ADRENERGIC-CHOLINERGIC INTERACTIONS IN THE HEART

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ABSTRACT

In the mammalian atrial myocardium the muscarinic receptor agonist carbachol can inhibit the positive inotropic responses to isoproterenol, a β-adrenoceptor agonist, forskolin, a direct activator of adenylate cyclase, IBMX, a phosphodiesterase inhibitor, and phenylephrine, an α-adrenoceptor agonist. While the inhibitory effect of carbachol on the isoproterenol-stimulated cAMP generation is believed to contribute to the negative inotropic effect of carbachol in the presence of isoproterenol, it is not known how carbachol inhibits the positive inotropic responses to phenylephrine, forskolin and IBMX in the atrial myocardium. One of the objectives of the present study was to investigate whether the reversal by carbachol of the positive inotropic responses of left atria to phenylephrine, forskolin and IBMX is related to the ability of carbachol to open potassium channels.

In rabbit left atria, carbachol exerted a direct negative inotropic response and inhibited the positive inotropic response to phenylephrine. Carbachol also promoted the efflux of ^{86}Rb in the presence and absence of phenylephrine from left atria. The ability of carbachol to increase the rate-constant of ^{86}Rb-efflux was attenuated by atropine (100 nM), 4-aminopyridine (50 and 500 μM), a potassium channel blocker, and pre-treatment of rabbits with pertussis toxin (0.5 and 1 μg/kg), an uncoupler of muscarinic receptors from potassium channels. Although both 4-aminopyridine and pertussis toxin attenuated the negative inotropic response to carbachol, both agents had a greater attenuating effect on the carbachol-stimulated ^{86}Rb-efflux than on the carbachol-induced negative inotropy. The abilities of carbachol to promote the ^{86}Rb-efflux and to exert a negative inotropic response in the presence of phenylephrine were attenuated by pertussis toxin

pre-treatment of rabbits. Although 4-aminopyridine was able to attenuate the inhibitory effect of carbachol on the phenylephrine-induced positive inotropy, only 500 μM 4-aminopyridine slightly reduced the carbachol-stimulated increase in the rate constant of ^{86}Rb efflux in the presence of phenylephrine.

4-Aminopyridine did not have any effect on the carbachol-induced inhibition of the isoproterenol-stimulated cAMP generation suggesting that 4-aminopyridine was not acting as a muscarinic receptor antagonist. 4-Aminopyridine inhibited only modestly the inhibitory effect of carbachol on the isoproterenol-induced positive inotropy, a cAMP-dependent response. The potassium channel openers, pinacidil and cromakalim, did not have any inhibitory effect on the isoproterenol-induced positive inotropy but inhibited in a concentration-dependent manner the positive inotropic responses to phenylephrine.

Uncoupling of muscarinic receptors from adenylate cyclase using pertussis toxin (2.2 μg/kg) attenuated only partially the negative inotropic responses of left atria to carbachol in the presence of forskolin and IBMX suggesting that at least part of the reversal by carbachol of positive inotropic responses to forskolin and IBMX occurs by a cAMP-independent mechanism. 4-Aminopyridine attenuated in a concentration-dependent manner the negative inotropic responses to carbachol in the presence of forskolin and IBMX in left atria from both saline and pertussis toxin pre-treated rabbits.

These results suggest that the ability of carbachol to open potassium channels may not explain completely the direct negative inotropic response to carbachol but may contribute to the negative inotropic responses to carbachol in the presence of phenylephrine, forskolin and IBMX.

It is established that muscarinic receptors are linked to potassium channels and adenylate cyclase in the atrium and only to adenylate cyclase in the ventricle by means of

pertussis toxin sensitive G-protein(s). The second objective of this study was to investigate if inhibition of the isoproterenol-stimulated adenylate cyclase by carbachol in the rabbit atrium is more sensitive to pertussis toxin than either the same response to carbachol in the ventricle or the ability of carbachol to open potassium channels in the atrium. Injection of rabbits with 0.5 μg/kg pertussis toxin, a dose which ADP-ribosylated 60 % of atrial and ventricular G-proteins, resulted in complete uncoupling of muscarinic receptors from the atrial adenylate cyclase. On the other hand, in rabbits injected with 1 μg/kg pertussis toxin, the ability of carbachol to inhibit the ventricular adenylate cyclase was not altered and to open the atrial potassium channels was only partially attenuated. These data suggest that the muscarinic receptor-mediated inhibition of adenylate cyclase in the atrium is more sensitive to pertussis toxin than either the muscarinic receptor-mediated activation of potassium efflux in the atrium or inhibition of adenylate cyclase in the ventricle. This suggests that there are differences in the coupling of muscarinic receptors to adenylate cyclase and potassium channels in the atrium and ventricle.

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4-AP	4-Aminopyridine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
[α-^{32}P]ATP	ATP where the α-phosphate group is radioactive
C	Centigrade
cAMP	Adenonsine 3',5' cyclic monophosphate
cpm	Counts per minute
[^{3}H]-cAMP	Tritium labelled cAMP
DMSO	Dimethyl sulphoxide
dpm	Disintegrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
EGTA	Ethyleneglycol-bis-(β-amino ethyl ether)-N, 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_p	G-protein mediating hormonal stimulation of phospholipase C
G_s	Stimulatory G-protein
$G_{s\alpha}$	α-Subunit of the stimulatory G-protein
G_o	Other G-protein
$G_{o\alpha}$	α-Subunit of the other G-protein
G_z	A pertussis toxin-insensitive G-protein

GTP	Guanosine triphosphate
GTPγS	Guanosine 5'-O-(3-thiotriphosphate)
cGMP	Guanosine 3',5' cyclic monophosphate
Hz	Hertz
IBMX	Isobutylmethyl xanthine
I_f	Pacemaker current
IP_3	Inositol trisphosphate
IP_4	Inositol tetrakisphosphate
IP_5	Inositol pentakisphosphate
IP_6	Inositol hexakisphosphate
kDa	Kilodalton
kg	Kilogram
l	Litre
M	Molar
MCK	Modified Chenoweth-Koelle
mAMP	Milliampere
mg	Milligram
ml	Millilitre
mM	Millimolar
μCi	Microcurie
μg	Microgram
μl	Microlitre
μM	Micromolar
mRNA	messenger RNA
min	Minute
N	Normal
NAD	Nicotinamide adenine dinucleotide

[^{32}P]-NAD	Nicotinamide adenine dinucleotide where one phosphorus is radioactive
ng	nanogram
nm	Nanometer
nM	Nanomolar
PMSF	Phenylmethylsulphonyl Fluoride
pmol	Picomole
^{86} Rb	Radioactive rubidium
R_{sp}	Specific radioactivity (dpm/pmol)
SDS	Sodium dodecylsulphate
S.E.M.	Standard error of the mean
TCA	Trichloroacetic acid
TEMED	N, N, N', N'-Tetra methyl ethylenediamine

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INTRODUCTION

1.1. OVERVIEW

The force and rate of myocardial contraction is subject to modulation, depending upon physiological needs, by the sympathetic and parasympathetic branches of the autonomic nervous system. The effects of sympathetic stimulation in the heart are mediated by the neurotransmitter norepinephrine which acts on the α- and β-adrenoceptors. The parasympathetic neurotransmitter acetylcholine on the other hand acts on muscarinic cholinoceptors. Stimulation of the α- and β- adrenoceptors by norepinephrine increases the rate and force of myocardial contraction. The consequences of muscarinic receptor stimulation in the heart, however, are very complex and seem to vary from one part of the myocardium to the other. Depending upon the region of the heart and the animal species involved, muscarinic agonists can exert a direct negative inotropic and chronotropic response, reverse positive inotropic and chronotropic responses to both cAMP-independent α-adrenoceptor agonists and cAMP-generating β-adrenoceptor agonists, and can also exert a positive inotropic response. The biochemical and/or electrophysiological changes accompanying muscarinic receptor stimulation include inhibition of adenylate cyclase activity and lowering of cAMP levels elevated by β-adrenoceptor agonists, elevation of cGMP levels, activation of protein phosphatase, increase in an outward potassium current (seen only in atrium), inhibition of the pacemaker current (atrium only) and the slow inward calcium current and promotion of phosphoinositide breakdown (see Loffelholz and Pappano, 1985; Hartzell, 1988; Pappano, 1990). It has been fairly well established that the inhibitory effect of muscarinic agonists on the β-adrenoceptor agonist-mediated positive inotropy and chronotropy is

mediated largely through the adenylate cyclase and cAMP pathway, although the existence of a cAMP-independent mechanism of action of muscarinic agonists has also been suggested (Endoh et al., 1985; MacLeod, 1986). In addition, it has also been shown that the negative inotropic responses to muscarinic agonists in the presence of cAMP-elevating agents like forskolin or isobutylmethyl xanthine (IBMX) are not always associated with a reduction in accompanying increases in cAMP levels (MacLeod and Diamond, 1986; Ray and MacLeod, 1992). On the other hand, muscarinic agonists have been reported to antagonize or reverse the cAMP-independent positive inotropic responses to α-adrenoceptor stimulation without altering the basal cAMP levels (MacLeod, 1986; 1987). It has been suggested that the ability of muscarinic agonists to activate a potassium current may contribute to the cAMP-independent negative inotropic response to muscarinic receptor stimulation (Ten Eick et al., 1976; Loffelholz and Pappano, 1985). However, it remains to be seen if activation of a potassium current may also play a role in the cAMP-independent functional interaction of muscarinic agonists with different positive inotropic agents.

It is well known that the α- and β- adrenoceptors and muscarinic cholinoceptors in the heart are linked to their effectors by guanine nucleotide binding proteins (Robishaw and Foster, 1988; Fleming et al., 1982). The guanine nucleotide binding proteins (G-proteins) consist of three subunits, α-, β- and γ. The α-subunit of a stimulatory G-protein (G_s) mediates the stimulation of adenylate cyclase in response to β-adrenoceptor stimulation. On the other hand, the message of muscarinic receptor occupancy to adenylate cyclase and potassium channels is conveyed by a group of pertussis toxin-sensitive G-proteins. These G-proteins can be further divided into two groups, G_i which is a mixture of three different proteins G_{i1}, G_{i2} and G_{i3} and G_o. It is

not clear how and which of these G-proteins connect muscarinic receptors to adenylate cyclase and potassium channels. It is well known that pertussis toxin can uncouple muscarinic receptors from adenylate cyclase and potassium channels in atria and ventricles (Fleming et al., 1992). In a previous study from this laboratory (Ray and MacLeod, 1992) it was observed that in left atria from rabbits pre-treated with pertussis toxin the muscarinic receptor agonist carbachol lost its inhibitory effect on the β-adrenoceptor agonist isoproterenol-induced increases in the force of contraction and cAMP levels but the direct negative inotropic response to carbachol was only partially attenuated. The same dose of pertussis toxin did not have any uncoupling effect on the ability of carbachol to inhibit the isoproterenol-stimulated positive inotropic response in the right ventricular papillary muscle of rabbits. This suggested that pertussis toxin uncoupled in a differential manner muscarinic receptors from potassium channels and adenylate cyclase in the atrium and muscarinic receptors from adenylate cyclase in the atrium and ventricle.

The purpose of the present study was to investigate, using pharmacological and biochemical techniques, (a) the role of muscarinic receptor-mediated potassium-efflux in the functional interaction of a muscarinic agonist with various positive inotropic agents in atria, and (b) whether pertussis toxin uncouples in a differential manner (i) muscarinic receptors from adenylate cyclase and potassium channels in atria, and (ii) muscarinic receptors from adenylate cyclase in atria and ventricles.

In the following pages an attempt will be made to describe in brief (i) the different types of adrenoceptors and muscarinic cholinoceptors in the heart, (ii) functional, biochemical and electrophysiological consequences of adrenoceptor and muscarinic cholinoceptor stimulation in the heart, with special reference to the

mechanism(s) of interaction of muscarinic cholinoceptor agonists with α- and β-adrenoceptor agonists in the heart, and (iii) the G-proteins and their role in the adrenergic-cholinergic interactions in the heart.

1.2. TYPES OF ADRENOCEPTORS IN THE HEART

1.2.1 β-Adrenoceptors

In the mammalian myocardium, stimulation of the sympathetic nervous system results in the release of norepinephrine. Although norepinephrine is capable of stimulating both α- and β- adrenoceptors, it is believed that under normal physiological conditions it is the β-adrenoceptors which mediate the effects of sympathetic stimulation on myocardial function. The presence of β-adrenoceptors in the heart can be demonstrated using a variety of different techniques, including pharmacological, ligand binding and autoradiographic localization (Stiles et al., 1984; Hartzell, 1988). Based on these studies, we know that in the mammalian myocardium there exists at least two different types of β-adrenoceptors, namely the β_1 and β_2, while the presence of a third type, β_3, is being debated (Kaumann, 1988). It has been shown that in the mammalian myocardium the ratio of β_1/β_2 receptors varies with the species and the region of the heart. Results of binding studies show that in rat, guinea pig, rabbit, cat, dog and human hearts, it is the β_1-adrenoceptors which predominate, usually constituting 80% or more of the total β-adrenoceptor population (Stiles et al., 1984). The number of β_2-adrenoceptors is usually very low or absent in the ventricle but represents 20 - 30 % of the total β-adrenergic receptors in the atrial myocardium (Stiles et al., 1984). Using selective agonists and antagonists it can be shown that the β_1-adrenoceptors play an equally

important role in atria and ventricles, whereas the β_2-adrenoceptors are more important in the sinoatrial node, less so in the left atrium and even less so in the ventricular myocardium (Kaumann, 1988).

1.2.2. α-Adrenoceptors

In contrast to the β-adrenoceptors, the role played by the α-adrenoceptors in the heart is not very clear. The presence of α-adrenoceptors can be demonstrated, using ligand binding techniques, in both atria and ventricles from different species of animals (Williams and Lefkowitz, 1978; Karliner et al., 1979; Yamada et al., 1980; Mukherjee et al., 1983). The number of α-adrenoceptors vary among different species of animals and between different regions of the heart. It has been shown that in rabbit and rat hearts, the number of α-adrenoceptor binding sites are almost equal to the number of β-adrenoceptor binding sites (Mukherjee et al. 1983). Much lower numbers of α-adrenoceptor binding sites, approximately 3 - 60 times less, were reported in dog, guinea pig and hamster hearts (Karliner et al., 1979; Mukherjee et al., 1983). The regional distribution of the α-adrenoceptors has been investigated by Yamada et al. (1980), who reported that in the rat heart the number of α-adrenoceptors are at least two times higher in ventricles compared to atria. The fact that α-adrenoceptor stimulation in the heart, using selective agonists, results in dose-dependent positive inotropic and chronotropic responses suggests that these receptors are functionally coupled to some effector(s) (Benfey, 1980; 1982; Scholz et al., 1986). The α-aderenoceptors can be divided into two groups, α_1 and α_2, which have been further subdivided into α_{1A}, α_{1B}, α_{1C} and α_{2A}, α_{2B}, α_{2C}, α_{2D} categories (Bylund, 1992). It has been established using selective agonists and antagonists that the α-adrenoceptors in the heart are of the α_1 subtype (Bruckner et al.,

1985; Minneman, 1988). More recently, in the rat heart, the messenger RNAs (mRNA) were reported for both the α_{1A}- and α_{1B}- adrenoceptors (Bylund, 1992). The functional significance of these different types of α-adrenoceptors and their distribution in different animal species, however, is not known at present.

1.3. CHOLINOCEPTORS IN THE HEART

The receptors which bind the neurotransmitter acetylcholine in the heart are of the muscarinic cholinergic subtype. With the help of pharmacological and radioligand binding studies it has been established that the muscarinic receptors in the heart are mostly of the M_2 subtype (Loffelholz and Pappano, 1985; Mei et al., 1989). When the myocardium from different species of animals was screened for the presence of mRNA for different muscarinic receptor subtypes, the mRNA for only the M_2 subtype was detected (Peralta et al., 1987; Maeda et al., 1988; Mei et al., 1989). Unlike the adrenoceptors, the number of binding sites for muscarinic cholinoceptors is much higher in the atrial myocardium compared to that in the ventricular myocardium from different species of animals (Fields et al. 1978; Wei and Sulakhe, 1978; Loffelholz and Pappano, 1985).

1.4. FUCNTIONAL CONSEQUENCES OF α- AND β- ADRENOCEPTOR AND MUSCARINIC CHOLINOCEPTOR STIMULATION IN THE HEART

The functional consequences of the adrenoceptor stimulation in the heart have been investigated widely and it is known that both α- and β- adrenoceptor stimulation can increase the rate and force of myocardial contraction (Bruckner et al., 1985; Hartzell,

1988). However, the functional effects of muscarinic receptor stimulation are not only very diverse, but they seem to vary from one part of the myocardium to another (Loffelholz and Pappano, 1985). In the atrial myocardium, muscarinic receptor agonists have been shown to exert a direct negative inotropic and chronotropic response and can also antagonize the positive inotropic responses to both α- and β- adrenoceptor stimulation. In contrast, in the ventricular myocardium the negative inotropic response to muscarinic receptor stimulation is minimal and muscarinic receptor agonists can inhibit the positive inotropic responses to β- but not to α- adrenoceptor stimulation. However, it is important to note that the inhibitory effect of muscarinic receptor stimulation on both the atrial and ventricular contractility becomes more pronounced in the presence of a β-adrenoceptor agonist or a background of sympathetic activity. This phenomenon of enhanced muscarinic response in the presence of a background sympathetic tone has been termed "accentuated antagonism" or the "anti-adrenergic effect" of muscarinic stimulation (Loffelholz and Pappano, 1985). In addition to the inhibition of atrial and ventricular contractility, it has been shown that in both atrial as well as in ventricular myocardium muscarinic receptor agonists in high concentrations can exert a positive inotropic response (Loffelholz and Pappano, 1985; Pappano, 1990).

1.5. BIOCHEMICAL AND/OR ELECTROPHYSIOLOGICAL CONSEQUENCES OF ADRENOCEPTOR STIMULATION IN THE HEART

1.5.1. β-Adrenoceptor

1.5.1.1. Adenylate cyclase and cAMP

The biochemical basis of the β-adrenoceptor-mediated positive inotropy has been investigated and reviewed very extensively (Drummond and Severson, 1979; Stiles et al.,

1984; Hartzell, 1988; Lindemann and Watanabe, 1990). It is well established that β-adrenoceptor agonists stimulate adenylate cyclase activity and increase intracellular cyclic AMP (cAMP) levels in a variety of different tissue and cell types including myocardial cells. Cyclic AMP in turn facilitates the entry of calcium through voltage-operated calcium channels (Hartzell, 1988; Brown, 1990), decreases the sensitivity of contractile proteins to calcium (Hartzell, 1988) and stimulates the uptake of calcium by the sarcoplasmic reticulum (Lindemann and Watanabe, 1990). Cyclic AMP has also been shown to stimulate the pacemaker current (I_f) in the sinoatrial node and to stimulate the delayed rectifier potassium current. Most of the effects of cAMP are believed to be related to its ability to activate a cAMP-dependent protein kinase which in turn phosphorylates various proteins. It has been shown that the cAMP-dependent protein kinase can phosphorylate the calcium channel (Trautwein and Hescheler, 1990), phospholamban (Lindemann and Watanabe, 1985) and proteins associated with the contractile machinery, namely troponin-I and tropomyosin (Hartzell, 1988). Although there is no direct evidence that cAMP-dependent protein kinase phosphorylates the potassium channel, it has been shown that at least part of the effects of β-adrenoceptor-stimulation on the delayed rectifier current are phosphorylation-dependent (Szabo and Otero, 1990). However, in a recent study DiFrancesco and Tortora (1991) have shown that cAMP can stimulate I_f channels without activating the cAMP-dependent protein kinase.

1.5.1.2. cAMP-independent effects of β-adrenoceptor stimulation

β-Adrenoceptor agonists have been shown to modulate potassium (Freeman et al., 1992; Szabo and Otero, 1990), calcium (Yatani et al., 1988; Yatani and Brown, 1990; Trautwein and Hescheler, 1990) and I_f channels (Yatani et al., 1990; Brown, 1990) without increasing cAMP levels or activating the cAMP-dependent protein kinase (see also, Brown, 1990, Brown and Birnbaumer, 1990). However, it is worth mentioning that Hartzell and his colleagues were unable to detect the cAMP-independent effects of the β-adrenoceptor stimulation on calcium currents (Hartzell et al., 1991). It has been argued that activation of the delayed rectifier current by a β-adrenoceptor agonist occurs secondary to activation of the calcium current (Hume, 1985; see also Hartzell, 1988). However, the fact that calcium and delayed rectifier currents are independent of each other was later confirmed by the differential temperature sensitivity of the two currents (Walsh and Kass, 1988), and by the ability of β-adrenoceptor agonists and cAMP-dependent protein kinase to activate the potassium current in the presence of a calcium channel blocker (Walsh and Kass, 1988; Walsh et al., 1991; Hartzell, 1988; Szabo and Otero, 1990).

1.5.2. α-Adrenoceptor

1.5.2.1. α-Adrenoceptors and ion channels

Unlike the β-adrenoceptors, very little is known about the mechanism of the α-adrenoceptor-mediated positive inotropy in the myocardial tissue. The involvement of cAMP or cGMP can be ruled out because the α-adrenoceptor agonists do not increase the intracellular cAMP and cGMP levels (Inui et al., 1982; Buxton and Brunton, 1986; MacLeod, 1986; see also Bruckner et al., 1985; Hartzell, 1988). α-Adrenoceptor agonists

were reported to stimulate the voltage-dependent slow inward calcium current (Miura et al., 1978; Bruckner and Scholz, 1984) and to increase the sensitivity of the myofilaments to calcium (Endoh and Blinks, 1988; Puceat et al., 1992). However, it has been shown that the increase in the slow inward calcium current in response to α-adrenoceptor stimulation is not only very small in magnitude (Handa et al., 1982), but it can be abolished in the presence of a β-adrenoceptor antagonist (Sanchez-Chapula, 1981; Hartman et al. 1988). Many others have failed to observe any change in the slow inward calcium current in response to α-adrenoceptor stimulation (Hescheler et al., 1988a; Ertl et al., 1991). Handa et al. (1982) reported that α-adrenoceptor agonists prolong the action potential duration by blocking an outward potassium current. This was later confirmed by many others (Apkon and Nerbonne, 1988; Fedida et al., 1989; Ravens et al., 1989; Braun et al., 1990; Fedida and Bouchard, 1992) and may contribute to the α-adrenoceptor-mediated positive inotropy by prolonging the action potential duration. It is, however, not known how α-adrenoceptor agonists increase the sensitivity of myofilaments to calcium or block the outward potassium current in the heart.

1.5.2.2. α-Adrenoceptors and phosphoinositide turnover

In many tissues, including the myocardium, α-adrenoceptor agonists have been shown to promote the turnover of a membrane phospholipid, phosphatidylinositol (Quist and Sanchez, 1983; Sekar and Roufogalis, 1984). The consequences of phosphatidylinositol turnover have been reviewed extensively (see Brown and Jones, 1986; Rana and Hokin, 1990). Briefly, phosphatidylinositol is sequentially phosphorylated to phosphatidylinositol mono- and bis- phosphates. Two different second messengers are generated as a result of phosphatidylinositol bisphosphate breakdown

under the influence of phospholipase C, namely inositol 1,4,5 trisphosphate (IP_3), and diacylglycerol. While IP_3 is believed to release calcium from intracellular stores, diacylglycerol in turn activates protein kinase C, a calcium- and phospholipid- dependent protein kinase, which phosphorylates a variety of intracellular proteins. In addition to IP_3, various products of phosphorylation of IP_3, such as inositol tetrakis (IP_4)-, pentakis (IP_5)- and hexakis (IP_6)- phosphates, are also believed to have second messenger functions.

In the heart, α-adrenoceptor agonists have been shown to promote the breakdown of phosphatidylinositol bisphosphate (Otani et al., 1988) and accumulation of different products of phosphatidylinositol breakdown namely, inositol mono-, bis- and tris-phosphates (Brown et al., 1985; Otani et al., 1988; Edes et al., 1991) and more recently tetrakis-, pentakis- and hexakis- phosphates (Scholz et al., 1992). Scholz et al. (1992) showed that the α-adrenoceptor-mediated generation of IP_3 precedes contraction and that of IP_4 coincides with increases in the force of contraction. They proposed that it is the IP_3 which initiates, and IP_4 which maintains, the α-adrenoceptor-mediated myocardial contractility. However, exactly how IP_3 and various phosphorylation products of IP_3 bring about changes in myocardial contractility is not known. Some workers have shown that IP_3 can release calcium from the sarcoplasmic reticulum (Nosek et al., 1986; Vites and Pappano, 1990) while others either failed to see any effect of IP_3 on calcium release from the sarcoplasmic reticulum (Movsesian et al. 1985) or were unable to ascribe any physiological significance to the IP_3 -induced calcium release in the heart (Fabiato, 1986).

While the presence of protein kinase C has been demonstrated in the heart by Nishizuka and colleagues using selective antibodies (see Shearman et al., 1989), the

functional significance of the protein kinase C pathway in the α-adrenoceptor-mediated positive inotropy in the heart is not clear. Both α-adrenoceptor agonists and phorbol esters, direct activators of protein kinase C, have been demonstrated to cause translocation of protein kinase C from the cytosol to sarcolemma (Yuan et al., 1987; Edes and Kranius, 1990; Edes et al., 1991; Talosi and Kranius, 1992). However, the similarity probably ends there. While α-adrenoceptor agonists have been shown to exert a positive inotropic response which is associated with an inhibition of the outward potassium current and an increase in intracellular calcium levels, activators of protein kinase C have very diverse effects on the myocardial contractility. For example, depending on the animal species and the type of activator used, phorbol esters can increase (Teutsch, 1987), decrease (Leatherman et al., 1987; Yuan et al., 1987; Nakanishi et al., 1989; Capogrossi et al., 1990; Karmazyn et al., 1990) or have no effect (Otani et al., 1988; Kushida et al., 1988) on myocardial contractility. Similarly, some phorbol esters have been shown to stimulate (Lacerda et al., 1988) or inhibit (Leatherman et al., 1987) calcium influx and intracellular calcium levels, and activate an outward potassium current (Tohose et al., 1987; Walsh and Kass, 1988).

Dissimilarity also exists at the level of protein substrates phosphorylated by α-adrenoceptor agonists and purified protein kinase C. In <u>in vivo</u> experiments α-adrenoceptor agonists and phorbol esters have been shown to promote phosphorylation of a 15 kDa sarcolemmal and/or a 28 kDa cytosolic protein (Lindemann, 1986; Edes and Kranius, 1990; Edes et al., 1991; Talosi and Kranius, 1992) in perfused guinea pig and rabbit hearts. On the other hand, purified protein kinase C has been shown to phosphorylate a variety of proteins in the sarcolemma and sarcoplasmic reticulum, most notably phospholamban (Movsesian et al., 1984; Yuan and Sen, 1986), and proteins

associated with contractile proteins, namely troponin C and T (Katoh et al., 1981; see also Hartzell, 1988; Rana and Hokin, 1989; Shearman et al., 1989). However, the significance of phosphorylation of these proteins is not clearly understood.

1.6 BIOCHEMICAL CONSEQUENCES OF MUSCARINIC RECEPTOR STIMULATION

Fig. 1 is a schematic representation of various electrophysiological and biochemical responses to muscarinic receptor stimulation in the heart. Muscarinic agonists exert their negative inotropic and chronotropic effects both in the presence and absence of α- and β- adrenoceptor agonists by a variety of mechanisms. These include: (a) inhibition of the β-adrenoceptor agonist-stimulated increases in adenylate cyclase activity and intracellular cAMP levels, and (b) mechanisms independent of adenylate cyclase inhibition. Muscarinic inhibition of the β-adrenoceptor agonist-stimulated cAMP generation is believed to contribute, at least in part, to the inhibitory effect of muscarinic agonists on the slow inward calcium current and the pacemaker current. The adenylate cyclase independent effects of muscarinic receptor stimulation include: (i) elevation of cGMP levels, which in turn may activate the cGMP-dependent protein kinase and cGMP-stimulated phosphodiesterase, (ii) activation of a protein phosphatase, and (iii) altering various ion channels and currents such as an outward potassium current, the pacemaker current (I_f) and the calcium current. In addition, muscarinic agonists also promote phosphoinositide breakdown in the heart. While this does not play a role in the adrenergic-cholinergic interactions, it is believed to be responsible for the positive inotropic responses to muscarinic stimulation.

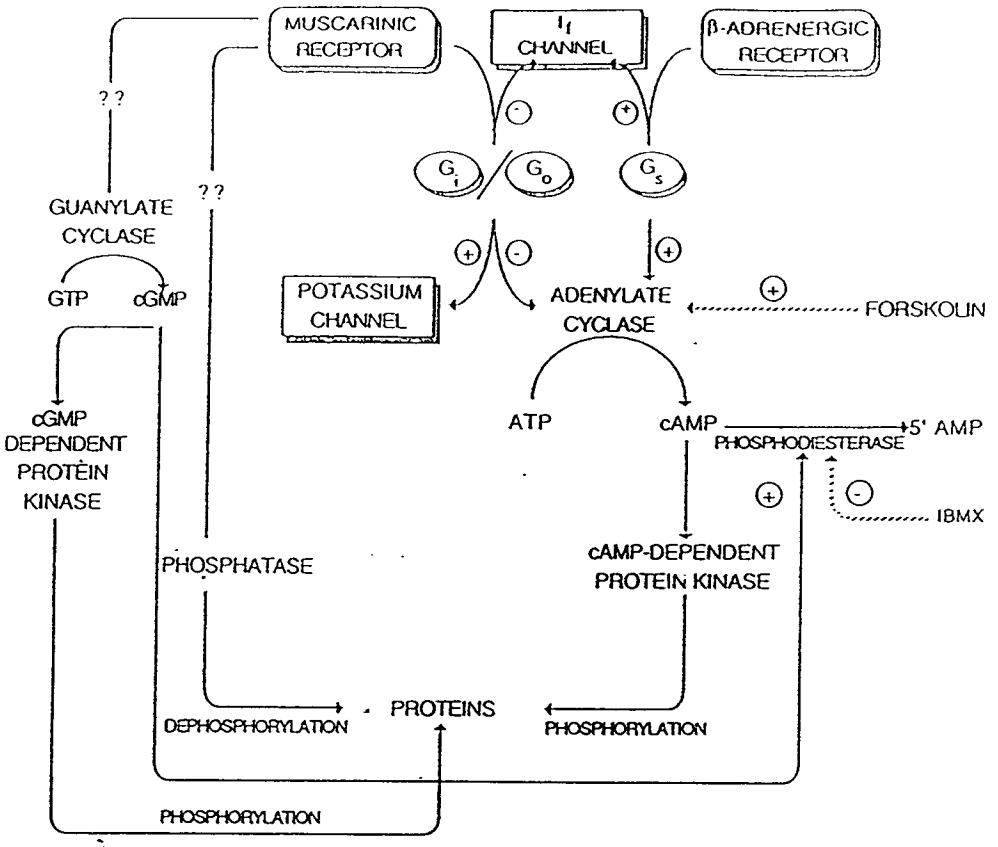


Fig. 1: A schematic representation of the possible consequences of muscarinic receptor stimulation in the heart.

(??) Indicates the mechanism of activation not known. (+) Represents stimulation and (-) represents inhibition of activity. G_s, G_i and G_o represent the stimulatory, inhibitory and the "other" guanine nucleotide binding proteins, respectively. G_s, G_i and G_o couple β-adrenoceptors and muscarinic cholinoceptors to adenylate cyclase and potassium channels respectively. In addition to inhibiting the β-adrenoceptor agonist-stimulated adenylate cyclase, muscarinic agonists can open potassium channels (atrium only), inhibit pacemaker (I_f) channels in the sinoatrial nodal cells, elevate cGMP levels and increase phosphatase activity (for details see text). cAMP-dependent protein kinase can stimulate the pacemaker channel (not shown in the figure).

1.6.1. Muscarinic receptors and adenylate cyclase

Since the first report by Murad et al. (1962), numerous workers have shown that muscarinic agonists can inhibit the β-adrenoceptor-stimulated adenylate cyclase activity, cAMP levels and positive inotropic responses in atria and ventricle from a variety of different animals (Watanabe et al., 1978; Jacobs et al., 1979; Sulakhe et al., 1985; Keely and Lincoln, 1978; Endoh, 1980; Endoh et al., 1985; MacLeod, 1985; 1986; see also Loffelholz and Pappano, 1985; Hartzell, 1988). In addition, in the ventricular myocardium a good correlation also exists between the muscarinic antagonism of the β-adrenoceptor-mediated cAMP generation and inhibition of various processes activated by an increase in cAMP levels, such as the cAMP-dependent protein kinase activity (Keely et al., 1978; Ingbretsen, 1980), slow inward calcium current (Biegon et al., 1980; Hescheler et al. , 1986; Rardon and Pappano, 1986), pacemaker current (DiFrancesco and Tromba, 1987; 1988) and phospholamban phosphorylation (Lindemann and Watanabe, 1985).

1.6.2. Adenylate cyclase-independent responses to muscarinic receptor stimulation

Although it has been shown that a good correlation sometimes exists between the ability of muscarinic agonists to inhibit the β-adrenoceptor-stimulated cAMP generation and force of contraction, many others have reported that the inhibition of cAMP generation does not always correlate with the inhibitory effect of muscarinic agonists on the β-adrenoceptor agonist-mediated positive inotropy in atria and ventricles (Keely et al. 1978; Endoh et al., 1985; MacLeod, 1986) and sometimes muscarinic agonists can inhibit the positive inotropic responses to β-adrenoceptor stimulation without having any

inhibitory effect on the elevated levels of cAMP (Watanabe and Besch 1975; Schmied and Korth, 1990). In addition, in atria and ventricles, muscarinic agonists have been shown to inhibit the positive inotropic responses to various cAMP-elevating agents including cholera toxin (Brown, 1980; Pappano et al, 1982), forskolin (Lindemann and Watanabe, 1985; MacLeod, 1985; MacLeod and Diamond, 1986) and isobutylmethyl xanthine (Brown, 1979; Biegon et al., 1980; Schmied and Korth, 1990), without lowering the accompanying increases in cAMP levels. This suggested that some mechanism other than, or in addition to, inhibition of cAMP generation may contribute to the functional interaction of muscarinic and cAMP-elevating agonists.

It is not clear how muscarinic receptor agonists produce a direct negative inotropic response or antagonize the cAMP-independent positive inotropic response to α-adrenoceptor stimulation in the atrial myocardium. Although it has been reported by some that muscarinic agonists can inhibit the basal adenylate cyclase activity in the atrial myocardium (Sulakhe et al., 1985), many others have shown that muscarinic agonists do not alter either the basal adenylate cyclase activity (Watanabe et al., 1978; Fleming et al., 1987) or the basal cAMP levels, in the absence (Keely and Lincoln, 1978; Linden and Brooker, 1979; Biegon et al., 1980; Pappano et al., 1982; Endoh et al., 1985; MacLeod and Diamond, 1986) or presence of an α-adrenoceptor agonist (Inui et al., 1982; MacLeod, 1986).

1.6.2.1. Contribution of cGMP

In the search of an alternative mechanism, it was proposed that the ability of muscarinic agonists to elevate cGMP levels may explain the cAMP-independent responses to muscarinic receptor stimulation in both atria and ventricles. In early studies

(George et al., 1970; 1973; 1975; Fink et al., 1976), a good correlation was reported between the negative inotropic response to muscarinic agonists in atria and ventricles and the ability of muscarinic agonists to elevate cGMP levels. Subsequently many others have shown that the negative inotropic responses to muscarinic receptor stimulation in the absence and presence of a β-adrenoceptor stimulation were associated with an increase in cGMP levels in both atria (Endoh and Yamshita, 1981) and ventricles (Watanabe and Besch, 1975; Endoh, 1980; Ingbretsen, 1980; Keely and Lincoln, 1978; Lincoln and Keely, 1980). It has also been shown that cGMP and various analogs of cGMP are capable of mimicking the electrophysiological and contractile responses to muscarinic receptor stimulation in the presence or absence of different cAMP-generating agents in atria (Kohlhardt and Haap, 1978; Endoh and Yamashita, 1981) and ventricles (Watanabe and Besch, 1975; Wahler and Sperelakis, 1985; 1986).

However, in the atrial myocardium several lines of evidence suggest that elevation of cGMP levels is probably not causally related to the electrophysiological and contractile responses to muscarinic receptor stimulation: (a) muscarinic agonists were able to exert their functional and electrophysiological responses without elevating cGMP levels (Brooker, 1977; Mirro et al., 1979; Brown, 1980), (b) interference with the ability of muscarinic agonists to elevate cGMP levels, using LY 83583 and methylene blue, did not affect the negative inotropic responses to muscarinic receptor stimulation in the presence and absence of the cAMP-elevating agent forskolin (Diamond and Chu, 1985; MacLeod and Diamond, 1986; Groschner et al., 1986), (c) although the lipid soluble analogs of cGMP and muscarinic receptor agonists were both capable of exerting a direct negative inotropic response in the mammalian atrial myocardium, Nawrath (1977) and Linden and Brooker (1979) have shown that the mechanism of the negative inotropic

response to a muscarinic receptor agonist differs from that of a cGMP-derivative, (d) and lastly, certain activators of the guanylate cyclase, such as sodium nitroprusside, in spite of producing a massive increase in cGMP levels, did not decrease the atrial contractility in the absence (Diamond et al., 1977) or presence of the various cAMP-elevating agents (Linden and Brooker, 1979).

In spite of reports to the contrary (Linden and Brooker, 1979; Endoh and Yamashita, 1981; Pappano et al., 1982), in the ventricular myocardium evidence exists to suggest that cGMP elevation contributes to the negative inotropic responses to muscarinic agonists in the presence of cAMP-elevating agents. MacLeod and Diamond (1986) have demonstrated that in the rabbit right ventricular papillary muscle interference with the ability of carbachol to elevate cGMP levels, using LY 83583, results in loss of the inhibitory effect of carbachol on the forskolin-induced increases in force of contraction. Several mechanisms have been suggested to account for the functional responses to cGMP elevation as described below.

1.6.2.1.1. Activation of cGMP-dependent protein kinase

Lincoln and Keely (1980) first demonstrated that the muscarinic receptor-stimulated elevation of cGMP levels in the rat heart was associated with activation of the cGMP-dependent protein kinase. It is believed that cGMP-dependent protein kinase antagonizes the effects of cAMP-dependent protein kinase by phosphorylating different proteins. In in vitro studies, the purified cGMP-dependent protein kinase has been shown to phosphorylate different sarcolemmal, sarcoplasmic reticular and cytosolic proteins including phospholamban (see Lohmann et al., 1991). However, the physiological significance of this phosphorylation is not known. Evidence has been provided that

muscarinic agonists, cGMP, and cGMP analogs can inhibit the calcium current in ventricles from different animal species, both in the presence and absence of cAMP-elevating agents and cAMP-analogs (Wahler and Sperelakis, 1985; 1986; Thakkar et al, 1988; Levi et al., 1989; Wahler et al., 1989; Mery et al., 1991). The lowering of intracellular calcium levels by cGMP, although so far not demonstrated experimentally, is proposed to be related to phosphorylation of the calcium channel protein in the ventricle (Lohman et al., 1991; Mery et al., 1991).

1.6.2.1.2. Activation of phosphodiesterase

Cyclic GMP and cGMP analogs have been shown to promote the hydrolysis of cAMP in the frog heart by activating a cGMP-stimulated phosphodiesterase (Hartzell and Fischmeister, 1986; Fischmeister and Hartzell, 1987). Although this cGMP-stimulated phosphodiesterase has been shown to be present in frog, rat and bovine hearts and has been purified from the bovine heart (see Hartzell, 1988; Lohman et al., 1991), its contribution to the inhibitory effects of muscarinic stimulation in the ventricle of species other than the frog is not clear at present (Lohman et al., 1991). In the hatched chick ventricle (Biegon et al., 1980) and in rat ventricular myocytes (Mery et al., 1991) muscarinic agonists did not alter the phosphodiesterase activity. Similarly, muscarinic-inhibition of the pacemaker current in the rabbit sinoatrial nodal cells was not affected by phosphodiesterase or phosphodiesterase inhibitors (DiFrancesco and Tromba, 1987; 1988b).

1.6.2.2. Activation of phosphatase

It is well established that elevation of cAMP levels result in activation of cAMP-dependent protein kinase which phosphorylates various proteins such as the calcium

channel, phospholamban, troponin I and cardiac C protein (Hartzell, 1988; Trautwein and Hescheler, 1990; Lindemann and Watanabe, 1990). Muscarinic agonists have been shown to antagonize the protein-phosphorylating effects of several cAMP-elevating agents (Loffelholz and Pappano, 1985). However, the mechanisms of these muscarinic responses are not clear, because muscarinic agonists do not always reduce cAMP levels increased by different cAMP-elevating agents such as IBMX and forskolin, as discussed above. On the other hand, the cGMP-dependent protein kinase has been shown to phosphorylate and not dephosphorylate proteins (discussed above). Thus, an alternative possibility could be activation of protein phosphatases. Previously, Trautwein and colleagues (Hescheler et al., 1987; 1988b; see also Trautwein and Hescheler, 1990) had shown that a protein phosphatase could inhibit, and the inhibitor of the protein phosphatase could enhance, the isoproterenol-induced increases in the calcium current. More recently, Watanabe and his group (Ahmad et al., 1989) have demonstrated that carbachol can also activate a type-I protein phosphatase in the guinea pig ventricle. However, the mechanism of muscarinic agonist-mediated activation of the protein phosphatase is not known and it is also not known if a cause and effect relationship exists between the activation of protein phosphatase and the ability of muscarinic agonists to antagonize the cAMP-dependent phosphorylation of proteins and inhibit the positive inotropic responses to cAMP-elevating agents.

1.6.2.3. Alteration of ion channel activity

1.6.2.3.1. Outward potassium current

It has been known for a long time that the negative inotropic and chronotropic responses to muscarinic receptor stimulation in the atrial myocardium are associated with

shortening of the action potential duration, hyperpolarization of the membrane, a decrease in the influx of calcium and an increase in the permeability to potassium (Burgen and Terroux, 1953; Raynor and Weatherall, 1959; Grossman and Furchgott, 1964; Van Zwitten, 1968; Jakobs et al., 1989). Ten Eick et al. (1976) reported the muscarinic activation, in low concentrations, of an outward potassium current in the atrial myocardium without inhibition of the slow inward calcium current. This was supported by others (DiFrancesco et al., 1980; Inoue et al., 1983; Soejima and Noma, 1984, Iijima et al., 1985; see also Hartzell, 1988; Pappano, 1990). It has also been shown that activation of the outward potassium current is independent of changes in intracellular cAMP, cGMP and calcium levels (Trautwein et al., 1982; Soejima and Noma, 1984; see also Loffelholz and Pappano, 1985; and Hartzell, 1988 for a review). More recently it has been shown that muscarinic receptors are linked to potassium channels by means of a pertussis toxin-sensitive guanine nucleotide binding protein (Breitwieser and Szabo, 1985; Pffafinger et al., 1985; Sorota et al., 1985; Hartzell, 1988; Brown and Birnbaumer, 1990; Pappano, 1990; Szabo and Otero, 1990).

Ten Eick et al (1976) proposed that the ability of muscarinic agonists to activate the outward potassium current may contribute to their negative inotropic effect by shortening the duration of action potential and reducing the influx of calcium. It has also been shown that the negative inotropic response to muscarinic agonists in the atrial myocardium is associated with an efflux of potassium (^{42} K) (Nawrath, 1977) or rubidium (^{86} Rb), a tracer for potassium (Raynor and Weatherall, 1959; Van Zwitten, 1968; Quast et al., 1988; Jakobs et al., 1989; Urquhart et al., 1991). Uncoupling of muscarinic receptors from potassium channels, using pertussis toxin, results in attenuation of the ability of muscarinic agonists to increase the outward potassium

current (Pffafinger et al., 1985; Sorota et al., 1985), promote the efflux of ^{86}Rb (Martin et al., 1985; Quast et al., 1988; Urquhart et al., 1991) and exert a direct negative inotropic response (Endoh et al., 1985; Sorota et al., 1985; Ray and MacLeod, 1992).

1.6.2.3.2. Pacemaker current (I_f)

This current, also known as the hyperpolarization-activated current, has been studied very extensively by DiFrancesco and colleagues (DiFrancesco and Tromba, 1987; 1988a; 1988b; DiFrancesco et al., 1989; DiFrancesco and Tortora, 1991; see also Hartzell, 1988; DiFrancesco, 1990 for a review). I_f is an inward current activated by hyperpolarization of the sinoatrial nodal cells and is believed to be the principal current responsible for the automaticity of the sinoatrial nodal cells. According to DiFrancesco et al. (1989), inhibition of the I_f is responsible for vagal slowing of the heart rate because inhibition of the I_f could be seen at a concentration at least 20 times less than that needed to promote the outward potassium current. However, it has been argued (Hirst et al., 1992) that whereas vagal stimulation produces cardiac arrest, inhibition of the I_f current alone by a muscarinic agonist merely slows the heart rate, suggesting that the contribution of some current in addition to inhibition of the I_f current cannot be ruled out in the process of vagal slowing of the heart rate. Muscarinic receptors are linked to the I_f channel by means of a pertussis toxin-sensitive guanine nucleotide binding protein (DiFrancesco and Tromba, 1987; Yatani et al., 1990). Evidence exists to suggest that muscarinic agonists can inhibit the pacemaker current both directly, without involving any second messenger (Yatani et al., 1990), as well as by inhibiting the basal and β-adrenoceptor agonist-stimulated adenylate cyclase activity (DiFrancesco and Tromba, 1987; 1988b; Yatani et al., 1990).

1.6.2.3.2. Calcium current

Muscarinic agonists can inhibit the slow inward calcium current stimulated by a β-adrenoceptor agonist in atria and ventricles by decreasing the β-adrenoceptor agonist-stimulated cAMP levels. In addition, the ability of muscarinic agonists to elevate cGMP levels may also contribute to the inhibitory effect of muscarinic stimulation on the basal calcium current (Hino and Ochi, 1981; Wahler et al., 1989) or the calcium current stimulated by different cAMP-elevating agents including isoproterenol, forskolin and IBMX (Wahler and Sperelakis, 1985; Rardon and Pappano, 1986; Thakkar et al., 1988). However, the mechanism of muscarinic inhibition of the basal calcium current in the mammalian atrial myocardium is not clear (Ten Eick et al. 1976; Iijima et al., 1985; Cerbai et al. 1988; DiFrancesco and Tromba 1988b; see also Hartzell, 1988). Iijima et al. (1985) have suggested that the muscarinic-inhibition of the calcium current is mostly secondary to activation of the outward potassium current. However, both Iijima et al. (1985) and Cerbai et al. (1988) have shown that a reduction of the calcium current can be measured even when the outward potassium current is blocked. An alternative possibility suggested by Cerbai et al. (1988) is that the ability of muscarinic agonists to inhibit the basal calcium current is related to the muscarinic-inhibition of the basal adenylate cyclase activity in atria. However, as discussed earlier, muscarinic agonists do not consistently inhibit either the basal adenylate cyclase activity or cAMP levels in the atrial myocardium.

1.6.2.4 Promotion of phosphoinositide turnover

Like the α-adrenoceptor agonists, high concentrations of muscarinic receptor agonists, usually 10 μM and higher, have been shown to promote phosphoinositide breakdown in the heart (Brown and Jones, 1986; Tajima et al., 1987; Pappano, 1990). This effect is believed to contribute to the pertussis toxin-insensitive positive inotropic and chronotropic (Tsuji et al. 1987; Tajima et al. 1987a; 1987b; Agnarsson et al., 1988; see also Pappano, 1990) responses to muscarinic receptor stimulation in the heart and not to the adrenergic-cholinergic interactions, and will not be discussed any further.

1.7. G-PROTEINS

The subject of the guanine nucleotide binding proteins has been reviewed extensively (see Gilman, 1987; Robishaw and Foster, 1988; Birnbaumer et al., 1990; Brown, 1990; Brown and Birnbaumer, 1990; Fleming et al., 1992; Lefkowitz, 1992)). It is well established that α- and β- adrenoceptors and muscarinic cholinoceptors are linked to their effectors including phospholipase C, adenylate cyclase and ion channels (potassium, calcium and I_f), by means of G-proteins. In addition, evidence also exists that G-proteins can activate phospholipase A_2 , phospholipase D, ATP-dependent potassium channels and sodium channels.

G-proteins are heterotrimeric proteins consisting of three different subunits α, β and γ. G-proteins usually have common β and γ subunits, whereas the α-subunits vary among G-proteins. The α-subunit of G-proteins are hydrophilic in nature and have a binding site for GTP, GTPase activity, sites for ADP-ribosylation by different bacterial toxins, namely cholera toxin and pertussis toxin, and myristoylation sites, although G-protein α-subunits which lack the ADP-ribosylation site for cholera toxin also lack the myristoylation site. However, in recent years G-proteins which lack the ADP-ribosylation

sites for either toxin have been identified . In contrast to the α subunit, the β and γ subunits are very hydrophobic in nature and are usually closely associated. In addition to helping anchor the α-subunits to the membrane, βγ subunits have been shown to increase the receptor-stimulated GTPase activity of α-subunits and may also have some physiological effects of their own. For example βγ subunits have been reported to inhibit adenylate cyclase, open potassium channels, and activate phospholipase C and phospholipase A_2.

Bacterial toxins have been of great help to identify, classify and study the functional significance of different G-proteins (Wregget, 1986). More recently with the help of molecular biological techniques, the genes for many G-protein α-subunits have been cloned and expressed and their amino acid sequences determined. Using α-subunit-specific antibodies raised against peptide sequences typical of a particular α-subunit, many different G-protein α-subunits have been identified both in cardiac and extracardiac tissues. On the basis of the molecular weight of the α-subunits, the G-proteins present in the heart can be grouped into the following (Fleming et al., 1992):

a. G_s or the stimulatory G-protein Two different subtypes of the α-subunits, of the molecular weight 45 and 52 kDa, have been identified. $G_{s\alpha}$ is known to mediate the agonist-stimulation of adenylate cyclase and voltage-dependent calcium channels. $G_{s\alpha}$ is a substrate for ADP-ribosylation by cholera toxin.

b. G_i or the inhibitory G-protein, and G_o the 'other' G protein α-Subunits of both of these proteins are substrates for ADP-ribosylation by pertussis toxin. $G_{i\alpha}$ consists of three different proteins namely $G_{i\alpha1}$ and $G_{i\alpha3}$ each 40 kDa and the 41 kDa $G_{i\alpha2}$. At least two different α-subunits of G_o, each of molecular weight 39 kDa, have been identified in the canine heart and bovine brain and purified from the bovine brain

(Birnbaumer et al., 1990; Kobayashi et al., 1990). Functions of the different α-subunits of G_i in vivo are not entirely clear, but it is believed that the $G_{i\alpha 2}$ mediates the hormonal-inhibition of adenylate cyclase (McClue et al., 1992), while the $G_{i\alpha 3}$ opens potassium channels (see Birnbaumer et al., 1990). Recently, it has been shown that the $G_{o\alpha}$ subunit is responsible for the hormonal-inhibition of the I_f channel in the heart (Brown, 1990; Yatani et al., 1990) and calcium channels in pituitary cells (Trautwein and Hescheler, 1990; Kleuss et al., 1991).

c. G_p/G_q This protein has recently been identified in the heart and has a molecular weight of 42 kDa. This protein lacks the site for ADP-ribosylation by bacterial toxins. Its exact function is not known.

d. G_z/G_x has also been shown to be present in the heart (Spicher et al., 1988; Foster et al., 1990). However, according to Foster et al. (1990) this protein is present in the membrane of non-myocardial cells. G_z is not ADP-ribosylated by bacterial toxins and a 43 kDa subunit has been found in the cell membrane (Foster et al., 1990) and another 40 kDa subunit in the cytosol (Spicher et al., 1988). The functional significance of G_z in the heart is not known. However, in transfection studies the wild type and mutationally-activated $G_{z\alpha}$ inhibited the agonist-stimulated adenylate cyclase in the presence and absence of various inhibitory receptor agonists (Wong et al., 1992).

The remaining section on the G-proteins will focus mainly on the mechanism of muscarinic inhibition of adenylate cyclase and activation of potassium channels in the heart. The mechanism of hormonal, eg. β-adrenoceptor agonist, stimulation of adenylate cyclase has been elucidated very clearly (review Gilman, 1987; Birnbaumer, 1990). Briefly, as shown in fig. 2 (Fleming et al., 1987), occupation of the receptor by an agonist facilitates exchange of GTP for GDP, and the GTP-bound $G_{s\alpha}$-subunit is dissociated

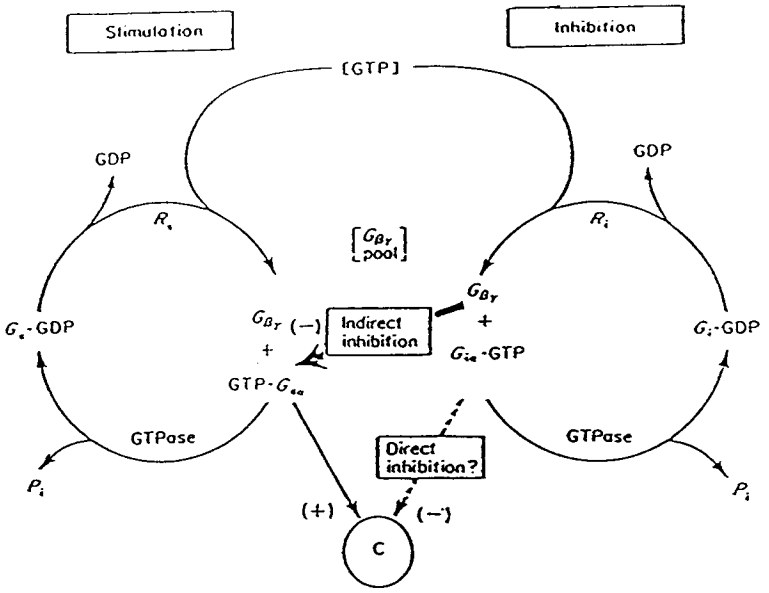


Fig. 2: A schematic representation of the role of G-proteins in the β-adrenoceptor-mediated activation and the muscarinic cholinoceptor-mediated inhibition of adenylate cyclase in the heart (Fleming et al., 1987). For details see text.

from $\beta\gamma$ subunits. The GTP-bound $G_{s\alpha}$ in turn activates adenylate cyclase. The effect is terminated when GTP is hydrolyzed by the GTPase of the α-subunit, and the GDP-bound $G_{s\alpha}$ unites with the $\beta\gamma$ subunits. The purified, pre-activated, native and recombinant $G_{s\alpha}$ has been shown to stimulate adenylate cyclase (see Birnbaumer et al., 1990). However, the mechanism of muscarinic inhibition of adenylate cyclase and activation of potassium channels in the heart is less well understood.

In reconstitution studies it has been shown that purified muscarinic receptors can interact with G_o and all three G_i proteins (Ikegaya et al., 1990). However, in the rat heart the agonist-stimulated muscarinic receptors have been shown to associate preferentially with G_{i2} and G_o in ventricles and only with G_o in atria (Matesic et al., 1991). The question then arises as to which G-protein(s) really connect muscarinic receptors to adenylate cyclase and potassium channels? According to Liang and Galper (1988), it is the G_i-like protein(s) which mediate the muscarinic inhibition of adenylate cyclase in the chick ventricular myocytes. In reconstitution (Kobayashi et al., 1990) and transfection (Wong et al., 1992) studies, it was shown that the α-subunits of all three G_i proteins, but not G_o, were able to inhibit adenylate cyclase. However, the exact G_i protein which conveys the message of muscarinic receptor occupancy to adenylate cyclase in the heart is not known. In extra-cardiac tissues, it has been shown that the $G_{i\alpha 2}$ is the most likely candidate that couples inhibitory receptors to adenylate cyclase (McClue et al., 1992). A similar problem exists regarding the identity of G-protein(s) that link muscarinic receptors to potassium channels. According to Brown, Birnbaumer and group (Yatani et al., 1988; Birnbaumer et al., 1990), all three pure and recombinant $G_{i\alpha}$ subunits, but not the $G_{o\alpha}$, can open potassium channels, and the authors believe that in intact tissue it is the $G_{i\alpha 3}$ which opens the agonist-stimulated potassium channels (see Birnbaumer et al.,

1990). In contrast, Kobayashi et al. (1990) have shown that the $G_{o\alpha}$ subunits, along with all three $G_{i\alpha}$ subunits, are also capable of opening the atrial potassium channels.

The second problem concerns which subunits of G-protein, α or $\beta\gamma$, convey the message of muscarinic receptor occupancy to adenylate cyclase and potassium channels. Watanabe and group (Fleming et al., 1987) in agreement with the observation of Katada et al. (1984) have suggested that the agonist-stimulated inhibition of adenylate cyclase in cardiac tissue is indirect and seen only when adenylate cyclase is stimulated previously by a stimulatory receptor agonist. According to this hypothesis, the $\beta\gamma$ subunit of inhibitory G-proteins, released as a result of inhibitory agonist stimulation, quenches the GTP-bound $G_{s\alpha}$ and prevents it from stimulating adenylate cyclase (fig. 2). In their study, Fleming et al. (1987) failed to observe any direct inhibitory effect of methacholine, a muscarinic receptor agonist, and GPP(NH)P, a non-hydrolyzable GTP analog, on the basal (in the absence of GTP) and forskolin-stimulated adenylate cyclase. Because the forskolin-induced activation of adenylate cyclase occurs independently of the stimulatory G-protein, the lack of inhibitory effect of methacholine and GPP(NH)P on the forskolin-stimulated adenylate cyclase was considered to be evidence that the α-subunits of G_i and G_o do not have any direct inhibitory effect on adenylate cyclase. However, several lines of evidence suggest that the α-subunits of inhibitory G-proteins may also contribute to the hormonal-inhibition of adenylate cyclase in cardiac and extra- cardiac tissues:

(a) In mutant S49 lymphoma cells which lack a stimulatory G protein, somatostatin, an inhibitory agonist for adenylate cyclase, was able to inhibit the forskolin-stimulated adenylate cyclase (see Birnbaumer, 1990 for a review). This observation was used to support the hypothesis that the hormonal-inhibition of adenylate cyclase is mediated by the α-subunit of inhibitory G-proteins.

(b) Hildebrandt and Kohnken (1990) have shown that in normal S49 lymphoma cells the mechanism of somatostatin-induced inhibition of the isoproterenol-stimulated adenylate cyclase is different from that of the βγ subunit-mediated inhibition of adenylate cyclase.

c) More recently, McClue et al. (1992) have shown that inhibition of the GTPase activity of the $G_{i\alpha 2}$ by a selective antibody resulted in loss of the ability of an α_2-adrenergic agonist to inhibit adenylate cyclase activity. Bourne and his group have also shown that the mutationally-activated α-subunits of inhibitory G-proteins, when expressed in mouse fibroblast cells were capable of inhibiting the forskolin-stimulated adenylate cyclase (Wong et al., 1991; 1992).

d) Reithman et al. (1989) reported that prolonged exposure of the rat heart cells to norepinephrine resulted in a loss of the ability of isoproterenol, forskolin and GTPγS to stimulate adenylate cyclase. This was associated with an increase in the immunoreactive $G_{i\alpha}$ subunit but not the β-subunit, suggesting that the α-subunit of inhibitory G-proteins play an important role in the inhibition of adenylate cyclase.

e) Many workers have shown that muscarinic agonists can inhibit both basal and forskolin-stimulated adenylate cyclase in the heart (Jakobs et al., 1979; Martin et al., 1985; Sulakhe et al., 1985).

Similar to adenylate cyclase, the mechanism of muscarinic receptor-mediated activation of potassium current is also not very clear. Birnbaumer, Brown and colleagues have demonstrated that the purified and recombinant (Codina et al., 1987; Yatani et al., 1987; Kirsch et al., 1988; Yatani et al., 1988a; see also Birnbaumer et al., 1990; Brown, 1990) α-subunits of inhibitory G-proteins open potassium channels and the βγ subunit of inhibitory G-proteins was shown to inhibit potassium channels (Okabe et al., 1988). A

specific antibody to the purified α-subunit of the inhibitory G-protein, also called α_k or α_{i3} by Brown, Birnbaumer and group, that opened potassium channels was shown to abolish the ability of carbachol and the pre-activated α-subunit to stimulate the potassium current (Yatani et al. 1988b).

Neer, Clapham and colleagues (Logothetis et al., 1987), on the other hand, were the first to report that the βγ subunits of inhibitory G-proteins are also capable of opening potassium channels. However, the same group later proposed that the mechanism of the βγ subunit-mediated activation of potassium current is related to stimulation of phospholipase A_2 and generation of lipoxygenase metabolites of arachidonic acid (Kim et al., 1989). In a recent study, however, Ito et al. (1992) have reaffirmed the original observation that the βγ subunits of G-proteins open potassium channels. In their study, Ito et al. (1992) reported that the purified α-subunits of $G_{i\ 1,\ 2}$ and $_3$ were very weak activators of potassium channels and failed to see any involvement of phospholipase A_2 and lipoxygenase metabolites as mediators of the βγ-induced channel opening.

Thus, further work is required to settle the issue of which particular G-protein(s), and which subunit of the G-proteins, conveys the message of muscarinic receptor occupancy to potassium channels and adenylate cyclase.

1.8. OBJECTIVES

In previous sections, evidence has been discussed that in both atrium and ventricle muscarinic agonists can antagonize the positive chronotropic and inotropic responses to β-adrenoceptor stimulation by inhibiting the β-adrenoceptor agonist-induced

increases in cAMP generation. However, it is not clear how muscarinic receptor agonists reverse the cAMP-independent positive inotropic responses to α-adrenceptor agonists in the atrial myocardium. In addition, previous reports from this laboratory have demonstrated that the negative inotropic responses to carbachol in the presence of forskolin and IBMX were not associated with any reduction in accompanying increases in cAMP levels (MacLeod and Diamond, 1986; Ray and MacLeod, 1992). It is known that muscarinic agonists can open potassium channels in the atrial myocardium and this is believed to contribute to the direct negative inotropic response to muscarinic agonists by shortening the action potential duration and reducing the influx of calcium (Ten Eick et al., 1976; Cerbai et al., 1988). One of the objectives of the present study was to test the hypothesis that the ability of carbachol to open potssium channels contributes to the cAMP-independent negative inotropic responses to carbachol in the presence of different cAMP-elevating and cAMP-independent positive inotropic agents in the rabbit left atrial myocardium.

In a previous study from this laboratory, it was shown that the cAMP-independent negative inotropic responses of rabbit left atria to carbachol in the presence of phenylephrine were attenuated by pre-treatment of rabbits with pertussis toxin (Ray and MacLeod, 1990). It is well established that pertussis toxin can uncouple muscarinic receptors from potassium channels in the atrial myocardium (Wregget, 1986). It has been shown that pre-treatment of animals with pertussis toxin results in the loss of the ability of muscarinic agonists to open potassium channels (Pffafinger et al., 1985; Sorota et al. 1985), to promote the efflux of ^{86}Rb (Martin et al., 1985; Quast et al., 1988; Urquhart et al., 1991) and to exert a direct negative inotropic effect (Endoh et al., 1985; Quast et al., 1988; Ray and MacLeod, 1990; 1992). Thus, attenuation by pertussis toxin of the cAMP-

independent negative inotropic response to carbachol in the presence of phenylephrine suggested a role of the carbachol-stimulated potassium current in this process. In order to investigate the contribution of potassium channels, we studied the ability of 4-aminopyridine, a potassium channel blocker, to antagonize the inhibitory effect of carbachol on the phenylephrine-induced positive inotropy. Evidence from electrophysiological studies suggests that in cardiac tissue, 4-aminopyridine can prolong the action potential duration by inhibiting the outward potassium current responsible for repolarization of the membrane (Van Bogaert et al., 1982; Gilmour et al., 1986; see also Rudy, 1988). 4-Aminopyridine has also been shown to antagonize the direct negative inotropic (Freeman, 1979; De Biasi et al., 1989; Urquhart and Broadley, 1991) and action potential shortening effects of carbachol (Freeman, 1979). We reasoned that if carbachol exerts its inhibitory effect on the phenylephrine-induced positive inotropy by opening potassium channels then 4-aminopyridine, by antagonizing the potassium channel opening effect of carbachol, should decrease the ability of carbachol to inhibit the phenylephrine-induced positive inotropy. At the same time, we also studied the effect of 4-aminopyridine on the ability of carbachol to inhibit the isoproterenol-stimulated positive inotropy, a cAMP-dependent response.

An attempt was also made to obtain more direct evidence for the muscarinic receptor-mediated opening of potassium channels and its contribution to the negative inotropic responses to carbachol in the presence and absence of phenylephrine. This was achieved by measuring the ability of carbachol to promote the efflux of ^{86}Rb-labelled rubidium chloride and to exert a negative inotropic response in the absence and presence of phenylephrine, in the same electrically-stimulated rabbit left atrium. Rubidium has been known for a long time to pass through potassium channels and it has been shown

that the contractile responses of guinea pig atria in the presence and absence of positive inotropic agents do not change if potassium is replaced by rubidium (Van Zwitten, 1968). In addition, radioactive rubidium (^{86} Rb) has a half life of 18.8 days compared to radioactive potassium (^{42} K) which has a half life of 12 hours. Thus, in spite of the criticism that the passage of ^{86} Rb across the membrane is slower than that of potassium (Smith et al., 1986; Jahnel and Nawrath, 1989), ^{86} Rb has been used quite extensively as a tracer for potassium to study the effect of various drugs on potassium channel activity in the heart (Raynor and Weatherall, 1959; Van Zwitten, 1968; Hunter and Nathanson, 1985; Quast et al., 1988; Kemmer et al., 1989; Urquhart et al., 1991) as well as in other tissues (Bolton and Clark, 1981; Smith et al.,1986). The effects of 4-aminopyridine and pertussis toxin were also tested on the contractile and ^{86} Rb-efflux promoting effects of carbachol in the presence and absence of phenylephrine.

Using a different approach, the effects of the potassium channel openers, pinacidil and cromakalim, were tested on the ability of phenylephrine and isoproterenol to exert a positive inotropic response. A previous study from this laboratory has shown that carbachol antagonized the development of α- and β- adrenoceptor-mediated positive inotropic responses in rabbit left atria (MacLeod, 1987). It was reasoned that if these inhibitory effects of carbachol on the positive inotropic responses to α- and β- adrenoceptor agonists were related to the ability of carbachol to open potassium channels, then potassium channel agonists should also be able to antagonize the development of the positive inotropic responses to α- and β- adrenoceptor agonists.

Previous reports from this laboratory have shown that the negative inotropic responses of left atria to carbachol in the presence of isoproterenol, but not in the presence of forskolin or IBMX, are associated with a reduction in the accompanying

increases in cAMP levels (MacLeod, 1986; MacLeod and Diamond, 1986; Ray and MacLeod, 1992). The contribution of the carbachol-stimulated potassium current to these cAMP-independent negative inotropic responses to carbachol in the presence of forskolin and IBMX was investigated using 4-aminopyridine. Again, very similar to the carbachol-phenylephrine interaction, the reasoning was if the ability of carbachol to open potassium channels contributes to the negative inotropic responses of left atria to carbachol in the presence of forskolin and IBMX, then antagonism by 4-aminopyridine of the carbachol-stimulated potassium current should attenuate the negative inotropic responses to carbachol in the presence of forskolin and IBMX.

However, it has been suggested that carbachol inhibits the forskolin-stimulated adenylate cyclase activity or cAMP generation in a compartment linked to the contractile machinery and a small reduction in cAMP levels is usually not detectable when total tissue cAMP levels are measured (Hartzell, 1988). In addition, methacholine, a muscarinic receptor agonist, was shown to inhibit the IBMX-stimulated cAMP levels in rat atria (Brown et al., 1980). In order to rule out the involvement of adenylate cyclase in the functional interaction of carbachol with forskolin and IBMX, pertussis toxin was used to uncouple muscarinic receptors from adenylate cyclase (Wregget, 1986; Fleming et al., 1988). The ability of carbachol to inhibit the isoproterenol-stimulated adenylate cyclase activity and cAMP levels was used as an index of uncoupling of muscarinic receptors from adenylate cyclase. The effect of pertussis toxin pre-treatment of rabbits, alone and in combination with 4-aminopyridine, was investigated on the negative inotropic responses to carbachol in the presence of forskolin and IBMX. The effects of 4-aminopyridine and pertussis toxin, alone and in combination, were also studied on the negative inotropic responses to carbachol in the presence of isoproterenol. Since

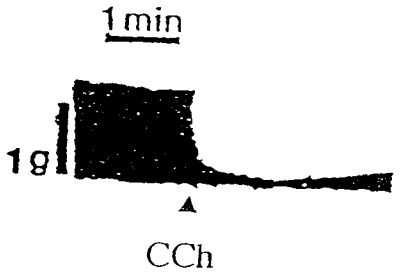
inhibition of the isoproterenol-stimulated cAMP generation is believed to contribute to the negative inotropic response to carbachol in the presence of isoproterenol (Ray and MacLeod, 1992), we reasoned that potassium channel blockade by 4-aminopyridine should have very little effect on the isoproterenol-carbachol interaction. In contrast, we expected that uncoupling of muscarinic receptors from adenylate cyclase by pertussis toxin would have a very pronounced inhibitory effect on the negative inotropic response to carbachol in the presence of isoproterenol.

It has been reported that 4-aminopyridine is capable of displacing muscarinic agonists from their receptor binding sites (Drukarch et al., 1988; Urquhart and Broadley, 1991). Therefore, in some experiments the effect of 4-aminopyridine on the ability of carbachol to inhibit the isoproterenol-stimulated cAMP generation was tested.

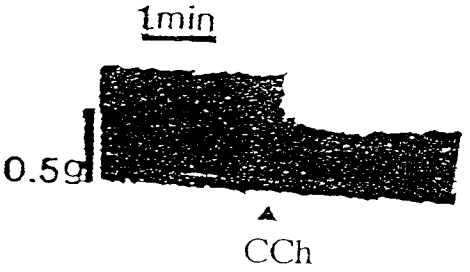
It has been discussed that muscarinic receptors are linked to potassium channels and adenylate cyclase in atria and ventricles by means of pertussis toxin-sensitive G-protein(s). However, it is not known very clearly exactly which G-protein(s), and which subunit(s) of the G-protein(s), link muscarinic receptors to different effectors, namely potassium channels and adenylate cyclase, in atria and ventricles. In a recent study from this laboratory (Ray and MacLeod, 1992), it was observed that a dose of pertussis toxin (1.75 μg/kg) completely attenuated the ability of carbachol to inhibit the isoproterenol-stimulated positive inotropic response (fig. 3 II B) and cAMP generation in rabbit left atrial strips, attenuated only partially the direct negative inotropic response to carbachol (fig. 3 I B). The negative inotropic response to carbachol, as discussed earlier, is believed to be related to the ability of carbachol to open potassium channels. Thus, our results suggested that in the rabbit left atrium pertussis toxin uncouples in a differential manner muscarinic receptors from potassium channels and adenylate cyclase. In addition,

Fig. 3 Tracings showing the effect of pertussis toxin pre-treatement of rabbits on the direct negative inotropic response to carbachol in the presence and absence of isoproterenol in rabbit left atrium.

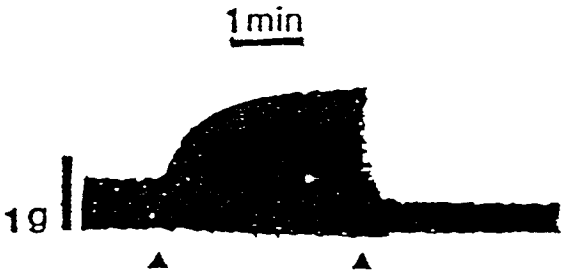
(I). Carbachol (3 μM) exerted a direct negative inotropic response (A) in rabbit left atrium which was attenuated by pre-treatment of rabbits with pertussis toxin (1.75 μg/kg) (B). (II). Carbachol (3 μM) attenuated the positive inotropic response to 100 nM isoproterenol (A). This inhibitory effect of carbachol on isoproterenol-induced positive inotropy was completely attenuated by pre-treatment of rabbits with 1.75 μg/kg pertussis toxin (B).



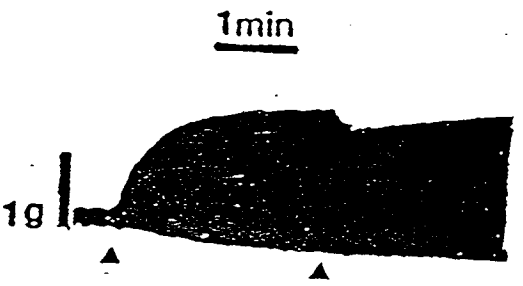
I A



B



II A



B

ISO = Isoproterenol (100 nM)

CCh = Carbachol (3 μM)

the same dose of pertussis toxin that completely attenuated the inhibitory effect of carbachol on the isoproterenol-stimulated cAMP generation in the left atrium did not have any uncoupling effect on the ability of carbachol to inhibit the positive inotropic response to isoproterenol in the right ventricular papillary muscle of rabbits. Thus, the second objective of the present study was to test the hypothesis that the muscarinic-inhibition of the isoproterenol-stimulated adenylate cyclase in the atrial myocardium is more sensitive to uncoupling by pertussis toxin than either the muscarinic-inhibition of the isoproterenol-stimulated adenylate cyclase in the ventricle or the muscarinic-activation of potassium channels in the left atrium.

The following were the specific goals of the present study:

(a) Study the effects of 4-aminopyridine on contractile responses of rabbit left atria to carbachol in the presence and absence of phenylephrine or isoproterenol.

(b) Correlate the negative inotropic response to carbachol in the presence and absence of phenylephrine with the ability of carbachol to promote the efflux of ^{86}Rb. Study the effects of 4-aminopyridine and pertussis toxin on these responses to carbachol.

(c) Study the effects of cromakalim and pinacidil on the positive inotropic responses to phenylephrine and isoproterenol in rabbit left atria.

(d) Study the effects of 4-aminopyridine and pertussis toxin on the inhibitory effect of carbachol on the isoproterenol-stimulated cAMP generation and adenylate cyclase activity in rabbit left atria.

(e) Study the effects of 4-aminopyridine and pertussis toxin, alone and in combination, on the negative inotropic responses to carbachol in the presence and absence of the cAMP-elevating agents isoproterenol, forskolin and IBMX in rabbit left atria.

(f) Measure adenylate cyclase activity in atria and ventricles.

(g) Study the effect of pertussis toxin pre-treatment of rabbits on the ADP-ribosylation of G-proteins. Compare the degree of ADP-ribosylation of G-proteins and the degree of uncoupling of muscarinic receptors from (i) adenylate cyclase in atria and ventricles and (ii) potassium channels in left atria.

MATERIALS AND METHODS

2.1. MATERIALS

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

Aldrich Chemical Co, Wisconsin, U.S.A.

Carbamylcholine chloride

Amersham Canada Ltd., Canada

[α-^{32} P]-ATP,

[^{86}] Rb-labelled rubidium chloride

BDH Chemical Co., Canada

Calcium chloride dihydrate,

Diethyl ether,

Dimethyl sulphoxide,

Glacial acetic acid,

d-Glucose,

Glycerol,

Hydrochloric acid,

Magnesium chloride hexahydrate,

Methanol,

Potassium chloride,

Sodium bicarbonate,

Sodium chloride,

Trichloroacetic acid

BIO-RAD Laboratories, Ontario, Canada

Acrylamide,

Ammonium persulphate,

N'-N'-methylene-bisacrylamide,

Bromophenol blue,

Coomasie blue,

Dithiothreitol,

Dowex Resin (H^+ form)

Glycine,

Protein assay kit,

Protein standards for electrophoresis,

Sodium dodecylsulphate,

TEMED

Tris base,

Tris hydrochloride,

Calbiochem Corporation, California, U.S.A.

Forskolin,

Isobutylmethylxanthine,

Du Pont Canada Inc., Ontario, Canada

Aquasol,

[^{32}P]-NAD,

Protosol

ICN Biomedicals Canada Ltd., Canada

[^{3}H]-cAMP

List Biological Laboratories, California, U.S.A.

Pertussis toxin

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

Adenosine deaminase,

Alamethicin,

Alumina (neutral; activity grade I)

4-Aminopyridine,

cAMP sodium salt,

Ascorbic acid,

ATP disodium salt,

Benzamidine hydrochloride,

Benzethonium chloride,

Creatine phosphate disodium salt,

Creatine phosphokinase,

Dithiothreitol,

EDTA,

EGTA,

Film, Kodak X-OMAT RP

GTP sodium salt,

GTPγS tetralithium salt,

Imidazole hydrochloride,

Isoproterenol hydrochloride,

Myokinase,

Nicotinamide adenine dinucleotide sodium salt,

Phenylephrine hydrochloride,

Phenylmethylsulphonylfluoride,

Processing chemicals kit, Kodak,

Sodium dodecylsulphate

Thymidine,

Timolol maleate,

Tris base,

Tris hydrochloride,

Trypsin inhibitor,

Pinacidil and cromakalim were gifts from Eli-Lilly and Co., Indianapolis, U.S.A. and Beecham Pharmaceuticals, Surrey, U.K., respectively.

2.2. PREPARATION OF SOLUTIONS

2.2.1. Modified Chenoweth-Koelle solution

A ten times concentrated stock solution of the modified Chenoweth Koelle (MCK) solution was prepared by dissolving sodium chloride (140 g), potassium chloride (8.4 g), calcium chloride (6.4 g) and magnesium chloride (3.6 g) in 1000 ml of distilled water. On the day of the experiment, 200 ml of the stock solution was diluted to 2000 ml with water after adding sodium bicarbonate (3.2 g) and glucose (3.6 g) to obtain a buffer of the final composition in mM : NaCl 120, KCl 5.7, $CaCl_2$ 2.2, $MgCl_2$ 0.9, $NaHCO_3$ 25 and glucose 10.

2.2.2. Pertussis toxin solution

Pertussis toxin was dissolved in distilled water to obtain a stock solution of 50 µg/500 µl for injecting into rabbits. For electrophoresis experiments, a stock solution of 50 µg/150 µl was prepared in distilled water.

2.2.3. Drug solutions

Solutions of carbachol, phenylephrine, timolol, atropine and 4-aminopyridine were prepared in distilled water. Isoproterenol was dissolved in distilled water in the presence of ascorbic acid (5 mg/ml) to prevent oxidation of isoproterenol. Forskolin was dissolved in 90 % ethanol to obtain a stock solution of 10 mM, from which subsequent dilutions were made using water. Cromakalim and pinacidil were dissolved in dimethyl sulphoxide (DMSO).

2.2.4. Solutions for the ADP-ribosylation experiments

2.2.4.1. Pertussis toxin activation buffer

A double strength pertussis toxin activation buffer was prepared by dissolving 60.5 mg Tris HCl, 7.4 mg EDTA, 6 mg Dithiothreitol (DTT), 12.4 mg ATP and 1 ml lubrol (1.0%) in a final volume of 10 ml in water. The pH of this solution was adjusted to 7.6 and stored in 500 µl aliquots at - 40 ^{o} C. On the day of the experiment, 30 µl of the activation buffer was incubated at 37 ^{o} C for 15 min with an equal volume of pertussis toxin (50 µg/ 150 µl) to obtain a final composition (in mM) : Tris HCl, 25; EDTA, 2; DTT, 20; ATP, 1 and Lubrol, 0.5%. A 9 µl aliquot of the mixture provided 30 µg/ml pertussis toxin in a final reaction volume of 50 µl for in vitro ADP-ribosylation of G-proteins.

2.2.4.2. ADP-ribosylation mixture

A 10 times concentrated ADP-ribosylation mixture was prepared by dissolving 1.2 g Tris base, 37.2 mg EDTA, 62.2 mg ATP, 15.3 mg DTT, 242 mg thymidine, 6.2 mg GTP and 2 mg DNAase I in 10 ml of distilled water. The pH of this solution was adjusted to 8 and it was stored in 500 µl aliquots at - 40 ^{o} C. A 5 µl aliquot of this reaction mixture was added to a final volume of 50 µl to obtain the final composition (in mM) : Tris, 100; EDTA, 1; ATP, 1; DTT, 1; Thymidine, 10, GTP, 0.1 and DNAase 20 µg/ml.

2.2.4.3. Nicotinamide adenine dinucleotide (NAD)

A stock solution of 10 mg/ml (14.5 mM) of the sodium salt of nicotinamide adenine dinucleotide was prepared in water and stored at - 40 ^{o} C. On the day of the experiment, a 4 µl aliquot of NAD was mixed with 2.5 µCi of radioactive NAD in a final volume of 10 µl adjusted with distilled water. In a final reaction volume of 50 µl, the amount of non-radioactive NAD present was 50 µM.

2.2.4.4. Solubilization buffer

The double strength solubilization buffer was prepared by mixing 1 ml Tris (0.5 M, pH 6.8), 1.6 ml sodium dodecylsulphate (10%), 0.8 ml glycerol, 0.4 ml 2-mercaptoethanol (5%) and bromophenol blue in sufficient quantity with 4 ml of water. This solution was stored at -40 ^{o} C. On the day of the experiment, the solubilization buffer was diluted with an equal volume of water and 50 µl was added to each tube to solubilize the precipitated G-proteins.

2.2.5. Solutions for electrophoresis

2.2.5.1. Electrode buffer

A ten times concentrated electrode buffer was prepared by dissolving 30 g Tris base, 144 g glycine and 10 g sodium dodecylsulphate in water to make a final volume of 1000 ml. The stock solution was stored at room temperature. On the day of the experiment, 400 ml of the stock was diluted to 4000 ml with water.

2.2.5.2. Protein standards

Bio-Rad low molecular weight protein standards (phosphorylase B, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa and lysozyme, 14.4 kDa) were used as the molecular weight markers. The stock solution of the standard proteins was diluted 20 times with the solubilization buffer and heated in a boiling water bath for 3 min before loading on the gel.

2.2.5.3. Staining solution

Staining solution was prepared by dissolving 2.5 g Coomassie blue in a mixture of 100 ml acetic acid, 450 ml methanol and 450 ml water. The staining solution was stored at room temperature.

2.2.5.4. Destaining solution

Destaining solution consisted of a mixture of methanol 450 ml, acetic acid 100ml and water 450 ml and was stored at room temperature.

2.2.5.5. Gel drying solution

One litre of gel drying solution contained 20 ml glycerol and 980 ml of water and stored at room temperature.

2.2.6. Solutions for the adenylate cyclase assay

2.2.6.1. Reaction mixture

The five times concentrated reaction mixture for the adenylate cyclase assay was prepared by dissolving 155 mg magnesium sulphate, 41.3 mg cAMP, 18.8 mg DTT, 725 mg sodium chloride, 27.5 mg isobutylmethyl xanthine and 988 mg Tris hydrochloride in sufficient water to make up the volume to 25 ml. The pH was adjusted to 7.8, and the reaction mixture was stored at - 40 ^{o} C prior to use. A 30 µl aliquot of this reaction mixture, when diluted with other components of the assay mixture to 150 µl, contained in mM: magnesium sulphate, 5; cAMP, 1.0; DTT, 1.0; sodium chloride, 100; isobutylmethyl xanthine, 1 and Tris hydrochloride, 50.

2.2.6.2. Regeneration mixture

A 100 mM solution of creatine phosphate was prepared by dissolving 255 mg creatine phosphate in 10 ml of distilled water. A 15 µl aliquot of this solution was diluted with other components of the assay mixture to 150 µl, so that the final concentration of creatine phosphate in each tube was 10 mM. Each tube also contained 1.6 U/150 µl of creatine phosphokinase (250 U/mg), 6 U/150 µl myokinase (200 U/mg) and 0.75 U/150 µl adenosine deaminase (200 U/mg).

2.2.6.3. GTP solution

A 500 µM GTP solution was prepared by dissolving 2.62 mg GTP sodium salt in sufficient water to make up the volume to 10 ml. A 15 µl aliquot of this solution, when

diluted with other components of the assay mixture to 150 µl gave a final concentration of 50 µM in each tube.

2.2.6.4. ATP solution

A 5 mM solution of disodium salt of ATP was prepared by dissolving 27.6 mg of ATP in 10 ml of water. Tris HCl was used to adjust the pH of this solution to 7.8. A 15 µl aliquot of this solution was added to each tube to obtain a final concentration of 0.5 mM when diluted with other components of the assay mixture. ATP solution was stored in 500 µl aliquots at - 40 ^{o} C prior to use. On the day of the experiment, 15 µl of non-radioactive ATP was mixed with 500,000 dpm of radioactive ATP and water to make up the volume to 30 µl. Each 30 µl ATP solution was diluted with other components of the assay mixture to obtain a final volume of 150 µl.

2.2.6.5. Stop solution

The stop solution was prepared by dissolving 200 mg sodium dodecylsulphate, 248.8 mg ATP and 4.61 mg cAMP in sufficient water to make up the volume to 10 ml. The pH was adjusted to 7.5 with Tris base. The solution was stored at -40 ^{o} C prior to use. Each 100 µl aliquot of the stop solution when added to individual tubes to stop the adenylate cyclase activity contained 2 % sodium dodecylsulphate, 40 mM ATP and 1.4 mM cAMP.

2.2.6.6. 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.

2.2.6.7. 0.1 M Imidazole hydrochloride buffer

Imidazole hydrochloride (10.04 g) was dissolved in 1000 ml water to obtain a 0.1 M solution. The pH of this solution was adjusted to 7.5.

2.3. GEL CASTING

The separating gel was prepared by dissolving 4.25 g acrylamide and 56 mg BIS (N', N'-methylene-bis-acrylamide) in distilled water containing 8.75 ml Tris base (1.5M ; pH 8.8), 350 µl SDS (10% w/v) and 875 µl ammonium persulphate (20 mg/ml) to a final volume of 35 ml. The solution was poured in a BIO-RAD Protean II (16 cm) gel casting apparatus after adding 12 µl TEMED, a catalyst that facilitates polymerization of acrylamide, and allowed to stand for 6 hours for the acrylamide solution to gel.

The stacking gel was prepared by dissolving 350 mg acrylamide and 9.3 mg BIS in a final volume of 10 ml distilled water containing 2.5 ml Tris base (0.5 M, pH 6.8), 100 µl SDS (10 % w/v) and 250 µl ammonium persulphate (20 mg/ml). The reaction was started by adding 10 µl TEMED and the solution was poured over the separating gel and allowed to stand for an hour to gel.

2.4. ISOLATED TISSUE PREPARATION

Rabbits of either sex were housed individually in cages and had free access to food and water. For experiments involving pertussis toxin, animals were injected with a single dose of pertussis toxin in a volume not exceeding 1 ml through the ear vein 48

hours before the experiments. It should be pointed out that in this study two different batches of pertussis toxin were used. For the contractility study, rabbits were injected with 2.2 μg/kg pertussis toxin (potency 0.1 ng/ml in CHO cell assay). On the other hand, rabbits were injected with 0.5, 1, 2 and 3 μg/kg pertussis toxin of a different batch (potency 0.03 ng/ml in CHO cell assay) for the ADP-ribosylation study, adenylate cyclase assay and rubidium efflux measurements. Control rabbits were injected with normal saline. On the day of the experiment, animals were sacrificed by an injection of pentobarbitone sodium (65 mg/kg) through the ear vein followed by exsanguination. Hearts were rapidly removed and placed in M. C. K. solution at room temperature, aerated with 95% O_2 and 5% CO_2. Left and right atria and the right ventricular free wall were removed from the heart. In some experiments, left atria were used for the contractility and ^{86} Rb efflux measurement studies. In other studies left and right atria and the right ventricle were frozen in liquid nitrogen for adenylate cyclase assay and ADP-ribosylation study.

2.5. CONTRACTILITY STUDY

Left atria were cut into 4-5 strips, approximately 2 mm in width and 5 mm in length, and one end of each strip was attached to a bipolar platinum electrode which was placed in a 20 ml tissue bath containing the M. C. K. solution maintained at 35^{o} C and aerated with 95 % O_2 and 5 % CO_2. The other end of the muscle preparation was attached by means of a cotton thread to a Grass FT .03 force displacement transducer. Tissues were stimulated to contract with pulses of 5 msec duration at a frequency of 1 Hz and a voltage two times threshold. Responses were recorded on a Grass polygraph

(model 7E). Atrial strips were placed under a resting tension of 0.5 g and the resting tension was adjusted throughout the 60 min equilibrium period to give the optimal basal developed tension. This usually occurred at resting tensions between 1.0 and 1.5 g. Atrial strips that developed contracture and/or arrhythmias during the equilibration period were discarded.

Cumulative concentration-response curves to carbachol alone or in the presence of 100 nM isoproterenol, 100 μM phenylephrine, 3 μM forskolin and 50 μM IBMX were obtained under various conditions. All experiments involving phenylephrine were conducted in tissues pre-treated with 1 μM timolol in order to block the β-adrenoceptor-mediated component of the phenylephrine-response. To determine the influence of 4-aminopyridine on responses to carbachol, tissues were randomly assigned to receive either 4-aminopyridine (50 or 500 μM) or vehicle for 10 min, before the concentration-response curves to carbachol alone or in the presence of isoproterenol (100 nM) or phenylephrine (100 μM) were obtained. For experiments involving forskolin and IBMX, tissues were exposed to 3 μM forskolin or 50 μM IBMX for 15 min each with 4-aminopyridine (50 and 500 μM) being added for the final 10 min, before obtaining the carbachol concentration-response curves.

Cumulative concentration-response curves to isoproterenol and phenylephrine were also obtained in the absence and presence of pinacidil and cromakalim. Tissues were pre-treated with either vehicle (DMSO), pinacidil or cromakalim for 10 min before the concentration-response curves to isoproterenol or phenylephrine were obtained.

In a separate set of experiments, atria were frozen for cAMP assay. For this purpose, atrial strips were set up and equilibrated as described above, and then divided into seven groups. The first and second groups were treated with vehicle and 4-

aminopyridine (500 μM), respectively and served as controls. The third and fourth groups were treated with vehicle or 4-aminopyridine (500 μM) for 10 min, with isoproterenol (100 nM) being added for the final 6 min. The fifth and sixth groups were exposed to either vehicle or 4-aminopyridine (500 μM) in combination with 100 nM isoproterenol (as described above) for 10 min with carbachol (3 μM) being added for the final 3 min. The seventh group was exposed to carbachol (3 μM) for 3 min. At the specified times tissues were frozen with clamps cooled in liquid nitrogen and stored at -70°C prior to assay for cAMP levels.

2.6. CYCLIC AMP ASSAY

Tissues were homogenized in 1 ml of 6% w/v trichloroacetic acid and centrifuged at 7000 rpm for a period of 40 min. Trichloroacetic acid was removed by extraction of the supernatant four times with 5 ml water-saturated ether. Cyclic AMP levels in the supernatant were determined using a radioimmunoassay kit obtained from New England Nuclear (MacLeod, 1986).

2.7. ^{86}Rb-EFFLUX MEASUREMENT

After an hour of equilibration, an atrial strip was exposed to 3 - 5 μCi / ml of ^{86}Rb (in a total volume of 17 ml) for another 2 hour period, during which time the force of contraction was monitored. At the end of the 2 hour loading period, the tissue was washed with the non-radioactive M.C.K. buffer every 2 min for 20 min. The atrial strip was then exposed to drugs dissolved in the M.C.K. buffer for various lengths of time

during which the tissue was washed every 2 min with the non-radioactive M.C.K. buffer containing the drug, and the force of contraction was also monitored. In order for the contractile response of the atrial strip to stabilize to the pre-drug treatment level, the atrial strip was re-equilibrated for 40 min in 17 ml of the non-radioactive M.C.K. buffer which was changed and collected for counting every 20 min. After the 40 min washout period, the tissue was again washed every 2 min with the non-radioactive M.C.K. buffer for another 10 min, followed by exposure to drugs as described above.

The contents of the tissue bath were collected in polystyrene scintillation vials and ^{86} Rb was counted by scintillation counting in the Cerenkov mode without the addition of any scintillation fluid (Urquhart et al., 1991). The radioactivity remaining in the atrial strip at the end of the experiment was obtained by dissolving the atrial strip in 2 ml of Protosol for 24 hours followed by liquid scintillation counting after adding 11 ml of Aquasol. In order to permit comparison between the aqueous counts (each 2 min sample) and the counts obtained in the presence of Aquasol, a non-radioactive atrial strip was spiked with a known amount of radioactivity and counted, after dissolution in Protosol and addition of scintillation fluid. At the same time, a 17 ml volume of M.C.K. solution was also spiked with same amount of radioactivity and was counted without adding any scintillation fluid. The ratio of the aqueous to organic counts was obtained (Urquhart et al., 1991), which was 0.29 in our experiments. The counts present in the atrial strips were multiplied by 0.29, and compared with other aqueous counts.

The efflux rate constant for rubidium was calculated using the formula

$$k_t = E_t/(C_t X\ t) \text{ where}$$

k_t = efflux rate constant at time t,

E_t = efflux (measured as cpm) at time t,

C_t = total tissue counts (cpm) of rubidium at time t,

t = collection time in min.

C_t was calculated by back addition of radioactivity remaining in the tissue at the end of the experiment plus all the radioactivity that was released during the experiment from time t to the end.

The efflux rate constant reflects the net movement of ^{86}Rb in inward and outward directions across the membrane. However, because the M.C.K. solution used to wash the tissues did not contain any rubidium, the efflux of ^{86}Rb predominated over the influx in these experiments.

Drugs were dissolved in 17 ml of warm M. C. K. solution containing 1 μM timolol to block the β-adrenoceptor-mediated component of the phenylephrine response. When the carbachol concentration-response curve was obtained, each concentration of carbachol was kept in contact with the tissue for an eight min period, with the solution being changed every 2 min. When the carbachol-response was measured in the presence of 4-aminopyridine or phenylephrine, tissues were exposed to them for a period of 16 min, with carbachol being added for the final 8 min. When the carbachol-response was measured in the presence of both 4-aminopyridine and phenylephrine, tissues were exposed to 4-aminopyridine for 24 min with phenylephrine and carbachol being added for the last 16 and 8 min, respectively.

In each experiment, the average of the final four values of the efflux rate constant or tension obtained immediately prior to addition of drug was considered the basal or initial response. In order to obtain the response to drug the four final values of the rate constant and tension obtained in the presence of each concentration of drug were averaged. Average values from four or more such experiments were used to calculate the

mean ± S.E.M. In some experiments, data were expressed as a percent of the basal response using the formula

% of basal response = (drug response/basal response)X100

2.8. HOMOGENIZATION OF TISSUE

Frozen atria and ventricles were ground under liquid nitrogen and homogenized in 10 volumes of ice cold 10 mM Tris hydrochloride buffer (pH 7.5), in the presence of protease inhibitors (EGTA 1 mM, PMSF 174 μg/ml, trypsin inhibitor 100 μg/ml, benzamidine 20 μg/ml, and benzethonium chloride 20 μg/ml), using Polytron (setting 6) twice for 5 sec each. The homogenate was filtered through several layers of cheese cloth. A portion of the homogenate was used for the ADP-ribosylation study and another portion was incubated with alamethicin (100 μg/ml) for 10 min at 37°C and used for the adenylate cyclase assay. The third portion of the homogenate was used for the protein assay.

2.9. ADP-RIBOSYLATION STUDY

In vitro pertussis toxin-catalyzed incorporation of the [^{32}P]-labelled ADP-ribose from [^{32} P]-NAD into G-proteins was studied by the method of Liang and Galper (1988) with certain modifications. Pertussis toxin was activated by incubation at 37°C for 15 min with equal volumes of the activation buffer at pH 7.8. Atrial and ventricular homogenates were incubated with the pre-activated pertussis toxin (30 μg/ml) and [^{32}P]-

NAD (2.5 μCi) for 1 hour at 37 ^{o}C in a 50 μl reaction medium containing in mM: thymidine 10, EDTA 1, ATP 1, DTT 1, GTP 0.1, NAD 0.05, creatine phosphate 10 and creatine phosphokinase 0.84 U. The reaction was started by adding the [^{32}P]-labelled NAD and stopped by adding 1 ml ice-cold TCA (20 %) followed by centrifugation at 3000 rpm for 30 min at 4 ^{o} C. The supernatant was decanted and the precipitate was washed with 1 ml of ice-cold ether and again centrifuged at 3000 rpm for 30 min. The precipitate was solubilized in the solubilization buffer at room temperature for 30 min and subjected to electrophoresis (35 mA and 500 Volts) using a 12 % polyacrylamide gel. The gel was stained with Coomasie blue for 30 min and destained to visualize the molecular weight standards using a destaining solution. The destained gel, after drying, was subjected to autoradiography by aligning against a Kodak X-OMAT-RP film for 6 - 18 hours using an intensifying screen. The film was developed by immersing it in a developing solution for 2 min, followed by immersion in a fixing solution for 2 min in the dark. The [^{32}P]-labelled G-protein bands were cut from the gel, by aligning the autoradiogram against the gel, and the incorporation of [^{32} P]-labelled ADP-ribose was measured by liquid scintillation counting in a Packard-Tricarb liquid scintillation counter in the presence of Aquasol.

The incorporation of [^{32} P]-labelled ADP-ribose was calculated using the formula:

pmol ADP-ribose per mg = $[(S - B) / (R_{sp} \times E)] \times 1000$, where

S = counts in dpm from sample lanes,

B = background dpm,

R_{sp} = specific activity of NAD in dpm/pmol,

E = protein concentration in μg,

The blank value was obtained by calculating the radioactivity incorporated in the G-proteins in the absence of pertussis toxin. The blank count was subtracted from the sample count to obtain the pmol ADP-ribose per mg.

2.10. ADENYLATE CYCLASE ASSAY

Adenylate cyclase was assayed by the method of Salomon (1979). The alamethicin- treated atrial and ventricular homogenates were incubated in a final volume of 150 µl for 10 min at 37 ^{o} C with the [α-^{32}P]-ATP (500,000 dpm) in a reaction medium (pH 7.8) of the composition in mM : $MgSO_4$ 5, cAMP 1, DTT 1, NaCl 100, IBMX 1, Tris HCl 50, GTP 0.05, ATP 0.5, creatine phosphate 10 mM, creatine phosphokinase 1.6 U, myokinase 6 U, and adenosine deaminase 0.75 U. The reaction was started by adding the [^{32}P]-labelled ATP and stopped by adding 100 µl stop solution.

The [^{32}P]-cAMP was separated from [^{32}P]-ATP by sequential chromatography on the Dowex and Alumina columns using [^{3}H]-cAMP (10000 cpm) as recovery marker. Briefly, 50 µl of the [^{3}H]-cAMP and 800 µl of water was added to each tube after the addition of the stop solution. The contents of each tube were added to the regenerated Dowex columns and the effluent was collected as waste. Individual reaction tubes were washed with 2 ml of water and the contents of each tube were transferred to the Dowex columns and the effluent was again collected as waste. Dowex columns were then placed on top of the regenerated Alumina columns and washed with 3 ml of water. Water was allowed to run through the Alumina columns and was collected as waste. Alumina columns were eluted with 4 ml of 0.1 M imidazole buffer and the effluent

collected in polystyrene scintillation vials. The [^{32}P]- and [^{3}H]- labelled cAMP were counted in a Packard-Tricarb liquid scintillation counter after adding 11 ml of Aquasol. Recovery of cAMP ranged from 50 - 80%. Effects of different drugs (isoproterenol, carbachol, atropine, timolol, forskolin and GTPγS) were tested by dissolving them directly in the reaction medium.

Adenylate cyclase activity (pmol/min/mg) was calculated using the formula proposed by Salomon (1979):

cAMP (pmol/min/mg) = [H/S]X[(S'-B'-F)X1000]/R_{sp} X E X t

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

P and P'= ^{32} P ATP standard counts in ^{3} H and ^{32} P channels respectively,

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

B and B'= Background counts in ^{3} H and ^{32} P channels respectively,

F = (H'-B')/H X S

R_{sp} = P'(dpm)/pmoles of ATP per tube,

E = Protein concentration in μg,

t = time of incubation in min.

In one tube the [^{32}-P]- ATP was added after the stop solution. This tube was used as the blank. Blank value was calculated in the same way as the sample, and was subtracted from the sample value to obtain net cAMP values in pmol/min/mg.

2.11. PROTEIN ASSAY

Protein was assayed by the method of Bradford (1976) using bovine serum albumin as standard. Standard proteins, and proteins in the atrial and ventricular homogenates were made to react with the dye solution and the absorbance was measured at 594 - 596 nm using a spectrophotometer.

2.12. STATISTICS

The results of the contractility studies were compared by one-way analysis of variance followed by Neuman Keul's multiple range test. Data from the saline- and pertussis toxin- treated groups were compared by two way analysis of variance followed by Neuman Keul's multiple range test. A $P<0.05$ was considered significantly different.

Results obtained in the rubidium-efflux experiments and adenylate cyclase assays were analyzed by Student's t-test. Mean values of tension, efflux rate constant and adenylate cyclase activity obtained for different treatments within the same experiment were compared by paired t-test. Means obtained for the same treatments but from different experiments were analyzed by Student's unpaired t-test. A $P<0.05$ was considered significantly different.

RESULTS

3.1. INTERACTION OF CARBACHOL WITH PHENYLEPHRINE

3.1.1. Effects of 4-aminopyridine on negative inotropic responses to carbachol alone and in the presence of phenylephrine and isoproterenol

In preliminary experiments it was observed that 4-aminopyridine exerted a concentration-dependent positive inotropic effect in the rabbit atrium over the concentration range of 50 μM to 5 mM (fig 4). The effects of 50 and 500 μM 4-aminopyridine were determined on the negative inotropic responses to carbachol in the absence and presence of positive inotropic agents.

The tension in left atrial strips in the presence of 50 μM 4-aminopyridine (0.8 ± 0.2 g) was not significantly different from that in its absence (0.6 ± 0.1 g). In comparison, 500 μM 4-aminopyridine produced a much greater increase (1.2 ± 0.1 g) in the contractile force (fig. 5A). Pre-treatment of left atria with 4-aminopyridine for 10 min caused a small rightward shift of the carbachol concentration-response curve and depressed the maximum response to carbachol (table 1). Increasing the concentration of 4-aminopyridine to 500 μM produced a greater shift in the carbachol concentration-response curve although the overall percentage tension remaining in the presence of a maximum concentration of carbachol was not further increased (table 1).

Phenylephrine (100 μM) plus timolol produced a positive inotropic effect in atria, which was completely reversed by carbachol (fig. 5B). The left atrial tension in the presence of 50 μM 4-aminopyridine plus phenylephrine was not different from that obtained with phenylephrine alone. However, in the presence of the combination of 50

TABLE 1. Effects of 4-aminopyridine on the maximum negative inotropic responses and pD_2 values of carbachol in the presence and absence of phenylephrine and isoproterenol.

Treatment		Control	4-Aminopyridine 50 μM	4-Aminopyridine 500 μM
Carbachol	Tension[a]	31.0±3.5 (6)	50.7±6.0 (8)	57.2±8.8 (9)
	pD_2	7.26±0.03 (6)	6.94±0.05 (8)	6.36±0.2* (7)
Carbachol + Phenylephrine	Tension	23.4±3.2 (10)	40.1±5.8* (9)	79.5±5.0* (5)
	pD_2	6.80±0.17 (10)	6.48±0.07 (9)	5.86±0.24* (4)
Carbachol + Isoproterenol	Tension	12.6±2.5 (6)	21.8±3.1 (8)	26.3±5.7* (8)
	pD_2	7.25±0.2 (5)	7.07±0.18 (8)	6.73±0.31 (6)

(*) Represents significantly different from control within the same treatment group (one-way ANOVA).

([a]) Contractile response is expressed as percent of the initial tension.

Each data point represents the mean ± S.E.M. of number of experiments shown in parentheses.

μM 4-aminopyridine and phenylephrine, the carbachol concentration-response curve was shifted slightly to the right and the maximum negative inotropic response to carbachol was significantly attenuated (fig. 5B, table 1). The positive inotropic effect of 500 μM 4-aminopyridine was additive with that of phenylephrine, and in the presence of the combination of phenylephrine plus 500 μM 4-aminopyridine, the carbachol concentration-response curve was shifted further to the right and the maximum inhibitory effect of carbachol was further reduced (fig. 5B, table 1).

Carbachol also antagonized the positive inotropic response to 100 nM isoproterenol in a concentration-dependent manner (fig. 5C). The tension in the presence of the combination of 100 nM isoproterenol and 50 μM 4-aminopyridine was not significantly different from that in the presence of isoproterenol alone and this concentration of 4-aminopyridine had very little effect on either the carbachol pD_2 value, or the maximum inhibitory effect of carbachol in the presence of isoproterenol (fig 5C; table 1). The combination of 500 μM 4-aminopyridine and 100 nM isoproterenol produced a further increase in tension (fig. 5C). Despite the high initial tension, there was only a small reduction in the magnitude of the maximum negative inotropic response to carbachol in the presence of isoproterenol plus 500 μM 4-aminopyridine (fig 5C; table 1). No significant difference in the potency of carbachol in the presence of isoproterenol and either concentration of 4-aminopyridine was detected (table 1).

3.1.2. Effects of pinacidil and cromakalim

Both pinacidil and cromakalim were dissolved in DMSO. Tissues were treated with either DMSO alone (100 μl) or DMSO containing various concentrations of pinacidil or cromakalim for 10 min prior to obtaining the concentration-response curves

to phenylephrine or isoproterenol. DMSO itself reduced the basal tension of left atrial strips, and no significant difference in the initial tensions, prior to obtaining the phenylephrine- or isoproterenol- concentration-response curves, was observed when tissues were treated with either DMSO alone or with pinacidil or cromakalim (data not shown). Phenylephrine alone produced a concentration-dependent positive inotropic effect which was not affected by DMSO. Pre-treatment of tissues with pinacidil antagonized the maximum positive inotropic response to phenylephrine in a concentration-dependent manner, without affecting the phenylephrine pD_2 value (fig. 6; table 2). The maximum inhibitory effect was observed in the presence of 1 mM pinacidil, which completely abolished the positive inotropic response to phenylephrine. Although cromakalim also attenuated the positive inotropic response to phenylephrine, in the concentration range tested, cromakalim was found to be less effective than pinacidil (fig. 7; table 2). Like pinacidil, cromakalim did not have any effect on the phenylephrine pD_2 value. The magnitude of the maximum positive inotropic response of left atria to isoproterenol was slightly less than double that to phenylephrine (fig. 8; table 2). Neither pinacidil (1 mM) nor cromakalim (1 mM) antagonized the contractile response to isoproterenol (fig. 8). In fact, pinacidil enhanced the maximum positive inotropic response to isoproterenol (table 2).

3.1.3. ^{86} Rb-Efflux studies

3.1.3.1. Time-dependence of the ^{86} Rb efflux rate constant

The efflux of rubidium from left atrial strips in the absence of any drug over a period of 60 min is shown in fig. 9. The rate-constant for rubidium-efflux declined from a

TABLE 2. Effects of pinacidil and cromakalim on the maximum positive inotropic responses and pD_2 values to phenylephrine and isoproterenol.

	Phenylephrine		Isoproterenol	
Treatment	Tension (g)	pD_2	Tension (g)	pD_2
Control	0.72±0.09 (9)	5.45±0.08 (9)	1.26±0.11 (7)	6.97±0.19 (7)
Pinacidil				
100 μM	0.61±0.07 (11)	5.31±0.23 (11)		
300 μM	0.42±0.05 (6)	5.21±0.23 (5)		
1 mM	0.18±0.05*(5)	N.D.	1.72±0.1* (6)	7.08±0.07 (5)
Cromakalim				
300 μM	0.65±0.10 (6)	5.60±0.16 (6)		
1 mM	0.40±0.05 (6)	5.68±0.12 (4)	1.41±0.11 (6)	7.14±0.23 (6)

(*) Represents significantly different from control within the same treament group (one way ANOVA).

(N.D.) Represents not determined.

Each data point represents the mean ± S.E.M. of number of experiments shown in parentheses.

very high value to a plateau of approximately 0.01/min within the first 10 min after loading and then remained stable for the remaining 60 min monitoring period.

3.1.3.2. Effects of carbachol on the ^{86} Rb-efflux-rate-constant and force of contraction

Carbachol produced an increase in the rate-constant of ^{86} Rb-efflux which reached a maximum (138 ± 2 % of basal) in the presence of 10 μM carbachol (fig. 10). Rubidium-efflux in response to 10 μM carbachol was attenuated by 100 nM atropine to 108 ± 2 % of basal (fig. 11). Atropine (100 nM) alone slightly reduced the basal efflux-rate-constant, to 88 ± 4% of basal. Carbachol also produced a negative inotropic response in rabbit left atria which reached a maximum at 1 μM carbachol (fig 10). A further increase in the carbachol concentration resulted in partial reversal of the negative inotropic response. The lowest concentration of carbachol used (100 nM) produced only a small increase in the efflux of ^{86}Rb (to 105 ± 3 % of basal) but produced a larger decrease (45 ± 7 %) in the basal developed force.

3.1.3.3. Effects of 4-aminopyridine on carbachol-induced increase in the rate-constant of ^{86} Rb-efflux and decrease in tension

4-Aminopyridine alone (50 and 500 μM) exerted a concentration-dependent positive inotropic effect in the rabbit left atrium (fig. 12A), but had no significant effect on the ^{86}Rb-efflux-rate-constant (fig. 12B). The same concentrations of 4-aminopyridine also attenuated the ability of carbachol to both reduce the tension (fig. 12A) and to increase the rate-constant of ^{86}Rb-efflux (fig. 12B). Carbachol alone (10 μM) produced a significant increase in the efflux-rate-constant to 128 ± 2% of the basal

and reduced the tension by 72 ± 4%. In the presence of 50 μM 4-aminopyridine, carbachol still produced a significant increase in the efflux-rate-constant to 120 ± 2%, while reducing the tension by 59 ± 6%. In the presence of the higher concentration of 4-aminopyridine (500 μM), carbachol had no significant effect on the efflux-rate-constant (fig. 12B), although it still produced a significant negative inotropic effect (fig. 12A), reducing the tension by 45 ± 7%.

3.1.3.4. Effects of carbachol on the rate-constant of ^{86}Rb-efflux and tension in the presence of phenylephrine

Phenylephrine alone (100 μM) had a positive inotropic effect in left atria but had no significant effect on the ^{86}Rb-efflux-rate-constant (table 3). In the presence of phenylephrine, 10 μM carbachol produced a significant decrease in the tension (fig. 13A) and increase in the ^{86}Rb-efflux-rate-constant (fig. 13B). When expressed as percentage of the corresponding values obtained in the presence of phenylephrine alone, carbachol produced a 65 ± 5% decrease in the tension elevated by phenylephrine, and increased the ^{86} Rb-efflux-rate-constant to 119 ± 5%.

3.1.3.5. Effects of 4-aminopyridine on responses to carbachol in the presence of phenylephrine

In this series of experiments, 4-aminopyridine alone (50 and 500 μM) produced only a very small positive inotropic response and had little effect on the positive inotropic response of left atria to phenylephrine (table 3). No significant effect on the ^{86}Rb-efflux was detected when phenylephrine was administered in the presence of either concentration of 4-aminopyridine (table 3).

Table 3. Effects of phenylephrine and 4-aminopyridine, alone and in combination, on the contractile response and the ^{86}Rb-efflux-rate-constant in electrically-stimulated rabbit left atria

	Tension (g)	Efflux Rate Constant (X 10^{-3})
Basal	0.7 ± 0.2	10.1 ± 0.6
Phenylephrine (100 μM)	1.2 ± 0.2*	10.4 ± 0.6
Basal	0.5 ± 0.2	10.0 ± 0.5
4–Aminopyridine (50 μM)	0.6 ± 0.1	10.0 ± 0.4
4–Aminopyridine (50 μM) + Phenylephrine (100 μM)	1.2 ± 0.2*	10.3 ± 0.4
Basal	0.6 ± 0.2	9.9 ± 0.5
4–Aminopyridine (500 μM)	0.8 ± 0.2	9.7 ± 0.5
4-Aminopyridine (500 μM) + Phenylephrine (100 μM)	1.4 ± 0.1*	9.7 ± 0.5

(*) Represents significantly different from the corresponding basal response (paired t-test).

Each data point represents the mean ± S.E.M. of 10 - 11 experiments.

4-aminopyridine attenuated the reversal by carbachol of the positive inotropic response to phenylephrine (fig. 13A). In the presence of 50 μM 4-aminopyridine, carbachol inhibited the positive inotropic response to phenylephrine by 51 ± 6%, while in the presence of 500 μM 4-aminopyridine, carbachol reduced the positive inotropic response to phenylephrine by 25 ± 5%. 4-Aminopyridine appeared to have somewhat less effect on the increase in the ^{86}Rb-efflux produced by carbachol in the presence of phenylephrine (fig. 13B). The lower concentration of 4-aminopyridine (50 μM) had no significant effect on the carbachol-induced increase in the efflux-rate-constant. The higher concentration of 4-aminopyridine (500 μM) reduced the magnitude of the carbachol-induced increase in the efflux-rate-constant to 113 ± 3%. However, the increase in the ^{86}Rb-efflux-rate constant produced by carbachol in the presence of the combination of phenylephrine and 500 μM 4-aminopyridine was still significant (fig. 13B).

3.1.3.6. Effects of pertussis toxin on carbachol-induced increase in the rate-constant of ^{86}Rb-efflux and decrease in tension

Pre-treatment of rabbits with 0.5 and 1 μg/kg pertussis toxin appeared to produce a rightward shift in the carbachol-concentration-response curve, while having little effect on the maximum stimulatory effect of carbachol on the efflux-rate-constant of ^{86}Rb (fig. 14B). In contrast, pertussis toxin pre-treatment inhibited the maximum negative inotropic response to carbachol, while having no effect on the reversal of the negative inotropic effect by 100 μM carbachol (fig. 14A). Responses to 1 μM carbachol, which produced a near-maximal increase in the rate-constant of ^{86}Rb-efflux and the maximum decrease in tension in tissues from saline-treated rabbits, were both markedly inhibited by

pertussis toxin pre-treatment of rabbits (fig. 14A, B). No statistically significant difference was observed in the abilities of 1 μM carbachol to increase the rate-constant of ^{86} Rb-efflux in atria from rabbits pre-treated with 0.5 and 1 μg/kg pertussis toxin (fig. 14B). However, 1 μM carbachol reduced the tension by 36 ± 8% and 20 ± 5 % in atria from 0.5 and 1 μg/kg pertussis toxin pre-treated rabbits, respectively (fig. 14A). No further reduction in tension of atria from pertussis toxin-treated rabbits was obtained by increasing the carbachol concentration to 10 μM, despite the fact that this concentration of carbachol increased the rate-constant of ^{86}Rb-efflux to almost the same extent as in atria from saline-treated rabbits (fig. 14 A,B).

3.1.3.7. Effects of pertussis toxin on responses to carbachol in the presence of phenylephrine

Pre-treatment of rabbits with pertussis toxin had no significant effect on the magnitude of the positive inotropic response of left atria to 100 μM phenylephrine. However, the ability of 10 μM carbachol both to reverse the positive inotropic response to phenylephrine and to increase the efflux-rate-constant in the presence of phenylephrine was essentially abolished by pertussis toxin pre-treatment (fig. 15 A,B). No carbachol-induced increase in the rate-constant of ^{86}Rb-efflux could be detected in the presence of 100 μM phenylephrine in atria from pertussis toxin pre-treated rabbits (fig. 15B). While carbachol still produced a small reduction in the tension elevated by phenylephrine in atria from pertussis toxin-treated rabbits, this difference was not significant (fig. 15A).

In the next series of experiments we studied the contribution of potassium channels to the cAMP-independent negative inotropic responses of left atria to carbachol

in the presence of forskolin and IBMX. In these studies pertussis toxin was used to uncouple muscarinic receptors from adenylate cyclase. However, the batch of pertussis toxin used in this study was less potent than the one used in the rubidium efflux study. In order to ascertain that pertussis toxin was uncoupling muscarinic receptors from adenylate cyclase, we measured the effect of pertussis toxin on the ability of carbachol to inhibit the isoproterenol-stimulated adenylate cyclase activity and cAMP generation. In addition, in order to ensure that 4-aminopyridine was not acting at the level of muscarinic receptors, we also studied the effect of 4-aminopyridine on the inhibitory effect of carbachol on the isoproterenol-stimulated cAMP generation.

3.2. EFFECTS OF 4-AMINOPYRIDINE AND PERTUSSIS TOXIN (2.2 μg/kg) ON THE INHIBITION BY CARBACHOL OF ISOPROTERENOL-STIMULATED ADENYLATE CYCLASE ACTIVITY AND cAMP GENERATION

In atrial strips from saline-treated rabbits, isoproterenol (100 nM) elevated cAMP levels from the basal value of 664 ± 17 pmol/g wet weight to a value of 1856 ± 102 pmol/g wet weight. Carbachol (3 μM) alone did not alter cAMP levels (590.8 ± 64.4 pmol/g wet weight) from the basal value, but when added in the presence of isoproterenol, carbachol inhibited the isoproterenol-stimulated cAMP generation (fig. 16A). Pre-treatment of rabbits with pertussis toxin (2.2 μg/kg) did not alter the basal cAMP levels (599 ± 70 pmol/g wet weight) or the ability of isoproterenol (100 nM) to stimulate cAMP generation (fig 16A). However, carbachol had no significant inhibitory effect on the isoproterenol-stimulated cAMP generation in atria from pertussis toxin pre-treated rabbits (fig 16A).

Basal adenylate cyclase activity in atrial homogenates from saline and pertussis toxin pre-treated rabbits was 115. 4 ± 18.5 (n=6) and 139.4 ± 7 (n=3) pmol/min/mg, respectively. Both isoproterenol and carbachol were able to stimulate and inhibit adenylate cyclase activity, respectively, although the potency of both agonists was markedly reduced compared to their effects on cAMP levels in intact tissues. Isoproterenol (100 μM) increased adenylate cyclase activity in atria from saline-treated rabbits and this increase was antagonized by 1 mM carbachol (fig. 17). Pertussis toxin pre-treatment of rabbits did not have any statistically significant effect on the ability of isoproterenol to increase adenylate cyclase activity but attenuated the ability of carbachol to inhibit the isoproterenol-stimulated adenylate cyclase (fig. 17).

We also investigated whether 500 μM 4-aminopyridine, the maximum concentration used in the study, had any effect on the ability of carbachol to inhibit the isoproterenol-induced increases in cAMP levels. Basal cAMP levels in the presence and absence of 4-aminopyridine were 825 ± 156 and 774 ± 60 pmol/g wet weight, respectively. 4-Aminopyridine did not have any effect on either the ability of isoproterenol to promote cAMP generation or on the inhibition by carbachol of isoproterenol-stimulated cAMP generation (fig. 16B).

3.3. INTERACTION OF CARBACHOL WITH cAMP-ELEVATING AGENTS: EFFECTS OF 4-AMINOPYRIDINE AND PERTUSSIS TOXIN

3.3.1. Effects of pertussis toxin and 4-aminopyridine on negative inotropic responses to carbachol in the absence and presence of isoproterenol

The effects of pertussis toxin and 4-aminopyridine were compared on the cAMP-independent direct negative inotropic response to carbachol, and the cAMP-dependent response to carbachol in the presence of isoproterenol. Pre-treatment of rabbits with pertussis toxin had no significant effect on the basal developed tension (fig. 18) or on the positive inotropic responses of rabbit left atrial strips to 100 nM isoproterenol (fig. 19). 4-Aminopyridine alone exerted a concentration-dependent positive inotropic response (fig. 18), but had little effect on the positive inotropic responses to isoproterenol (fig. 19) in atria from both control and pertussis toxin pre-treated rabbits. No significant differences in the magnitude of positive inotropic responses to 4-aminopyridine were observed between saline and pertussis toxin-treated rabbits (fig. 18).

In left atrial strips from control rabbits, carbachol exerted a concentration-related negative inotropic response (fig. 18A). Pre-treatment of rabbits with the dose of pertussis toxin which completely uncoupled muscarinic receptors from adenylate cyclase (fig. 16A, 17) had very little effect on the direct negative inotropic response to carbachol, producing only a slight reduction in the sensitivity, while having no effect on the maximum negative inotropic response, to this agonist (fig. 18, table 4). 4-Aminopyridine produced a concentration-dependent attenuation of the sensitivity and maximum negative inotropic response to carbachol (fig. 18; table 4). The antagonism by 4-aminopyridine of the direct negative inotropic response to carbachol was little affected by pertussis toxin

TABLE 4. Effects of 4-aminopyridine and pertussis toxin, alone and in combination, on the maximum negative inotropic responses and pD_2 values to carbachol in the presence and absence of isoproterenol, forskolin and IBMX.

Treatment		Saline-treated			Pertussis toxin		
		Control	4-Aminopyridine 50 μM	4-Aminopyridine 500 μM	Control	4-Aminopyridine 50 μM	4-Aminopyridine 500 μM
Carbachol	Tension[a]	31.4±3.30 (9)	45.3±4.90 (14)	57.1±6.2† (15)	34.0±8.6 (7)	51.9±9.3 (7)	66.2±7.1† (7)
	pD_2	7.08±0.09 (9)	6.84±0.05 (14)	6.29±0.12† (12)	6.68±0.15 (6)	6.49±0.08 (6)	6.18±0.12† (6)
Carbachol + Isoproterenol	Tension	11.7±3.1 (6)	18.9±3.5 (6)	35.1±4.9† (6)	68.7±10.6†+(4)	75.4±11.6+(4)	81.1±3.6+(5)
	pD_2	6.81±0.09 (6)	6.70±0.09 (7)	6.30±0.14† (6)	6.02±0.23+(4)	6.15±0.09+(4)	6.02±0.18+(5)
Carbachol + Forskolin	Tension	13.0±4 (11)	25.1±2.6†(10)	52.3±3.7†(9)	46.4±8.4+ (8)	60.5±5.9†+(7)	72.4±5.3†+(5)
	pD_2	6.62±0.08 (11)	6.37±0.07†(10)	6.03±0.07†(9)	6.51±.1 (7)	6.28±0.03 (7)	6.12±0.15†(4)
Carbachol + IBMX	Tension	7.70±3.7 (13)	16.9±4.4 (8)	34.6±4.4†(9)	22.1±8.8 (7)	37.6±8.1 (7)	65.5±7.2†+(5)
	pD_2	6.89±0.06 (13)	6.80±0.07 (8)	6.14±0.09†(9)	6.71±0.08 (4)	6.34±0.05†+(4)	6.11±0.21†(5)

(†) Represents significantly different from control within the same treatment group (one-way ANOVA).
(+) Represents significantly different from the respective treatment in saline-treated group (two-way ANOVA).
([a]) Contractile response is expressed as percent of the initial tension.
Each data point represents the mean ± S.E.M. of number of experiments shown in parentheses.

pre-treatment of rabbits. There was no significant difference between atria from control and pertussis toxin pre-treated rabbits in the maximum response or sensitivity to carbachol in the presence of either concentration of 4-aminopyridine (fig. 18, table 4).

Carbachol also completely reversed the positive inotropic responses of left atrial strips from saline-treated rabbits to isoproterenol (fig. 19A). The response to carbachol in the presence of isoproterenol was affected to a much greater extent by pertussis toxin pre-treatment of rabbits than was the direct negative inotropic response to carbachol (fig. 18B). Both the maximum response and the sensitivity to carbachol in the presence of isoproterenol were significantly reduced in atria from pertussis toxin-treated rabbits compared to atria from control rabbits (table 4). On the other hand, the response of atria from saline-treated rabbits to carbachol in the presence of isoproterenol was relatively resistant to inhibition by 4-aminopyridine, being blocked to a small extent by 500 μM but not by 50 μM 4-aminopyridine (fig. 19A, table 4). In atria from pertussis toxin pre-treated rabbits, 4-aminopyridine produced further concentration-dependent inhibition of the responses to carbachol in the presence of isoproterenol, although the carbachol response was not completely abolished by the combination of both pertussis toxin and 4-aminopyridine (fig.19B, table 4).

3.3.2. Effects of pertussis toxin and 4-aminopyridine on the negative inotropic responses to carbachol in the presence of forskolin and IBMX:

In atria from saline-treated rabbits, both forskolin (3 μM) and IBMX (50 μM) produced a positive inotropic response which was slightly smaller in magnitude than that produced by 100 nM isoproterenol (fig. 20A, 21A). Pre-treatment of rabbits with pertussis toxin had no significant effect on positive inotropic responses of left atria to

either forskolin or IBMX (fig. 20, 21). Overall, the contractile force in the presence of the combination of 4-aminopyridine plus forskolin or IBMX was greater than the positive inotropic effect of either agent alone, in atria from both saline and pertussis toxin pre-treated rabbits (fig. 20, 21). However, this difference was not consistently significant.

In left atrial strips from saline-treated rabbits, increasing concentrations of carbachol completely reversed the positive inotropic response to forskolin (fig. 20A). The ability of carbachol to overcome the response to forskolin was attenuated by pertussis toxin pre-treatment of rabbits (fig. 20B). While the carbachol pD_2 value was unaffected, the maximum decreases in tension produced by carbachol in the presence of forskolin was significantly reduced in atria from pertussis toxin pre-treated rabbits (fig. 20, table 4). In atria from saline-treated rabbits, 4-aminopyridine produced a concentration-dependent reduction of the response to carbachol in the presence of forskolin, reducing both the potency and maximum inhibitory effect of carbachol in the presence of forskolin. 4-Aminopyridine produced a further inhibition of the response to carbachol in the presence of forskolin in atria from pertussis toxin pre-treated rabbits, such that the maximum negative inotropic responses to carbachol in the presence of forskolin and 4-aminopyridine were less than those to carbachol in the presence of forskolin alone in atria from pertussis toxin-treated rabbits (fig. 20, table 4).

Carbachol also produced a concentration-dependent reversal of the positive inotropic response to 50 μM IBMX in left atrial strips from saline-treated rabbits. The negative inotropic response to carbachol in the presence of IBMX was very pronounced, and 7 out of 13 tissues stopped beating after the addition of 2 μM carbachol. In atria from rabbits pre-treated with pertussis toxin, the maximum negative inotropic response to carbachol in the presence of IBMX was only slightly attenuated (fig. 21, table 4), but

only one tissue stopped beating in the presence of the maximum concentration of carbachol. The lower concentration of 4-aminopyridine (50 μM) also had relatively little apparent effect on the response to carbachol in the presence of IBMX in atria from saline-treated rabbits, but only 2 out of 7 tissues stopped beating after 2 μM carbachol. The higher concentration of 4-aminopyridine (500 μM) significantly reduced both the potency and negative inotropic response to carbachol in the presence of IBMX in atria from saline-treated rabbits (fig. 21, table 4). In atria from pertussis toxin pre-treated rabbits, 4-aminopyridine produced further attenuation of the response to carbachol in the presence of IBMX (fig. 21, table 4). The maximum response to carbachol in the presence of IBMX plus 500 μM 4-aminopyridine in atria from pertussis toxin pre-treated rabbits was significantly different from the corresponding value in atria from saline-treated rabbits.

3.4. ADP-RIBOSYLATION EXPERIMENTS

Before describing the next series of experiments, it is important to mention that the batch of pertussis toxin used in this series was the same as that used in the rubidium-efflux experiment. The reason for this was we wanted to make a direct comparison of the uncoupling effects of pertussis toxin on the carbachol-mediated inhibition of adenylate cyclase and the carbachol-stimulated rubidium-efflux in atria and ventricles.

3.4.1. Selecting the concentration of pertussis toxin for in vitro ADP-ribosylation

Atrial and ventricular homogenates (50 - 100 μg protein) were incubated with increasing concentrations of pertussis toxin (10, 20, 30 and 100 μg/ml) in the presence of ^{32} P-NAD. Two bands in the molecular weight range 45 - 39 kDa were seen upon electrophoretic separation followed by autoradiography of the reaction mixture (fig. 22). The average molecular weight of the labelled proteins was 40.2 ± 0.9 and 41.5 ± 0.9 kDa (n=4) in the atrium and 41 ± 0.3 and 42.4 ± 0.2 kDa (n=4) in the ventricle. As shown in table 5, the maximum effect was observed in the presence of 30 μg/ml pertussis toxin. Increasing the concentration of pertussis toxin to 100 μg/ml did not result in any significant increase in the ADP-ribosylation of G-proteins.

3.4.2. Estimation of in vivo ADP-ribosylation of G-proteins by pertussis toxin

Figs. 23 and 24 are two representative autoradiograms showing the effect of injection of rabbits with pertussis toxin (0.5, 1, 2, 3 μg/kg) on the ability of exogenous pertussis toxin (30 μg/ml) to ADP-ribosylate the atrial and ventricular G-proteins. The degree of in vivo ADP-ribosylation was determined by comparing the ability of in vitro pertussis toxin (30 μg/ml) to transfer the [^{32} P]-ADP-ribose from [^{32} P]-NAD to G-proteins in the atrium and ventricle from saline and pertussis toxin pre-treated rabbits. In a typical experiment the amount of radioactivity incorporated by 30 μg/ml pertussis toxin in atrial and ventricular homogenates of rabbits injected with normal saline was 1258 dpm and 829 dpm respectively. In rabbits injected with 0.5 and 3.0 μg/kg pertussis toxin, the amount of radioactivity incorporated in the presence of 30 μg/ml pertussis toxin in atrial homogenates was 875 and 227 dpm respectively. In the presence of 30 μg/ml

Table 5. Invitro ADP-ribosylation of G-proteins by pertussis toxin in the rabbit atrial and ventricular homogenates.

Concentration	Atrium	(n)	Ventricle	(n)
(µg/ml)	(pmol ADP-Ribose/mg)		(pmol ADP-Ribose/mg)	
10	85.1±21.0	(6)	75.5±19.1	(4)
20			73.2±10.3	(3)
30	122.2±41.4	(6)	95.2±15.7	(4)
100	133.0±48.2	(6)	65.9±5.3	(4)

Each data point represents the mean ± S.E.M. Values in parentheses represent number of animals.

Table 6. ADP-Ribosylation by exogenous pertussis toxin (30 μg/ml) of G-proteins in atrial and ventricular homogenates from saline and pertusssis toxin (0.5, 1, 2 and 3 μg/kg) pre-treated rabbits.

		In vitro		In vivo	
		(pmol ADP-Ribose/mg)	(% of control)	(% of control)	(n)
Control	Atrium	238.6±48.5	100.0	0.0	(9)
	Ventricle	156.9±34.3	100.0	0.0	(9
Pertussis	Atrium	77.4±14.1	32.4±5.9	67.6±5.9	(4)
toxin	Ventricle	77.3±20.4	49.3±12.8	50.7±12.8	(5)
(0.5 μg/kg)					
Pertussis	Atrium	71.8±28.1	30.1±10	69.9±10.8	(6)
toxin	Ventricle	63.4±9.3	40.4±5.9	59.6±5.9	(6)
(1 μg/kg)					
Pertussis	Atrium	10.9±10.2	4.6±4.2	95.4±4.3	(5)
toxin	Ventricle	12.4±16.4	7.9±10.5	92.1±10.5	(6)
(2 μg/kg)					
Pertussis	Atrium	0	0	100.0	(5)
toxin	Ventricle	0	0	100.0	(5)
(3 μg/kg)					

Each data point represents the mean ± S.E.M. of number of rabbits shown in parentheses.

pertussis toxin, incorporation of radioactivity in ventricular homogenates from rabbits injected with 0.5 and 3.0 μg/kg pertussis toxin was 711 and 194 dpm respectively. Blank counts in atrial and ventricular homogenates typically were 105 and 196 dpm respectively. In the atrium and ventricle from control rabbits, the amounts of ADP-ribose incorporated were 238.6 ± 48.5 pmol/mg (n=9) and 156.9 ± 34.3 pmol/mg (n=9) respectively. Pre-treatment of rabbits with pertussis toxin resulted in a loss of the ability of exogenous pertussis toxin to ADP-ribosylate G-proteins (table 6). In the atrium, 0.5 μg/kg pertussis toxin ADP-ribosylated 68 % of all pertussis toxin-sensitive G-proteins. In the ventricle, the same dose of pertussis toxin ADP-ribosylated 51 % of all G-proteins. Increasing the dose of pertussis toxin to 1 μg/kg resulted in very small increase in the in vivo ADP-ribosylation of G-proteins in the atrium and ventricle. Pre-treatment of rabbits with 2 μg/kg pertussis toxin resulted in 95 % and 92 % ADP-ribosylation of G-proteins in the atrium and ventricle respectively. In the atrium and ventricle from rabbits pre-treated with 3 μg/kg pertussis toxin, external pertussis toxin was unable to ADP-ribosylate any G-protein.

3.5. ADENYLATE CYCLASE ASSAY

The alamethicin-treated atrial and ventricular homogenates were used to measure adenylate cyclase activity. The basal adenylate cyclase activity in the atrial and ventricular homogenates were found to be 121.9 ± 12.9 and 156.2 ± 15.5 pmol/min/mg, respectively. The basal adenylate cyclase activity was responsive to stimulation by forskolin and GTPγS (table 7). This suggested that the crude homogenates of atrium and

Table 7. Adenylate cyclase activity in the rabbit atrial and ventricular homogenates in the presence and absence of various activators.

		Adenylate cyclase activity (pmol/min/mg)	% of basal	(n)
Basal	Atrium	121.9 ± 12.9		(15)
	Ventricle	156.2 ± 15.5		(10)
Isoproterenol	Atrium	163.3 ± 13.3	144.2 ± 11	(15)
	Ventricle	252.4 ± 27.8	198.2 ± 22	(7)
Forskolin	Atrium	907.0 ± 92.1	689.4 ± 38	(14)
(1 μM)	Ventricle	1115 ± 92	780 ± 50	(11)
GTPγS	Atrium	245.6 ± 23.9	241.7 ± 23	(7)
(10 μM)	Ventricle	311.0 ± 47	215.0 ± 19	(5)

Each data point represents the mean ± S.E.M. of adenylate cyclase activity in the atrial and ventricular homogenates from number of animals shown in parentheses.

Concentrations of isoproterenol used were 100 μM in atrium and 1 μM in ventricle.

ventricle had a functioning adenylate cyclase enzyme which was stimulatory G-protein coupled.

3.5.1. β-Adrenoceptor stimulation of adenylate cyclase

Isoproterenol stimulated adenylate cyclase activity in a concentration-dependent manner in atrial and ventricular homogenates (fig. 25). In the concentration range used in the present study (100 nM - 1 mM) the ventricular adenylate cyclase was more responsive to isoproterenol, both in terms of potency and maximum stimulatory effect, than that of the atrium (fig. 25). Whereas 100 nM isoproterenol was able to stimulate adenylate cyclase activity in the ventricle, the same concentration of isoproterenol did not stimulate the basal adenylate cyclase activity in the atrium (fig. 25). Similarly, 100 μM isoproterenol increased adenylate cyclase activity 289.5 pmol/min/mg from the basal value in the ventricle. The same concentration of isoproterenol, however, produced only a 36.3 pmol/min/mg increase in the enzyme activity in the atrial homogenate (fig. 26).

In order to ascertain that the stimulatory effect of isoproterenol on adenylate cyclase was mediated through β-adrenoceptors, timolol, a β-adrenoceptor blocker was used to antagonize the isoproterenol response. Timolol (1 μM) alone did not have any effect on the basal adenylate cyclase activity in homogenates of atrium and ventricle. However, when combined with 100 μM isoproterenol, timolol (1 μM) attenuated the ability of isoproterenol to stimulate adenylate cyclase activity (fig. 26).

3.5.2. Effect of muscarinic receptor stimulation on adenylate cyclase activity in the atrium and ventricle

3.5.2.1. Basal adenylate cyclase

In both atrium and ventricle the inhibitory effect of carbachol on adenylate cyclase activity was seen at concentrations of 10 μM and higher. In the ventricle, carbachol, 10 μM - 1 mM, produced a concentration-dependent inhibitory effect on adenylate cyclase activity (fig. 27A). The maximum concentration of carbachol used in the study (1 mM) produced a 27.7 ± 2 % inhibition of the basal adenylate cyclase activity. The inhibitory effect of carbachol (1 mM) was sensitive to antagonism by atropine, a muscarinic receptor blocker. Atropine (10 μM), alone had a slight stimulatory effect on the basal adenylate cyclase activity (fig. 28A), but when used with carbachol, atropine antagonized the ability of carbachol to inhibit adenylate cyclase activity (fig. 28A).

In the atrium, on the other hand, a measurable inhibition of 11.8 ± 4.3 % (fig 27A) of the basal adenylate cyclase activity was seen only in the presence of 1 mM carbachol. In two atria, out of a total of eleven, carbachol did not inhibit the basal adenylate cyclase activity at all. In the remaining nine, inhibition of the basal adenylate cyclase activity ranged from 1.8 - 33 %. Atropine, 10 μM, did not have any statistically significant attenuating effect on the ability of carbachol to inhibit the basal adenylate cyclase activity (fig 28A).

3.5.2.2. Isoproterenol-stimulated adenylate cyclase

Carbachol (10 μM - 1 mM) exerted a concentration-dependent inhibitory effect on adenylate cyclase activity stimulated by 1 μM isoproterenol in the ventricle (fig. 27B).

In the concentration range used in the present study a maximum inhibition of 54.7 ± 5.7 % of the increase produced by isoproterenol was seen in the presence of 1 mM carbachol (fig 27B). Atropine (10 μM) completely blocked the inhibitory effect of carbachol on the isoproterenol-stimulated adenylate cyclase activity (fig 28B).

Carbachol also inhibited in a concentration-dependent manner the ability of isoproterenol to stimulate adenylate cyclase activity in the atrium (fig 27B). In the concentration range used in the present study (10 μM - 1 mM), a maximum of 57.2 ± 10.2 % inhibition of the isoproterenol-stimulated adenylate cyclase was observed in the presence of 1 mM carbachol. The inhibitory effect carbachol on the isoproterenol-stimulated adenylate cyclase activity (fig 28B) was attenuated by atropine (10 μM).

3.5.2.3. Forskolin- and GTPγS- stimulated adenylate cyclase

Since the maximum inhibitory effect on the basal and isoproterenol-stimulated adenylate cyclase activity was seen in the presence of 1 mM carbachol, this concentration of carbachol was used to study the interaction with forskolin and GTPγS.

In the ventricle, carbachol inhibited the forskolin-stimulated adenylate cyclase activity (fig. 29A). The inhibitory effect of carbachol (18.4 ± 3.6 %) on the forskolin-stimulated adenylate cyclase activity was attenuated by atropine (data not shown). Carbachol also exerted a 17 ± 2.4% inhibition of the GTPγS-stimulated adenylate cyclase activity (fig. 29B).

In contrast, in the atrium carbachol did not have any inhibitory effect on adenylate cyclase activity stimulated by either forskolin or GTPγS (fig. 29 A,B).

3.5.3. Pertussis toxin treatment of rabbits

Rabbits were treated with 4 different doses of pertussis toxin (0.5, 1, 2, 3 μg/kg) 48 hours prior to sacrifice. In the ventricle, the effect of 0.5 μg/kg pertussis toxin on adenylate cyclase activity was not tested. In both atrium and ventricle, pertussis toxin pre-treatment of rabbits did not have any effect on the basal adenylate cyclase activity (table 8). Similarly, the abilities of isoproterenol or forskolin to stimulate adenylate cyclase activity were not altered by pre-treatment of rabbits with pertussis toxin (table 9).

3.5.3.1. Muscarinic receptor-mediated response

3.5.3.1.1. Basal adenylate cyclase

In the ventricle, pertussis toxin pre-treatment of rabbits resulted in a dose-dependent attenuation of the ability of carbachol to inhibit the basal adenylate cyclase activity (fig 30A). In rabbits pre-treated with 1 μg/kg pertussis toxin, a dose that ADP-ribosylated 60 % of G-proteins, carbachol retained its ability to inhibit (28 ± 4%) the basal adenylate cyclase activity. Increasing the dose of the toxin to 2 and 3 μg/kg, which resulted in 92% and 100% ADP-ribosylation, respectively, attenuated the maximum inhibitory effect of carbachol. The inhibitory effects of carbachol (1 mM) in the ventricle from rabbits pre-treated with 2 and 3 μg/kg pertussis toxin were 21 and 7 %, respectively.

3.4.3.1.2. Interaction of carbachol with isoproterenol:

In the ventricle, pertussis toxin pre-treatment of rabbits resulted in a dose-dependent attenuation of the inhibitory effect of carbachol on the isoproterenol-stimulated adenylate cyclase activity. As shown in fig. 30B, pre-treatment of rabbits with 1 μg/kg pertussis toxin had no effect on the ability of carbachol to inhibit the

Table 8. Effects of pre-treatment of rabbits with different doses of pertussis toxin on basal adenylate cyclase activity in the atrium and ventricle.

	Adenylate cyclase activity (pmol/min/mg)			
Treatment	Atrium	(n)	Ventricle	(n)
Saline	116.5±16	(12)	156.0±16	(10)
Pertussis toxin (0.5 μg/kg)	128.5±23	(6)		
Pertussis toxin (1 μg/kg)	148.8±29	(6)	301.0±82	(6)
Pertussis toxin (2 μg/kg))	140.8±19	(7)	172.7±26	(6)
Pertussis toxin (3 μg/kg)	123.9±36	(6)	140.6±20	(5)

Each data point represents the mean ± S.E.M. of adenylate cyclase activity in the atrial and ventricular homogenates of number of animals shown in parentheses.

Table 9. Effects of pertussis toxin pre-treatment of rabbits on the isoproterenol (ISO)- and forskolin- stimulated adenylate cyclase activity in the atrium and ventricle.

	Adenylate cyclase activity (% of Basal)			
	Atrium		Ventricle	
Treatment	ISO	Forskolin	ISO	Forskolin
Saline	149±14	684±39	198±22	825±45
Pertussis toxin (0.5 μg/kg)	131±5	612±26		
Pertussis toxin (1 μg/kg)	131±9	542±60	198±32	810±115
Pertussis toxin (2 μg/kg))	148±11	563±62	181±7	794±67
Pertussis toxin (3 μg/kg)	139±10	553±48	245±30	975±87

Each data point represents the mean ± S.E.M. of adenylate cyclase activity in the atrial and ventricular homogentes from 5 - 15 animals.

Concentrations of isoproterenol used were 1 μM in ventricle and 100 μM in atrium.

isoproterenol-stimulated adenylate cyclase. In rabbits pre-treated with 1 μg/kg pertussis toxin carbachol produced 59.8 ± 8.5 % inhibition of the isoproterenol-stimulated adenylate cyclase which was not significantly different from the inhibitory effect of 1 mM carbachol (54.9 ± 5.7%) on the isoproterenol-stimulated adenylate cyclase observed in atria from saline-treated rabbits. Carbachol inhibited the isoproterenol-stimulated adenylate cyclase activity by 18 ± 11 % in rabbit pre-treated with 2 μg/kg pertussis toxin. In rabbits pre-treated with 3 μg/kg pertussis toxin the inhibitory effect of 10 μM carbachol (18.4±10%) on the isoproterenol-stimulated adenylate cyclase activity was higher than the inhibitory effect (-17.1 ± 9.9 %) observed in the presence of the same concentration of carbachol in saline-treated rabbits. This inhibitory effect of carbachol, however, was not concentration-dependent. Increasing the concentration of carbachol to 1 mM did not result in any further increase in the inhibitory effect of carbachol (27.4 ± 7.9%) on the enzyme activity in the presence of isoproterenol.

In the atrium, the effect of pertussis toxin pre-treatment of rabbits on the isoproterenol-carbachol interaction was very pronounced and complex (fig 31). In the atrium from rabbits pre-treated with 0.5, 1 and 3 μg/kg pertussis toxin, which resulted in 68, 70 and 100 % ADP-ribosylation of G-proteins respectively, the inhibitory effect of carbachol (1 mM) on the isoproterenol-stimulated adenylate cyclase activity was almost completely attenuated (fig 31). In contrast, in rabbits pre-treated with 2 μg/kg pertussis toxin, which ADP-ribosylated 95% of G-proteins, the inhibitory effect of carbachol on the isoproterenol-stimulated adenylate cyclase activity was very similar to that observed in saline-treated rabbits (fig. 31).

Fig. 4: Concentration-dependent positive inotropic effects of 4-aminopyridine in electrically-stimulated rabbit left atria.

Cumulative concentration-response curves to 4-aminopyridine were obtained in rabbit left atrial strips. Data are expressed as the developed tension in grams. Each data point represents the mean ± S.E.M. of 9 experiments.

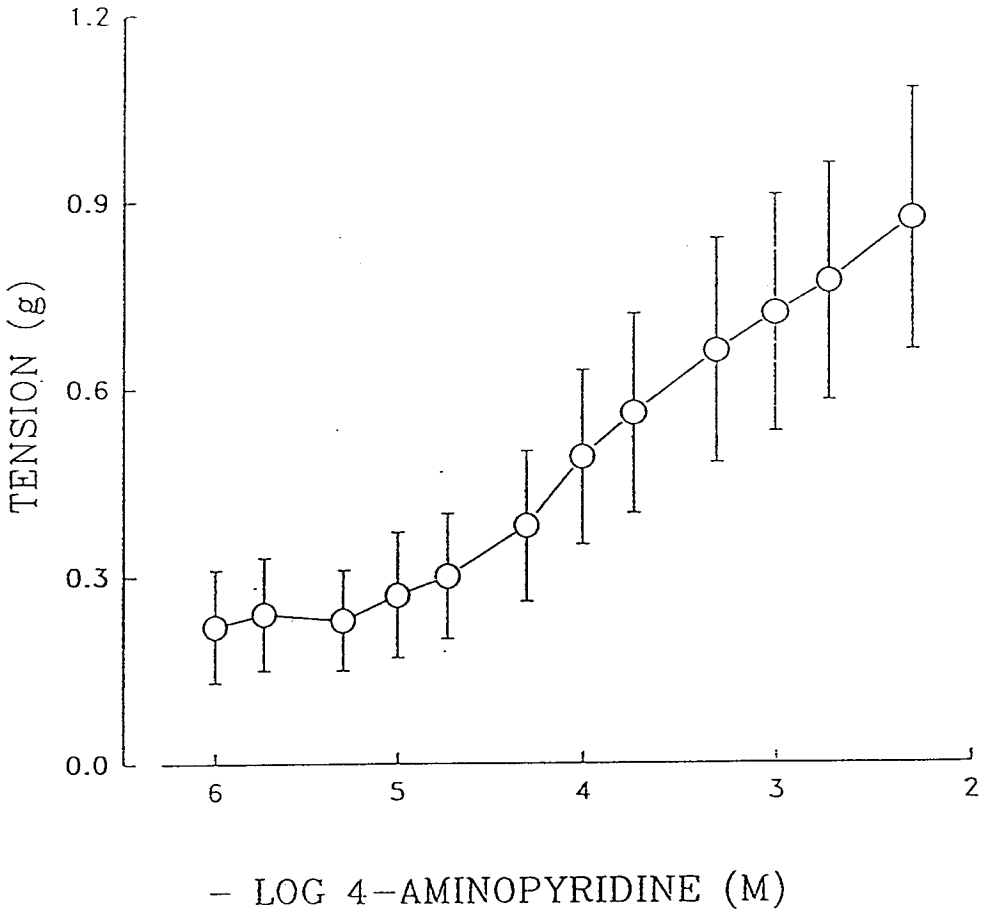

1.2
0.9
0.6
0.3
0.0
TENSION (g)
6
5
4
3
2
– LOG 4–AMINOPYRIDINE (M)

Fig. 5: Effects of 4-aminopyridine on negative inotropic responses to carbachol in the absence and presence of phenylephrine or isoproterenol in electrically-stimulated rabbit left atrial strips.

Left atrial strips were treated with normal saline (○), or 50 (△) or 500 μM (▽) 4-aminopyridine either alone (A) or in combination with 100 μM phenylephrine plus 1 μM timolol (B) or 100 nM isoproterenol (C) prior to obtaining cumulative concentration-response curves to carbachol. Tissues were treated with 4-aminopyridine for 10 min, with phenylephrine or isoproterenol being added for the final 3 min. Data are expressed as the the mean ± S.E.M. of developed tension in grams. B represents the basal developed tension prior to addition of any drug. In panel A, the contractile force of atria treated with saline, 50 and 500 μM 4-aminopyridine was 0.9 ± 0.2 (n=8), 0.8 ± 0.2 (n=8), and 1.2 ± 0.1 g (n=8), respectively. In panels B and C, P and I represent the phenylephrine- and isoproterenol- induced increases in force of contraction in the presence of saline or 4-aminopyridine. In panel B, the tension in the presence of phenylephrine alone and in combination with 50 and 500 μM 4-aminopyridine was 0.7 ± 0.1 g (n=9), 0.7 ± 0.1 g (n=9) and 1.2 ± 0.1 g (n=9) respectively. In panel C, the positive inotropic response to isoproterenol alone and in combination with 50 and 500 μM 4-aminopyridine was 1.1 ± 0.1 g (n=6) , 1 ± 0.1 (n=8) and 1.7 ± 0.2 g (n=6) respectively.

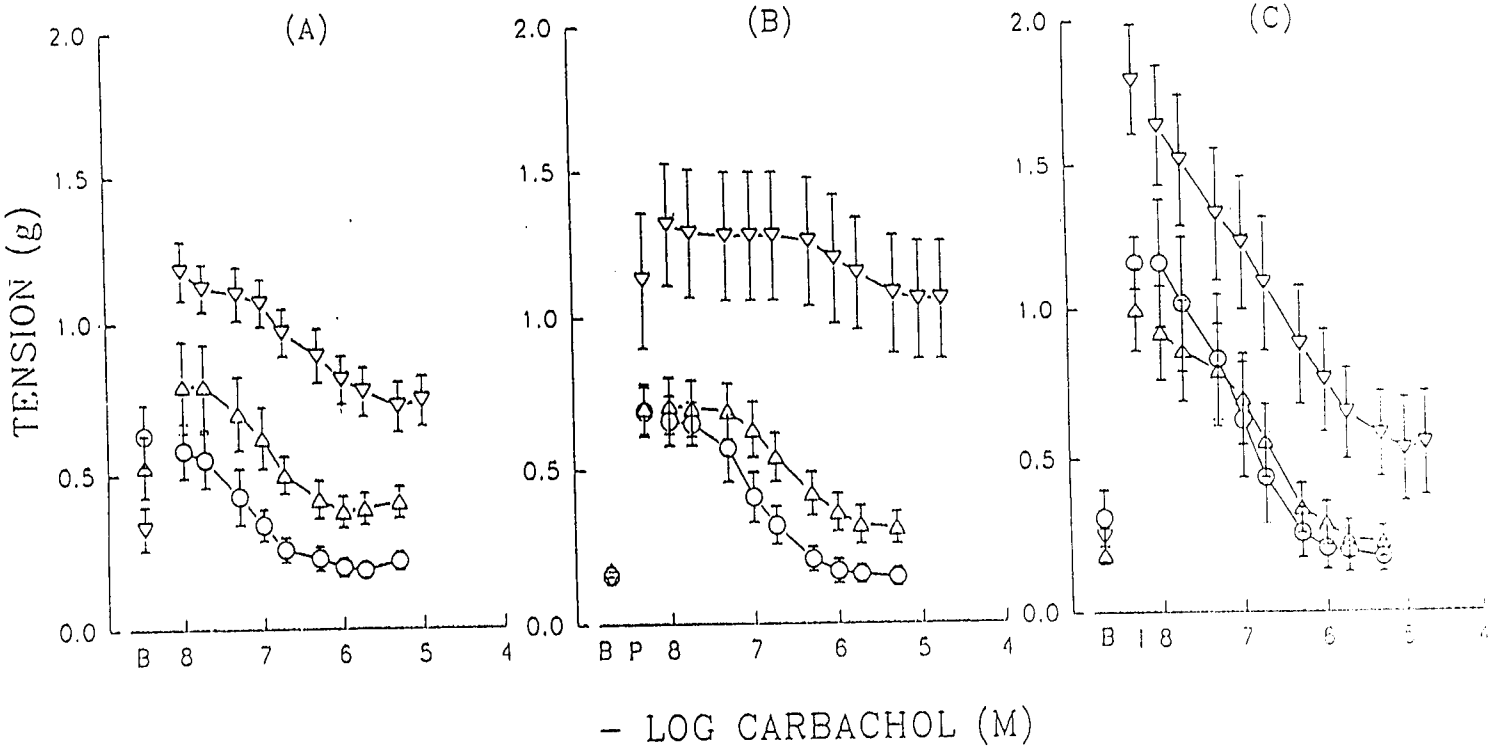

(A)
(B)
(C)
TENSION (g)
2.0
1.5
1.0
0.5
0.0
B 8 7 6 5 4
B P 8 7 6 5 4
B I 8 7 6
– LOG CARBACHOL (M)

Fig. 6: Effect of pinacidil on the phenylephrine-induced positive inotropic response in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to phenylephrine were obtained after treating left atrial strips with DMSO (○) (n=9) or 100 μM (●) (n=11), 300 μM (△) (n=6) or 1 mM (▲) (n=5) pinacidil. Atrial strips were exposed to DMSO or pinacidil for 10 min. Data are expressed as the mean ± S.E.M. of the developed tension in grams (g).

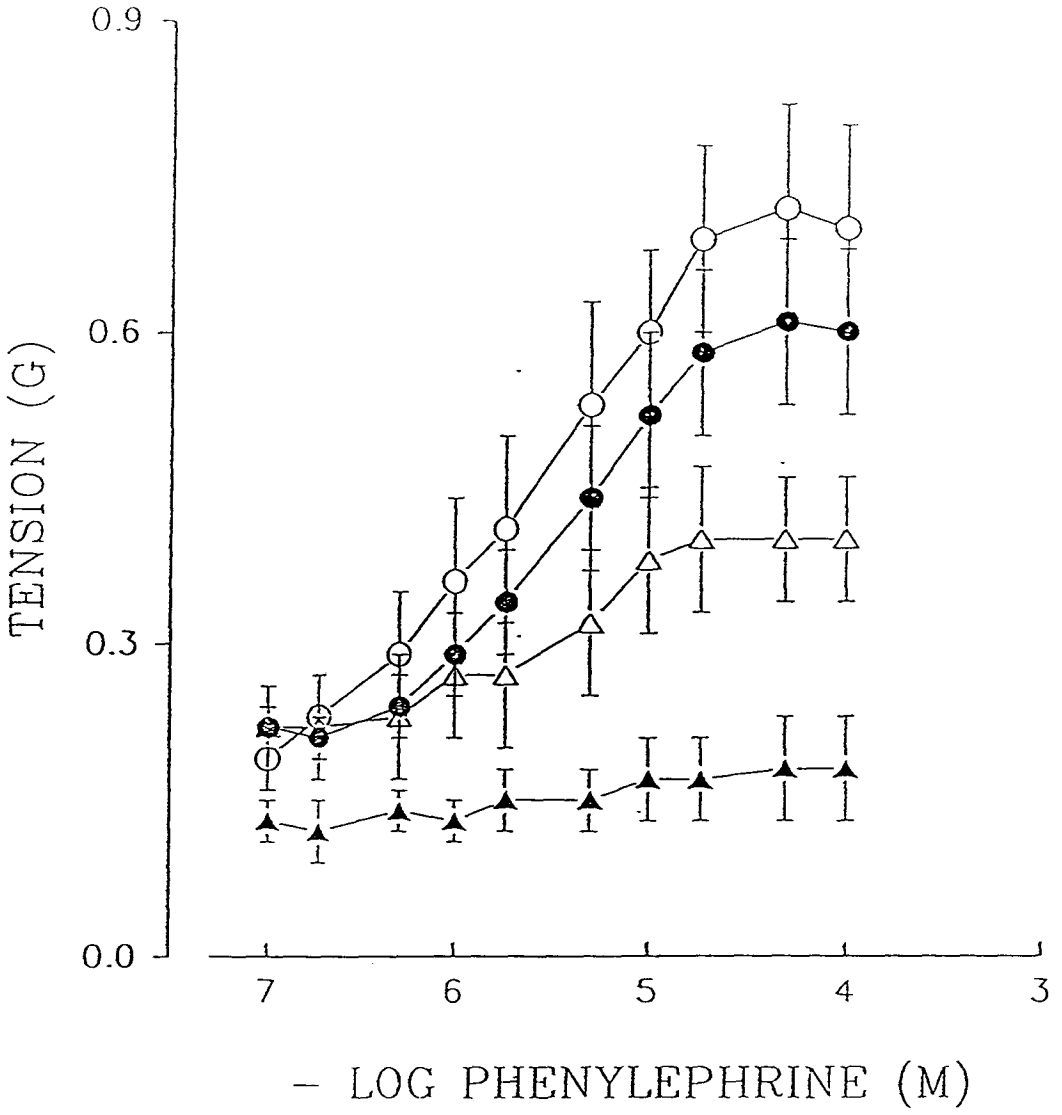

0.9
0.6
0.3
0.0
TENSION (G)
7
6
5
4
3
– LOG PHENYLEPHRINE (M)

Fig. 7: Effect of cromakalim on the phenylephrine-induced positive inotropic response in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to phenylephrine were obtained after treating left atrial strips with DMSO (○) (n=9) or 300 μM (△) (n=6) and 1 mM (▲) (n=6) cromakalim. Atrial strips were exposed to DMSO or cromakalim for 10 min. Data are expressed as the mean ± S.E.M. of attained tension in grams.

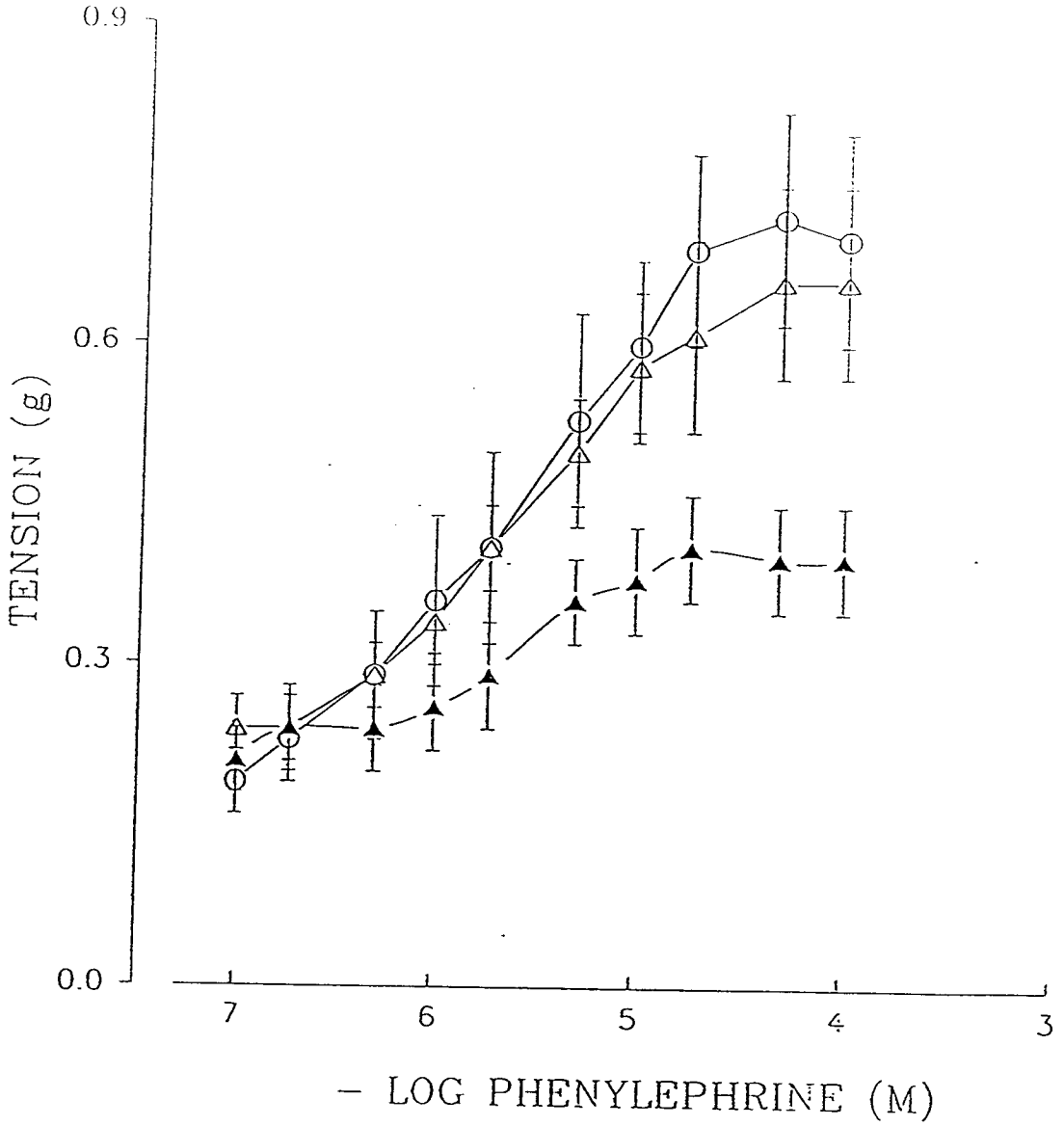

0.9
0.6
0.3
0.0
TENSION (g)
7
6
5
4
3
– LOG PHENYLEPHRINE (M)

Fig. 8: Effects of pinacidil and cromakalim on the isoproterenol-induced positive inotropic response in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to isoproterenol were obtained after treating left atrial strips with DMSO (○) (n=7), 1 mM pinacidil (△) (n=5) or 1 mM cromakalim (●) (n=6). Atrial strips were exposed to DMSO, pinacidil or cromakalim for 10 min. Data are expressed as the mean ± S.E.M. of the developed tension in grams.

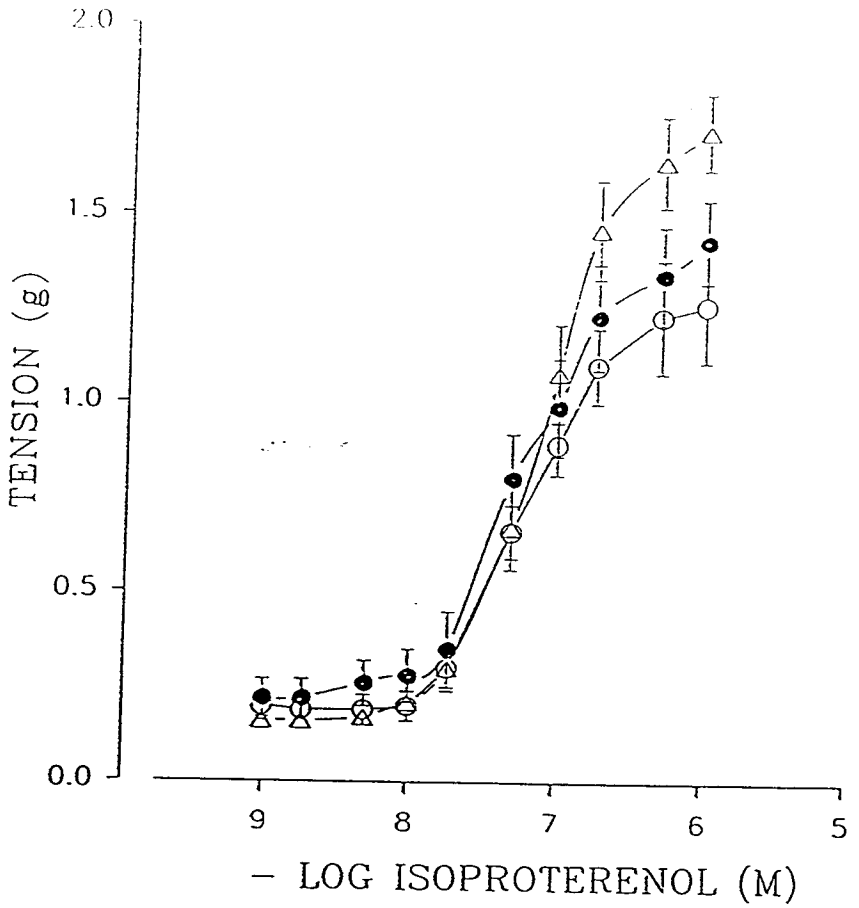

2.0
1.5
1.0
0.5
0.0
TENSION (g)
9
8
7
6
5
– LOG ISOPROTERENOL (M)

Fig. 9: Time-dependence of the ^{86} Rb efflux rate constant in electrically-stimulated rabbit left atrial strips.

Efflux of ^{86} Rb was monitored from pre-loaded and electrically-stimulated rabbit left atrial strips every 2 min for 60 min. Data are expressed as the efflux rate constant. Each data point represents the mean of 3 experiments.

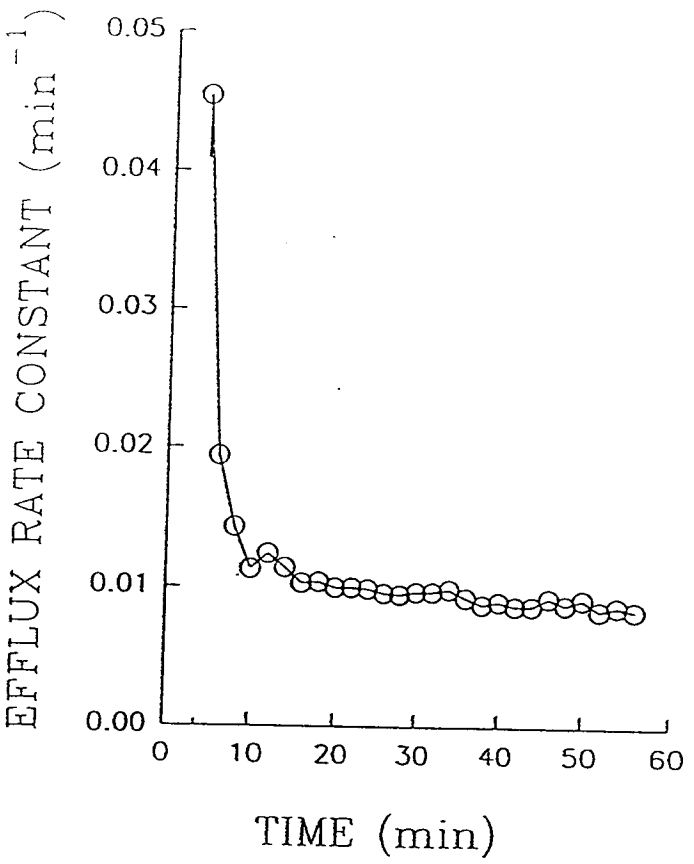

EFFLUX RATE CONSTANT (min^{-1})
0.05
0.04
0.03
0.02
0.01
0.00
0
10
20
30
40
50
60
TIME (min)

Fig. 10: Effects of carbachol on the rate constant of ^{86} Rb efflux and the force of contraction in electrically-stimulated rabbit left atria.

Concentration-response effects of carbachol on the rate constant of ^{86} Rb efflux (○) and force of contraction (●) were determined in left atrial strips. Atrial strips were exposed to each concentration of carbachol dissolved in 17 ml of M.C.K. buffer for 8 min during which time the tissue was washed every 2 min with non-radioactive M.C.K. solution containing the drug and the force of contraction of the tissue was also monitored. In order for the contractile response of the atrial strip to stabilize to the pre-drug treatment level, the atrial strip was equilibrated for 40 min in 17 ml of the non-radioactive M.C.K. solution which was changed and collected for counting every 20 min. After the 40 min washout period, the tissue was again washed every 2 min with the non-radioactive M.C.K. solution for another 10 min, followed by exposure to another concentration of carbachol as described above. Data are expressed as percent of the initial efflux or force of contraction immediately prior to addition of each concentration of carbachol. Each curve represents the mean ± S.E.M. of 5 experiments.

% INITIAL EFFLUX (○) OR
FORCE OF CONTRACTION (●)
140
120
100
80
60
40
20
0
8
7
6
5
4
3
– LOG CARBACHOL (M)

Fig 11: Effect of atropine on the rate constant of ^{86}Rb efflux in the presence of carbachol in electrically-stimulated rabbit left atrial strips.

The rate constant of ^{86}Rb efflux was monitored in the presence of 10 μM carbachol (A) alone, 10 μM carbachol plus 100 nM atropine (B) or 100 nM atropine alone (C). Atrial strips were exposed to atropine or carbachol alone for 8 min each. When atropine was combined with carbachol, tissues were exposed to atropine for 16 min, with carbachol being added for the final 8 min. Data are expressed as percent of the initial efflux-rate-constant prior to addition of any drug. The initial efflux-rate-constants prior to addition of carbachol (10 μM), atropine (100 nM) and atropine plus carbachol were 0.0078 ± 0.0006, 0.0094 ± 0.0006 and 0.0081 ± 0.0008 per min respectively. Data were analyzed by paired t-test. Each bar represents the mean ± S.E.M. of 4 experiments. (*) Represents significantly different from carbachol alone.

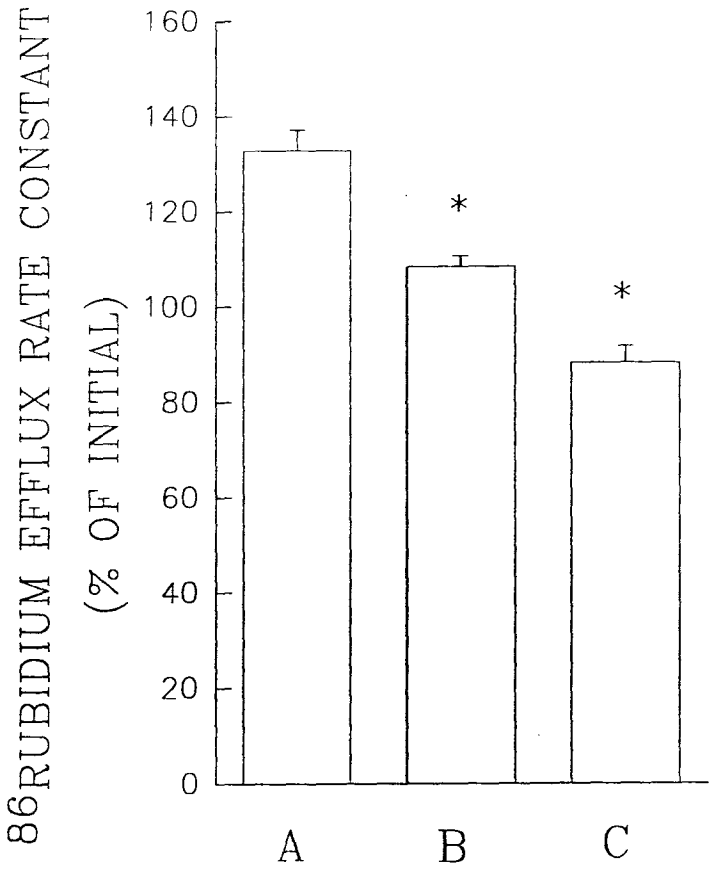

86RUBIDIUM EFFLUX RATE CONSTANT
(% OF INITIAL)
160
140
120
100
80
60
40
20
0
*
*
A
B
C

Figure 12: Effects of 4-aminopyridine on the negative inotropic response and the rate constant of ^{86}Rb efflux in the presence of carbachol in electrically-stimulated rabbit left atrial strips.

Effects of carbachol were monitored on the contractile force (A) and the rate constant of ^{86}Rb efflux (B) in electrically-stimulated rabbit left atrial strips in the absence (CON) and presence of 4-aminopyridine (4-AP, 50 and 500 μM). Open bars represent the basal response prior to addition of drug, cross-hatched bars represent the 4-aminopyridine response, and hatched bars represent the response to carbachol (10 μM). Atrial strips were exposed to carbachol for 8 min. When carbachol was combined with 4-aminopyridine, atrial strips were exposed to 4-aminopyridine for 16 min, with carbachol being added for the final 8 min. Contractile response is expressed as the attained tension in grams and efflux is expressed as the efflux rate constant. Each bar represents the mean ± S.E.M. of 6-8 experiments. (*) Represents significantly different from the corresponding response immediately prior to addition of 4-aminopyridine and carbachol ($P<0.05$, paired t-test). (+) Represents significantly different from carbachol response in the control group ($P<0.05$, t-test).

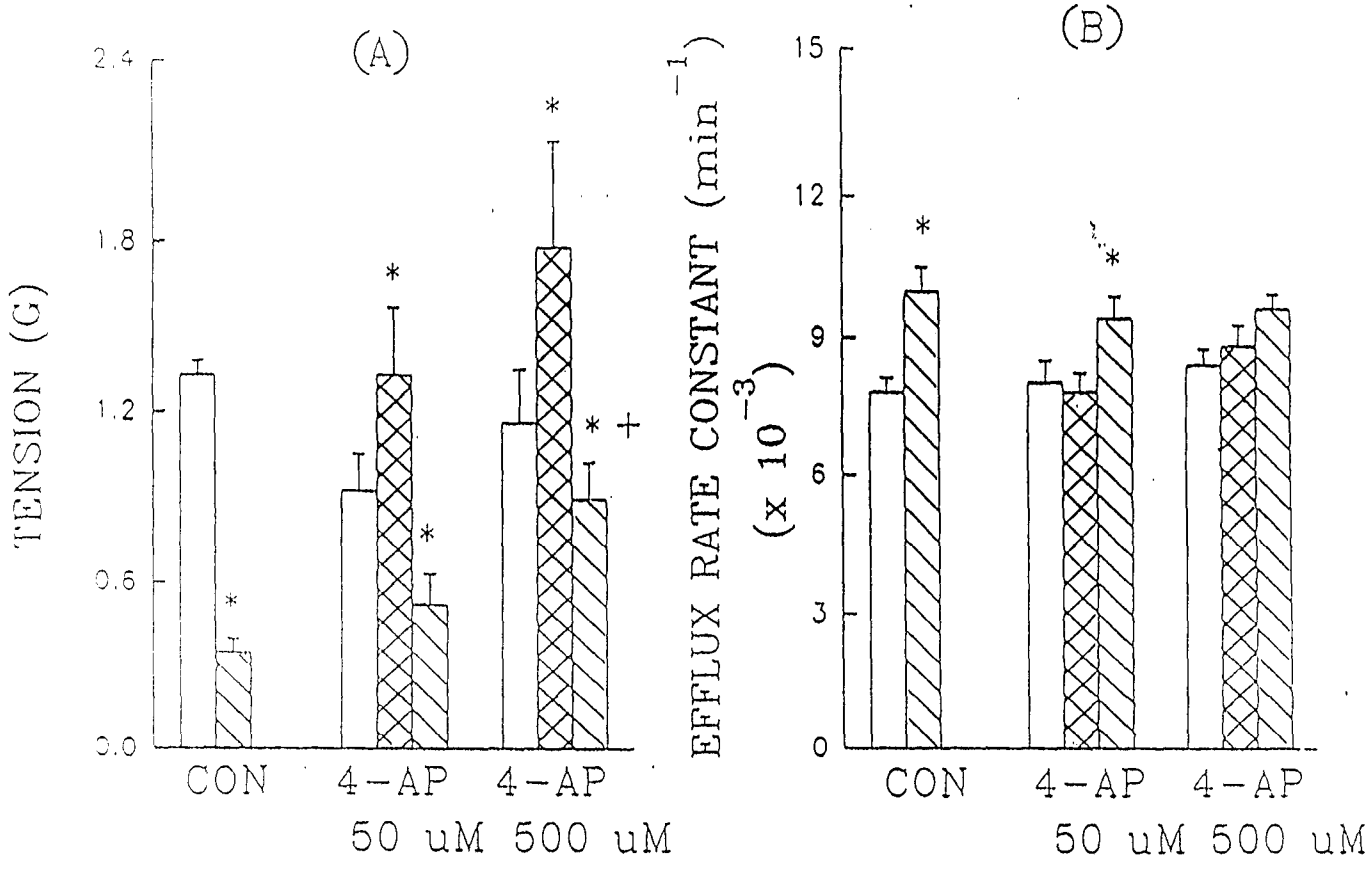
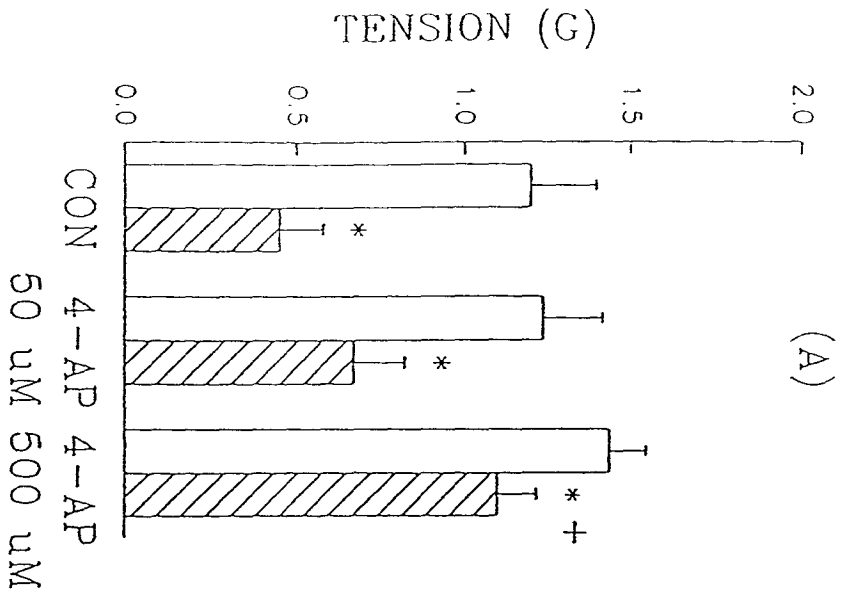

(A)
TENSION (G)
2.4
1.8
1.2
0.6
0.0
CON
4-AP
4-AP
50 uM 500 uM
(B)
EFFLUX RATE CONSTANT (min^{-1})
(x 10^{-3})
15
12
9
6
3
0
CON
4-AP
4-AP
50 uM 500 uM

Fig 13: Effects of 4-aminopyridine on the rate constant of ^{86}Rb efflux and the negative inotropic response to carbachol in the presence of phenylephrine in electrically-stimulated rabbit left atria.

Effects of carbachol were monitored in the presence of phenylephrine on the contractile force (A) and the rate constant of ^{86}Rb efflux (B) in electrically-stimulated rabbit left atrial strips. Open bars represent the response to phenylephrine (100 μM) alone and hatched bars represent the response to carbachol (10 μM) in combination with phenylephrine, in the absence (CON) and presence of 50 and 500 μM 4-aminopyridine (4-AP 50 and 500 μM). Tissues were treated with 4-aminopyridine for 24 min, and phenylephrine for 16 min, with carbachol being added for the final 8 min. Contractile response is expressed as the attained tension in grams and efflux is expressed as the efflux rate constant. Each bar represents the mean ± S.E.M. of 10-11 experiments. (*) Represents significantly different from the corresponding response immediately prior to addition of carbachol ($P<0.05$, paired t-test). (+) Represents significantly different from carbachol response in the control group ($P<0.05$, t-test).


TENSION (G)
0.0
0.5
1.0
1.5
2.0
CON
4-AP
50 uM
500 uM
4-AP
*
*
*
+
(A)

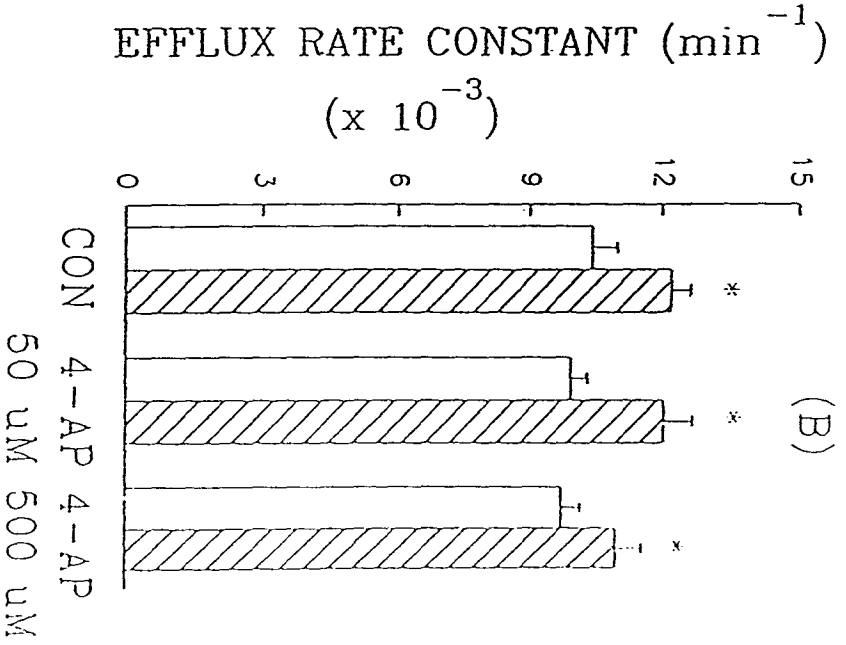

EFFLUX RATE CONSTANT (min^{-1})
(x 10^{-3})
0
3
6
9
12
15
CON
4-AP
50 uM
500 uM
4-AP
*
*
*
(B)

Fig 14: Effects of pre-treatment of rabbits with pertussis toxin on the rate constant of ^{86}Rb efflux and the negative inotropic response to carbachol in electrically-stimulated rabbit left atria.

Effects of carbachol were monitored on the contractile force (A) and the rate constant of ^{86}Rb efflux (B) in left atrial strips from saline (○), 0.5 (●) or 1 μg/kg (▽) pertussis toxin pre-treated rabbits. Atrial strips were exposed to a concentration of carbachol dissolved in 17 ml of M.C.K. solution for 8 min during which the tissue was washed every 2 min with non-radioactive M.C.K. buffer containing the drug and the force of contraction of the tissue was also monitored. In order for the contractile response of the atrial strip to stabilize to the pre-drug treatment level, the atrial strip was equilibrated for 40 min in 17 ml of the non-radioactive M.C.K. buffer which was changed and collected for counting every 20 min. After the 40 min washout period, the tissue was again washed every 2 min with the non-radioactive M.C.K. solution for another 10 min, followed by exposure to another concentration of carbachol as described above. Data are expressed as percent of the initial efflux or force of contraction immediately prior to addition of each concentration of carbachol. Each curve represents the mean ± S.E.M. of 4 - 5 experiments.

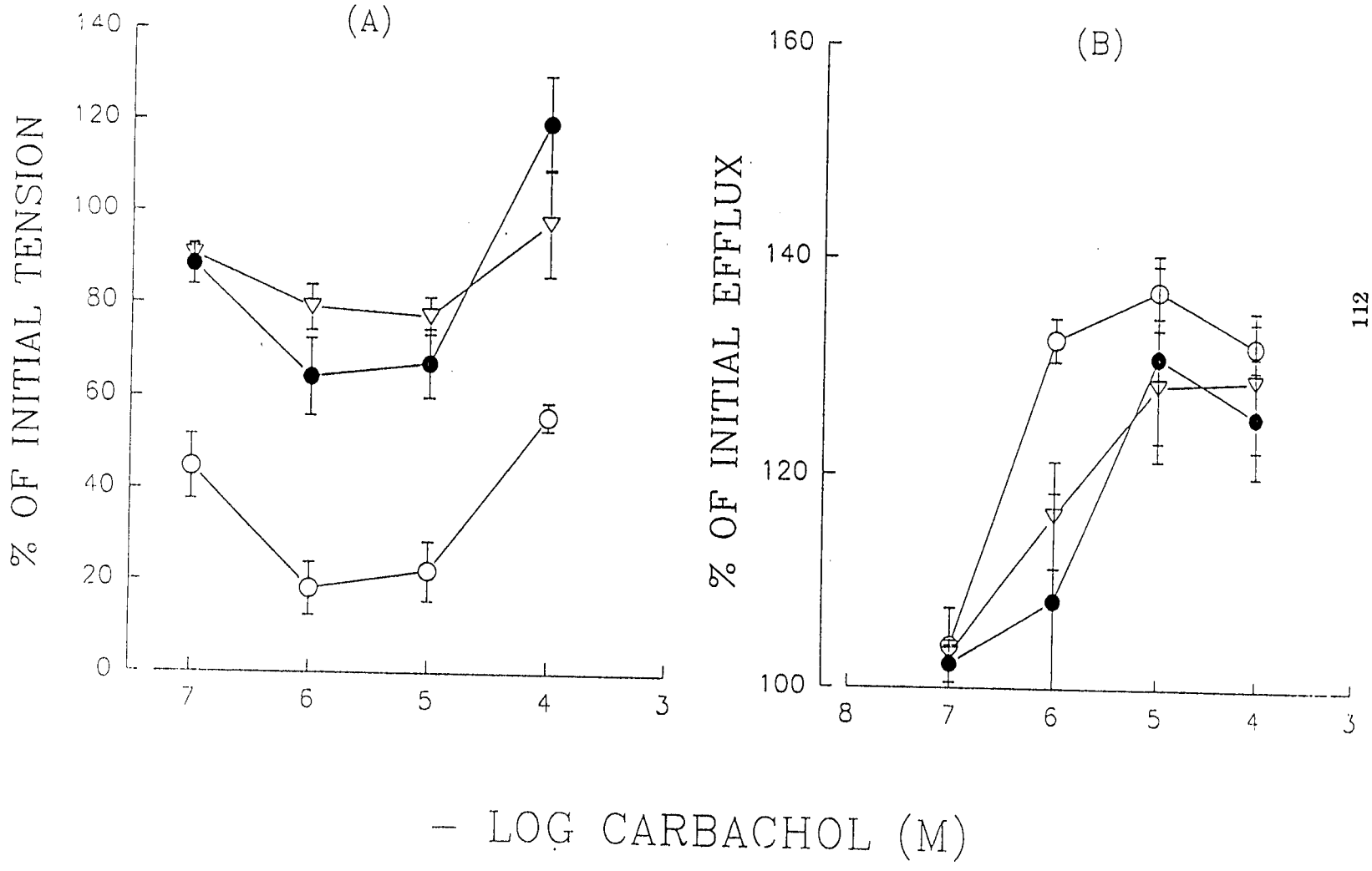

(A)
% OF INITIAL TENSION
140
120
100
80
60
40
20
0
7
6
5
4
3
(B)
% OF INITIAL EFFLUX
160
140
120
100
8
7
6
5
4
3
– LOG CARBACHOL (M)

Fig. 15: Effects of pre-treatment of rabbits with pertussis toxin on carbachol-induced changes in the contractile force and the rate constant of ^{86}Rb efflux in the presence of phenylephrine in electrically-stimulated rabbit left atrial strips.

Force of contraction (A) and the rate constant of ^{86}Rb efflux (B) were monitored in left atrial strips from saline (S), and pertussis toxin 0.5 (P 0.5) and 1 μg/kg (P 1.0) pre-treated rabbits in the presence of 100 μM phenylephrine alone (open bars) or with 10 μM carbachol in combination with phenylephrine (hatched bars). Atria were treated with phenylephrine for 16 min, with carbachol being added for the final 8 min. Contractile response is expressed as the attained tension in grams and rubidium efflux is expressed as the efflux rate constant. Each bar represents the mean ± S.E.M. of 4-6 experiments. (*) Represents significantly different from the response immediately prior to addition of carbachol ($P<0.05$, paired t-test).

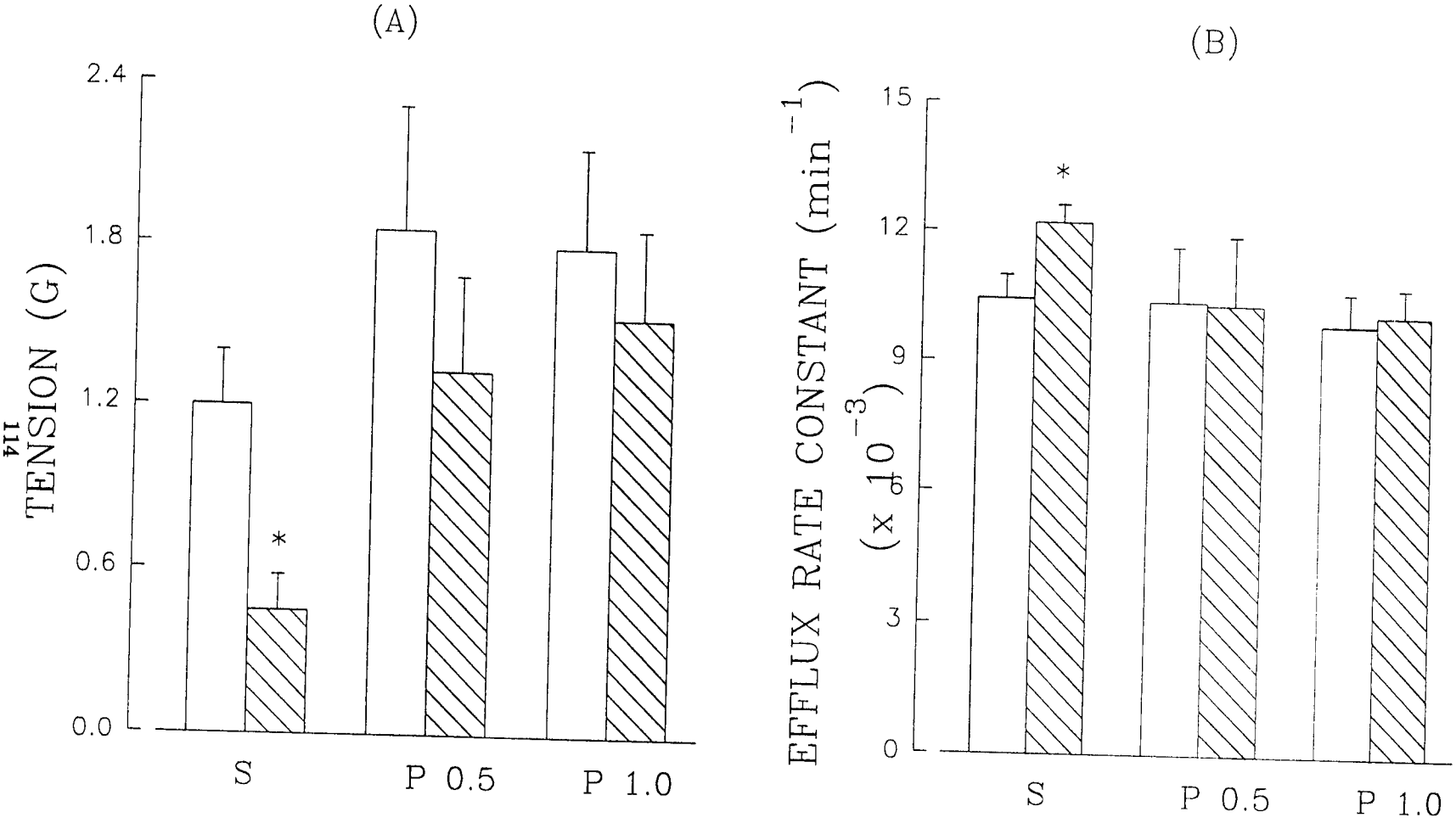
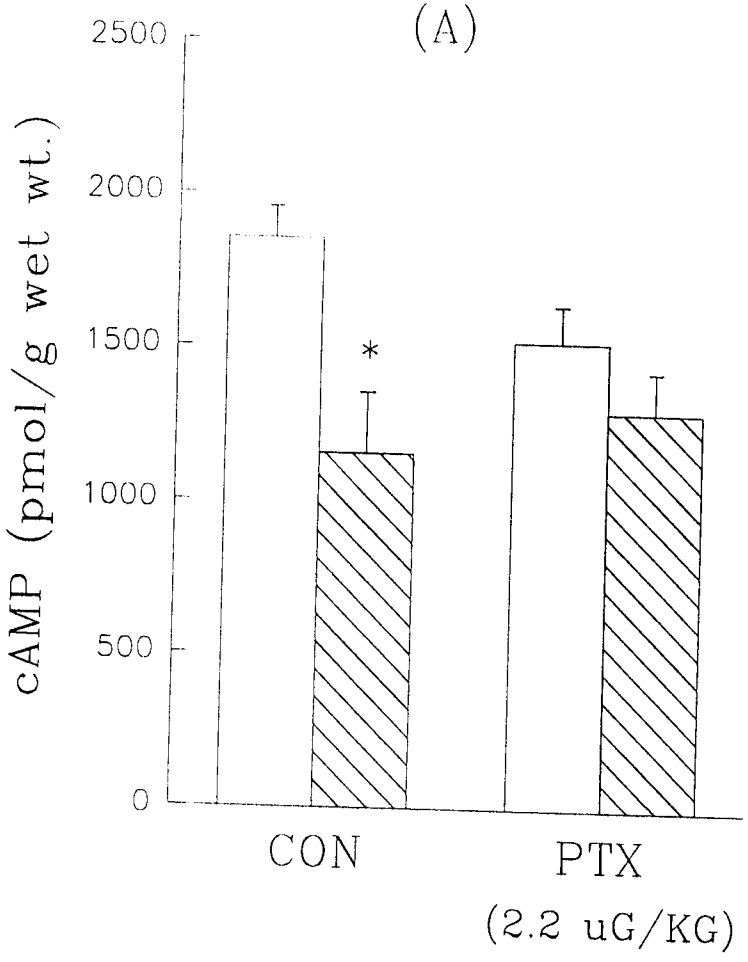

(A)
TENSION (G)
2.4
1.8
1.2
0.6
0.0
*
S
P 0.5
P 1.0
(B)
EFFLUX RATE CONSTANT (min^{-1})
(x 10^{-3})
15
12
9
6
3
0
*
S
P 0.5
P 1.0

Fig. 16: Effects of 4-aminopyridine and pertussis toxin on cAMP levels in electrically-stimulated left atrial strips from saline and pertussis toxin pre-treated rabbits in the presence of isoproterenol alone and in combination with carbachol.

(A). Atrial strips from saline (CON) and 2.2 μg/kg pertussis toxin pre-treated (PTX) rabbits were exposed to 100 nM isoproterenol alone for 6 min, or to 100 nM isoproterenol for 6 min with 3 μM carbachol being added for the final 3 min. (B) Atrial strips were exposed to 500 μM 4-aminopyridine (4-AP) or the M.C.K. solution (CON) for 10 min. Atria were then treated with isoproterenol and carbachol as described in part A. Data are expressed as pmol cAMP / g wet weight. Open bars represent the isoproterenol response and hatched bars represent responses in the presence of isoproterenol in combination with carbachol. Each bar represents the mean ± S.E.M. of 4 - 6 experiments. (*) Represents significantly different from isoproterenol alone.


(A)
2500
2000
1500
1000
500
0
cAMP (pmol/g wet wt.)
*
CON
PTX
(2.2 uG/KG)

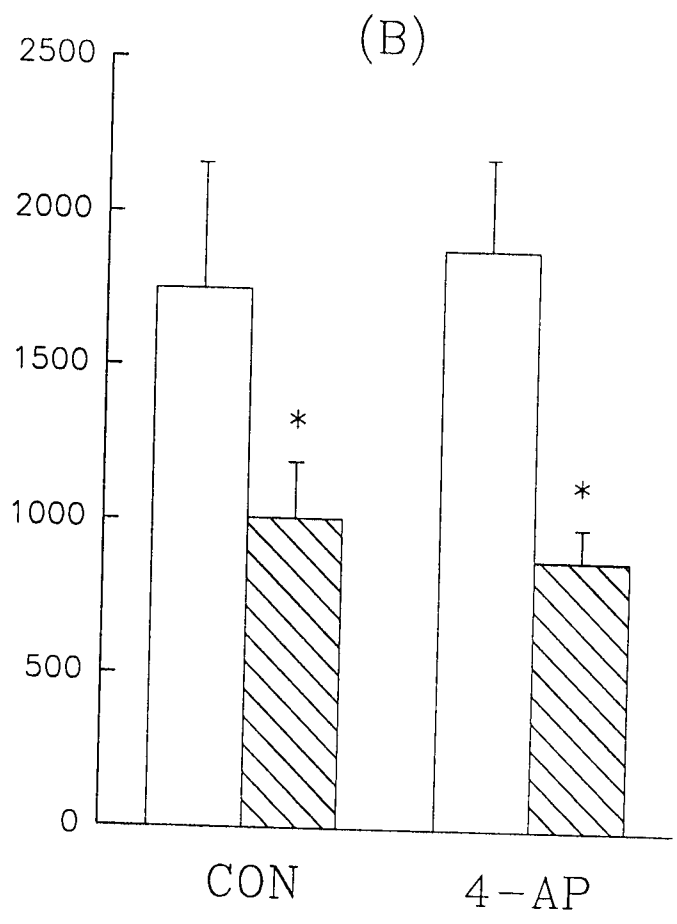

(B)
2500
2000
1500
1000
500
0
*
*
CON
4-AP

Fig. 17: Effects of carbachol on isoproterenol-stimulated adenylate cyclase activity in atrial homogenates from saline and pertussis toxin pre-treated rabbits.

Adenylate cyclase activity was measured in atrial homogenates from saline (CON) and 2.2 μg/kg pertussis toxin (PTX) pre-treated rabbits in the presence of isoproterenol (100 μM) alone and in combination with carbachol (1 mM). Open bars represent the isoproterenol response and hatched bars represent isoproterenol in combination with carbachol. Data are expressed as change from the basal adenylate cyclase activity which was 115.4 ± 18.5 (n=6) in saline and 139.4 ± 7 (n=3) in pertussis toxin pre-treated rabbits Each bar represents the mean ± S.E.M. (*) Represents significantly different from isoproterenol alone.

CHANGE FROM BASAL ACTIVITY
(pmol/min/mg)
45
30
15
0
*
CON
PTX
(2.2 ug/kg)

Fig. 18: Effects of 4-aminopyridine and pertussis toxin, alone and in combination, on negative inotropic responses to carbachol in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to carbachol were obtained in the absence (○) and presence of 4-aminopyridine 50 (▽) or 500 μM (△) in left atrial strips from saline (A) and pertussis toxin (B) pre-treated rabbits. Atrial strips were exposed to 4-aminopyridine for 10 min before carbachol concentration-response curves were obtained. B represents the basal developed tension prior to addition of any drug. In panel A, the contractile force of atria treated with normal saline, 50 and 500 μM 4-aminopyridine was 0.8 ± 0.2 g (n=9), 1.0 ± 0.1 g (n=14) and 1.3 ± 0.1 g (n=15). In panel B, the contractile response of atria treated with saline, 50 and 500 mM 4-aminopyridine was 0.6 ± 0.1 g (n=7), 1.1 ± 0.2 (n=7) and 1.4 ± 0.3 (n=7) respectively. Data are expressed as the developed tension in grams. Each data point represents the mean ± S.E.M.

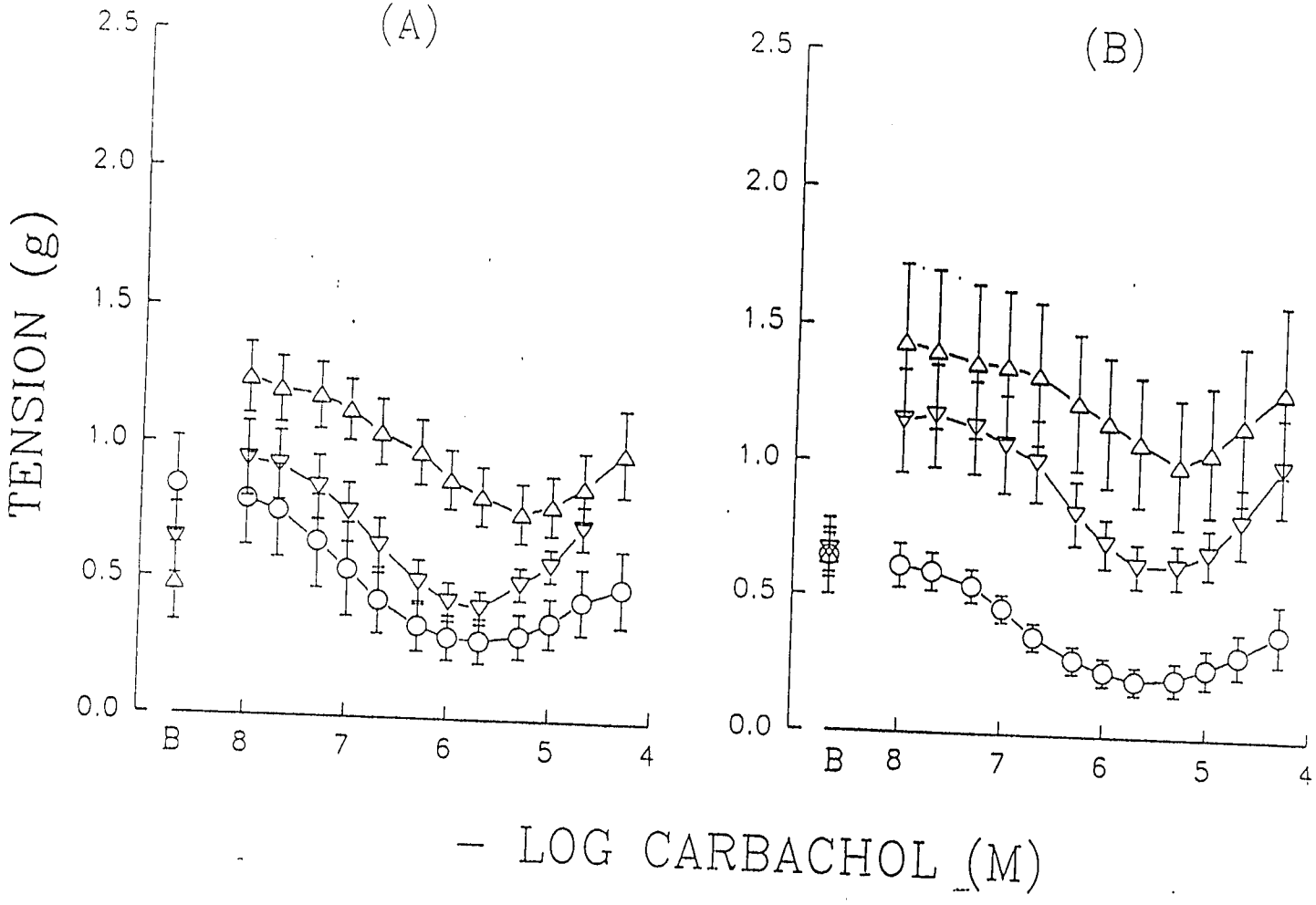

(A)
(B)
TENSION (g)
2.5
2.0
1.5
1.0
0.5
0.0
B 8 7 6 5 4
– LOG CARBACHOL (M)

Fig. 19: Effects of 4-aminopyridine and pertussis toxin, alone and in combination, on negative inotropic responses to carbachol in the presence of isoproterenol in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to carbachol were obtained in the presence of 100 nM isoproterenol alone (○) and in combination with 50 (▽) or 500 μM (△) 4-aminopyridine in left atrial strips from saline (A) and pertussis toxin (B) pre-treated rabbits. Atrial strips were exposed to 4-aminopyridine for 10 min with isoproterenol being added for the final 3 min. Data are expressed as the developed tension in grams. Each curve represents the mean ± S.E.M. B represents the basal developed tension prior to addition of any drug. I represents the isoproterenol-induced increases in contractile response prior to addition of carbachol. In panel A, the positive inotropic response to isoproterenol in atria treated with normal saline, 50 and 500 μM 4-aminopyridine was 1.9 ± 0.4 (n=6), 1.8 ± 0.3 (n=7) and 2.1 ± 0.4 (n=6) respectively. In panel B, the contractile response to isoproterenol in atria treated with saline, 50 and 500 μM 4-aminopyridine was 2.0 ± 0.3 g (n=4), 2.3 ± 0.4 g (n=4) and 2.5 ± 0.2 g (n=5) respectively.

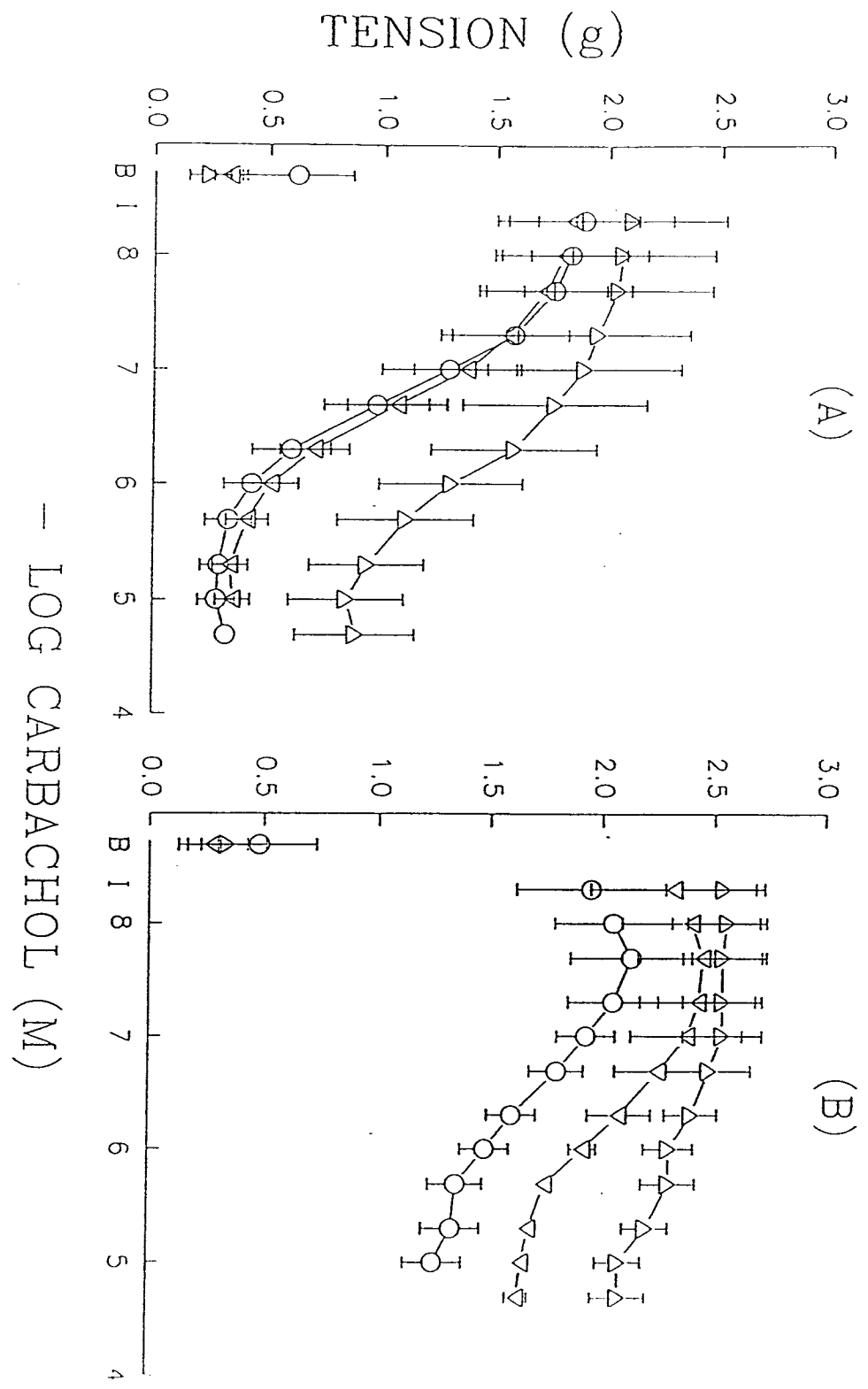

TENSION (g)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
B I 8
7
6
5
4
(A)
(B)
— LOG CARBACHOL (M)

Fig. 20: Effects of 4-aminopyridine and pertussis toxin, alone and in combination, on negative inotropic responses to carbachol in the presence of forskolin in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to carbachol were obtained in the presence of 3 μM forskolin alone (○) and in combination with 50 (▽) or 500 μM (△) 4-aminopyridine in atrial strips from saline (A) and pertussis toxin (B) pre-treated rabbits. Atrial strips were exposed to forskolin for 16 min with 4-aminopyridine being added for the final 10 min. Data are expressed as the developed tension in grams. Each curve represents the mean ± S.E.M. B represents the basal developed tension prior to addition of any drug. F represents the forskolin-induced increases in contractile response prior to addition of carbachol. In panel A, forskolin-induced positive inotropic response in atria treated with normal saline, 50 and 500 μM 4-aminopyridine was 1.2 ± 0.2 g (n=11), 1.7 ± 0.2 g (n=10) and 1.9 ± 0.2 g (n=9) respectively. In panel B, the contractile response of left atria to forskolin in the presence of normal saline, 50 and 500 μM 4-aminopyridine was 1.3 ± 0.2 (n=7), 1.6 ± 0.2 (n=7) and 1.7 ± 0.3 (n=6) respectively.

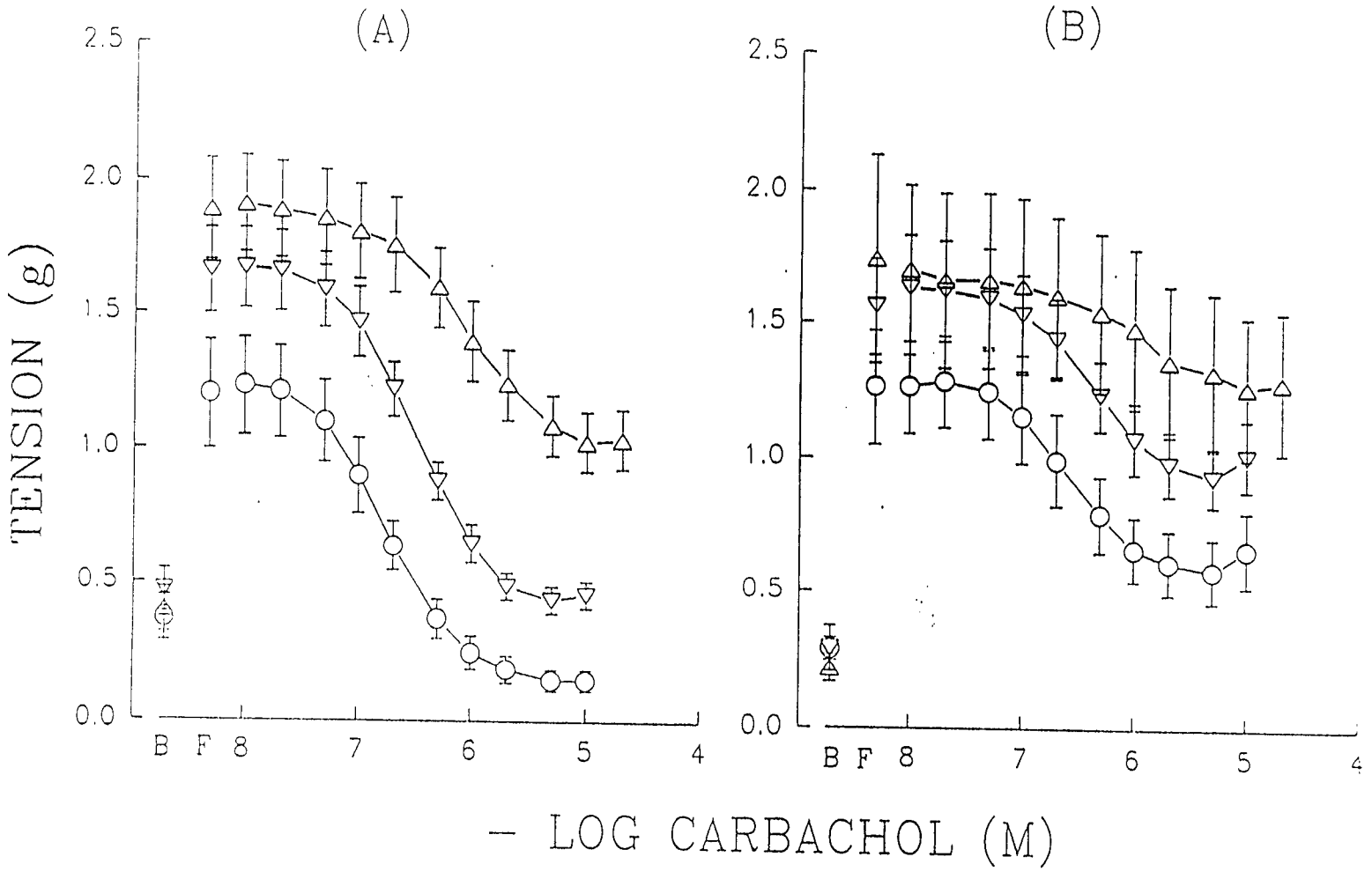

(A)
(B)
TENSION (g)
2.5
2.0
1.5
1.0
0.5
0.0
B F 8 7 6 5 4
– LOG CARBACHOL (M)

Fig. 21: Effects of 4-aminopyridine and pertussis toxin, alone and in combination, on negative inotropic responses to carbachol in the presence of IBMX in electrically-stimulated rabbit left atrial strips.

Cumulative concentration-response curves to carbachol were obtained in the presence of 50 μM IBMX alone (○) and in combination with 50 (▽) or 500 μM (△) 4-aminopyridine in atrial strips from saline (A) and pertussis toxin (B) pre-treated rabbits. Atrial strips were exposed to IBMX for 16 min with 4-aminopyridine being added for the final 10 min. Data are expressed as the developed tension in grams. Each curve represents the mean ± S.E.M. B represents the basal developed tension prior to addition of any drug. X represents the IBMX-induced increases in force of contraction prior to addition of carbachol. In panel A, the IBMX-induced positive inotropic response in atria treated with normal saline, 50 and 500 μM 4-aminopyridine was 1.1 ± 0.2 g (n=12) 1.0 ± 0.3 g (n=8) and 1.5 ± 0.1 g (n=10) respectively. In panel B, the positive inotropic response of atria to IBMX in the presence of saline, 50 and 500 μM 4-aminopyridine was 1.2 ± 0.2 g (n=7), 1.7 ± 0.1 g (n=7) and 1.9 ± 0.2 g (n=5) respectively.

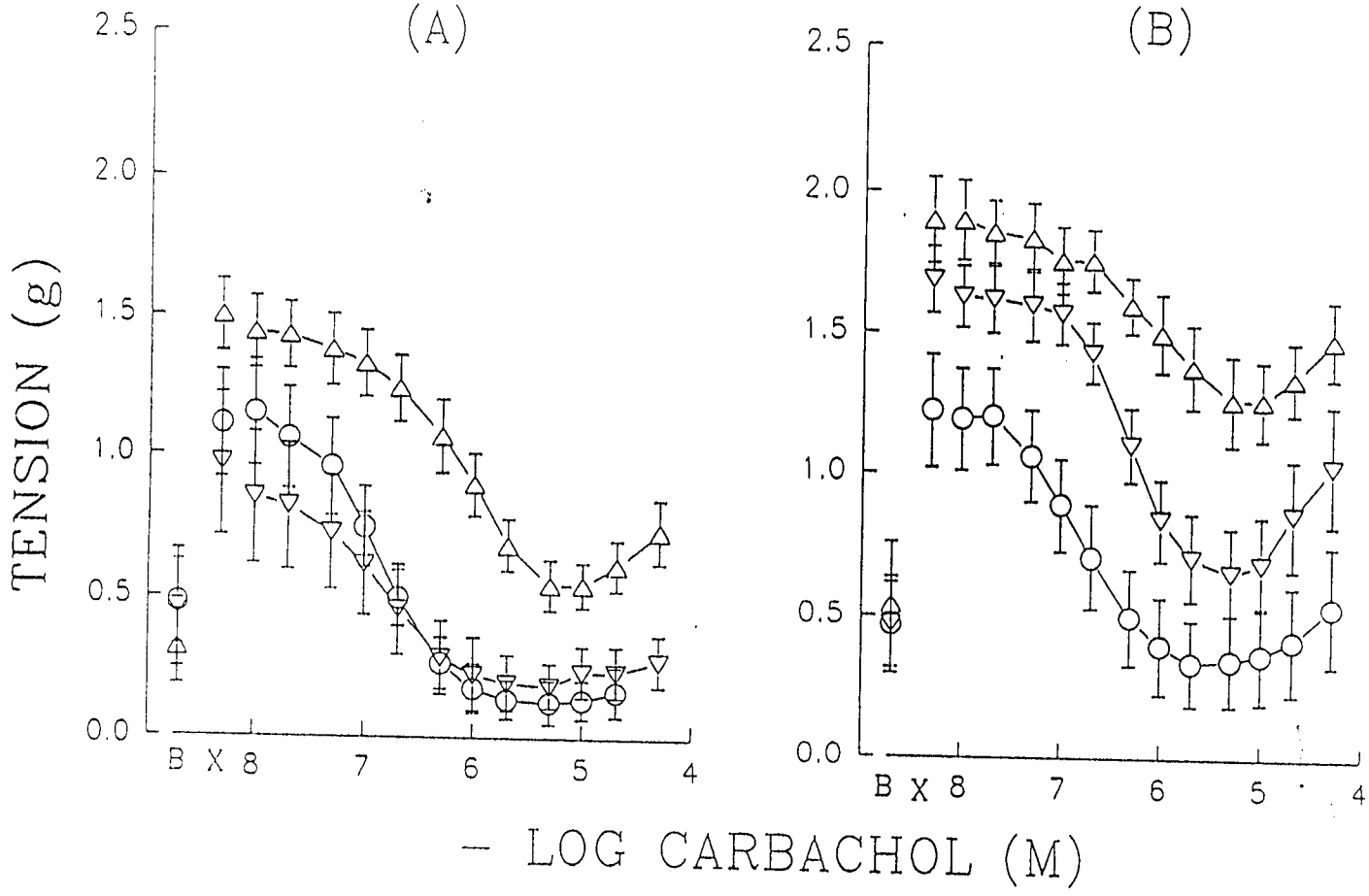

(A)
(B)
TENSION (g)
2.5
2.0
1.5
1.0
0.5
0.0
B X 8 7 6 5 4
– LOG CARBACHOL (M)

Fig. 22: Autoradiogram showing the ADP-ribosylating effect of exogenous pertussis toxin in rabbit atrial and ventricular homogenates.

Atrial and ventricular homogenates (50 - 70 μg protein) were incubated in the absence (-) or presence of increasing concentrations (10 - 100 μg/ml) of pertussis toxin . Reaction mixtures were subjected to gel electrophoresis followed by autoradiography. The numbers 97 - 31 indicate the position of proteins of known molecular weight (kDa) on the gel.

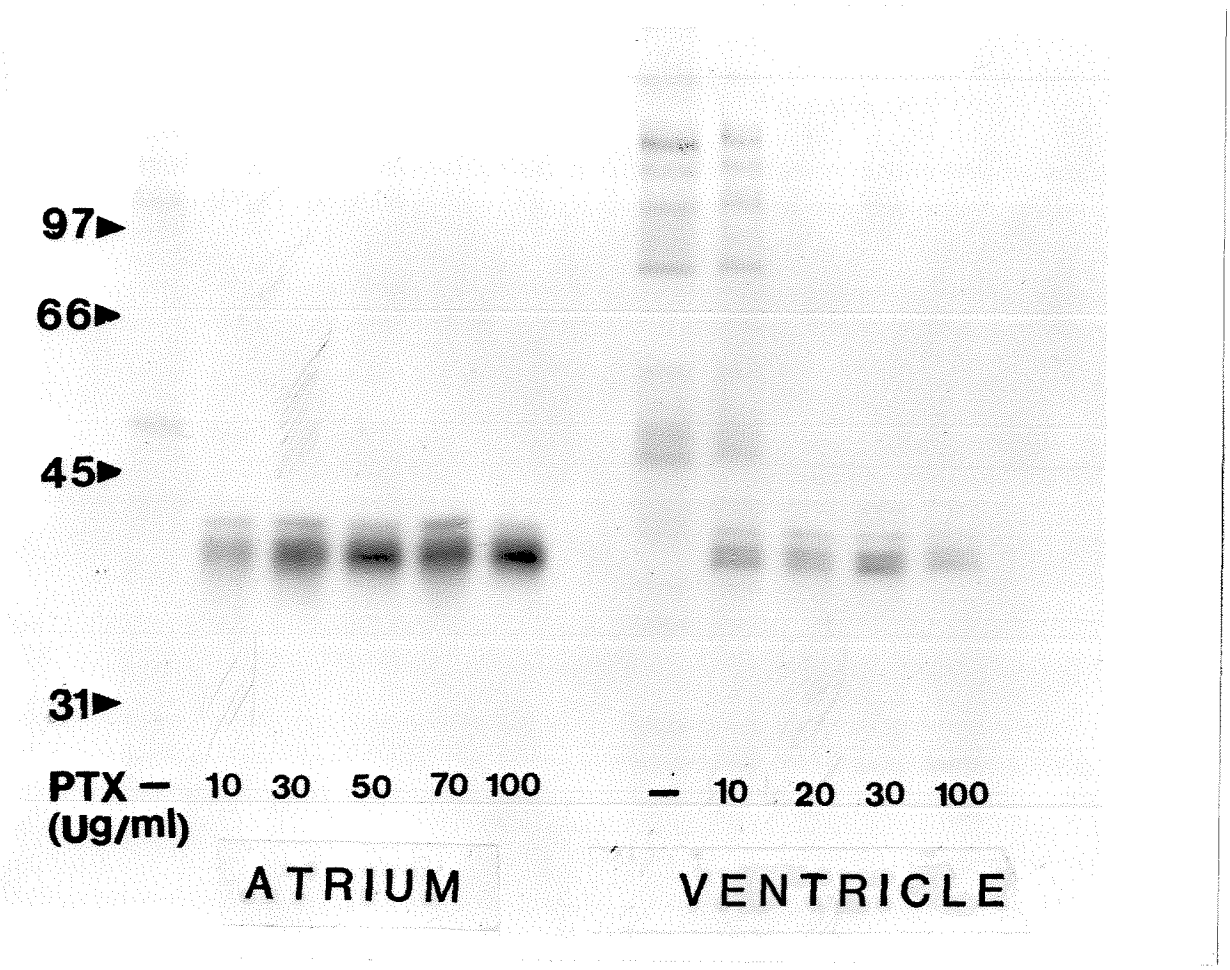

97►
66►
45►
31►
PTX — 10 30 50 70 100 — 10 20 30 100
(ug/ml)
ATRIUM
VENTRICLE

Fig. 23: Autoradiogram showing the effect of pre-treatment of rabbits with pertussis toxin on the ADP-ribosylation of G-proteins in atrial homogenates by exogenous pertussis toxin.

Atrial homogenates (50 - 70 μg protein) from rabbits pre-treated with normal saline (-) or 0.5, 1, 2 or 3 μg/kg pertussis toxin were incubated in duplicate in the absence (-) or presence of 30 μg/ml pertussis toxin. The reaction mixture was subjected to electrophoresis followed by autoradiography. The numbers 97 - 31 indicate the position of proteins of known molecular weight (kDa) on the gel.

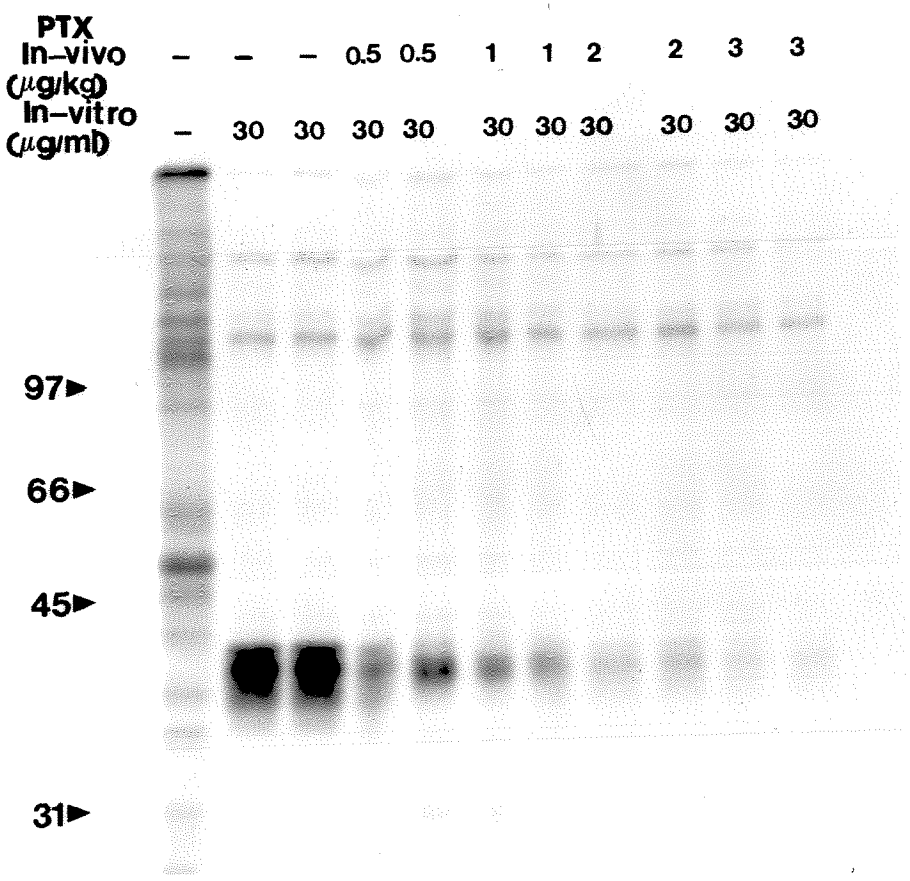

PTX
In-vivo
(μg/kg)
In-vitro
(μg/ml)
– – – 0.5 0.5 1 1 2 2 3 3
– 30 30 30 30 30 30 30 30 30 30
97►
66►
45►
31►

Fig. 24: Autoradiogram showing the effect of pre-treatment of rabbits with pertussis toxin on the ADP-ribosylation of G-proteins in ventricular homogenates by exogenous pertussis toxin.

Ventricular homogenates (50 - 70 μg protein) from rabbits pre-treated with normal saline (-) or 0.5, 1, 2 or 3 μg/kg pertussis toxin were incubated in duplicate in the absence (-) or presence of 30 μg/ml pertussis toxin. The numbers 97 - 31 indicate the position of proteins of known molecular weight (kDa) on the gel.

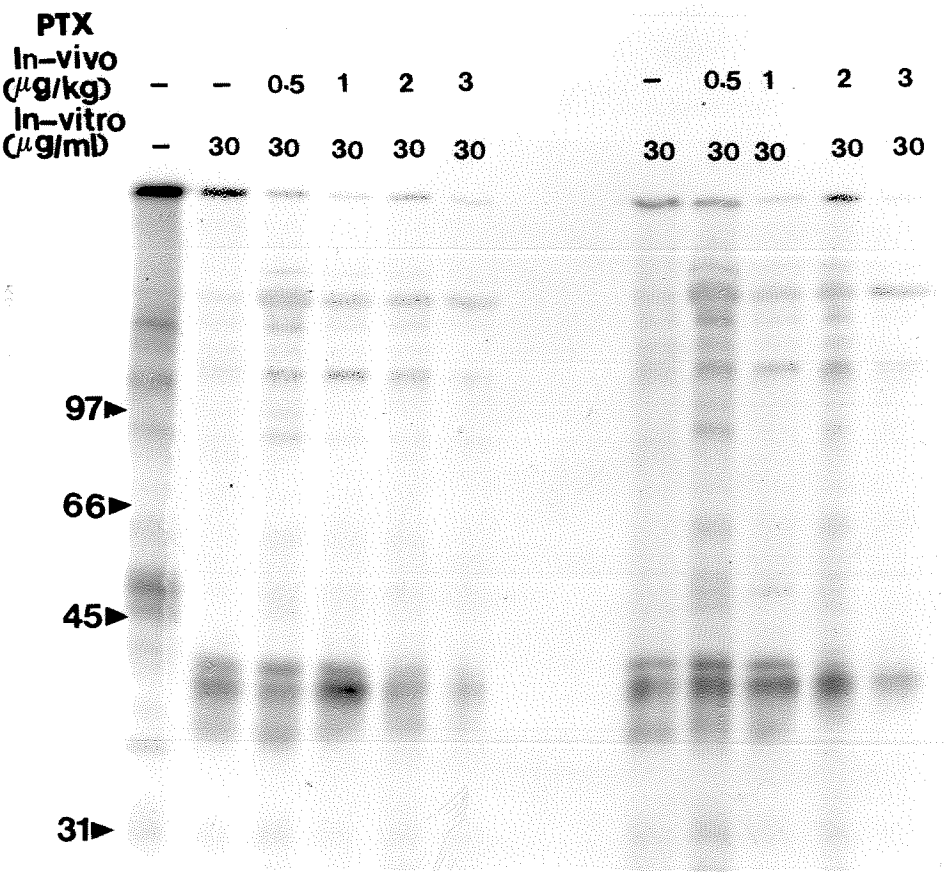

PTX
In-vivo
(μg/kg)
In-vitro
(μg/ml)
– – 0.5 1 2 3 – 0.5 1 2 3
– 30 30 30 30 30 30 30 30 30 30
97►
66►
45►
31►

Fig. 25: Concentration-response effects of isoproterenol on adenylate cyclase activity in atrial and ventricular homogenates or rabbit.

Atrial (▽) and ventricular (●) homogenates were incubated in the presence of increasing concentrations of isoproterenol for 10 min. Adenylate cyclase activity is expressed as percent of the basal activity which was 130 ± 26.8 in atria and 179.8 ± 29.2 (pmol/min/mg) in ventricles. Each curve represents the mean ± S.E.M. of 4 experiments done in duplicate using atrial and ventricular homogenates from 4 different rabbits.

ADENYLATE CYCLASE ACTIVITY
(% OF INITIAL)
400
350
300
250
200
150
100
7
6
5
4
3
– LOG ISOPROTERENOL (M)

Fig. 26: Effects of timolol on the isoproterenol-stimulated adenylate cyclase activity in rabbit atrial and ventricular homogenates.

Atrial and ventricular homogenates were incubated with 100 μM isoproterenol (solid bars), 1 μM timolol (open bars) or isoproterenol plus timolol (hatched bars), for 10 min. Data are expressed as change from the basal adenylate cyclase activity which was 130 ± 26.8 in atria and 179.8 ± 29.2 (pmol/min/mg) in ventricles respectively. Each bar represents the mean ± S.E.M. of 3 - 4 separate experiments done in duplicate in atrial and ventricular homogenates obtained from separate rabbits. (*) Represents significantly different from isoproterenol alone (paired t-test; $P<0.05$).

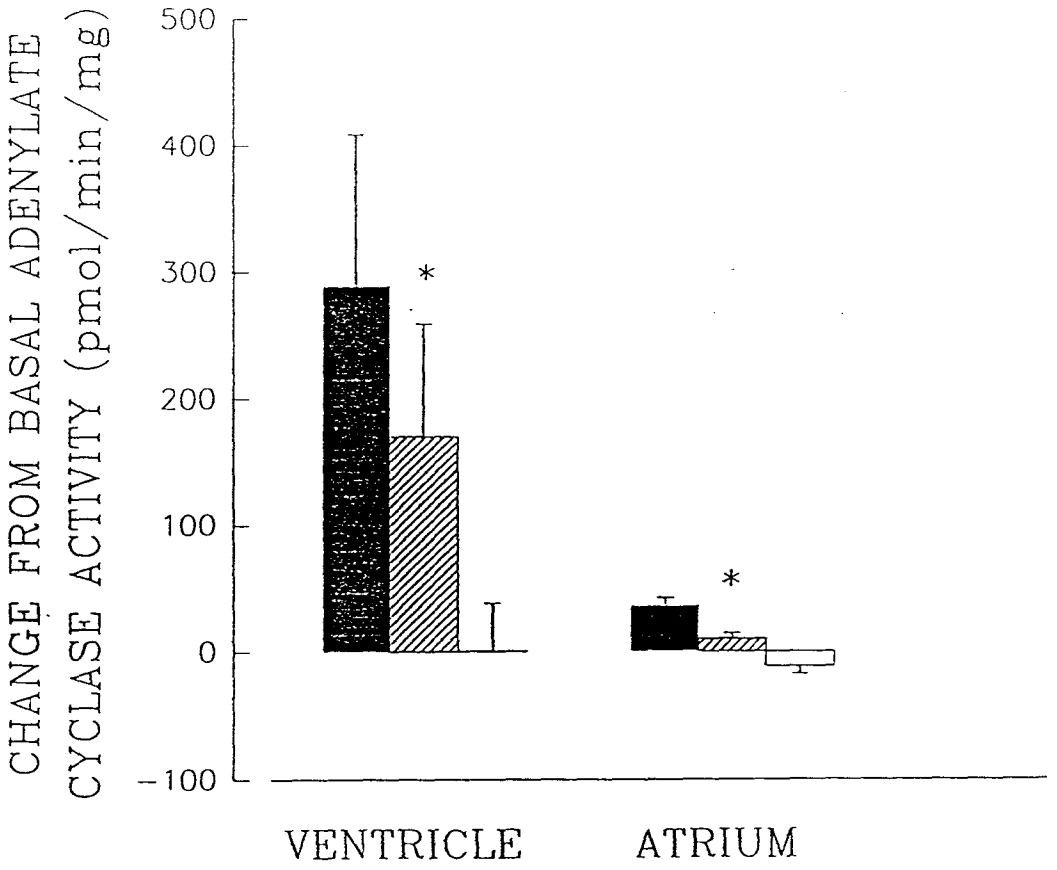

CHANGE FROM BASAL ADENYLATE CYCLASE ACTIVITY (pmol/min/mg)
500
400
300
200
100
0
-100
*
*
VENTRICLE
ATRIUM

Fig. 27: Concentration-response curves to carbachol in the absence and presence of isoproterenol in rabbit atrial and ventricular homogenates.

Atrial ($\triangle$) and ventricular ($\circ$) homogenates were incubated with increasing concentrations of carbachol in the absence (A) and in the presence (B) of 1 μM (ventricle) or 100 μM (atria) isoproterenol for 10 min. Data are expressed as percent of the basal adenylate cyclase activity, which is shown in Table 6. I represents the response to isoproterenol alone. The effect of carbachol on basal adenylate cyclase activity was determined in duplicate in atrial and ventricular homogenates from 13 and 10 different rabbits, respectively. The effect of carbachol on isoproterenol-stimulated adenylate cyclase was determined in duplicate in atrial and ventricular homogenates from 15 and 7 separate rabbits, respectively.

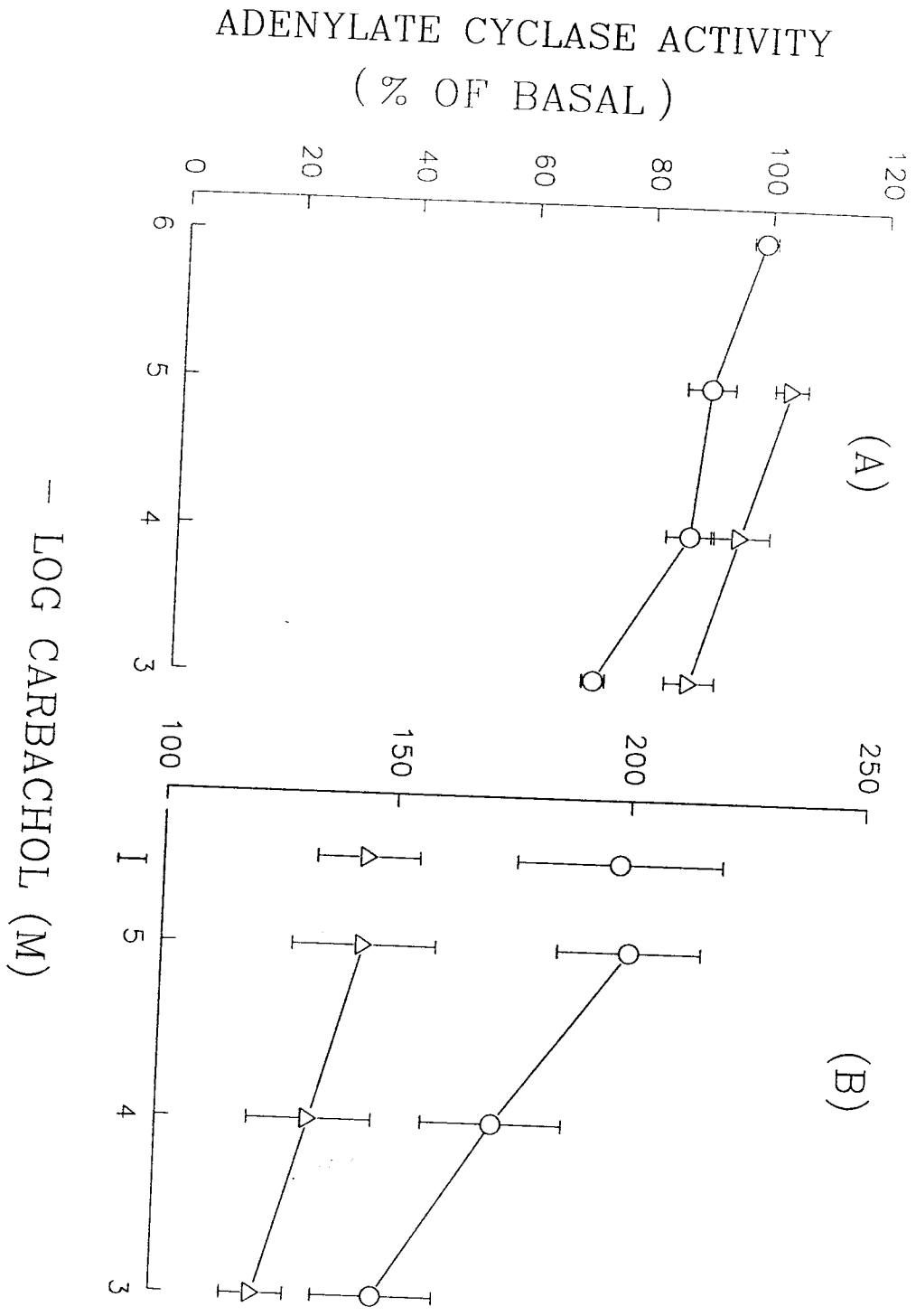

ADENYLATE CYCLASE ACTIVITY
(% OF BASAL)
0
20
40
60
80
100
120
6
5
4
3
(A)
100
150
200
250
I
5
4
3
(B)
– LOG CARBACHOL (M)

Fig. 28: Effects of atropine on the carbachol-induced inhibition of adenylate cyclase activities in the absence and presence of isoproterenol in rabbit atrial and ventricular homogenates.

(A). Atrial and ventricular homogenates were incubated with 1 mM carbachol (open bars) or 10 μM atropine (solid bars) alone or in combination (hatched bars) for 10 min. The effect of atropine was tested in duplicate in atrial and ventricular homogenates from 3 - 4 separate rabbits. The effect of atropine in combination with carbachol was tested in duplicate in 3 separate ventricles and 9 separate atria. (B). Atrial (n=11) and ventricular (n=5) homogenates were incubated with isoproterenol alone (open bars) or in combination with either carbachol alone (hatched bars) or carbachol plus atropine (solid bars) for 10 min. Each assay was done in duplicate. Concentrations of isoproterenol used were 1 μM in ventricle and 100 μM in atrium. Data are expressed as change from the basal adenylate cyclase activity (pmol/min/mg).

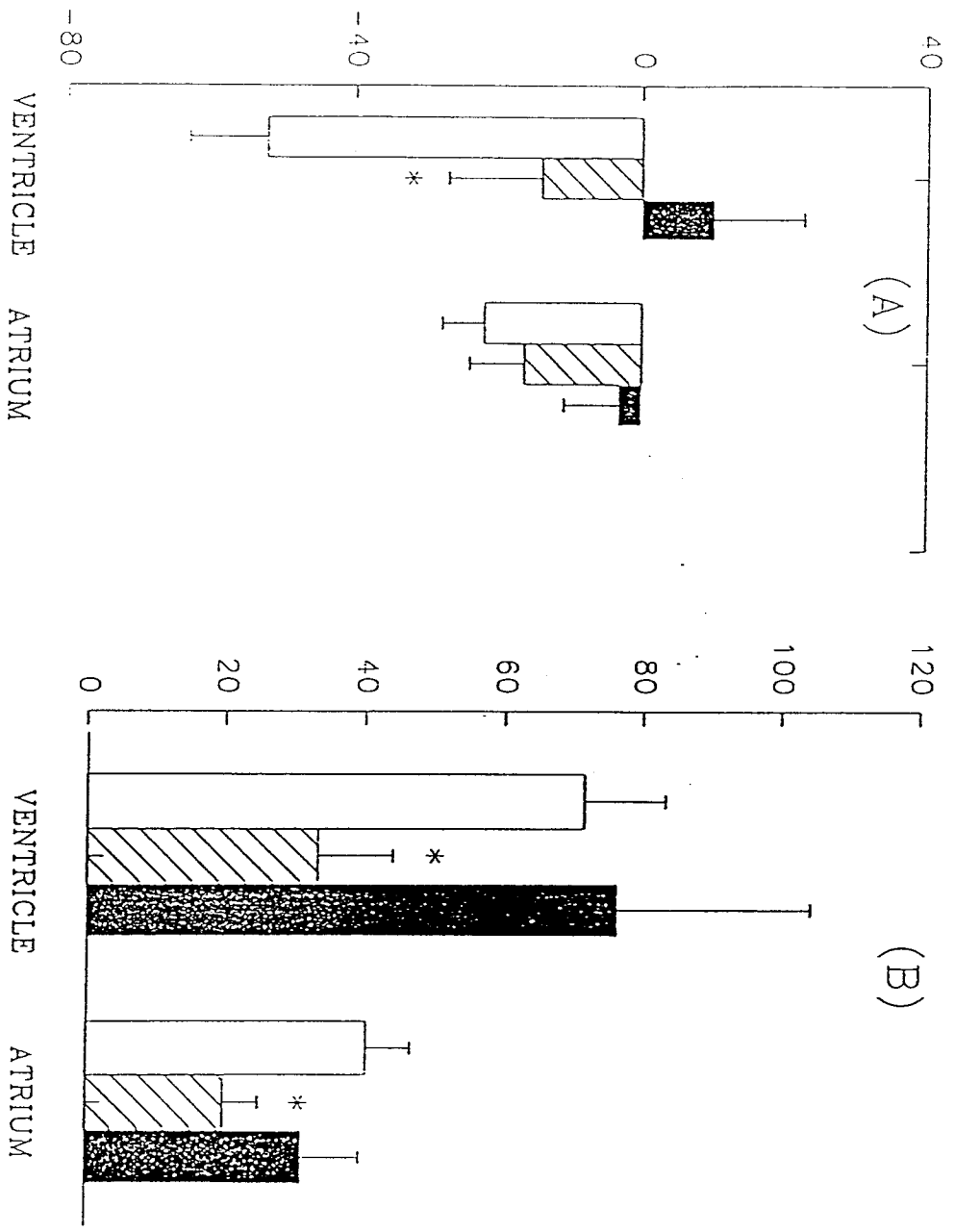

CHANGE FROM BASAL ADENYLATE CYCLASE ACTIVITY (pmol/min./mg)
-80
-40
0
40
VENTRICLE
ATRIUM
(A)
0
20
40
60
80
100
120
VENTRICLE
ATRIUM
(B)

Fig. 29. Effects of carbachol on forskolin- and GTPγS- stimulated adenylate cyclase activities in rabbit atrial and ventricular homogenates.

Atrial and ventricular homogenates were incubated with 1 μM forskolin or 10 μM GTPγS in the absence or presence of 1 mM carbachol. Data are expressed as change from the basal adenylate cyclase activity, which was 142.6 ± 15.3 (n=10) and 162.5 ± 22.2 (n=8) pmol/min/mg, in atrial and ventricular homogenates exposed to forskolin. The basal adenylate cyclase activity in atrial and ventricular homogenates exposed to GTPγS was 106.9 ± 17.7 (n=7) and 156.9 ± 34.3 (n=5) pmol/min/mg, respectively. Each assay was done in duplicate in atrial and ventricular homogenates prepared from separate rabbits.

(A)
(B)
CHANGE FROM BASAL ADENYLATE CYCLASE ACTIVITY (pmol/min/mg)
FORSKOLIN
FORSKOLIN + CARBACHOL
GTPγS
GTPγS + CARBACHOL
1200
900
600
300
0
200
160
120
80
40
0
*
*
VENTRICLE
ATRIUM
VENTRICLE
ATRIUM

Fig. 30: Effects of pertussis toxin pre-treatment of rabbits on the inhibitory effect of carbachol on adenylate cyclase activity in the absence and presence of isoproterenol in rabbit ventricular homogenates.

Ventricular homogenates from rabbits pre-treated with normal saline (○), 1 (△), 2 (▽) or 3 μg/kg (□) pertussis toxin were incubated with increasing concentrations of carbachol in the absence (A) and presence (B) of 1 μM isoproterenol. Data are expressed as percent of the basal activity, which is shown in table 8. Each curve represents the mean ± S.E.M. of 6 - 7 separate experiments each done in duplicate in ventricular homogenates from different rabbits.

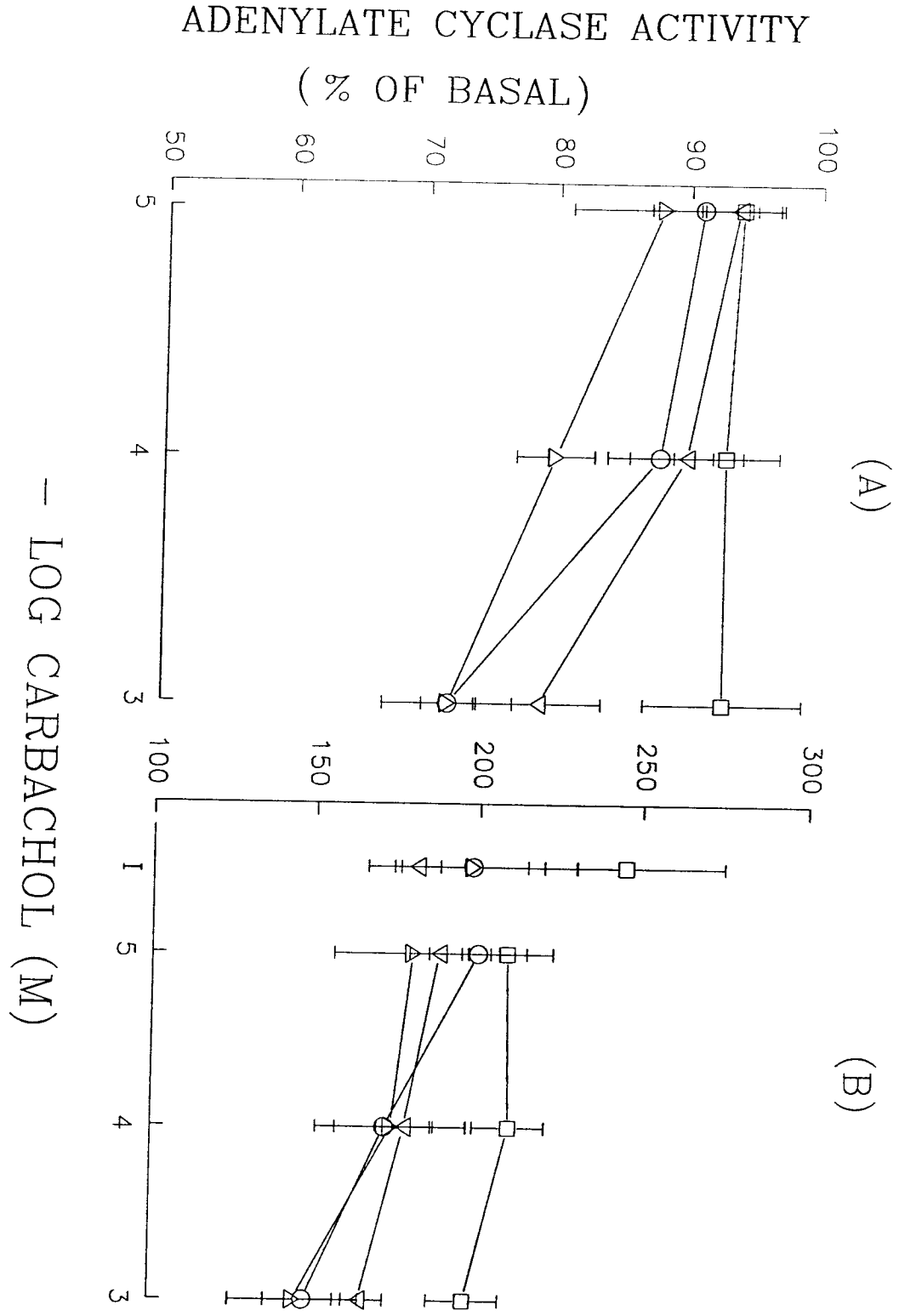
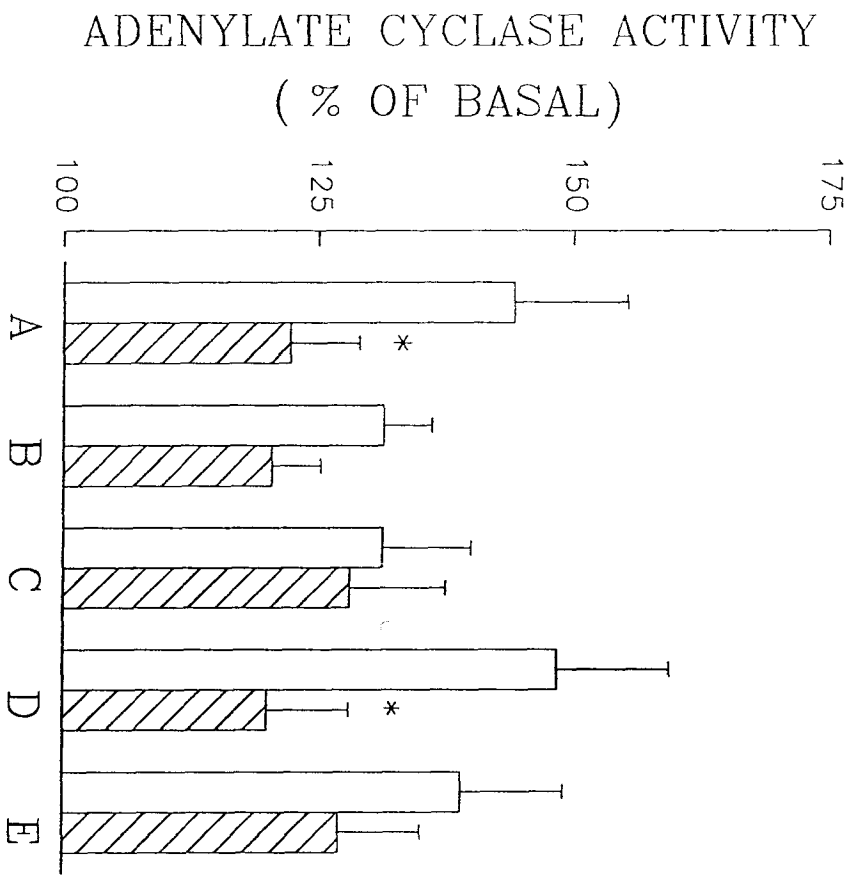

ADENYLATE CYCLASE ACTIVITY
(% OF BASAL)
50
60
70
80
90
100
5
4
3
(A)
100
150
200
250
300
I
5
4
3
(B)
– LOG CARBACHOL (M)

Fig. 31: Effects of pre-treatment of rabbits with different doses of pertussis toxin on the inhibitory effect of carbachol on isoproterenol-induced increases in adenylate cyclase activity in rabbit atrial homogenates.

Atrial homogenates from saline (A) (n=15), 0.5 (B) (n=6), 1 (C) (n=6), 2 (D) (n=7) and 3 μg/kg (E) (n=6) pertussis toxin pre-treated rabbits were incubated with 100 μM isoproterenol alone (open bars) or in combination with 1 mM carbachol (hatched bars) for 10 min. Each bar represents the mean ± S.E.M. of adenylate cyclase activity, expressed as percent of basal activity (table 8), done in duplicate in atrial homogenates from separate rabbits.


ADENYLATE CYCLASE ACTIVITY
(% OF BASAL)
100
125
150
175
A
B
C
D
E
*
*

DISCUSSION

The results of the present study suggest that: (a) the ability of carbachol to open potassium channels contributes to the negative inotropic responses to carbachol in the presence of phenylephrine, forskolin and IBMX in the rabbit left atrium, and (b) pertussis toxin is more effective in uncoupling muscarinic receptors from adenylate cyclase in atria than either muscarinic receptors from potassium channels in atria or muscarinic receptors from adenylate cyclase in ventricles.

It is well established that in the atrial myocardium muscarinic receptors are linked to potassium channels by means of a pertussis toxin-sensitive G-protein (Fleming et al., 1992). The cyclic nucleotide-independent direct negative inotropic response to muscarinic receptor stimulation in the atrial myocardium is believed to be related to the ability of muscarinic agonists to open potassium channels (Ten Eick et al., 1976; Cerbai et al., 1988). One of the objectives of the present study was to investigate the contribution of potassium channel opening in the functional interaction of carbachol with a variety of cAMP-elevating and cAMP-independent positive inotropic agents in rabbit left atria. Phenylephrine, an α-adrenoceptor agonist, was used as a cAMP-independent positive inotropic agent. The cAMP-elevating agents used in the study were isoproterenol, a β-adrenoceptor agonist, forskolin, a direct activator of adenylate cyclase and IBMX, a phosphodiesterase inhibitor.

4.1. DIRECT NEGATIVE INOTROPIC RESPONSE TO CARBACHOL

In this series of experiments, 4-aminopyridine and pertussis toxin were used to interfere with the ability of carbachol to open potassium channels. In the present study, 4-aminopyridine, in the concentration range of 1 μM - 5 mM exerted a concentration-dependent positive inotropic response in rabbit left atria, in agreement with previous reports (Yanagisawa and Taira, 1979; Wollmer et al., 1981). Based on preliminary experiments, the effects of two different concentrations of 4-aminopyridine (50 and 500 μM) were studied on the negative inotropic responses to carbachol in the presence and absence of various positive inotropic agents. It has been reported previously that 4-aminopyridine in high concentrations is capable of displacing muscarinic agonists from their receptor sites (Drukarch et al. ,1989; Urquhart and Broadley, 1991). In order to investigate this possibility, the effect of 500 μM 4-aminopyridine on the ability of carbachol to inhibit the isoproterenol-stimulated cAMP generation was tested. 4-Aminopyridine did not interfere with the ability of carbachol to inhibit the isoproterenol-stimulated cAMP generation, suggesting that 4-aminopyridine was not acting at the level of muscarinic receptors.

In the present study, carbachol exerted a concentration-dependent negative inotropic response which was attenuated by 4-aminopyridine in a concentration-dependent manner. This was in agreement with previous reports of De Biasi et al (1989) and Urquhart and Broadley (1991). However, 500 μM 4-aminopyridine did not completely attenuate the negative inotropic response to carbachol suggesting that either 500 μM 4-aminopyridine is not capable of completely blocking the carbachol-stimulated

potassium current or activation of the potassium current may not completely explain the direct negative inotropic response to carbachol in left atria.

In order to obtain more direct evidence for the carbachol-stimulated opening of potassium channels and its contribution to the negative inotropic response to carbachol, we monitored the effects of carbachol on the rate-constant of ^{86} Rb-efflux and contractile response in the same electrically-stimulated left atria. Carbachol produced an increase in the efflux-rate-constant of ^{86} Rb from rabbit left atrial strips which was blocked by atropine and was attenuated by pre-treatment of rabbits with pertussis toxin. In agreement with previous reports in rat (Quast et al., 1988) and guinea pig (Urquhart et al., 1991) atria, this suggests that in the rabbit left atrium, muscarinic receptors are coupled to potassium channels by means of a pertussis toxin-sensitive G-protein.

Carbachol exerted a direct negative inotropic response over the same concentration-range that it increased the ^{86}Rb-efflux-rate-constant. However, the lowest concentration of carbachol used caused a very marked decrease in the left atrial tension, while producing only a very small increase in the rate-constant of ^{86}Rb-efflux. The relative effects of carbachol on the tension and ^{86}Rb-efflux appear to be very similar to those reported in rat left atria (Quast et al, 1988). It is possible that either a very small increase in the potassium-efflux can produce a very marked effect on the tension, or that changes in the inotropic response can be measured with greater sensitivity than changes in the potassium-efflux with the methods employed, as has been suggested to occur in vascular smooth muscle (Smith et al, 1986; Quast and Baumlin, 1988). These possibilities seem unlikely, since Urquhart et al (1991) have shown that a good correlation exists between the negative inotropic response and increases in the rate-constant of ^{86}Rb-efflux in response to adenosine receptor stimulation in guinea pig left

atria. A third possibility is that mechanisms other than increases in the potassium-efflux contribute to the direct negative inotropic response to carbachol. To further investigate this, we used 4-aminopyridine and pertussis toxin to interfere with the ability of carbachol to open potassium channels.

4-Aminopyridine alone had no significant effect on the basal ^{86}Rb-efflux-rate constant, but it reduced the increases in the rate-constant of ^{86} Rb-efflux produced by carbachol in rabbit left atria. However, a concentration of 4-aminopyridine (500 μM) which completely blocked the carbachol-induced increase in the efflux-rate-constant, only partially attenuated the direct negative inotropic response to carbachol. This, in agreement with our observation in functional studies, suggests that some process in addition to the increased potassium-conductance contributes to the direct negative inotropic response of rabbit left atria to carbachol.

Pre-treatment of rabbits with 0.5 and 1 μg/kg pertussis toxin resulted in a marked reduction of the ability of 1 μM carbachol to exert a negative inotropic response and to increase the rate-constant of ^{86} Rb-efflux. However, 0.5 μg/kg pertussis toxin appeared to cause a relatively greater depression of the increase in the ^{86} Rb-efflux-rate-constant than the negative inotropic effect produced by 1 μM carbachol. Increasing the dose of pertussis toxin to 1 μg/kg did not result in any greater inhibition of the ability of 1 μM carbachol to promote the ^{86} Rb-efflux, but the negative inotropic response of left atria to 1 μM carbachol was further attenuated. These data are consistent with those obtained with 4-aminopyridine and further suggest that some mechanism in addition to increases in the potassium-conductance contributes to the direct negative inotropic response of left atria to muscarinic receptor stimulation. No further decrease in the tension was observed in presence of 10 μM carbachol in atria from the pertussis toxin-treated rabbits, although

this concentration of carbachol produced a much greater increase in the ^{86}Rb-efflux-rate-constant than 1 μM carbachol. Instead, 10 μM carbachol had a tendency to reverse the negative inotropic response observed in the presence of 1 μM carbachol in atria from both saline and pertussis toxin pre-treated rabbits. Increasing the concentration of carbachol to 100 μM produced an even greater reversal of the negative inotropic response to carbachol. It is well established that muscarinic agonists in concentrations of 10 μM and higher can promote phosphoinositide turnover and exert a positive inotropic response by a pertussis toxin-insensitive mechanism (Tajima et al, 1987; see Pappano, 1990). It is possible that this effect might have contributed to the reversal of the negative inotropic response seen with 10 and 100 μM carbachol in the present investigation.

The mechanism by which carbachol could produce a potassium channel-independent direct negative inotropic response is not known. Previous studies have ruled out the involvement of cAMP (Endoh et al., 1985; MacLeod, 1986; Ray and MacLeod, 1990) and cGMP (MacLeod and Diamond, 1986) in this process. It is possible that the pertussis toxin- and 4-aminopyridine- insensitive component of the direct negative inotropic response to carbachol may be related to the ability of muscarinic agonists to inhibit the calcium current directly (Ten Eick et al, 1976; Cerbai et al, 1988).

4.2 INTERACTION OF CARBACHOL WITH PHENYLEPHRINE AND ISOPROTERENOL

Carbachol completely reversed the positive inotropic response of rabbit left atria to phenylephrine. 4-Aminopyridine produced a concentration-dependent antagonism of the negative inotropic response to carbachol in the presence of phenylephrine, which may

suggest that the muscarinic receptor-induced activation of potassium current may explain the reversal by carbachol of the positive inotropic response to phenylephrine.

When the ability of carbachol to promote the ^{86} Rb-efflux was monitored in the presence of phenylephrine, carbachol produced an increase in the ^{86}Rb-efflux-rate constant and a reduction in tension in the presence of phenylephrine. 4-Aminopyridine (50 and 500 μM) attenuated the negative inotropic responses to carbachol in the presence of phenylephrine but were not very effective in blocking the increases in the carbachol-stimulated ^{86} Rb-efflux-rate-constant. The reason why the carbachol-stimulated ^{86}Rb-efflux was inhibited to a lesser extent in the presence of the combination of 4-aminopyridine and phenylephrine than in the presence of 4-aminopyridine alone is unknown.

The same concentration of pertussis toxin which had little effect on the increases in the ^{86}Rb-efflux-rate-constant in response to 10 μM carbachol alone, completely blocked the increases in the rate-constant of ^{86}Rb-efflux produced by 10 μM carbachol in the presence of phenylephrine. This was associated with almost complete loss of the reversal by carbachol of the positive inotropic responses to phenylephrine. These results are consistent with a role for the increases in the potassium-efflux in the inotropic responses to carbachol in the presence of phenylephrine.

4-Aminopyridine was found to exert a greater attenuating effect on the response to carbachol in the presence of phenylephrine than on either the direct negative inotropic response to carbachol or the negative inotropic responses to carbachol in the presence of isoproterenol, forskolin or IBMX (see below). A similar observation was also made in the present as well as in an earlier study (Ray and MacLeod, 1990) concerning the inhibitory effect of pertussis toxin on the negative inotropic response to carbachol in the presence of

phenylephrine. The explanation for this observation is unclear at present. However, it could be related to the ability of phenylephrine to block the outward potassium current (Apkon and Nerbonne, 1988; Braun et al., 1990; Fedida and Bouchard, 1992) which is believed to contribute to the α-adrenoceptor-mediated positive inotropy. The greater inhibitory effect of pertussis toxin and 4-aminopyridine on the responses to carbachol in the presence of phenylephrine may be due to an additive effect between pertussis toxin or 4-aminopyridine and phenylephrine, resulting in greater antagonism of the carbachol-stimulated potassium-efflux.

Carbachol also antagonized the positive inotropic responses of left atria to isoproterenol in a concentration-dependent manner. The response to carbachol in the presence of isoproterenol was only slightly inhibited by 4-aminopyridine. It is well established that carbachol can inhibit the isoproterenol-stimulated adenylate cyclase activity and cAMP generation (Endoh et al., 1985; Sorota et al., 1985; MacLeod, 1986; Ray and MacLeod, 1992; also the present study). Our results suggest that because the inhibitory effect of carbachol on the isoproterenol-stimulated cAMP generation contributes to the functional interaction between isoproterenol and carbachol, carbachol retained its inhibitory effect on the positive inotropic response to isoproterenol even in the presence of the potassium channel blockade.

4.3. EFFECTS OF PINACIDIL AND CROMAKALIM

Using a different approach, the effects of potassium channel openers on the ability of phenylephrine and isoproterenol to exert positive inotropic responses were studied. It was argued that if carbachol antagonized the effects of these positive inotropic

agents by opening potassium channels, then potassium channel agonists should also antagonize the development of positive inotropic responses to isoproterenol and phenylephrine. Pinacidil and cromakalim, two known agonists of the ATP-dependent potassium channel (Cook, 1988), were used in the study. Both pinacidil and cromakalim antagonized the phenylephrine-induced positive inotropic response in a concentration-dependent manner. This, in agreement with our 4-aminopyridine data, suggests that activation of the potassium current contributes to the negative inotropic responses of left atria to carbachol in the presence of phenylephrine. In contrast, neither pinacidil nor cromakalim had any effect on the positive inotropic responses to isoproterenol, suggesting that inhibition of the isoproterenol-stimulated adenylate cyclase activity by carbachol plays a more dominant role in this interaction.

4.4. INTERACTION OF CARBACHOL WITH FORSKOLIN AND IBMX

Results from this (MacLeod and Diamond, 1986; Ray and MacLeod, 1992) and other laboratories (Brown, 1979; Biegon et al., 1980; Pappano et al., 1982; Lindemann and Watanabe, 1985; Schmied and Korth, 1990) have shown that muscarinic agonists either do not inhibit the forskolin- or IBMX- induced increases in cAMP levels or that muscarinic antagonism of the positive inotropic responses of left atria to forskolin and IBMX occurs independently of changes in the forskolin- or IBMX- induced increases in cAMP levels. However, others have demonstrated that muscarinic agonists can reduce the forskolin- and IBMX- stimulated adenylate cyclase activity and cAMP levels in the atrial myocardium (Brown et al 1980; Sulakhe et al., 1985). It has also been argued that carbachol may only reduce adenylate cyclase activity and cAMP levels elevated by

forskolin in a small compartment of the heart, linked to the inotropic response, which is not detectable when total tissue levels of cAMP or adenylate cyclase activity are measured (Hartzell, 1988). In order to rule out the possible involvement of muscarinic inhibition of adenylate cyclase in the functional interaction of carbachol with forskolin and IBMX, muscarinic receptors were uncoupled from adenylate cyclase using pertussis toxin. In rabbits pre-treated with 2.2 μg/kg pertussis toxin, carbachol lost its inhibitory effect on the isoproterenol-induced increases in adenylate cyclase activity and cAMP levels, suggesting that muscarinic receptors were completely uncoupled from adenylate cyclase. Under this circumstance, consistent with previous reports (Endoh et al., 1985; Ray and MacLeod, 1992), pertussis toxin pre-treatment of rabbits resulted in a pronounced attenuation of the negative inotropic response to carbachol in the presence of isproterenol. At the same time, however, pertussis toxin had relatively little attenuating effect on the inhibition by carbachol of the positive inotropic responses to forskolin and IBMX, and pertussis toxin did not have any inhibitory effect on the direct negative inotropic response to carbachol. This, in agreement with a previous report from this laboratory (Ray and MacLeod, 1992), may suggest that inhibition of the forskolin- or IBMX- induced increases in cAMP levels does not play an important role in the functional interaction of carbachol with these positive inotropic agents.

The mechanism of this cAMP-independent component of the negative inotropic response to carbachol in the presence of forskolin and IBMX was investigated using 4-aminopyridine in saline and pertussis toxin pre-treated rabbits. 4-Aminopyridine produced a concentration-dependent attenuation of the response to carbachol in the presence of forskolin in atria from saline-treated animals. The magnitude of the inhibitory effect of 4-aminopyridine on both the direct negative inotropic response to

carbachol and on the response to carbachol in the presence of forskolin was very similar in atria from saline and pertussis toxin-treated animals. This is consistent with a role for activation of potassium channels in mediating the response to carbachol in the presence of forskolin. The response of atria from saline-treated rabbits to carbachol in the presence of IBMX appeared to be more resistant to inhibition by 4-aminopyridine than either the direct negative inotropic response to carbachol or the response to this agonist in the presence of forskolin. The reason for this is not clear. However, it is possible that an inhibitory effect of 4-aminopyridine was at least partially masked by the effectiveness of carbachol in reversing the response to IBMX, since the majority of atria (7/13 or 54%) from saline-treated rabbits stopped beating in the presence of the maximum concentration of carbachol plus IBMX. The fact that only 2 out of 7 atria (29%) stopped beating in response to carbachol in the presence of IBMX following treatment of atria with 50 μM 4-aminopyridine suggests that this may be the case. This is further supported by the data obtained in atria from pertussis toxin-treated rabbits, in which carbachol was still very effective in reversing the response to IBMX, but arrested only one atrium out of a total of seven atria used in the study. Under these circumstances, 4-aminopyridine exerted a more marked inhibitory effect on the response to carbachol in the presence of IBMX, similar in magnitude to the inhibitory effect of 4-aminopyridine on the direct negative inotropic response to carbachol. These data are also consistent with a role for the muscarinic receptor-mediated activation of potassium current in the reversal by carbachol of the positive inotropic response to IBMX.

However, it should be noted that the higher concentration of 4-aminopyridine used did not attenuate completely the response to carbachol in the presence of IBMX and forskolin. This suggests that some mechanism in addition to potassium channel

activation, eg. activation of protein phosphatase (Ahmad et al., 1989) and calcium channel inhibition (TenEick et al., 1976; Cerbai et al., 1988), may also contribute to these responses to carbachol.

It is not clear how pertussis toxin attenuated the negative inotropic responses to carbachol in the presence of forskolin and IBMX, although it did not have any inhibitory effect on the direct negative inotropic response to carbachol. There could be several possible explanations. Firstly, it is possible that a small uncoupling of muscarinic receptors from potassium channels might have been sufficient to attenuate the negative inotropic responses to carbachol in the presence of IBMX and forskolin but was inadequate to attenuate the direct negative inotropic response to carbachol. A second possibility could be that some effector other than adenylate cyclase and potassium channels, eg. phosphatase, might have been uncoupled from muscarinic receptors resulting in a greater attenuation of the inhibitory effect of carbachol in the presence of forskolin and IBMX. Lastly, it is possible that uncoupling of adenylate cyclase from muscarinic receptors in a compartment not detectable in the whole tissue cAMP assay might have contributed to this greater attenuation of the inhibitory responses to carbachol in the presence of forskolin and IBMX.

In contrast to forskolin or IBMX, the negative inotropic responses to carbachol in the presence of isoproterenol was affected very modestly by 4-aminopyridine. This is again consistent with our data discussed in the previous section and the pertussis toxin data discussed in this section, and suggests a dominant role of muscarinic inhibition of adenylate cyclase in the interaction. However, in the present investigation pertussis toxin, although completely uncoupling muscarinic receptors from adenylate cyclase, did not completely inhibit the ability of carbachol to reverse the positive inotropic response

to isoproterenol. The response to carbachol in the presence of isoproterenol that remained in pertussis toxin-treated tissues was further inhibited, in a concentration-dependent manner, by 4-aminopyridine. This suggests that although inhibition of the isoproterenol-stimulated cAMP generation plays an important role in the functional interaction of isoproterenol and carbachol, some of the negative inotropic response to carbachol in the presence of isoproterenol may depend on the ability of carbachol to open potassium channels. This is in agreement with previous reports that inhibition of τηε β-adrenoceptor-stimulated increases in adenylate cyclase activity is not sufficient to completely explain the inhibition by muscarinic agonists of the positive inotropic response to β-adrenoceptor stimulation (Brown et al., 1980; Endoh et al., 1985; MacLeod, 1986).

4.5. DIFFERENTIAL EFFECTS OF PERTUSSIS TOXIN

We have reported in an earlier study (Ray and MacLeod, 1992) that injection of rabbits with pertussis toxin resulting in complete loss of the ability of carbachol to inhibit the isoproterenol-stimulated increases in cAMP levels and positive inotropy only partially affected the cAMP-independent responses to carbachol such as the direct negative inotropic effect and the inhibitory effect on the forskolin-induced positive inotropy. In the present study, a similar result was obtained when it was observed that 2.2 μg/kg pertussis toxin uncoupled muscarinic receptors from adenylate cyclase but had no inhibitory effect on the direct negative inotropic response to carbachol and only partially inhibited the ability of carbachol to inhibit the forskolin- and IBMX- induced positive inotropy. Since our data suggests that these cAMP-independent responses to carbachol

are related, at least in part, to the ability of carbachol to open potassium channels, we investigated the possible differential uncoupling of muscarinic receptors from potassium channels and adenylate cyclase in the atrial myocardium. We had also found that a dose of pertussis toxin which completely attenuated the inhibitory effect of carbachol on the isoproterenol-stimulated increases in cAMP levels and positive inotropy in the atrial myocardium did not have any inhibitory effect on the negative inotropic response to carbachol in the presence of isoproterenol in the rabbit right ventricular papillary muscle (Ray and MacLeod, unpublished observations). This prompted us to investigate if the muscarinic inhibition of adenylate cyclase in the atrium is more sensitive to the uncoupling effect of pertussis toxin than the muscarinic receptor-mediated inhibition of adenylate cyclase in the ventricle. We have compared the effect of carbachol on adenylate cyclase activity in the presence and absence of isoproterenol in atrial and ventricular homogenates from saline and pertussis toxin pre-treated rabbits. The uncoupling effect of pertussis toxin on the carbachol-inhibited adenylate cyclase activity was compared with the effect of pertussis toxin on the ability of carbachol to promote the efflux of ^{86} Rb from the left atrium of rabbits. The attenuating effects of pertussis toxin on the carbachol-stimulated ^{86} Rb-efflux-rate-constant and on carbachol-mediated inhibition of adenylate cyclase activity in atria and ventricles were correlated with the degree of ADP-ribosylation of G-proteins in these tissues.

In the present study, carbachol did not have any effect on the basal adenylate cyclase activity but inhibited the enzyme activity stimulated by isoproterenol in the atrial homogenates. This is in agreement with previous reports from this and other laboratories that muscarinic agonists can inhibit the β-adrenoceptor agonist-stimulated adenylate cyclase activity and cAMP generation but not the basal enzyme activity or cAMP levels

in atria (Sorota et al., 1985; MacLeod, 1986; Ray and MacLeod, 1990). Carbachol also stimulated the rate constant of ^{86} Rb efflux in rabbit atrial strips (discussed in the previous section). Pre-treatment of rabbits with 0.5 and 1 μg/kg pertussis toxin, which ADP-ribosylated approximately 60 - 70% of the atrial G-proteins, only partially attenuated the ability of carbachol to increase the rubidium-efflux-rate-constant. However, the same dose of pertussis toxin completely blocked the inhibition by carbachol of the isoproterenol-stimulated adenylate cyclase in the atrium.

The identity of the pertussis toxin-sensitive G-proteins present in the rabbit heart has not been established, but studies have demonstrated the presence of four different pertussis toxin-sensitive G-proteins, the 3 isoforms of G_i, G_{i1}, G_{i2} and G_{i3}, and G_o in hearts of other mammalian species. Neither the identity of the G-protein(s) nor the mechanism by which the G-protein(s) couple muscarinic receptors to adenylate cyclase and potassium channels invivo are known. One possible explanation for our findings is that two or more different G-proteins, which are not equally susceptible to ADP-ribosylation by pertussis toxin, may link muscarinic receptors to adenylate cyclase and potassium channels. On the other hand, it is also possible that different subunits of the same G-protein couple muscarinic receptors to adenylate cyclase and potassium channels in the atrium, with the α-subunit directly activating the potassium channels and the βγ subunits inhibiting the isoproterenol-stimulated adenylate cyclase by quenching the α-subunit of the stimulatory G-protein, as suggested by Brown (1990) and Robishaw and Foster (1990). This possibility was also proposed by Pappano and Mubagwa (1992) to explain the differential desensitization they observed of the carbachol-activated potassium current and carbachol-mediated inhibition of the isoproterenol-stimulated slow inward calcium current in the guinea pig atrial myocardium. Recently, Matesic et al.

(1991) showed that muscarinic receptors in the rat atrium associate preferentially with G_o, supporting the hypothesis that a single G-protein, G_o, couples muscarinic receptors to both adenylate cyclase and potassium channels in this tissue. If this is the case, the differential sensitivity of muscarinic responses in the atrium to pertussis toxin that we observed could arise because on partial ADP-ribosylation of G_o, the number of $\beta\gamma$ subunits produced is less than is required to promote the quenching of $G_{s\alpha}$, while sufficient α-subunits are still available to activate potassium channels. However, at this time we cannot rule out the possibility of two different G-proteins coupling muscarinic receptors to potassium channels and adenylate cyclase in the atrium.

In the ventricle, carbachol inhibited both basal and isoproterenol-stimulated adenylate cyclase activity in a concentration-dependent and atropine-sensitive manner, in agreement with previous reports in rabbit (Jakobs et al. 1979) and rat (Sulakhe et al. 1985) ventricles. However, the muscarinic receptor-mediated inhibition of adenylate cyclase in the ventricle was less sensitive than that in the atrium to inhibition by pertussis toxin. There were no significant differences in the extent of invivo ADP ribosylation in atria and ventricles from rabbits injected with either 0.5 and 1 μg/kg pertussis toxin. However, carbachol completely lost its ability to inhibit the isoproterenol-stimulated adenylate cyclase in atria from rabbits treated with 0.5 μg/kg pertussis toxin, which ADP-ribosylated 68% of G-proteins. In contrast, pre-treatment of rabbits with 1 μg/kg pertussis toxin, which ADP-ribosylated 60 % of the ventricular G-proteins, did not have any effect on the inhibition by carbachol of either basal or isoproterenol-stimulated adenylate cyclase. Increasing the dose of pertussis toxin to 3 μg/kg, which resulted in complete ADP-ribosylation of the ventricular G-proteins, abolished the ability of carbachol to inhibit adenylate cyclase in the ventricle.

Matesic et al (1991) have demonstrated that in the rat ventricle, in contrast to the atrium, muscarinic receptors couple to two different G-proteins, G_{i2} and G_o. Both the α-subunit of G_{i2} and the $\beta\gamma$ subunits of both G_i and G_o could potentially contribute to the muscarinic receptor-mediated inhibition of adenylate cyclase in the ventricle. Thus, the differential sensitivity of the muscarinic receptor-mediated inhibition of adenylate cyclase found between the atrium and ventricle could arise because two different G-proteins and/or different subunits of the same G-protein convey the message of muscarinic receptor occupancy in the atrium and ventricle.

The observation that muscarinic agonists can inhibit the basal adenylate cyclase activity in the ventricle but not in the atrium suggests that the mechanism of muscarinic-inhibition of adenylate cyclase may differ between the two tissues. We have further investigated this possibility by comparing the inhibitory effects of carbachol on the forskolin- and GTPγS- stimulated adenylate cyclase activity in the atrium and ventricle. Consistent with our observation on the basal adenylate cyclase activity, carbachol inhibited both forskolin- and GTPγS- stimulated adenylate cyclase activity in the ventricle but not in the atrium. Watanabe and group (Fleming et al., 1987) have suggested that muscarinic inhibition of the myocardial adenylate cyclase is indirect in nature and occurs only when the enzyme activity is stimulated by a β-adrenoceptor agonist. According to this model, $\beta\gamma$ subunits released from the inhibitory G-proteins sequester the $G_{s\alpha}$ subunit and prevent it from stimulating the adenylate cyclase. However, the results of the present study suggest that Watanabe's model may be true in rabbit atria but not in the ventricle, where we cannot rule out the possibility of a direct inhibition of the enzyme by either the α- or $\beta\gamma$- subunits of the G_i proteins.

It is not clear why 2 μg/kg pertussis toxin did not attenuate the inhibitory effect of carbachol on the isoproterenol-stimulated adenylate cyclase activity in the atrium. This lack of effect cannot be attributed to the lack of ADP-ribosylating effect of pertussis toxin, because in these same rabbits pertussis toxin ADP-ribosylated nearly 95% of G-proteins. It is well established in the heart that there are at least three different G_i and one G_o protein, all of which can potentially link muscarinic receptors to adenylate cyclase. The functional significance of all of these G proteins has not been clearly elucidated and we cannot rule out a complicated interaction between different G proteins activated as a result of muscarinic receptor stimulation. It is possible that ADP-ribosylation of G-proteins by pertussis toxin might have unmasked the inhibitory effect of some G-protein whose effects are otherwise not expressed.

In summary, the results of the present study demonstrate that the muscarinic receptor-mediated inhibition of adenylate cyclase in the atrium is more sensitive to uncoupling by pertussis toxin than either the muscarinic receptor-mediated activation of potassium-efflux in the atrium or inhibition of adenylate cyclase in the ventricle. In addition, we have also observed that in the atrial myocardium carbachol can inhibit only the isoproterenol-stimulated adenylate cyclase, whereas in the ventricle carbachol inhibits not only the isoproterenol-stimulated but also basal, forskolin- and GTPγS- stimulated adenylate cyclase activity. This suggests that there are differences in the coupling of muscarinic receptors with the adenylate cyclase and potassium channels in the atrium and ventricle.

SUMMARY AND CONCLUSIONS

5.1. SUMMARY

1. The direct negative inotropic response to carbachol was attenuated in a concentration-dependent manner by 4-aminopyridine, suggesting a role for the muscarinic receptor-activated potassium current in this process. However, the highest concentration of 4-aminopyridine (500 μM) used in the study did not completely inhibit the direct negative inotropic response to carbachol.

2. Carbachol promoted the ^{86} Rb-efflux from left atrial strips in an atropine-, 4-aminopyridine- and pertussis toxin-sensitive manner. However, although 500 μM 4-aminopyridine completely attenuated the ability of carbachol to increase the rate-constant of ^{86} Rb-efflux, the direct negative inotropic response to carbachol was only partially inhibited by the same concentration of 4-aminopyridine. Similarly, pre-treatment of rabbits with pertussis toxin (0.5 and 1 μg/kg) shared almost complete attenuation of the ^{86} Rb-efflux-rate-constant increased by 1 μM carbachol, but the negative inotropic response to 1 μM carbachol was only partially attenuated. This suggests that only a part of the negative inotropic response to carbachol in rabbit left atrium is related to the ability of carbachol to open potassium channels.

3. The negative inotropic response to carbachol in the presence of phenylephrine was also attenuated in a concentration-dependent manner by 4-aminopyridine. Carbachol increased the rate constant of ^{86} Rb efflux in the presence of phenylephrine. Although 4-aminopyridine attenuated in a concentration-dependent manner the negative inotropic response to carbachol in the presence of phenylephrine, only 500 μM 4-aminopyridine slightly reduced the carbachol-induced increases in the ^{86} Rb-efflux-rate-constant in the presence of phenylephrine. In contrast, pre-treatment of rabbits with pertussis toxin (0.5

and 1 μg/kg) resulted in complete loss of the ability of carbachol to increase the ^{86}Rb-efflux-rate-constant and inhibit the positive inotropic responses of left atria to phenylephrine. Similarly, the potassium channel openers, pinacidil and cromakalim, attenuated in a concentration-dependent manner the positive inotropic responses of left atria to phenylephrine. Overall, the results suggest that the ability of carbachol to open potassium channels contributes to the negative inotropic response to carbachol in the presence of phenylephrine.

4. The negative inotropic responses of left atria to carbachol in the presence of isoproterenol was attenuated very modestly by 4-aminopyridine. Neither pinacidil nor cromakalim had any inhibitory effect on the isoproterenol-induced positive inotropy. This suggests that inhibition by carbachol of the isoproterenol-stimulated cAMP generation plays a dominant role in the functional interaction of carbachol with isoproterenol.

5. Injection of rabbits with 2.2 μg/kg pertussis toxin resulted in complete attenuation of the inhibitory effect of carbachol on the isoproterenol-stimulated adenylate cyclase activity and cAMP generation. Under this conditions, the negative inotropic effects of carbachol in the presence of forskolin and IBMX were only partially attenuated. Pertussis toxin pre-treatment, however, had a much greater inhibitory effect on the negative inotropic response to carbachol in the presence of isoproterenol. This suggests that inhibition of cAMP generation may play an important role in the isoproterenol-carbachol interaction, but not in the functional interaction of carbachol with forskolin and IBMX.

6. 4-Aminopyridine attenuated in a concentration-dependent manner the negative inotropic responses of left atria to carbachol in the presence of forskolin and IBMX,

suggesting that the ability of carbachol to open potassium channels contributes to the interaction of carbachol with forskolin and IBMX.

7. In atrial homogenates, carbachol inhibited the isoproterenol-stimulated adenylate cyclase in a concentration-dependent manner and this inhibition was sensitive to blockade by atropine. Carbachol did not have any inhibitory effect on the basal, forskolin- or GTPγS- stimulated adenylate cyclase activity. In contrast, in the ventricle carbachol inhibited not only the isoproterenol-stimulated but also basal, forskolin- and GTPγS- stimulated enzyme activity. This suggests that the mechanism of muscarinic inhibition of adenylate cyclase may differ between the atrium and ventricle.

8. In atrial homogenates from rabbits pre-treated with 0.5 and 1 μg/kg pertussis toxin, which ADP-ribosylated 68 - 70 % of G-proteins, carbachol lost its inhibitory effect on the isoproterenol-stimulated adenylate cyclase. The same dose of pertussis toxin shifted the carbachol-concentration-response curves for the ^{86} Rb-efflux to the right without inhibiting the maximum response. On the other hand 1 μg/kg pertussis toxin, which ADP-ribosylated 60 % of G-proteins in the ventricle, did not have any inhibitory effect on either basal or isoproterenol-stimulated adenylate cyclase activity in the presence of carbachol. These results suggest that inhibition of adenylate cyclase in atria by carbachol is more sensitive to uncoupling by pertussis toxin than either the same response to carbachol in ventricles or the ability of carbachol to open potassium channels in left atria.

5.2. CONCLUSIONS

The results obtained in the present study suggest that:

(a) the ability of carbachol to open potassium channels in left atria contributes to the cAMP-independent component of the negative inotropic response to carbachol in the presence of the cAMP-elevating and cAMP-independent positive inotropic agents.

(b) The direct negative inotropic response to carbachol in rabbit left atria is related, in part, to the ability of carbachol to open potassium channels. However, the contribution of some effector in addition to potassium channels to the negative inotropic response to carbachol cannot be ruled out.

(c) Inhibition by carbachol of adenylate cyclase in atria is more sensitive to uncoupling by pertussis toxin than either the ability of carbachol to open potassium channels in atria or the ability of carbachol to inhibit adenylate cyclase in the ventricle.

(d) Carbachol can inhibit only the isoproterenol-stimulated adenylate cyclase in the atrial myocardium, whereas in the ventricle carbachol inhibits not only the isoproterenol-stimulated but also basal, forskolin- and GTPγS- stimulated adenylate cyclase activity. This suggests that the mechanism of coupling of muscarinic receptors and adenylate cyclase may differ between the atrium and ventricle.

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