gamma$ subunits inhibit the dissociation of GDP from the α subunit, whereas in the presence of Mg^{2+} it promotes GTP:GDP exchange by destabilizing the α.GDP complex (reviewed by Birnbaumer and Birnbaumer, 1995). The dissociation of $\beta\gamma$ dimer upon activation of a G-protein is the result of a conformational change in α subunit when it binds to GTP. Bourne et al. (1991) have suggested that the α subunit maintains its high intrinsic GTPase activity due to a built-in GTPase-activating protein (GAP)-like domain. Since the rate of dissociation of receptor-agonist complex from G-protein is faster than the rate of inactivation of the G-protein (GTPase activity) each agonist-receptor complex catalytically activates multiple G-proteins, leading to signal amplification (Pedersen and Ross, 1982). In an in vitro study, Brandt and Ross (1986) demonstrated that 20 molecules of G_s are activated per purified β adrenergic receptor while Vuong et al. (1984) showed that about 1000 molecules of transducin are activated per rhodopsin receptor in the retina.

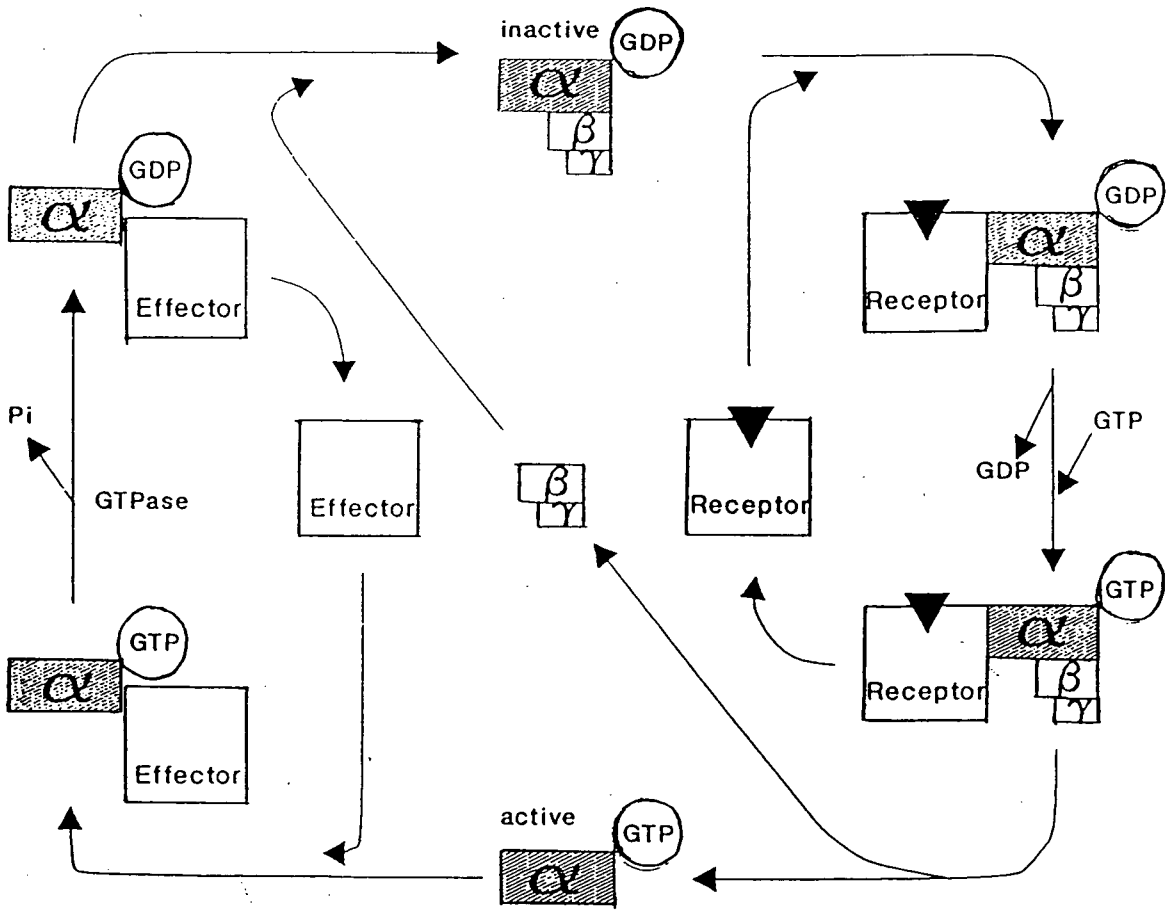

Fig. 2 Activation - deactivation cycle (GTPase cycle) of G-proteins (Eschenhagen, 1993). See text for details

1.5. ADENYLYL CYCLASE ISOFORMS

Adenylyl cyclase is the most extensively studied effector of G-proteins. For more than a decade, the existence of three isoforms of adenylyl cyclase has been well established. These include a calmodulin-sensitive form in the brain, a calmodulin and G_s-insensitive form in the testes and a G_s-stimulated isoform that is ubiquitously distributed. However, the cloning of six full length and two partial adenylyl cyclases has now enabled the identification of eight isoforms of the enzyme (Iyengar, 1993). Type I adenylyl cyclase was initially identified from bovine brain

membranes by purification of a 120 kDa G_s and Ca^{2+}-calmodulin-sensitive isoform (Pfeuffer and Metzger, 1982) and later by expression of cDNA encoding type I isoform (Tang et al., 1991). The cDNA for a Ca^{2+}-calmodulin-insensitive type II adenylyl cyclase was cloned from rat brain (Feinstein et al., 1991), while the cDNA for type IV adenylyl cyclase was identified from rat testes (Gao and Gilman, 1991). Type III adenylyl cyclase was cloned from rat olfactory tissue and has been suggested to be involved in olfactory signal transduction (Bakalyar and Reed, 1990). Ishikawa et al. (1992) and Katsushika et al. (1992) demonstrated the presence of isoforms V and VI of adenylyl cyclase in mammalian heart by cloning the respective cDNAs from canine myocardium. More recently, Wallach et al. (1994) cloned a novel type V isoform from rabbit myocardium.

A very high degree of sequence homology (> 50% in the transmembrane region) has been demonstrated between type V and VI isoforms of adenylyl cyclase (Katsushika et al., 1992).

Tissue distribution studies of adenylyl cyclase isoforms using Northern blotting, solution hybridization and polymerase chain reaction have demonstrated the presence of isoforms IV, V, VI and VII in heart (Iyengar, 1993). Manolopoulos et al. (1995) recently used reverse transcriptase - polymerase chain reaction (RT-PCR) and detected abundant message for isoforms V and VI in rat whole heart homogenate, no message for isoform II, traces of type III and no message for type IV adenylyl cyclase.

1.5.1. Muscarinic receptor-mediated inhibition of adenylyl cyclase in heart

It is well established that in mammalian myocardium muscarinic receptor-stimulation causes inhibition of adenylyl cyclase, especially when activated by β AR agonists such as Iso (Ransnas et al., 1986 ; Fleming and Watanabe, 1988 ; Watson et al., 1988) or a direct activator of adenylyl cyclase, forskolin (Watson et al., 1988). Binding of a muscarinic agonist such as Ach, Cch or Oxo to its receptors in the heart causes activation of an inhibitory G-protein, G_i which significantly increases high affinity GTPase activity and also inhibits adenylyl cyclase activity (Fleming et al., 1987 ; Fleming and Watanabe, 1988 ; Fleming et al., 1988 ; Hilf and Jakobs, 1989 ; Braun and Walsh, 1993). Muscarinic receptor-mediated effects on high affinity GTPase, adenylyl cyclase and cAMP levels in atrial and ventricular myocardium have been demonstrated to be PTX-sensitive (Fleming and Watanabe, 1988 ; Fleming et al., 1988 ; Ray and MacLeod, 1992 ; Braun and Walsh, 1993).

It is well established that stimulation of β ARs cause activation of the stimulatory G-protein, G_s which exchanges GDP for GTP and the active $G_{s\alpha.GTP}$ thus formed in turn stimulates adenylyl cyclase and produces an increase in the intracellular levels of cAMP. However, the exact mechanism of inhibition of adenylyl cyclase upon muscarinic receptor stimulation is not clearly established. Two models have been proposed by Gilman (1987) for G_i-mediated inhibition of adenylyl cyclase (fig. 3) :

(i) A subunit dissociation model, where Gβγ subunits released upon activation and dissociation of heterotrimeric Gi (into $G_{i\alpha.GTP}$ and $G_{\beta\gamma}$ subunits) reduce the

concentration of $G_{s\alpha.GTP}$ thereby causing **indirect inhibition** of adenylyl cyclase (Gilman, 1987 ; Katada et al., 1984a)

(ii) **Direct inhibition** of the catalytic subunit of adenylyl cyclase by $G_{i\alpha.GTP}$ (Katada et al., 1984b ; Hildebrandt et al., 1984 ; Codina et al., 1984)

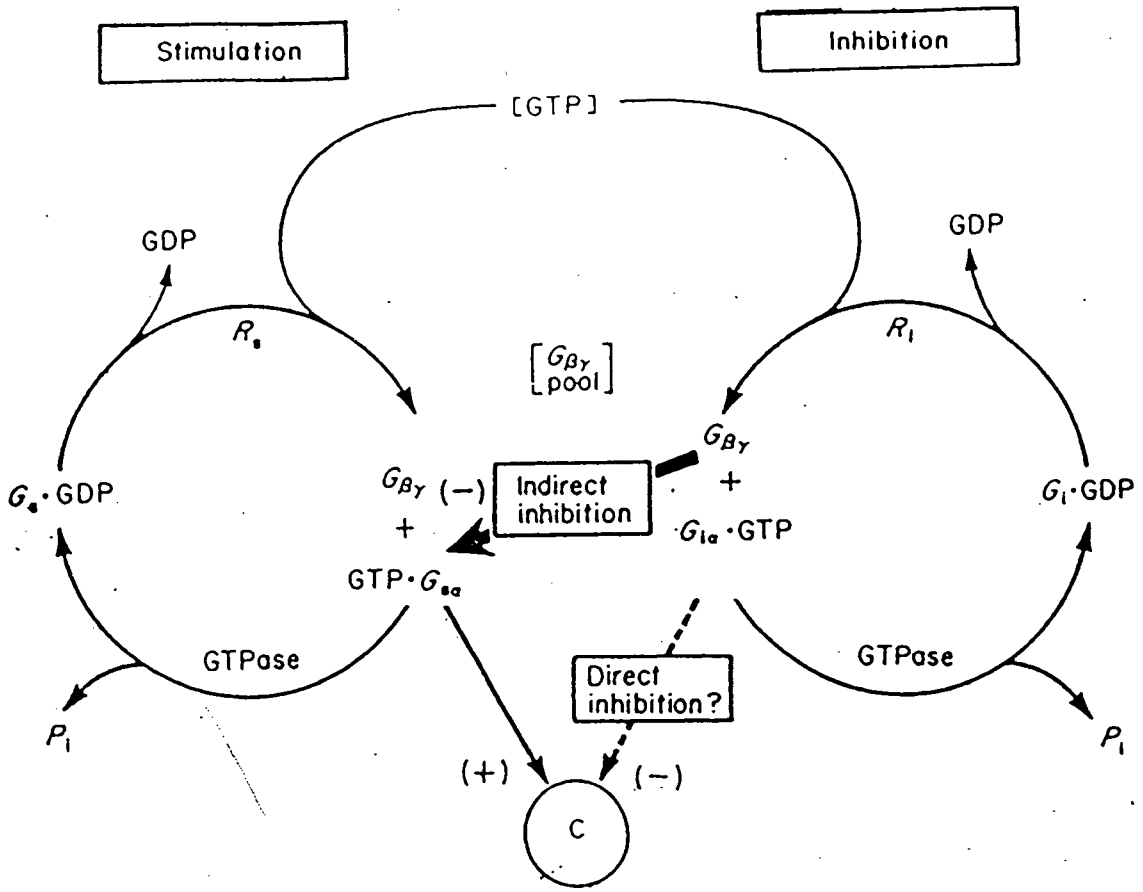

Fig. 3 Schematic representation of proposed mechanisms of muscarinic receptor-mediated inhibition of adenylyl cyclase in the heart (Fleming et al., 1987). See text for details.

The mechanisms proposed by Gilman (1987) do not suggest a role for βγ subunits in the direct inhibition of adenylyl cyclase. Although some in vitro studies on non-cardiac tissues and cell lines have provided evidence in favor of this suggestion they do not involve type V and VI adenylyl cyclases, the isoforms most abundantly expressed in the heart. Katada et al. (1987) demonstrated that type II and IV adenylyl cyclase isoforms are stimulated at very high concentrations of βγ subunits (1-100nM) in the presence of activated $G_{s\alpha}$ leading to elevation of $G_{s\alpha}$-stimulated adenylyl cyclase activity (Gao and Gilman, 1991 ; Tang and Gilman, 1991), while type I adenylyl cyclase is inhibited directly by βγ subunits in a concentration range of 1-50nM. In platelets and S49 cell membranes, exogenously added βγ subunits directly inhibit type I adenylyl cyclase, whereas even high concentrations of activated $G_{i\alpha}$ subunit only have a modest effect (Tang et al., 1991). Premont et al. (1992) demonstrated that βγ subunits have no direct effect on type V and VI adenylyl cyclase, the isoforms expressed abundantly in the heart (Manolopoulos et al., 1995) which would suggest that βγ subunits do not play a role in direct inhibition of adenylyl cyclase upon muscarinic receptor stimulation in the myocardium. One of the objectives of the present study was to investigate the mechanism of inhibition of adenylyl cyclase upon muscarinic receptor stimulation in mammalian atrial and ventricular myocytes.

The observations of a recent study from our laboratory (Ray, 1992) formed the basis for the present study. Ray (1992) demonstrated that in rabbit atrial homogenates, Cch did not affect basal adenylyl cyclase activity but inhibited Iso-stimulated cyclase activity, whereas forskolin and GTPγS-stimulated cyclase

activities were not affected. On the other hand in ventricular homogenates, Cch not only inhibited basal adenylyl cyclase activity but also decreased adenylyl cyclase activity elevated by Iso, forskolin and GTPγS. These observations suggested an interaction between adrenergic and cholinergic receptor regulated pathways in rabbit atrial but not ventricular myocardium. Therefore, another objective of the present study was to investigate whether different mechanisms exist for muscarinic receptor-mediated regulation of adenylyl cyclase activity in mammalian atrial and ventricular myocytes.

1.6 Summary, research hypothesis and research plan

It is well established that under normal physiological conditions, sympathetic stimulation of β AR in the heart causes activation of adenylyl cyclase, increases in intracellular cAMP levels, phosphorylation of intracellular proteins and Ca^{2+} channels and thereby produces positive inotropic and chronotropic effects. On the other hand, parasympathetic stimulation of muscarinic receptors inhibits adenylyl cyclase, may or may not decrease cAMP levels, inhibits phosphorylation of contractile proteins, activates ion channels such as atrial muscarinic K^+ channels and thereby produces negative inotropic and chronotropic effects.

From the background literature it is clear that the inhibitory effects of muscarinic receptor stimulation are prominent in the presence of sympathetic stimulation. Moreover, in the atrial myocardium muscarinic receptor stimulation exerts a direct negative inotropic effect, which is absent or minimal in ventricular myocardium. It is also known that the sympathetic and parasympathetic effects in the heart are mediated via stimulatory (G_s) and inhibitory (G_i) G-proteins respectively and

that the G_i-mediated effects are PTX-sensitive. Extensive information on cardiac regulation of adenylyl cyclase has demonstrated specific roles for G_s and G_i in the stimulation and inhibition of the enzyme. The mechanism of the G_s-mediated stimulation of adenylyl cyclase in mammalian myocardium has been extensively studied and clearly established. However, what is not known is the exact mechanism of muscarinic receptor-stimulated inhibition of adenylyl cyclase in the heart. It is also not known whether there are differences in the mechanism of muscarinic receptor-mediated inhibition of adenylyl cyclase activity in different regions of the heart and whether different G-proteins are present in atrial and ventricular myocytes.

The present study was designed to test the hypothesis that there are differences in the mechanism of inhibition of adenylyl cyclase by inhibitory G-proteins coupled to muscarinic receptors in the rabbit atrial and ventricular cardiomyocytes. The following experiments were performed to investigate the research hypothesis :

(a) The effects of β AR and muscarinic receptor stimulation alone on G-protein activation in atrial and ventricular myocytes were determined by measuring Iso and Cch-stimulated high affinity GTPase activity.

(b) The functional interaction between G_s and G_i-mediated effects in atrial and ventricular myocytes was studied by measuring the effects of Cch and Iso together on high affinity GTPase activity.

(c) The effects of β AR and muscarinic receptor stimulation alone and in combination, on adenylyl cyclase activity in atrial and ventricular myocytes was measured.

(d) The inhibitory (G_i and G_o) G-protein subtypes in atrial and ventricular myocyte membranes were identified by Western blotting.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

The materials used in this study were obtained from the following sources :

Amersham Canada Ltd., Canada

[α ^{32}P] ATP

[γ ^{32}P] ATP

BDH Chemical Co., Canada

Hydrochloric acid

Magnesium Chloride hexahydrate

Methanol

Potassium chloride

Sodium bicarbonate

Sodium chloride

BIO-RAD Laboratories, Ontario, Canada

Acrylamide

Ammonium persulphate

N'-N'-methylene bisacrylamide

Bromophenol blue

Dowex resin (H^+ form)

Glycine

Protein assay kit

Sodium dodecyl sulphate

N, N, N', N' - tetramethylethylenediamine (TEMED)

CALBIOCHEM CORPORATION, California, U.S.A.

Anti $G_{i\alpha1}/G_{i\alpha2}$ antibody (AS/7)

Anti $G_{i\alpha1}$

Anti $G_{i\alpha3}$

Anti$G_{i\alpha3}/G_{o\alpha}$ (EC/2)

GIBCO Laboratories, Ontario, Canada

Minimum Essential Medium (MEM-JOKLIK)

ICN Pharmaceutical Inc., Canada

[^{3}H] cAMP

NEN-DuPont Canada Inc., Ontario, Canada

Anti $G_{o\alpha}$ antiserum (GC/2)

[γ ^{32}P] GTP

Sigma Chemical Co., St. Louis, U.S.A.

Activated charcoal

Adenosine deaminase

Alamethicin

Alumina (neutral; activity grade I)

cAMP sodium salt

Ascorbic acid

ATP disodium salt

Atropine sulphate

Benzamidine hydrochloride

Carbamylcholine chloride

Creatine phosphate disodium salt

Creatine phosphokinase

Dithiothreitol

EDTA disodium salt

EGTA

Film, Kodak X-OMAT RP

GTP sodium salt

Imidazole hydrochloride

Isobutyl methyl xanthine

Isoproterenol hydrochloride

Leupeptin

Myokinase

Ouabain

Oxotremorine sesquisulphate

Phenyl methyl sulphonyl fluoride

Processing chemicals kit, Kodak

Sodium azide

Timolol maleate

Trizma base

Tris hydrochloride

Trypsin inhibitor

Anti $G_{i\alpha 2}$ antibody (J-883) was a generous gift from Dr. Suzanne Mumby, University of Texas, Dallas, TX, U.S.A.

2.2. PREPARATION OF SOLUTIONS

2.2.1. Drug solutions

Solutions of carbachol, timolol, atropine and oxotremorine were prepared in distilled water. Isoproterenol was dissolved in distilled water in the presence of ascorbic acid (1mg/ml) to reduce oxidation.

2.2.2. Homogenization buffer for preparation of sarcolemmal membranes

A ten times concentrated homogenization buffer was prepared by dissolving 15.8 g Tris HCl, 2.1 g EDTA, and 376 mg DTT in sufficient water to make up the final volume to 500 ml. The pH of the solution was adjusted to 7.8 with Tris base. On the day of the experiment, 10 ml of the stock solution was diluted to 100 ml with distilled water and had the following composition (in mM) : Tris HCl 20.0, EDTA 1.0 and DTT 1.0. The homogenization buffer also contained trypsin inhibitor (100µg/ml), PMSF (1 mM), benzamidine (3 mM), pepstatin (1µM) and leupeptin (1µM).

2.2.3 Solutions for GTPase assay

2.2.3.1. GTPase reaction mixture

A ten times concentrated reaction mixture was prepared by dissolving 1.5 g Tris HCl, 508 mg $MgCl_2$ and 104 mg disodium EDTA in 25 ml distilled water. The pH of the solution was adjusted to 7.8 and the solution was stored at -30°C prior to use. A 10 µl aliquot of this solution, when diluted with other components contained in mM : Tris HCl 40.0, $MgCl_2$ 10.0 and EDTA 1.0. The reaction mixture also contained 5 U/ml creatine phosphokinase.

2.2.3.2 Phosphocreatine solution

A stock solution of 50 mM phosphocreatine was prepared by dissolving 255 mg phosphocreatine in sufficient distilled water to make a final volume of 20 ml. A 10 µl aliquot of this stock solution, when mixed with other components of the reaction mixture to a final volume of 100 µl contained 5 mM phosphocreatine.

2.2.3.3. App(NH)p, ATP solution

A ten times concentrated stock solution of a mixture of ATP and App(NH)p was prepared by dissolving 146 mg ATP and 151 mg App(NH)p in sufficient distilled water to make up the final volume to 25 ml. The solution was stored in aliquots of 1.5 ml at -30°C. A 10 µl aliquot of this solution, when added to other components of the reaction mixture contained in mM : ATP 1.0 and App(NH)p 5.0.

2.2.3.4. GTP solution

A 1 mM stock solution of GTP was prepared by dissolving 5.2 mg GTP sodium in sufficient distilled water to make up the final volume to 10 ml. The solution was stored in aliquots of 1 ml at -30°C. A 10 µl aliquot of this solution, when added to other components of the reaction mixture contained 100 µM GTP.

2.2.4. Solutions for adenylyl cyclase assay

2.2.4.1. Adenylyl cyclase reaction mixture

A five times concentrated reaction mixture was prepared by dissolving 155 mg $MgSO_4$, 41.3 mg cAMP, 18.8 mg DTT, 725 mg sodium chloride, 27.5 mg IBMX and 988 mg Tris hydrochloride in sufficient water to make up the volume to 25 ml. The

pH of the solution was adjusted to 7.8 and stored at -30°C prior to use. A 30µl aliquot of this mixture, when diluted with other components of the assay mixture to 150 µl contained in mM : $MgSO_4$ 5.0, cAMP 1.0, DTT 1.0, NaCl 100, IBMX 1.0 and Tris hydrochloride 50.0

2.2.4.2 Regeneration mixture

A 100µl solution of creatine phosphate was prepared by dissolving 255 mg creatine phosphate in 10 ml water. A 15 µl aliquot of this solution, when diluted with other components of the reaction mixture to a final volume of 150 µl contained 10 mM creatine phosphate. Other components of regeneration mixture included creatine phosphokinase (1.6 U/150 µl), myokinase (6 U/150 µl, 200 U/mg) and adenosine deaminase (0.75 U/150 µl ; 200U/mg).

2.2.4.3. GTP solution

A stock solution of 500 µM GTP was prepared by dissolving 2.6 mg GTP sodium salt in sufficient water to make up the final volume to 10 ml. A 15 µl aliquot of this stock solution, when diluted with other components of the reaction mixture in a final volume of 150 µl gave a final concentration of 50 µM in each tube.

2.2.4.4. ATP solution

A 5 mM stock solution of disodium salt of ATP was prepared by dissolving 27.6 mg of ATP in sufficient water to make up the final volume to 10 ml. The pH of the solution was adjusted to 7.8 with Tris HCl. A 15 µl aliquot of the solution, when added to other components of the reaction mixture gave a final concentration of 0.5

mM. On the day of the experiment, 15 µl of this solution was mixed with 500,000 dpm ($\alpha^{32}P$) ATP and sufficient water to make up the volume to 30 µl.

2.2.4.5. Stop solution

Stop solution was prepared by dissolving 200 mg SDS, 248.8 mg ATP and 4.6 mg cAMP in sufficient water to make up the volume to 10 ml. The pH of the solution was adjusted to 7.5 with Tris base and stored at -30°C until further use. A 100 µl of this solution, when added to stop adenylyl cyclase activity contained 2% SDS, 40 mM ATP and 1.4 mM cAMP.

2.2.4.6. 0.1 M Imidazole hydrochloride solution

A 0.1 M imidazole hydrochloride solution was prepared by dissolving 10.04 g imidazole hydrochloride in sufficient distilled water to make up the final volume to 1000 ml. The pH of the solution was adjusted to 7.4 with Tris base.

2.2.4.7. 0.1 N Hydrochloric acid

Concentrated hydrochloric acid (12 N) was diluted 120 times with distilled water to obtain approximately 0.1 N hydrochloric acid.

2.2.5. Solutions for Na^+ K^+ ATPase assay

2.2.5.1. Na^+ K^+ ATPase reaction mixture

A ten times concentrated reaction mixture was prepared by dissolving 157.6 mg Tris HCl, 117 mg NaCl, 14.9 mg KCl, 121.9 mg $MgCl_2$ and 7.6 mg EGTA in sufficient distilled water to make up the final volume to 20 ml. A 50 µl aliquot of this mixture, when diluted with other components of the mixture contained in mM : Tris HCl 50.0, NaCl 100.0, KCl 10.0, $MgCl_2$ 3.0 and EGTA 1.0.

2.2.5.2 Sodium azide solution

A ten times concentrated solution of sodium azide was prepared by dissolving 195 mg sodium azide in 20 ml distilled water. The solution was stored in aliquots at -30°C. A 10 µl aliquot of this solution, when mixed with other components of the reaction mixture contained 15 mM of sodium azide.

2.2.5.3. Ouabain solution

A 100 mM stock solution of ouabain was prepared by dissolving 7.3 mg ouabain in 10% methanol. Since the drug is extremely light sensitive, the solution was prepared fresh before each experiment with minimum exposure to light. A 10 µl aliquot of the stock solution, when added to a final volume of 100 µl contained 10 mM ouabain.

2.3 Isolation of atrial and ventricular myocytes

Adult White New Zealand rabbits (1.5-2.5 kg) of either sex were housed in cages and had free access to food and water. The animals were injected with sodium pentobarbital (65 mg/kg body weight) and heparin (1000 U) through the ear vein. After exsanguination, the heart was removed immediately and placed in ice cold Minimum Essential Medium (MEM-JOKLIK) buffer composed of the following : MEM-JOKLIK modified 1 pack, $NaHCO_3$ 2.0 g, $MgSO_4.6H_2O$ 144 mg, L-carnitine 198 mg and sufficient double distilled water to make 1000 ml. The buffer was constantly oxygenated with a mixture of 95% O_2 and 5% CO_2 to maintain a pH of 7.4.

The heart was mounted on a Langendorff apparatus and perfused in a retrograde fashion for 10 min with Ca^{2+}-free MEM-JOKLIK buffer at a perfusion flow rate of 30 - 35 ml/min. to ensure that the coronary vessels were free of blood. At the

end of perfusion, the heart was digested with 50 ml collagenase (300 U/ml) solution for 10 min with the addition of 25 µl Ca^{2+} (100 mM) at 5 and 7 min respectively. For separation of atrial and ventricular myocytes, the atria and ventricles were separated and minced in a weigh boat containing 15 and 50 ml MEM/BSA (BSA 1 mg/ml) respectively. The contents of the weigh boat were transferred to a flask and shaken for 10 min at 37°C with constant oxygenation. After 10 min the suspensions of atrial and ventricular tissues were filtered through a 200-220 µm nylon filter. The dispersed atrial and ventricular tissues were resuspended in 30 ml (30 U/ml) and 50 ml (50 U/ml) collagenase solution respectively and agitated for 10 min. The pellets were centrifuged at low speed, supernatants were discarded and the dispersed pellets were resuspended in 20 ml MEM/BSA (Ca^{2+} concentration 200 µM). The process of incubation, filtration and resuspension of cells in collagenase solution was repeated 6-7 times to ensure complete digestion of the atrial and ventricular tissue.

Atrial and ventricular cells obtained after the final digestion were pooled separately, washed 3-4 times to remove dead cells and their viability estimated using the Trypan blue exclusion method. Cells were determined to be viable if they retained rod shaped morphology and excluded the dye, Trypan blue (0.2% w/v). Using this procedure an average of 2 - 4 x 10^6 atrial and 8 - 10 x 10^6 ventricular myocytes could be isolated simultaneously from each rabbit with an average viability greater than 65%. The supernatants were finally discarded and the myocytes were stored at -70°C until further use.

2.4 Preparation of sarcolemmal membrane

The procedure for preparation of sarcolemmal membrane from atrial and ventricular myocytes has been described by Braun and Walsh (1993). Atrial and ventricular myocytes were thawed, resuspended in homogenization buffer at a concentration of 2-3 x 10^6 cells/ml and homogenized using a glass/teflon homogenizer for 14 strokes at a setting of 6.0. The homogenate was centrifuged at 500 x g for 10 min at 4°C to separate large tissues. The supernatant was then carefully layered on top of a discontinuous sucrose gradient consisting of 5% layered over 30% sucrose and centrifuged at 70000 x g for 60 min at 4°C. The membranes present at the interface of 5% and 30% sucrose and in the 5% layer were removed and stirred on ice for 10 min at 4°C after the addition of 0.6M solid KCl. The solution was centrifuged at 120,000 x g for 60 min at 4°C and the membrane pellet was finally resuspended in homogenization buffer at a concentration of 1-2 mg/ml and stored in aliquots at -70°C until futher use. The protein content of the membrane was determined by the Bradford (1976) method of protein determination.

2.5. Assay for GTPase activity

The procedure for GTPase assay was slightly modified from the method described by Fleming et al. (1987). To each reaction tube 40 µl of reaction mixture was added. Sarcolemmal membranes from atrial and ventricular myocytes were incubated with the ionophore, alamethicin (0.5 µg/µg protein) for 20 min at room temperature and 10 µl of the membrane was added to each tube. After the addition of 10 µl of either agonist and/or antagonist to the respective tubes, the reaction was

initiated by the addition of (γ^{32}P) GTP (0.034 µCi/tube ; 3000 cpm/pmol) to each tube and incubated for 15 min at 37°C. At the end of the incubation period, the reaction was stopped by adding 0.9 ml ice cold, activated charcoal solution (5% w/v in 20 mM phosphoric acid) and the tubes were centrifuged at 2700 rpm for 15 min at 4°C. From the supernatant, 0.5 ml was transferred to scintillation vials, 2.5 ml of scintillation fluid was added and the ^{32}P-labeled Pi, released upon hydrolysis of ^{32}P-labeled GTP was quantitated in liquid scintillation counter. High affinity GTPase activity was calculated as the difference between total and non-specific GTP hydrolysis (determined in the presence of 100 µM GTP).

2.6. Assay for adenylyl cyclase activity

The details of the procedure for adenylyl cyclase assay have been described by Salomon (1979). Sarcolemmal membranes from atrial and ventricular myocytes were incubated with (α^{32}P)ATP (500,000 dpm) for 10 min at 37°C in a final volume of 150 µl containing 30 µl of reaction mixture, 30 µl of regeneration mixture, 15 µl of the agonist and/or antagonist, 15 µl GTP, 15 µl unlabeled ATP, 10 µl of the membrane and made up to volume with distilled water. At the end of the incubation period, 100 µl of stop solution was added to each tube to stop the reaction.

Using sequential chromatography with Dowex and Alumina columns the (^{32}P)-cAMP formed was separated from (^{32}P)-ATP using (^{3}H)-cAMP (10000 cpm/tube) as the recovery marker according to the following procedure :

To the reaction tubes, 800 µl of water was added and the contents were transferred to Dowex columns. The tubes were rinsed with 2 ml water, the contents transferred to Dowex columns and the effluent collected as waste. The Dowex

columns were mounted on top of Alumina columns previously regenerated with 0.1 M imidazole solution and 3 ml water was added to each column. The effluent from the Alumina columns was collected as waste. The Alumina columns were washed with 4 ml 0.1 M imidazole solution and the effluent was collected into 20 ml polyethylene scintillation vials. Finally, 11 ml of scintillation fluid (Aquasol ®) was added to each vial and the ^{3}H and ^{32}P-labeled cAMP were quantitated in a liquid scintillation counter.

Adenylyl cyclase activity was determined as the amount of cAMP formed (pmol/min/mg protein) using the formula described by Salomon (1979)

cAMP (pmol/min/mg) = [H/S] x [(S'-B'-F)x1000)]/Rsp x E x t, where

F = (H'-B')/HxS

Rsp = P'(dpm)/pmoles of ATP per tube

H and H' = (^{3}H) cAMP standard counts in ^{3}H and ^{32}P channels respectively

P' = (^{32}P) ATP standard counts in ^{32}P channels

B' = Background counts in ^{32}P channels

S and S' = sample counts in ^{3}H and ^{32}P channels respectively

E = Protein concentration in mcg

t = Incubation time in min

In the blank tubes, (^{32}P) ATP was added after stopping the reaction with the stop solution. To determine net cAMP formed (pmol/min/mg), the blank value was subtracted from the sample value.

2.7. Na^{+} K^{+} ATPase assay

The procedure for measuring ouabain-inhibitable $Na^{+}K^{+}$ATPase activity was modified from Braun and Walsh (1993). The assay was performed in minimal light

conditions to prevent breakdown of ouabain. The final reaction volume of 100 µl in each tube had the following composition : Tris HCl 50 mM, NaCl 100 mM, KCl 10 mM, $MgCl_2$ 3 mM, EGTA 1 mM, NaN_3 15 mM and alamethicin (0.5 µg/µg protein). The reaction tubes were initially warmed for 5 min at 37°C and then allowed to react with ($\gamma^{32}P$) ATP (5 mM ; 3000 cpm/pmol) for 15 min at 37°C. At the end of incubation, the reaction was stopped by adding 0.9 ml activated charcoal solution (5% w/v in 20 mM phosphoric acid) to each tube and tubes were kept on ice for 5 min. The tubes were centrifuged at 3000 x g for 10 min at 4°C and 0.5 ml of the supernatant was transferred to scintillation vials. Finally, 2.5 ml of Aquasol® was added to each vial and ^{32}P-labeled Pi released upon hydrolysis of ($\gamma^{32}P$) ATP was measured in a liquid scintillation counter. Specific activity was determined by subtracting the non-specific activity in the presence of 1 mM ouabain.

2.8 Western Blotting of atrial and ventricular myocyte membranes

Atrial and ventricular myocyte sarcolemmal membrane proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis using the method of Laemmli (1970) on a 10% acrylamide/0.8% *N,N'*-methylenebisacrylamide gel run overnight at 500 volts and 10 mA constant current. To each lane on the gel, 20 µg of membrane protein was added. The gel was then transferred electrophoretically onto nitrocellulose membrane according to the method of Towbin et al. (1979) at 0.4A for 2 hr at 4°C and blocked with 5% skim milk powder in Tris buffered saline (pH=7.5) with Tween 20 (0.1%) for 2 hr at 4°C. The blot was then incubated with 1:1000 dilution of AS/7, EC/2, anti$G_{i\alpha1}$, anti$G_{i\alpha3}$ or anti$G_{o\alpha}$ or 1:5000 dilution of anti $G_{i\alpha2}$ (J-883, Mumby et al., 1986) for 1 hr at room temperature and subsequently incubated with horse

radish peroxidase conjugated donkey anti-rabbit secondary antibody (1:10000) and finally detected using enhanced chemiluminescence. The exposure time ranged from 1-10 min. The film was developed in a developing solution (1:5 dilution in distilled water) for 1-2 min, rinsed in distilled water and fixed by placing in a fixing solution (1:5 dilution in distilled water) for 1 min.

2.9 PROTEIN ASSAY

The protein content in atrial and ventricular myocyte membranes was determined by the Bradford (1976) method using BSA as standard. Proteins from atrial and ventricular myocyte membranes were allowed to react with with dye reagent (previously diluted 1 : 4 with distilled water and filtered to remove any impurities) and the absorbance was measured at 594-596 nm using the ultraviolet spectrophotometer.

2.10 STATISTICS

The results from studies on GTPase activity and adenylyl cyclase activity were analyzed by repeated measures one - way analysis of variance followed by a Bonferroni post-hoc test. The data from Na^+ K^+ ATPase assay and substrate saturation (kinetic analysis) experiments were analyzed by paired t-test. A $p< 0.05$ was considered significantly different.

3.0 RESULTS

3.1 Assay for Na^+ K^+ ATPase activity

The measurement of ouabain-inhibitable Na^+ K^+ ATPase activity was used as a marker to determine the degree of enrichment of membranes prepared from atrial and ventricular myocytes by discontinuous sucrose density gradient ultracentrifugation. Sarcolemmal membranes from atrial and ventricular myocytes displayed 63 and 117 fold enrichment from their respective homogenates as determined by measuring ouabain-inhibitable Na^+ K^+ ATPase activity (Table 1).

3.2 CHARACTERIZATION OF β ADRENOCEPTOR AND MUSCARINIC RECEPTOR-STIMULATED HIGH AFFINITY GTPase ACTIVITY

3.2.1 Kinetic Analysis - Substrate saturation curves

In ventricular myocyte sarcolemmal membranes, GTP saturation curves revealed high affinity GTPase activity with basal V_{max} values of 24.7 ± 6.7 pmolPi/min/mg (for experiments with Iso) and 58.0 ± 11.8 pmolPi/min/mg (for experiments with Cch) and K_m values of 0.36 ± 0.07 μm and 0.65 ± 0.1 μm respectively. Both Iso (100μM) and Cch (100μM) increased the V_{max} without affecting K_m values (Table 2, fig. 4 and 5). In separate experiments, Oxo (10μM) also increased the V_{max} from the basal value (35.3 ± 4.4 pmolPi/min/mg) significantly while having no significant effect on basal K_m (0.33 ± 0.05 μm) (fig. 6, Table 2).

In a preliminary experiment on atrial myocyte membranes, Oxo (10μM) produced a 40% increase in V_{max} from the basal value (28.3 pmolPi/min/mg) without affecting the K_m value (data not shown). Substrate saturation experiments using Iso and Cch and more experiments using Oxo could not be performed on atrial myocyte

membranes due to very low membrane protein content obtained by sucrose density gradient ultracentrifugation.

3.2.2 Concentration - dependent effects of β-adrenoceptor and muscarinic receptor agonists on high affinity GTPase activity

In ventricular myocyte membranes, Iso (1μM-1mM) produced a concentration-dependent increase in high affinity GTPase activity with a maximum stimulation of 35% above the basal value (17.06 ± 2.7 pmolPi/min/mg) at 100μM (fig. 7). Similarly, Cch (100nM - 1mM) and Oxo (10nM-1mM) also increased the high affinity GTPase activity in a concentration-dependent manner with maximum stimulation of 106% and 122% above their respective basal values (19.11 ± 4.9 and 6.98 ± 1.1 pmolPi/min/mg) at 100μM and 10μM respectively (fig. 8 and 9).

In a preliminary experiment on atrial myocyte membranes Cch (100nM - 1mM) produced a concentration-dependent increase in high affinity GTPase activity with a maximum stimulation of 66% above the basal value (22.6 pmolPi/min/mg) at 100μM (data not shown). However, more experiments could not be performed due to very low membrane protein yield.

Based on the observations from preliminary characterization experiments, Iso and Cch were used at a concentration of 100μM while Oxo was used at a concentration of 10μM in all further experiments.

3.3 Interaction of β adrenoceptor and muscarinic receptor-stimulated high affinity GTPase activity

In ventricular myocyte membranes Iso and Cch alone produced 31.0 ± 4.2% and 115.0 ± 9.9% increases in high affinity GTPase activity above the basal value

(10.39 ± 1.4 pmolPi/min/mg). In the presence of Iso and Cch in combination the GTPase activity was elevated to 143.0 ± 10.9%. The measured activity was very close to the value predicted if the combined effects of Iso and Cch on high affinity GTPase activity were additive (146%) (fig. 10). In different experiments, Iso and Oxo alone produced 28.2 ± 3.2% and 97.0 ± 9.7% increases in high affinity GTPase activity from their respective basal values. In the presence of a combination of Iso and Oxo, the GTPase activity was increased to 123.4 ± 9.0% above the basal, which is very close to the predicted value for an additive effect (125%) (fig. 11)

In atrial myocyte membranes Iso and Cch alone produced 26.0 ± 5.4% and 57.0 ± 6.4% increases in high affinity GTPase activity above the basal value (17.4 ± 3.3 pmolPi/min/mg). However, in the presence of Iso and Cch in combination, there was no further elevation in GTPase activity (52.0 ± 6.7%) compared to Cch alone (fig. 12). In a different set of experiments, Iso and Oxo alone produced 26.4 ± 6.6% and 53.2 ± 7.7% increases in high affinity GTPase activity from the basal value (17.84 ± 4.03 pmol Pi/min/mg). In the presence of Iso and Oxo in combination there was no further increase in GTPase activity (50.4 ± 8.3%) (fig. 13). If the effects of Iso and Cch or Iso and Oxo on high affinity GTPase activity were additive, the predicted value in the presence of both agonists in combination would have been 83% and 79.6% respectively above their basal values.

3.4 β Adrenoceptor and muscarinic receptor-stimulated adenylyl cyclase activity

In ventricular myocyte membranes, Iso (100μM) produced a 46.0 ± 5.8% increase in adenylyl cyclase activity above the basal value (2904.8 ± 539.8 pmol cAMP formed/min/mg protein), while Cch (100μM) caused a 36.0 ± 4.7% inhibition of basal adenylyl cyclase activity. In the presence of Iso and Cch, in combination the adenylyl cyclase activity remained close to the basal value (101.0 ± 3.1%). The measured activity was very close to the value predicted if the stimulatory effects of Iso and inhibitory effects of Cch on adenylyl cyclase activity were occurring independently (110%) (fig. 14).

We had difficulties in reliably measuring β-adrenoceptor and muscarinic receptor-stimulated adenylyl cyclase activity in atrial myocyte membranes. However, in preliminary experiments Iso produced a 39.7 ± 12.8% increase in adenylyl cyclase activity while Cch did not affect basal cyclase activity (8% above basal). However, in the presence of Iso and Cch in combination, the Iso-stimulated adenylyl cyclase activity was inhibited by 51% but still remained 20% above the basal value (fig. 15). Although none of the measured effects were statistically significant, they were qualitatively consistent with previous observations from this laboratory on atrial homogenates (Ray, 1992) where Cch (1mM) alone did not affect the basal cyclase activity but in combination with Iso (100μM) produced a 57.0 ± 10.2% inhibition of Iso-stimulated adenylyl cyclase activity (fig. 16).

3.5 Identification of G-proteins by immunoblotting

In atrial myocyte sarcolemmal membranes, immunoblotting with AS/7 and EC/2 produced distinct bands at approximately the expected molecular weights. The antibody for $G_{i\alpha1}$ did not produce any band whereas anti $G_{i\alpha2}$ and anti $G_{i\alpha3}$ antibodies produced distinct bands. Anti $G_{o\alpha}$ produced a distinct band at the expected molecular weight of the $G_{o\alpha}$ subtype. We were, therefore able to identify directly and indirectly $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{o\alpha}$ subtypes of G-proteins in atrial myocyte membranes (fig. 17).

Western blot of ventricular myocyte membranes with AS/7 and anti $G_{i\alpha1}$ produced distinct bands while anti $G_{i\alpha2}$ did not reveal any band at the expected molecular weight of the $G_{i\alpha2}$ subtype. The selective antibody for $G_{i\alpha3}$, anti $G_{i\alpha3}$ produced a faint band at approximately the expected molecular weight while EC/2 produced a distinct band. However, the anti $G_{o\alpha}$ antiserum exhibited high non specific activity resulting in several bands which could not be distinguished from the band for $G_{o\alpha}$ subtype at the expected molecular weight. We were therefore unable to conclusively identify $G_{o\alpha}$ subtype in ventricular myocyte membranes. Thus, in ventricular myocyte membranes we were able to identify $G_{i\alpha1}$ and $G_{i\alpha3}$ subtypes of inhibitory G-proteins, both directly and indirectly (fig. 18).

Table 1. $Na^+ K^+$ ATPase activity in atrial and ventricular sarcolemmal membranes. Results are from (n) different membrane preparations and are expressed as µmol Pi/min/mg protein ± S.E.M.

Cells	$Na^+ K^+$ ATPase activity		Enrichment[@]
	Homo genate	Sarcolemmal membranes	
	µmol Pi. mg protein^{-1} .min^{-1}		
Atrial myocytes (n=3)	0.0036 ± 0.0012	0.225 ± 0.067	63
Ventricular myocytes (n=3)	0.0026 ± 0.00085	0.304 ± 0.074	117

[@] equals the $Na^+ K^+$ ATPase activity measured in sarcolemmal membranes divided by the activity in the homogenate

TABLE 2. Kinetic Analysis : Substrate saturation analysis for high affinity GTPase activity in the presence of isoproterenol (Iso), carbachol (Cch) and oxotremorine (Oxo) in ventricular myocyte membranes

Treatment	Vmax (pmol Pi/min/mg)	Km (μM)
Iso (100μM) (n=5)	31.1 ± 6.9 * (24.7 ± 6.7)	0.47 ± 0.08 (0.36 ± 0.07)
Cch (100μM) (n=5)	113.4 ± 28.9 * (58.0 ± 11.8)	0.51 ± 0.07 (0.65 ± 0.1)
Oxo (10μM) (n=4)	79.8 ± 17.3 * (35.3 ± 4.4)	0.43 ± 0.058 (0.33 ± 0.047)

* Significantly different from basal value ($p<0.05$; paired t - test)

Data is expressed as mean ± S.E.M

Numbers in parantheses indicate basal values

Results are from 'n' different membrane preparations

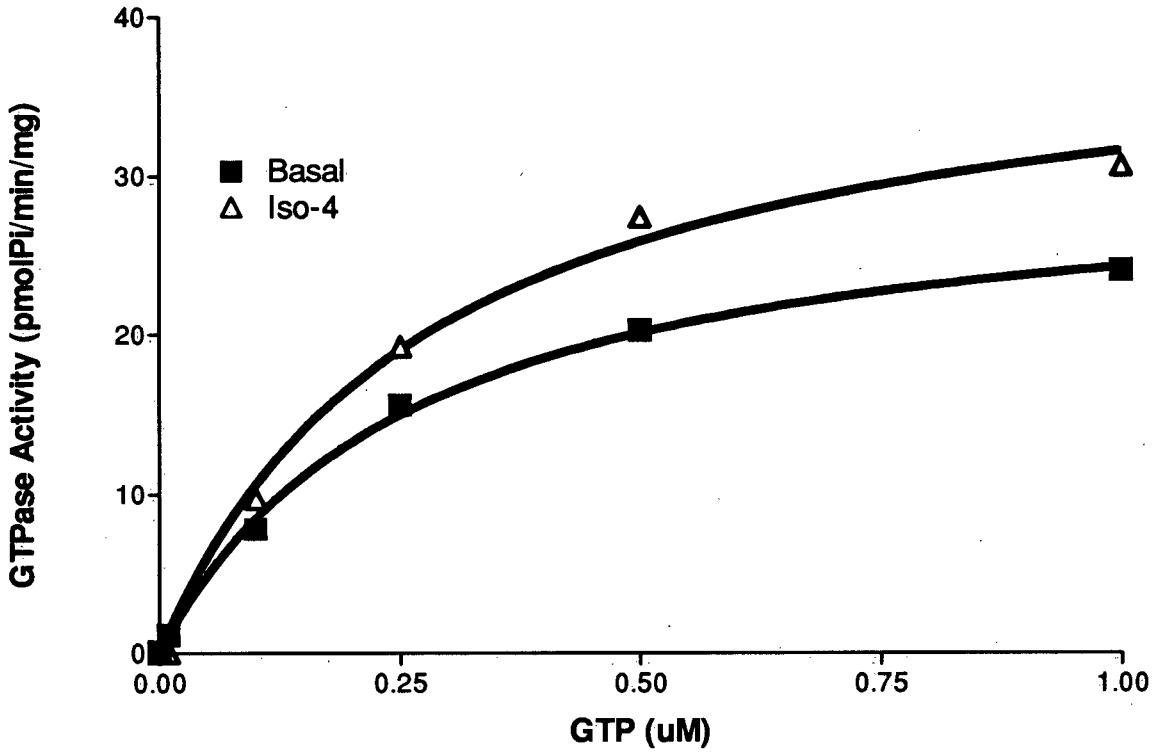

Fig. 4 Substrate saturation curve for high affinity GTPase activity in ventricular myocyte membranes in the presence (open triangles) and absence (closed squares) of isoproterenol (Iso ; 100μM). A representative curve from assay of one membrane preparation is shown. Results are the mean of duplicates.

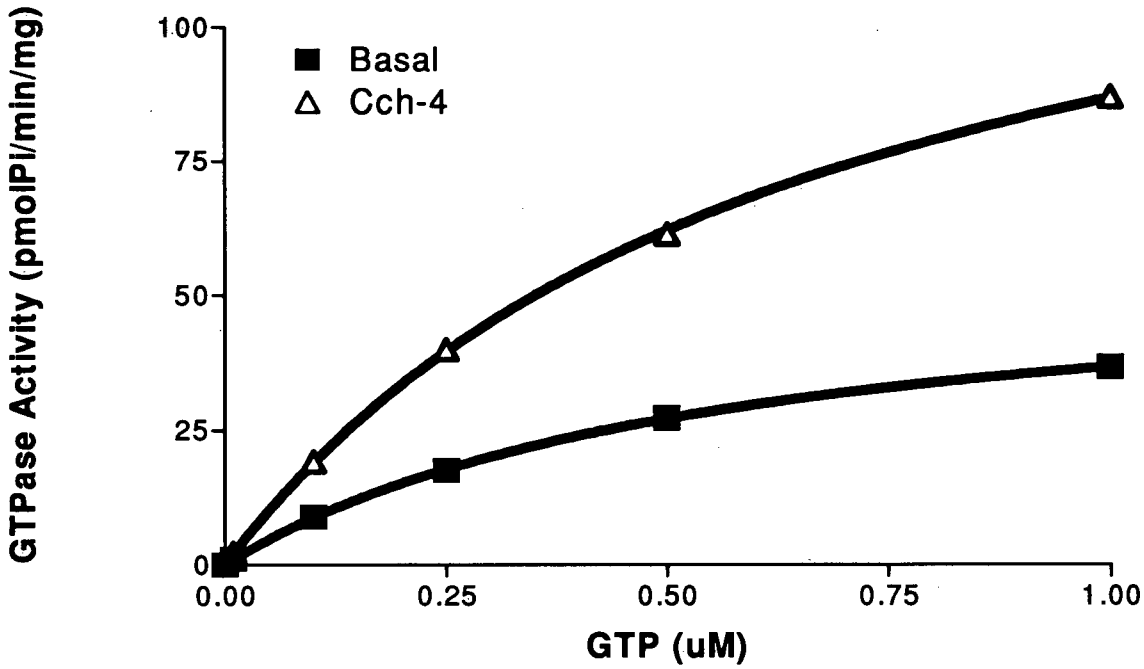

Fig. 5 Substrate saturation curve for high affinity GTPase activity in ventricular myocyte membranes in the presence (open triangles) and absence (closed squares) of carbachol (Cch ; 100μM). A representative curve from assay of one membrane preparation is shown. Results are the mean of duplicates.

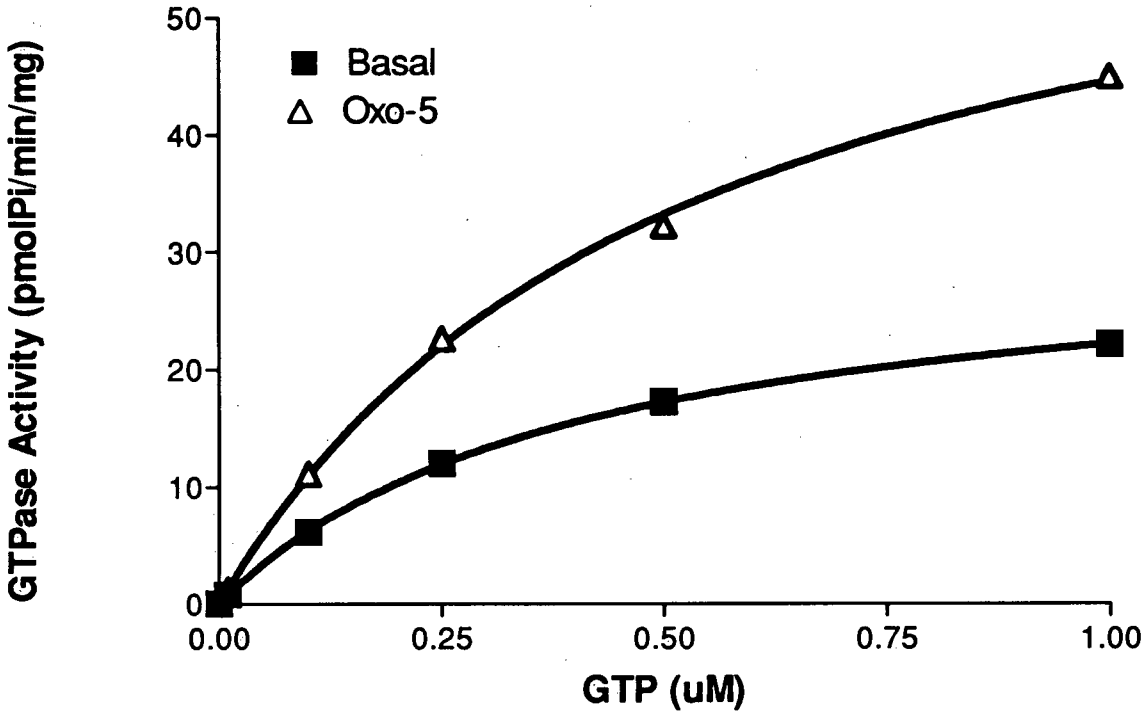

Fig. 6 Substrate saturation curve for high affinity GTPase activity in ventricular myocyte membranes in the presence (open triangles) and absence (closed squares) of oxotremorine (Oxo ; 10μM). A representative curve from assay of one membrane preparation is shown. Results are the mean of duplicates.

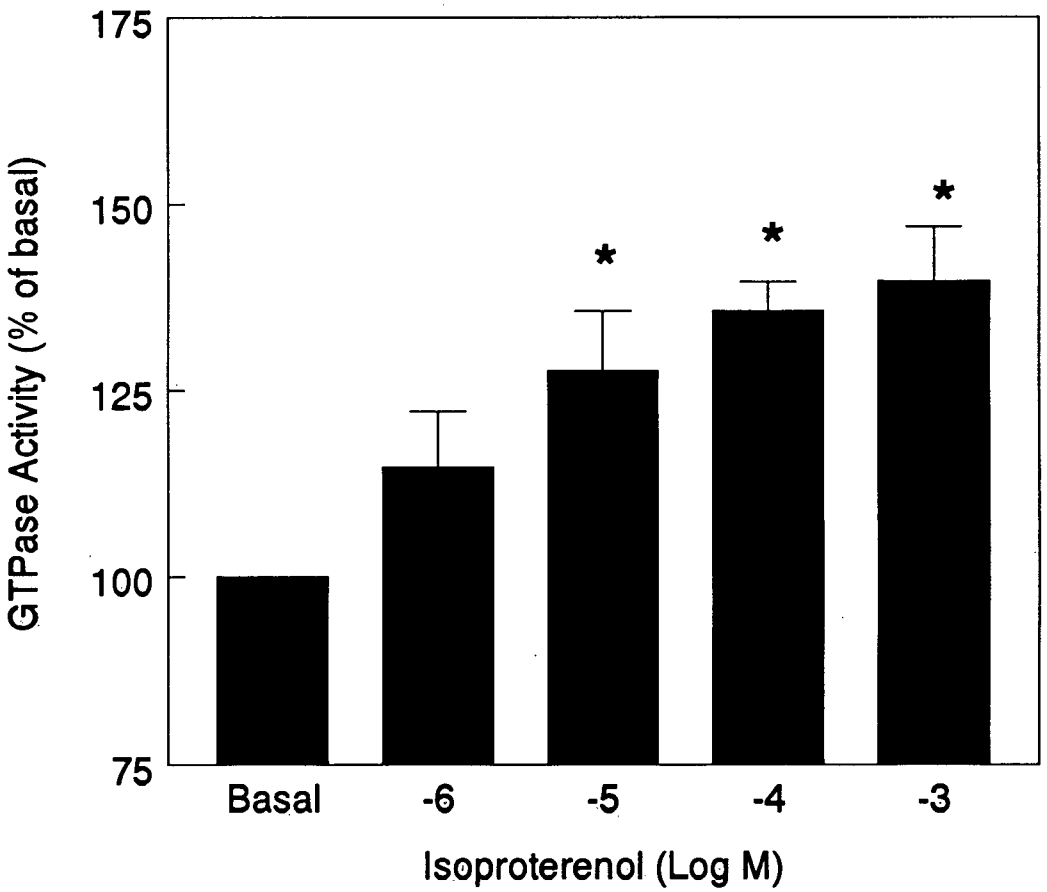

Fig. 7 Concentration-dependent effects of isoproterenol (1μM - 1mM) (n=3) on high affinity GTPase activity in ventricular myocyte membranes.

* Significantly different from basal

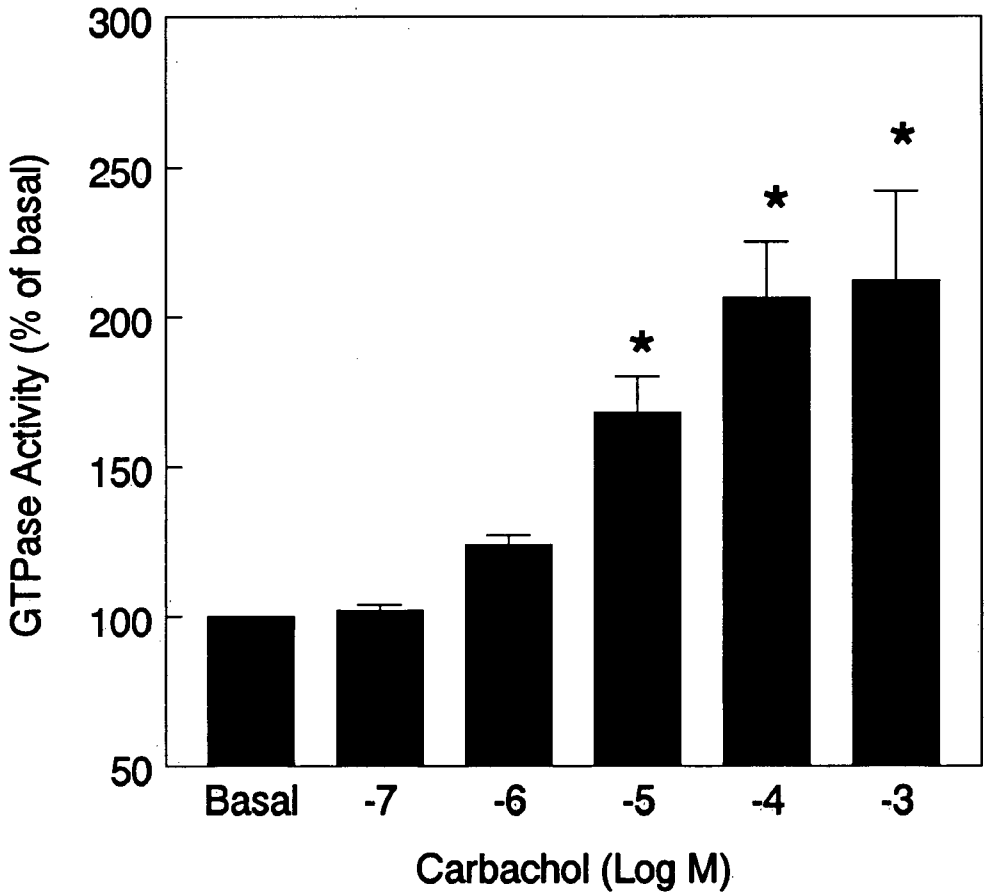

Fig. 8 Concentration-dependent effects of carbachol (100nM - 1mM) (n=3) on high affinity GTPase activity in ventricular myocyte membranes.

* Significantly different from basal

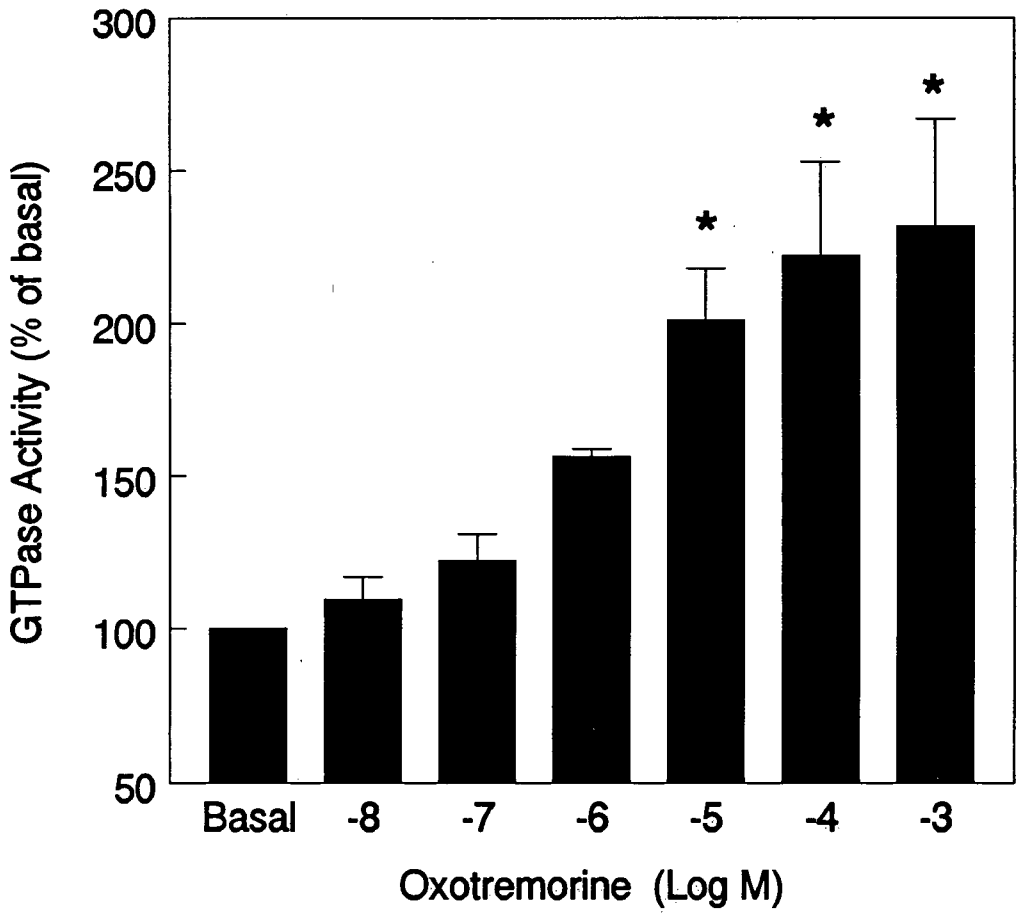

Fig. 9 Concentration-dependent effects of oxotremorine (10nM - 1mM) (n=3) on high affinity GTPase activity in ventricular myocyte membranes.

* Significantly different from basal

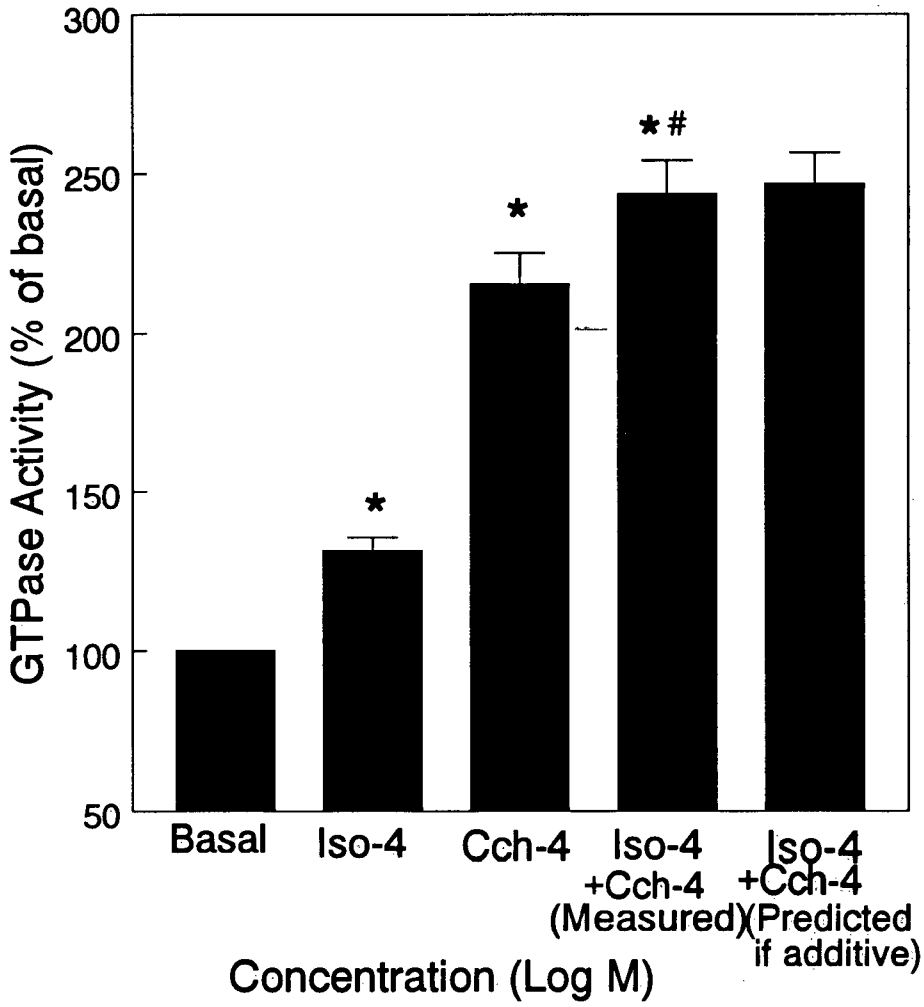

Fig 10 Effect of isoproterenol (Iso ; 100μM) and carbachol (Cch 100μM), alone and in combination on high affinity GTPase activity in ventricular myocyte membranes (n=9).

* Significantly different from basal

Significantly different from Cch alone

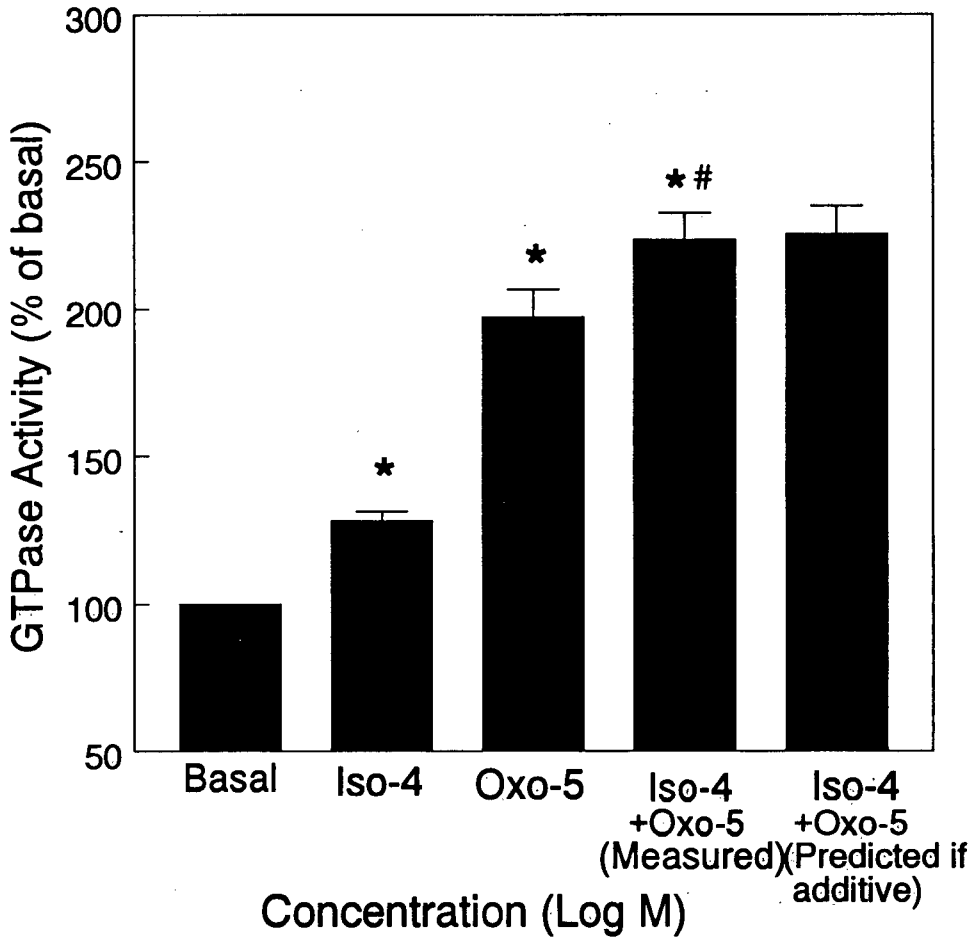

Fig. 11 Effect of isoproterenol (Iso ; 100μM) and oxotremorine (Oxo ; 10μM), alone and in combination on high affinity GTPase activity in ventricular myocyte membranes (n=9)

* Significantly different from basal

\# Significantly different from Oxo alone

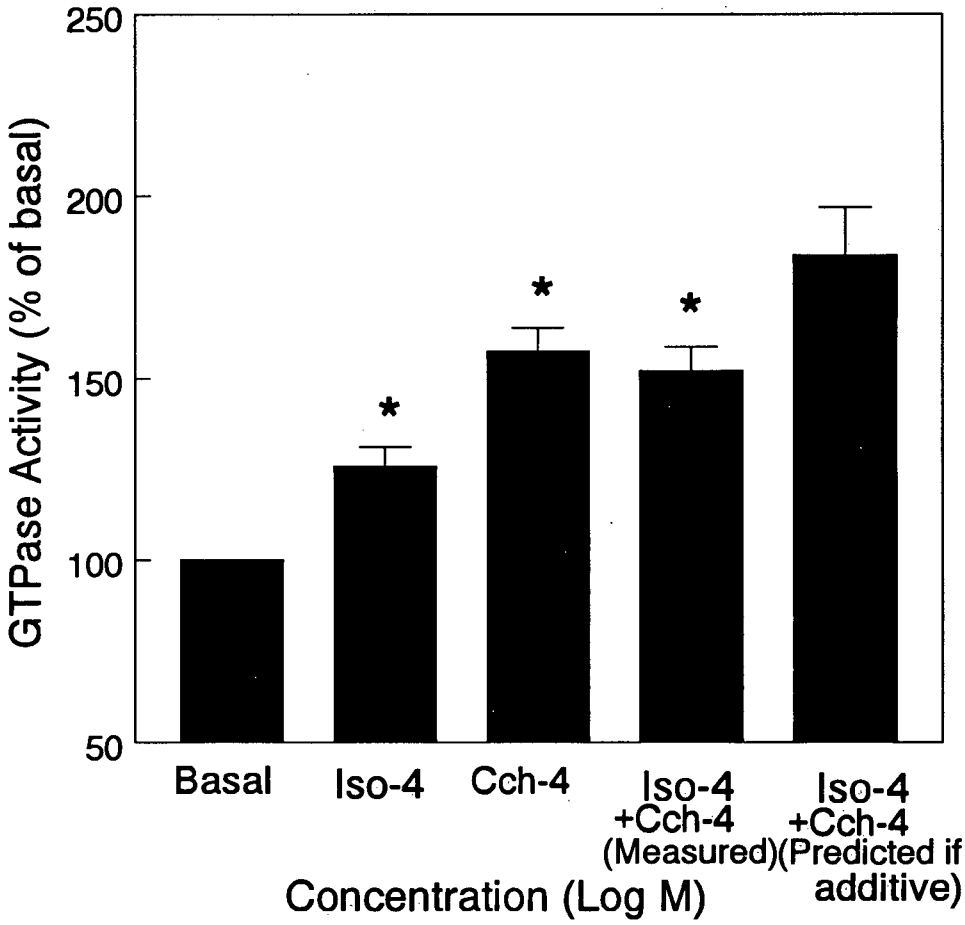

Fig 12 Effect of isoproterenol (Iso ; 100μM) and carbachol (Cch ; 100μM), alone and in combination on high affinity GTPase activity in atrial myocyte membranes (n=6)

* Significantly different from basal

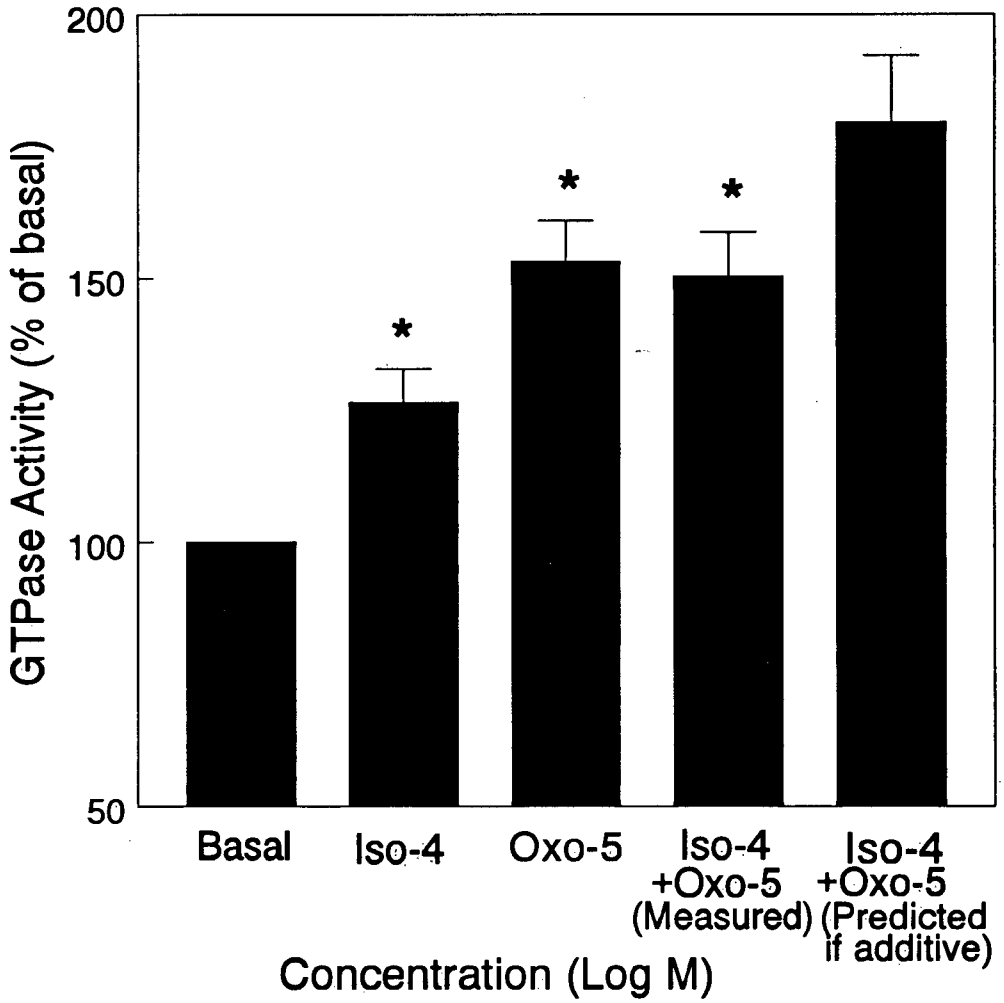

Fig. 13 Effect of isoproterenol (Iso ; 100μM) and oxotremorine (Oxo ; 10μM), alone and in combination on high affinity GTPase activity in atrial myocyte membranes (n=5)

* Significantly different from basal

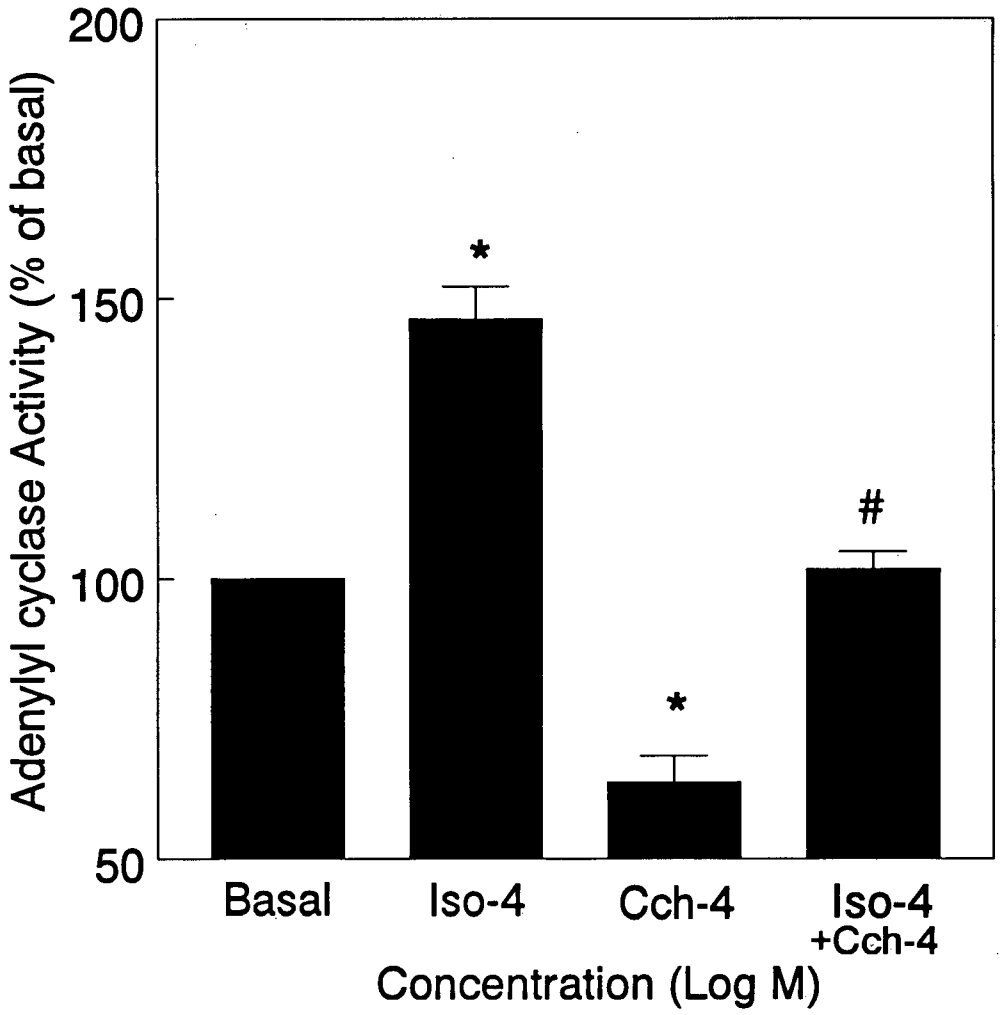

Fig. 14 Effect of isoproterenol (Iso ; 100μM) and carbachol (Cch ; 100μM), alone and in combination on adenylyl cyclase activity in ventricular (n=5) myocyte membranes

* Significantly different from basal

\# Significantly different from Iso or Cch alone

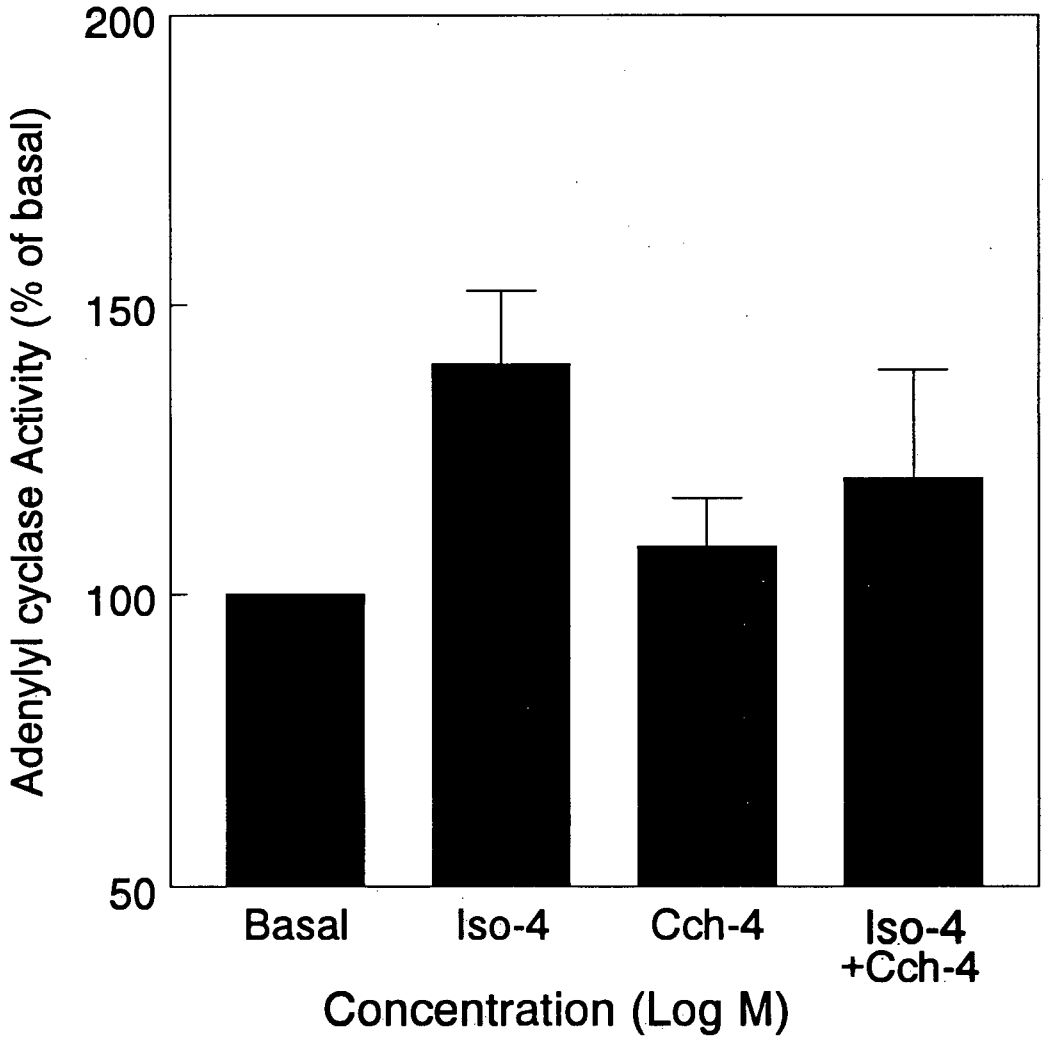

Fig. 15 Effect of isoproterenol (Iso ; 100μM) and carbachol (Cch ; 100μM), alone and in combination on adenylyl cyclase activity in atrial (n=3) myocyte membranes

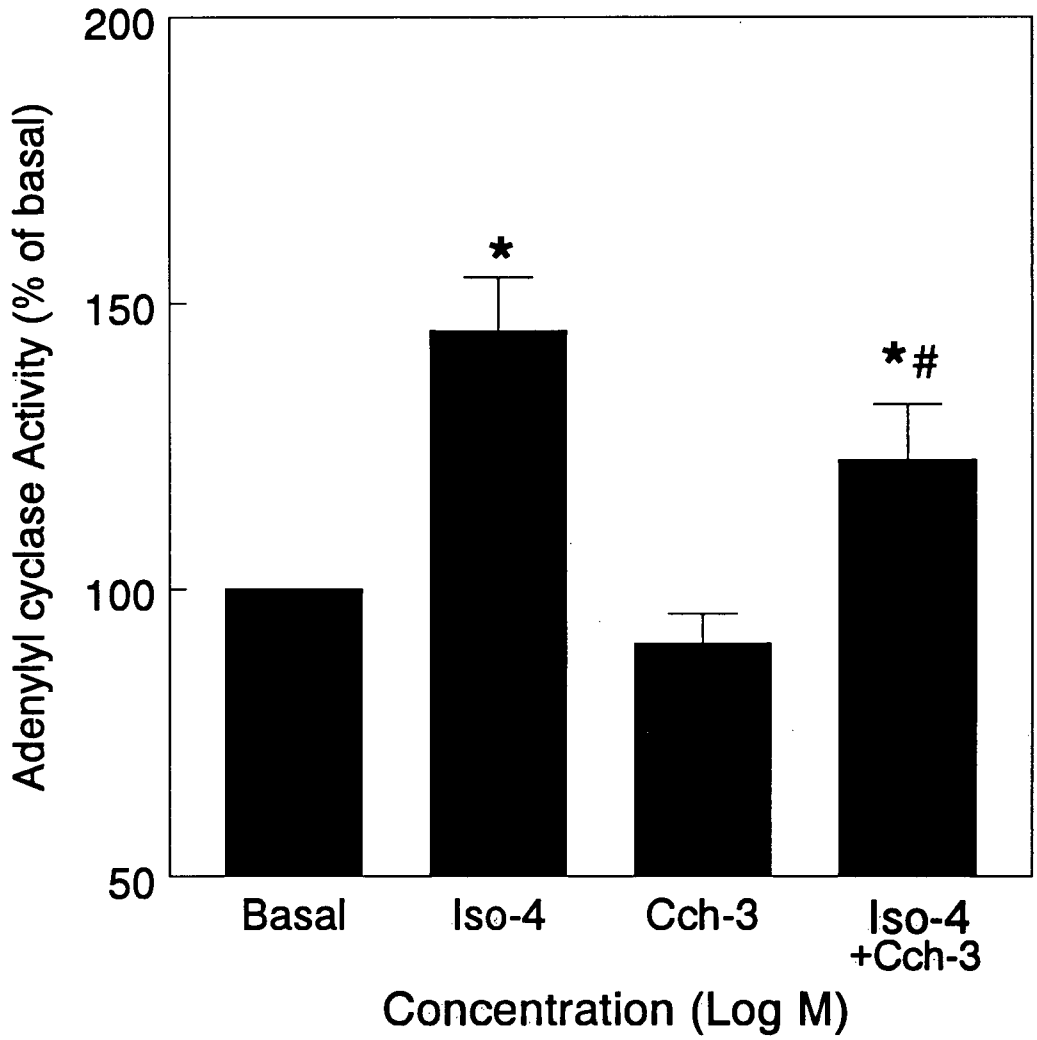

Fig. 16 Effect of isoproterenol (Iso ; 100μM) and carbachol (Cch ; 1mM), alone and in combination on adenylyl cyclase activity in rabbit atrial homogenates (n=8) *(Data compiled from Ray, 1992)*

* Significantly different from basal

\# Significantly different from Iso or Cch alone

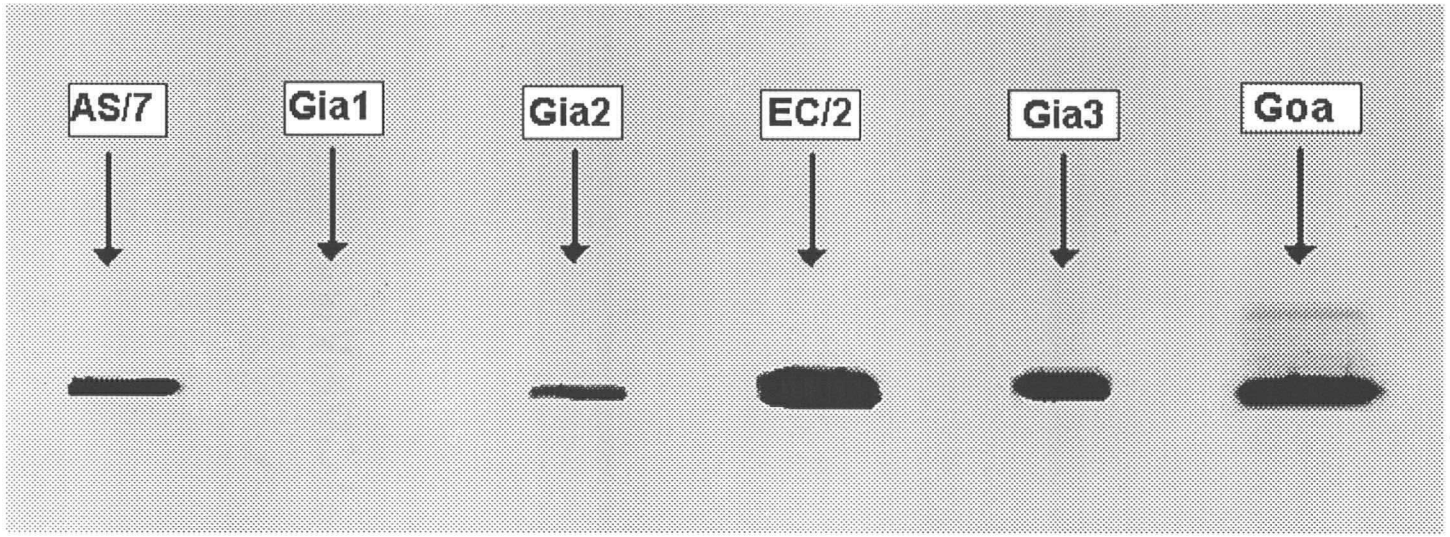

Fig. 17 Western blot of atrial myocyte membranes using antibodies specific for $G_{i\alpha1}$ and $G_{i\alpha2}$ (AS/7), $G_{i\alpha3}$ and $G_{o\alpha}$ (EC/2), $G_{i\alpha1}$ (anti $G_{i\alpha1}$), $G_{i\alpha2}$ (J - 883 or anti $G_{i\alpha2}$), $G_{i\alpha3}$ (anti $G_{i\alpha3}$) and $G_{o\alpha}$ (anti $G_{o\alpha}$)

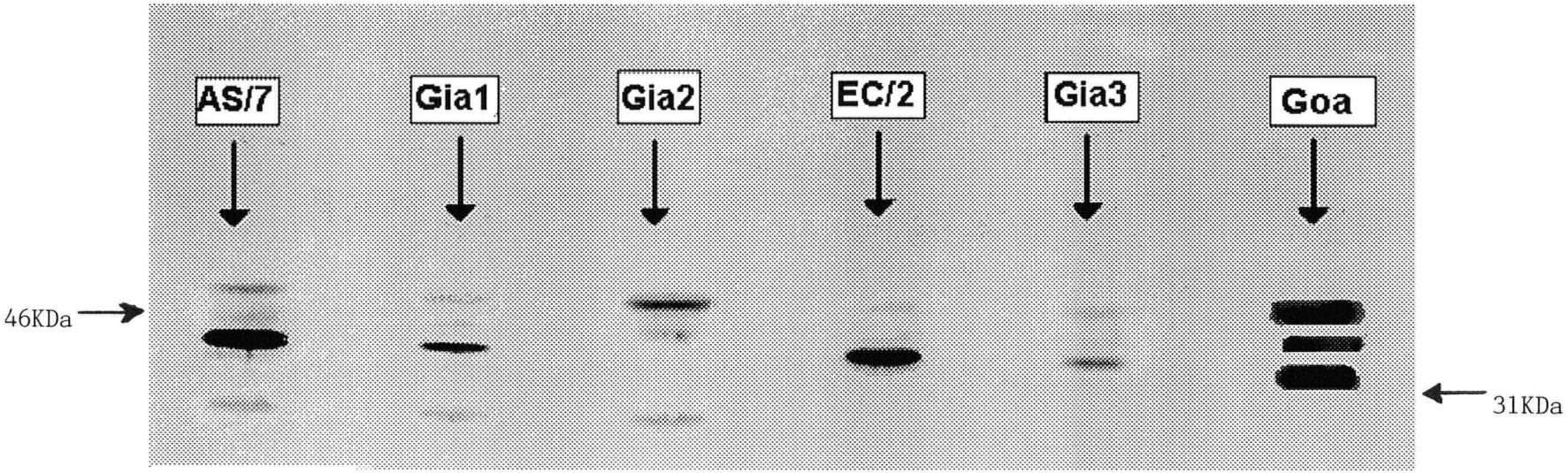

Fig. 18 Western blot of ventricular myocyte membranes using antibodies specific for $G_{i\alpha1}$ and $G_{i\alpha2}$ (AS/7), $G_{i\alpha3}$ and $G_{o\alpha}$ (EC/2), $G_{i\alpha1}$ (anti $G_{i\alpha1}$), $G_{i\alpha2}$ (J - 883 or anti $G_{i\alpha2}$), $G_{i\alpha3}$ (anti $G_{i\alpha3}$) and $G_{o\alpha}$ (anti $G_{o\alpha}$)

4.0 DISCUSSION

The results from this study suggest that (a) there are differences in the mechanism of inhibition of adenylyl cyclase by muscarinic receptor stimulation in atrial and ventricular myocardium and (b) this is associated with the presence of different isoforms of the inhibitory G-protein, G_i in these two tissues.

In the present study atrial and ventricular myocyte membranes prepared by sucrose density gradient ultracentrifugation were highly enriched in sarcolemma as determined by measuring ouabain-inhibitable Na^+ K^+ ATPase activity. The degree of enrichment of atrial and ventricular myocyte membranes obtained in our study (63 and 117 fold respectively) was much higher than that obtained by Braun and Walsh (1993) in an earlier study on rabbit atrial and ventricular myocyte sarcolemmal membranes (15.5 and 12 fold respectively). However, the Na^+ K^+ ATPase activity measured by Braun and Walsh in crude homogenates was 200 fold higher, while the activity in the sarcolemmal membranes was 30 - 40 fold greater than our study. The reason for the differences between their results and our own are not clear, since we employed similar procedures for isolating sarcolemmal membranes and assaying Na^+ K^+ ATPase activity.

Preliminary characterization studies on ventricular myocyte membranes to determine substrate kinetics using GTP (100 pM - 1μM) revealed high affinity GTPase activity. Iso (100μM), Cch (100μM) and Oxo (10μM) significantly increased V_{max} from the basal value without affecting the K_m. The K_m values obtained in the absence (0.3 - 0.65 μM) and presence (0.4 - 0.5 μM) of muscarinic agonists (Cch and Oxo) in our study are greater than the values reported in an earlier study (Fleming

and Watanabe, 1988) on canine cardiac sarcolemma (0.14 - 0.23 μM in the absence of muscarinic agonist and 0.2 - 0.3 μM in the presence of 10 μM Oxo). Our observations are, however qualitatively consistent with the earlier observations (Fleming and Watanabe, 1988) since the K_m was not affected with either Cch or Oxo. The Oxo-stimulated increase in V_{max} measured in our study is also consistent with the observations of Fleming and Watanabe (1988) on canine cardiac sarcolemma. In a preliminary experiment on atrial myocyte membranes in our study, Oxo produced a 40% increase in V_{max} from the basal value (28.3 pmol Pi/min/mg) without affecting the K_m. In an earlier study on sucrose density purified porcine atrial membranes, Hilf and Jakobs (1989) reported a 60-70% increase in V_{max} with 10μM Oxo without any effect on the K_m value. We are unable to compare our observations on atrial myocyte membranes with the earlier study by Hilf and Jakobs (1989) since more experiments could not be performed due to extremely low membrane protein yield.

In ventricular myocyte membranes, Iso (1μM - 1mM), Cch (100 nM - 1mM) and Oxo (10 nM-1mM) produced concentration-dependent increases in high affinity GTPase activity with maximum stimulation of 35%, 106% and 100% at 100μM, 100 μM and 10 μM respectively. To our knowledge this is the first study that has measured β AR-stimulated increases in high affinity GTPase activity in cardiac membranes. In a classical study on turkey erythrocyte membranes, Cassel and Selinger (1976) demonstrated a 52% increase in GTPase activity above the basal value (10.3 ± 1.2 pmol Pi/min/mg) with 100μM L-isoproterenol. In a subsequent study on turkey erythrocyte membranes, Bitonti et al. (1980) also reported a 56% increase in 100 μM Iso-stimulated GTPase activity above the basal value. In other

tissues such as hamster adipocytes (Aktories et al., 1982) and rat liver (Kimura and Shimada, 1980), no increase in GTPase activity could be detected even at a high concentration (200 - 300μM) of Iso.

Fleming and Watanabe (1988) demonstrated concentration-dependent increases in high affinity GTPase activity with Oxo (10 pM - 1mM) in canine cardiac sarcolemma with a maximum stimulation of 100% at 10 μM Oxo and our observations appear to be very consistent with their values. In a preliminary experiment on atrial myocyte membranes, Cch (100 nM - 1mM) produced concentration-dependent increases in high affinity GTPase activity with a maximum stimulation of 66% above the basal value at 100μM Cch. In an earlier study on purified porcine atrial membranes, Hilf and Jakobs (1989) also observed concentration-dependent increases in high affinity GTPase activity with Cch and Oxo (10 nM-100μM), with maximum stimulation of 60 - 70% at 100μM and 10μM respectively. The Cch and Oxo-stimulated high affinity GTPase activities measured in our study are very similar to the reported values.

In an effort to determine if there is a functional interaction of β AR and muscarinic receptor-stimulated G-proteins, the effects of Iso (100μM), Cch (100μM) and Oxo (10μM) alone and in combination on high affinity GTPase activity were studied in atrial and ventricular myocyte membranes. In ventricular myocyte membranes Iso, Cch and Oxo alone produced significant increases in high affinity GTPase activity above the basal value. In an earlier study on rabbit ventricular myocyte sarcolemmal membranes, Braun and Walsh (1993) reported a 125% increase in Cch (100μM)-stimulated high affinity GTPase activity above the basal

value and the activity measured in our study is very consistent with their observations. In the presence of Iso and Cch or Iso and Oxo in combination, an additive effect on high affinity GTPase activity was observed. The GTPase activity measured in the presence of either Iso and Cch or Iso and Oxo together was very close to the value predicted if the effects of the two agonists were independent of each other. These observations suggest that in ventricular myocyte membranes, G_s and G_i-mediated pathways are independent of each other and there is no functional interaction between β-adrenoceptor and muscarinic receptor-stimulated G-proteins.

In atrial myocyte membranes, Iso (100μM), Cch (100μM) and Oxo (10μM), alone also produced significant increases in high affinity GTPase activity from the basal value. It is interesting to note that although Iso-stimulated GTPase activity was similar in both atrial and ventricular myocyte membranes, the Cch and Oxo-stimulated activities were lower in atrial myocyte membranes. This difference in muscarinic receptor-stimulated GTPase activity could be due to uncoupling of the inhibitory G-proteins from the muscarinic receptors during the preparation of sarcolemmal membranes or high basal GTPase activity which may have reflected a smaller increase in muscarinic receptor-stimulated activity. In the presence of either Iso and Cch or Iso and Oxo in combination, there was no further increase in GTPase activity when compared to Cch or Oxo alone. The GTPase activity measured in the presence of Iso and Cch or Iso and Oxo in combination, were not close to the values predicted for an additive effect of these agonists (83% and 79% respectively) which suggests an interaction between β AR and muscarinic receptor-stimulated G-proteins. If there was no functional interaction and the effects of β AR and

muscarinic agonists together on high affinity GTPase activity were independent of each other we would have expected an additive effect on GTPase activity as seen in ventricular membranes. The functional interaction observed in atrial myocyte membranes could be due to binding of excess βγ subunits generated upon dissociation of heterotrimeric G_i to activated $G_{s\alpha.GTP}$, which would lead to a decrease in GTP hydrolysis by $G_{s\alpha}$ GTPase.

This is the first time to our knowledge that an interaction study measuring high affinity GTPase activity upon β AR and muscarinic receptor stimulation in combination has ever been performed in cardiac myocytes. Our next objective was to determine whether differences between atria and ventricles in the interaction of β agonist and muscarinic agonists on GTPase activity was reflected in differences in their regulation of adenylyl cyclase activity. In our study 100μM Iso produced a 46% increase in adenylyl cyclase activity above the basal value in ventricular myocyte membranes. In an earlier study on rat ventricular myocyte membranes, Romano et al. (1989) demonstrated a 4 fold increase in Iso (100μM)-stimulated adenylyl cyclase activity from the basal value. In another study on highly purified canine ventricular sarcolemmal membranes, Quist et al. (1994) showed a concentration-dependent increase in Iso (50-200 nM)-stimulated adenylyl cyclase activity with maximum stimulation of 40 - 50% at 150 nM Iso. A 250% increase in 10 μM Iso -stimulated cyclase activity above basal has been observed in adult rabbit ventricular myocytes (Kumar et al., 1994). A possible reason for higher Iso-stimulated cyclase activity in some of the earlier studies (Romano et al., 1989, Kumar et al., 1994) could be that these studies used either intact ventricular membranes or ventricular myocyte

membrane homogenates, whereas we used sucrose density gradient purified atrial and ventricular myocyte membranes. We may have lost some adenylyl cyclase activity during ultracentrifugation. Further, the animal species used in most of these studies except Kumar et al. (1994) were different from our study. In our study, Cch (100μM) significantly inhibited basal adenylyl cyclase activity, which is in good agreement with the observations from earlier studies on rabbit (Jakobs et al., 1979; Watson et al., 1988; Ray, 1992) and canine (Quist, 1992; Niroomand et al., 1993) ventricular myocardium. In our own study the cyclase activity measured in the presence of Iso and Cch in combination (101%) was very close to the value predicted if the stimulatory effects of Iso and the inhibitory effects of Cch were independent of each other (predicted value of 110%). This is also consistent with the observations of an earlier study from our laboratory on rabbit ventricular homogenates (Ray, 1992), where the adenylyl cyclase activity measured in the presence of Iso and Cch together (133%) was very close to the value predicted for an independent effect of the two agonists (124%). These observations suggest that in the ventricular myocardium, muscarinic receptor-stimulated inhibition of adenylyl cyclase probably occurs by a direct mechanism. According to direct mechanism of muscarinic receptor-stimulated inhibition of adenylyl cyclase proposed by Gilman (1987) the activated $G_{i\alpha.GTP}$ acts directly on the catalytic subunit of the enzyme.

In rabbit ventricular homogenates (Ray, 1992) and ventricular myocyte membranes (Watson et al., 1988), Cch significantly inhibited forskolin (1μM)-stimulated adenylyl cyclase activity. Since it is well known that forskolin activates adenylyl cyclase directly, the observations of Watson et al. (1988) and Ray (1992)

provide further evidence for a direct mechanism of muscarinic receptor-mediated inhibition of adenylyl cyclase activity.

We had difficulties in reliably measuring adenylyl cyclase activity in purified atrial myocyte membranes and in four out of seven experiments neither Iso nor Cch had any effect on adenylyl cyclase activity when present alone or in combination. It is quite possible that we may have lost the enzyme activity during the preparation of sarcolemmal membranes. Although cyclase activity could be measured in three different membrane preparations in our study the values obtained were not statistically significant. Since we are not aware of other reports of measurement of adenylyl cyclase activity in purified atrial myocyte sarcolemmal membranes, we do not know if other groups have encountered similar problems. In three experiments, Iso produced a 40% increase in adenylyl cyclase activity above the basal value, whereas Cch did not affect the basal activity (8% above basal). In the presence of Iso and Cch in combination, the Iso-stimulated adenylyl cyclase activity was inhibited by 51%. Although none of the measured effects are statistically significant they are qualitatively consistent with earlier observations on atrial homogenates (Ray, 1992) where Cch (1mM), alone did not affect the basal cyclase activity but in combination with Iso (100μM) produced significant inhibition of Iso-stimulated adenylyl cyclase activity.

Since Cch did not affect basal adenylyl cyclase activity but inhibited Iso-stimulated cyclase activity in both atrial homogenates (Ray, 1992) and atrial myocyte membranes (present study), we suggest that in mammalian atria muscarinic receptor-stimulated inhibition of adenylyl cyclase occurs by an indirect mechanism, probably

by decreasing the amount of $G_{\alpha s.GTP}$ available for activation of the enzyme as proposed by Gilman (1987). In the present study we have also provided evidence for functional interaction between β AR and muscarinic receptor-stimulated G-proteins in atrial myocyte membranes by demonstrating lack of additive effect of Iso and Cch or Iso and Oxo together, on high affinity GTPase activity. Taken together, the results from our study suggest that there are differences in muscarinic receptor-mediated regulation of adenylyl cyclase in mammalian atria and ventricles. It would be interesting to know whether these differences in interaction between β AR and muscarinic receptor-stimulated G-proteins and adenylyl cyclase regulation in atria and ventricles have any physiological significance and whether there is any correlation between GTPase activity and adenylyl cyclase activity in the myocardium.

In an early study on turkey erythrocyte membranes, Lester et al. (1982) demonstrated a positive correlation between prostaglandin E_1-stimulated GTPase and activation of adenylyl cyclase activity. PGE_1 (1nM - 1μM)-stimulated GTPase activity displayed strikingly similar dose-response characteristics with adenylyl cyclase activity. We are unable to make such a correlation from our study on atrial or ventricular myocyte membranes as we did not measure concentration-dependent effects of either Iso, Cch or Oxo on adenylyl cyclase activity. However, in an earlier study from our laboratory, Ray (1992) demonstrated concentration-dependent increases in Iso (100 nM - 100μM)-stimulated adenylyl cyclase activity with a maximum stimulation at 100μM in rabbit atrial and ventricular homogenates. In the present study, a concentration-dependent increase in high affinity GTPase activity with Iso (100 nM - 1mM) has been demonstrated in ventricular myocyte membranes.

Although the experimental systems used in both the studies (Ray, 1992 and the present study) are different, the observations derived from them suggest a positive correlation between high affinity GTPase and adenylyl cyclase activity in response to Iso in ventricular myocardium. A similar correlation cannot be drawn for atrial myocardium from the present study since concentration-dependent effects of Iso on high affinity GTPase activity were not measured. A negative correlation appears to exist between muscarinic receptor-stimulated high affinity GTPase and adenylyl cyclase activity in both atria and ventricles. An inhibitory effect on basal adenylyl cyclase activity in the presence of muscarinic agonists has been observed in rabbit (Watson et al., 1988; Ray, 1992 and the present study) and canine (Watanabe et al., 1979; Fleming et al., 1988) ventricular myocardium. In rabbit atrial homogenates, Ray (1992) demonstrated a small but not significant inhibition of basal and a significant concentration-dependent inhibition of 100μM Iso-stimulated adenylyl cyclase activity by Cch. In preliminary experiments on atrial myocyte membranes in our study, we did not observe any effect of Cch (100μM) on basal cyclase activity, but the Iso (100μM)-stimulated cyclase activity was inhibited.

On the other hand concentration-dependent increases in Cch and Oxo-stimulated high affinity GTPase activity have been demonstrated in porcine atrial membranes (Hilf and Jakobs, 1989), rabbit atrial and venticular myocyte membranes (present study) and canine ventricular myocardium (Fleming and Watanabe 1988 ; Fleming et al., 1988). These observations on atrial and ventricular myocardium suggest that muscarinic receptor-stimulated increases in high affinity GTPase activity are associated with a decrease in adenylyl cyclase activity.

Physiologically, β AR stimulation in atrial and ventricular myocardium causes activation of adenylyl cyclase via stimulation of G_s, which in turn causes an elevation in cAMP levels eventually leading to a positive inotropic effect (Bristow et al., 1982 ; Kaumann et al., 1982 ; Ikezono et al., 1987 ; Kumar et al., 1994 ; MacDonell et al., 1995). In ventricular myocardium a decrease in both basal (Watson et al., 1988; Quist et al., 1992; Ray, 1992 and the present study) and β agonist - stimulated cyclase activity (Ransnas et al., 1986; Fleming and Watanabe, 1988; Watson et al., 1988; Quist, 1992; Ray, 1992) along with a decrease in cAMP levels and PKA activity upon muscarinic receptor stimulation were suggested to be responsible for antagonism of positive inotropic responses to cAMP elevating agents (Keely et al., 1978; Ingebretsen, 1980; Inui et al., 1982; Katano and Endoh, 1993). However other studies demonstrate that muscarinic agonists antagonize the positive inotropic effects of β agonists and adenylyl cyclase activators such as forskolin without any changes in total cAMP levels or PKA activity in the tissue (Watanabe and Besch, 1975; MacLeod, 1984; Schmied and Korth, 1990; Gupta et al., 1994; MacDonell et al., 1995). Several lines of evidence have indicated the existence of discrete pools of adenylyl cyclase in the heart and compartmentalization of cAMP and PKA activity (Corbin et al., 1977; Hayes and Mayer, 1980; Buxton and Brunton, 1983; England and Shahid, 1987). In a recent interaction study on rat ventricular myocardium, Zhang and MacLeod (1996) demonstrated that perfusion of rat hearts with 3μM Cch and 0.1μM Iso concurrently for 1.5 min completely abolished the increase in left ventricular pressure (LVP) developed by Iso without affecting the total and particulate cAMP levels and soluble and particulate PKA activity. On the other hand,

preperfusion with 3μM Cch for 1 min followed by perfusion with 3μM Cch and 0.1μM Iso for 1.5 min not only abolished the increases in LVP produced by Iso but also reversed the increase in total and particulate cAMP levels and soluble and particulate PKA activity. Using the same protocol it was also demonstrated that 3μM Cch abolished forskolin (1μM for 2 min and 5 min)-stimulated increases in LVP without affecting cAMP (total and particulate) and PKA (soluble and particulate) activities. The authors (Zhang and MacLeod, 1996) suggest that inhibition of adenylyl cyclase activity may not be necessary for the inhibitory effects of muscarinic agonists on β AR-stimulated positive inotropic effects in rat ventricles. These observations raise questions about the physiological significance of the decrease in adenylyl cyclase activity produced by Cch in the presence of β agonists.

In the atrial myocardium, a direct negative inotropic effect due to activation of atrial muscarinic K^+ channels has been reported in several studies (Ten Eick et al., 1976 ; Bellardinelli and Isenberg, 1983 ; Cerbai et al., 1988 ; Ray, 1992). Studies on intact atrial tissues from different animal species have indicated that the decrease in contractile response upon muscarinic receptor stimulation occurs at low concentrations of the agonists (Diamond et al., 1977; MacLeod, 1986; Ray and MacLeod, 1992). However, in purified porcine atrial membranes (Hilf and Jakobs, 1989) and in a preliminary experiment on purified rabbit atrial myocyte membranes (present study), maximum high affinity GTPase activity was detected at a concentration of 100μM Cch. Further, the basal adenylyl cyclase activity was not affected even in the presence of 100μM Cch in atrial myocyte membranes (present study) or with 1mM Cch in rabbit atrial homogenates (Ray, 1992). Taken together,

these observations suggest that maximum stimulation of high affinity GTPase activity may not be necessary for muscarinic agonists to elicit a direct negative inotropic effect in the atrial myocardium. Further, a change in basal adenylyl cyclase activity may not be required for direct negative inotropic effects of muscarinic agonists in atrial myocardium.

In order to identify different G-protein subtypes that are possible candidates for coupling to the muscarinic receptors, sarcolemmal membranes from atrial and ventricular myocytes were immunoblotted with AS/7, EC/2, anti $G_{i\alpha1}$, anti $G_{i\alpha2}$ (J-883) and anti $G_{i\alpha3}$ antibodies after resolving the G-proteins by SDS-PAGE. In atrial myocyte membranes, we were able to identify $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{o\alpha}$ subtypes using anti $G_{i\alpha2}$, anti $G_{i\alpha3}$, EC/2 and AS/7 antibodies. AS/7, with relative selectivity for both $G_{i\alpha1}$ and $G_{i\alpha2}$ produced only one band while anti $G_{i\alpha1}$ antibody did not produce any band. On the other hand, anti $G_{i\alpha2}$ and anti $G_{i\alpha3}$ produced distinct bands for $G_{i\alpha2}$ and $G_{i\alpha3}$ subtypes. EC/2, which is selective for both $G_{i\alpha3}$ and $G_{o\alpha}$ subtypes produced a distinct band, while anti $G_{o\alpha}$ also produced a prominent band for $G_{o\alpha}$ subtype at the expected molecular weight of $G_{o\alpha}$ subtype. In ventricular myocyte membranes, we identified $G_{i\alpha1}$ and $G_{i\alpha3}$ subtypes using anti $G_{i\alpha1}$, anti $G_{i\alpha3}$, EC/2 and AS/7 antibodies. AS/7 and EC/2 produced distinct bands at approximately the expected molecular weights. The $G_{i\alpha1}$ -specific antibody, anti $G_{i\alpha1}$ produced a clear band, whereas anti $G_{i\alpha2}$ did not produce any band at the expected molecular weight for $G_{i\alpha2}$ subtype. Anti $G_{i\alpha3}$ produced a faint band, while $G_{o\alpha}$ subtype could not be distinctly identified in ventricular myocyte membranes.

Kumar et al. (1994) used anti $G_{i\alpha1}$, anti $G_{i\alpha2}$ (Mumby et al., 1986) and anti $G_{i\alpha3}$ antibodies to identify different G-protein subtypes in washed particulate membranes from adult rabbit whole ventricles and isolated ventricular myocytes. The $G_{i\alpha1}$ subtype was identified in both whole ventricles and ventricular myocytes, whereas $G_{i\alpha2}$ and $G_{i\alpha3}$ were present in whole ventricular membranes but not ventricular myocyte membranes. Using two dimensional densitometry, the authors (Kumar et al., 1994) demonstrated that the amount of $G_{i\alpha1}$ in whole ventricles and isolated ventricular myocytes were not significantly different from each other, whereas the $G_{i\alpha2}$ content was significantly lower, if present in ventricular myocyte membranes as compared to whole ventricular membranes. Although our observations on ventricular myocyte membranes appear to be in agreement with Kumar et al., we were unable to detect any $G_{i\alpha2}$, whereas we could distinctly identify $G_{i\alpha3}$ subtype in our membranes using anti $G_{i\alpha3}$. It should be noted, however that Kumar et al. used washed particulate fraction of ventricular myocytes, whereas we used highly purified ventricular myocyte membranes. Some other differences in experimental protocols in the two studies (Kumar et al., 1994 and the present study) may account for the discrepancies in observations. For example, Kumar et al. alkylated their membrane samples before SDS-PAGE, whereas we did not do so. Secondly, Kumar et al. used 1 : 500 dilution of anti $G_{i\alpha2}$, whereas we used 1 : 5000 dilution of the same antibody in TBS-T buffer. Further, Kumar et al. incubated the membranes with primary antibodies for 4 hours at room temperature, while we incubated the myocyte membranes for 2 hours at 37°C

The observations from the present study do not indicate whether the differences in functional interaction between β AR and muscarinic receptors in atrial and ventricular myocardium are related to differences in G-protein subtypes in the two tissues. However, It is a possibility that the excess βγ subunits liberated upon muscarinic receptor-stimulation of both G_i and G_o in the atrial myocytes (where we were able to detect $G_{i\alpha 2}$, $G_{i\alpha 3}$ and $G_{o\alpha}$) reduce the concentration of free $G_{s\alpha.GTP}$ thereby causing indirect inhibition of adenylyl cyclase.

FUTURE EXPERIMENTS

In order to understand the mechanism of interaction of β AR and muscarinic receptor-stimulated G-proteins, it may be worthwhile to study the effects of in vivo PTX pretreatment on high affinity GTPase and adenylyl cyclase activity in the presence of β agonist and muscarinic agonists alone or in combination. PTX ADP ribosylates the α subunit of inhibitory G proteins and uncouples them from the muscarinic receptor (Spiegel et al., 1992). Functionally, ADP ribosylation of G_i or G_o would cause inactivation of the G protein and therefore, loss of GTPase activity. Therefore in the presence of PTX, muscarinic agonists alone would not produce any increase in GTPase activity above the basal value (Fleming et al., 1988 ; Fleming and Watanabe, 1988), whereas in the presence of either Iso and Cch or Iso and Oxo in combination, the GTPase activity will not be expected to be different from Iso alone if an interaction exists. PTX pretreatment also reverses the muscarinic receptor-mediated inhibition of adenylyl cyclase activity without affecting β AR-stimulated cyclase activity (Fleming et al., 1988 ; Fleming and Watanabe, 1988).

Functional interaction between β AR and muscarinic receptor-stimulated G-proteins can also be studied by determining the effects of β AR and muscarinic receptor agonists, alone and in combination on high affinity GTPase and adenylyl cyclase activity in the presence of anti G-protein antibodies which interfere with the activation of G-proteins. Such a study would also provide valuable information regarding the specific subtypes of inhibitory G-proteins involved in the biochemical responses.

The technique of radioligand binding can be used to study functional interaction between β AR and muscarinic receptor-stimulated G proteins. One way of initiating such a study would be to determine the concentration-dependent effects of a β AR agonist such as Iso on the displacement of a radiolabeled antagonist (such as (^{125}I) iodocyanopindolol) from the β adrenoceptor. The effect of muscarinic agonists (Cch or Oxo) on Iso displacement of a radiolabeled antagonist can then be determined in the absence and presence of GTP or its non-hydrolyzable analogues such as Gpp(NH)p. If an interaction exists between β AR and muscarinic receptor-stimulated G proteins, the muscarinic agonist would be expected to prevent a rightward shift in the Iso curve for displacement of the antagonist in the presence of non-hydrolyzable GTP analogue.

The results obtained from the present study form the basis for further research in investigating the mechanistics of adenylyl cyclase regulation upon muscarinic receptor stimulation under normal and compromised physiological conditions in the mammalian myocardium.

5.0 SUMMARY AND CONCLUSIONS

5.1 SUMMARY

1. In ventricular myocyte membranes, Iso (100μM), Cch (100μM) and Oxo (10μM) produced significant increases in V_{max} without affecting the K_m value of high affinity GTPase activity.

2. In ventricular myocyte membranes, Iso (1μM - 1mM), Cch (100 nM - 1mM) and Oxo (100 pM - 1mM) produced concentration-dependent increases in high affinity GTPase activity with maximum stimulation of 35%, 106% and 122% at 100μM, 100μM and 10μM respectively.

3 In ventricular myocyte membranes, Iso (100μM), Cch (100μM) and Oxo (10μM), alone produced a significant increase in high affinity GTPase activity from the basal value. In the presence of either Iso and Cch or Iso and Oxo in combination, the high affinity GTPase activity was further elevated as compared to Cch or Oxo alone. In atrial myocyte membranes, Iso, Cch and Oxo, alone produced significant increases in high affinity GTPase activity from the basal level. However, in the presence of either Iso and Cch or Iso and Oxo, in combination there was no further increase in GTPase activity as compared to Cch or Oxo alone.

4. In ventricular myocyte membranes, Iso (100μM) produced a significant increase in adenylyl cyclase activity while Cch (100μM) significantly decreased basal cyclase activity. In the presence of Iso and Cch in combination, the adenylyl cyclase activity was close to the value that was predicted if the stimulatory effects of Iso and inhibitory effects of Cch were independent of each other. In preliminary experiments on atrial myocyte membranes, Iso caused an increase in cyclase activity from the

basal, whereas Cch did not affect basal cyclase activity. in the presence of both agonists in combination the Iso-stimulated adenylyl cyclase activity was inhibited.

5. Atrial and ventricular myocyte membranes were highly enriched in sarcolemma as determined by measuring ouabain-inhibitable Na^+ K^+ ATPase activity.

6. Western blot of atrial and ventricular myocyte membranes using AS/7, anti $G_{i\alpha1}$, anti $G_{i\alpha2}$ and anti $G_{i\alpha3}$ antibodies identified $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha}$ subtypes in atrial membranes, whereas in ventricular myocyte membranes $G_{i\alpha1}$ and $G_{i\alpha3}$ subtypes were identified.

5.2 CONCLUSIONS

In conclusion, the results from the present study suggest that

I. In atrial myocytes there is a functional interaction between β AR and muscarinic receptor-stimulated G-proteins, and muscarinic receptor-stimulated inhibition of adenylyl cyclase occurs by an indirect mechanism.

ii. In ventricular myocytes, β AR-stimulated (G_s-mediated) and muscarinic receptor-stimulated (G_i-mediated) high affinity GTPase and adenylyl cyclase activities are independent of each other and muscarinic receptor-mediated inhibition of adenylyl cyclase occurs by a direct mechanism.

iii. Atrial myocytes contain $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{o\alpha}$ subtypes of inhibitory G-proteins whereas ventricular myocytes contain $G_{i\alpha1}$ and $G_{i\alpha3}$ subtypes.

Thus, inhibition of adenylyl cyclase upon muscarinic receptor stimulation in mammalian atria and ventricles appears to occur by different mechanisms.

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