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AGONIST-INDEPENDENT EFFECTS OF MUSCARINIC ANTAGONISTS ON Ca^{2+} AND K^+ CURRENTS IN FROG AND RAT CARDIAC CELLS

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SUMMARY

1. The whole-cell patch clamp and intracellular perfusion techniques were used for studying the effects of atropine and other muscarinic acetylcholine receptor (mAChR) antagonists on the L-type calcium currents (I_{Ca}) in frog and rat ventricular myocytes, and on the mAChR-activated K^+ current ($I_{K(ACh)}$) in frog atrial myocytes.

2. In frog ventricular myocytes, atropine (0·1 nM to 1 μM) reversed the inhibitory effect of acetylcholine (ACh, 1 nM) on I_{Ca} previously stimulated by isoprenaline (Iso, 2 μM), a β-adrenergic agonist. However, in the concomitant presence of Iso, ACh and atropine, I_{Ca} was > 50% larger than in Iso alone.

3. The effects of atropine were then examined in the absence of mAChR agonists. After a preliminary stimulation of I_{Ca} with Iso (0·1 or 2 μM), atropine induced a dose-dependent stimulation of I_{Ca}. EC_{50} (i.e. the concentration of atropine at which the response was 50% of the maximum) and E_{max} (i.e. maximal stimulation of I_{Ca} expressed as percentage increase in I_{Ca} with respect to the level in Iso alone) were respectively 0·6 nM and 35%. The stimulatory effect of atropine on I_{Ca} was not voltage dependent.

4. Atropine (1 μM) had no effect on frog I_{Ca} (i) under basal conditions, (ii) upon stimulation of I_{Ca} by the dihydropyridine agonist (−)-Bay K 8644 (1 μM), or (iii) when I_{Ca} had been previously stimulated by intracellular perfusion with cyclic AMP (3 μM). However, atropine increased I_{Ca} after a stimulation by forskolin (0·3 μM). Therefore, an increased adenylyl cyclase activity was required for atropine to produce its stimulatory effect on I_{Ca}.

5. The order of potency of mAChR antagonists to reverse the inhibitory effect of ACh on Iso elevated I_{Ca} in frog ventricle was atropine > AF-DX 116 ≫ pirenzepine. In the absence of ACh, mAChR antagonists produced their stimulatory effect on Iso elevated I_{Ca} with the same order of potency.

6. Intracellular substitution of Gpp(NH)p (5′-guanylylimidiphosphate) for GTP (420 μM) induced a strong inhibition of frog I_{Ca} in the presence of Iso (2 μM). This

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effect was attributed earlier to the spontaneous and irreversible activation of the GTP-binding regulatory protein (G protein), G_i, responsible for adenylyl cyclase inhibition. Atropine (1 μM) slowed down by a factor of 2 the rate of I_{Ca} inhibition induced by Gpp(NH)p.

7. In frog atrial myocytes, intracellular perfusion with 1 mM Gpp(NH)p induces spontaneous activation of $I_{K(ACh)}$. This effect was attributed earlier to the spontaneous and irreversible activation of the G protein, G_K. Atropine (1 μM) or propylbenzyl choline mustard (1 μM) slowed down by a factor of 2–3 the rate of spontaneous activation of $I_{K(ACh)}$.

8. In rat ventricular cells, atropine (1 μM) exerted also a stimulatory effect on I_{Ca}. However, unlike in frog myocytes, atropine enhanced both basal and Iso-stimulated I_{Ca} in rat.

9. It is concluded that, in the absence of mAChR agonist, atropine and other 'M2-selective' mAChR antagonists exert a stimulatory effect on I_{Ca} and an inhibitory effect on $I_{K(ACh)}$ after binding to the mAChR. Both effects are due to a reduction in the spontaneous activation of the G proteins, G_i and G_K, respectively. Therefore, in addition to displacing the agonist from its binding site, mAChR antagonists may induce a conformational change of the receptor which impairs spontaneous G protein activation by the receptor.

INTRODUCTION

The cardiac negative inotropic effect of acetylcholine (ACh) is due to two major phenomena: (i) activation of an inwardly rectifying K^+ channel current ($I_{K(ACh)}$) (Szabo & Otero, 1990) and (ii) inhibition of the L-type Ca^{2+} channel current (I_{Ca}) consecutive to adenylyl cyclase inhibition (Hartzell, 1988). Both effects are likely to be linked to a common muscarinic ACh receptor (mAChR), the M2 receptor (Hulme, Birdsall & Buckley, 1990). The binding of a muscarinic agonist to its receptor promotes the association between the mAChR and a GTP-binding regulatory protein (G protein), G_K or G_i, which induces the GDP–GTP exchange on the G protein and permits the dissociation of its subunits and the consecutive activation of $I_{K(ACh)}$ or inhibition of adenylyl cyclase, respectively (Gilman, 1987; Holmer & Homcy, 1991).

The classical mAChR antagonist atropine counteracts the effects of ACh on $I_{K(ACh)}$ and I_{Ca}. Although it is generally assumed that atropine behaves as a competitive antagonist, i.e. it displaces agonists from their binding site but does not alter the receptor–G protein interaction, there is evidence, however, that mAChR occupancy by atropine or other muscarinic antagonists is not entirely passive with regard to this interaction.

A first set of evidence comes from early electrophysiological studies of the effect of ACh on I_{Ca} in a multicellular preparation of frog atrium (Giles & Noble, 1976). These authors found that in the presence of ACh, which strongly inhibited basal I_{Ca}, atropine increased I_{Ca} to a level that was substantially larger than control (Giles & Noble, 1976). In this study, Giles & Noble (1976) also reported an increase in I_{Ca} upon application of atropine (10 nM to 10 μM) in the absence of ACh. They concluded that these effects were probably due to a blocking action of atropine on the muscarinic action of some endogenous ACh, which was possibly released from nerve terminals

present in the preparation (Giles & Noble, 1976). However, in a later study performed on isolated cardiac myocytes, atropine was also found to produce mAChR agonist-independent effects (Soejima & Noma, 1984) which could not be easily explained by a contamination by endogenous ACh. Indeed Soejima & Noma (1984) found that in isolated myocytes from rabbit atrium, atropine inhibits the spontaneous $I_{K(ACh)}$ single channel activity. However, this finding is contradicted by the observation that in guinea-pig atrial cells, basal activity of $I_{K(ACh)}$ channels was not affected by atropine (Okabe, Yatani & Brown, 1991). A second set of evidence for an intrinsic action of atropine comes from biochemical studies. For example, atropine has been found to dissociate complexes of mAChR and G protein in rat (Matesic & Luthin, 1991) and porcine heart membranes (Hilf, 1991), which could indicate a possible interaction between antagonist binding and basal mAChR activation of G proteins.

We decided to test this hypothesis in frog cardiac myocytes, because these cells have a strong agonist-independent activity of mAChR associated G proteins, G_K and G_i. Indeed, intracellular perfusion of frog atrial myocytes with hydrolysis-resistant GTP analogues (Gpp(NH)p or GTPγS (guanosine 5′-O-(3-thiotriphosphate)) produces a strong activation of $I_{K(ACh)}$ (Breitweiser & Szabo, 1988; Otero, Li & Szabo, 1991). Similarly, intracellular perfusion of frog ventricular myocytes with Gpp(NH)p or GTPγS, which has no effect on basal I_{Ca} (Fischmeister & Shrier, 1989; Parsons, Lagrutta, White & Hartzell, 1991), exerts a profound inhibition on I_{Ca} when adenylyl cyclase activity has been previously stimulated by isoprenaline (Iso) or forskolin (Parsons *et al.* 1991). In the present study, we examined the agonist-independent effects of atropine, as well as other mAChR antagonists, pirenzepine and AF-DX 116, on I_{Ca} measured in isolated ventricular myocytes from frog and, to a lesser extent, from adult rat. We also examined the effect of atropine on $I_{K(ACh)}$ and I_{Ca} in frog ventricular and atrial myocytes, respectively, under conditions when agonist-independent activities of the G proteins involved in both systems were amplified by intracellular perfusion with Gpp(NH)p. 'M2-selective' muscarinic antagonists were found to produce stimulatory effects on I_{Ca} and inhibitory effects on $I_{K(ACh)}$. Characterization of these effects unveils an inhibitory action of mAChR antagonists on basal G protein mediated inhibition of adenylyl cyclase or activation of $I_{K(ACh)}$. Thus, muscarinic antagonists interfere with the basal mAChR–G protein interaction, which challenges classical models of drug–receptor interactions. A preliminary report of some of these results has appeared earlier (Hanf, Li, Szabo & Fischmeister, 1992).

METHODS

Solutions and drugs

For the preparation of frog ventricular cells, the ionic composition of Ca^{2+}-free Ringer solution was (mM): 88·4 NaCl; 2·5 KCl; 23·8 $NaHCO_3$; 0·6 NaH_2PO_4; 1·8 $MgCl_2$; 5 creatine; 10 D-glucose; 1 mg ml^{-1} fatty acid-free bovine serum albumin (BSA; Sigma Chemical Co., St Louis, MO, USA), 50 i.u. ml^{-1} penicillin (Sigma Chemical Co.); 50 μg ml^{-1} streptomycin (Sigma Chemical Co.), pH 7·4 maintained with 95% O_2–5% CO_2. Storage Ringer solution was Ca^{2+}-free Ringer solution to which was added 0·9 mM $CaCl_2$; 10 μl ml^{-1} non-essential amino acid and vitamin solution (MEM; Sigma Chemical Co.). Dissociation medium was composed of Ca^{2+}-free Ringer solution to which was added 0·3 mg ml^{-1} trypsin (Sigma Chemical Co., Type III), 1–1·5 mg ml^{-1} collagenase B (Sigma Chemical Co.), and 10 μl ml^{-1} M199 medium (Sigma Chemical Co.).

For the preparation of frog atrial cells, the ionic composition of Ca^{2+}-free Ringer solution was (mM): 110 NaCl; 5·4 KCl; 1·0 $MgCl_2$; 10 Hepes; pH 7·2 adjusted with NaOH. Dissociation medium was composed of Ca^{2+}-free Ringer solution to which was added 1·2 mg ml^{-1} collagenase (Boehringer Mannheim Biochemicals, FRG) and 0·08 mg ml^{-1} protease (Type XIV, Sigma Chemical Co.).

For I_{Ca} recording, frog ventricular cells were bathed in a caesium Ringer solution which contained (mM): 88·4 (frog) or 108·4 (rat) NaCl; 20 CsCl; 10 Hepes; 0·6 NaH_2PO_4; 1·8 $MgCl_2$; 1·8 $CaCl_2$; 5 D-glucose; 5 sodium pyruvate; 3×10^{-4} (frog) or 5×10^{-2} (rat) tetrodotoxin (Sigma Chemical Co.); pH 7·4 adjusted with NaOH. The patch pipette (0·6–2·5 MΩ resistance) was filled with a caesium internal solution which contained (mM): 119·8 (frog) or 139·8 (rat) CsCl; 5 K_2EGTA; 4 $MgCl_2$; 5 Na_2CP; 3·1 Na_2ATP; 0·062 $CaCl_2$; 10 Hepes; pH 7·1 adjusted with KOH; 0·42 Na_2GTP or Na_2Gpp(NH)p added as indicated; pCa was 8·5.

For $I_{K(ACh)}$ recording, frog atrial cells were bathed in a potassium Ringer solution containing (mM): 90 NaCl; 2·5 KCl; 5·0 $MgCl_2$; 2·5 $CaCl_2$; 5×10^{-3} TTX; 20 Hepes; pH 7·4 adjusted with NaOH. The composition of the patch pipette solution was (mM): 80 potassium asparate; 30 KCl; 0·5 EGTA; 5·0 Hepes; 2·0 MgATP; 1 Gpp(NH)p; pH 7·4 adjusted with KOH.

The drugs used in the experiments were (−)-isoprenaline, acetylcholine, atropine, pirenzepine, forskolin, Gpp(NH)p (5′-guanylylimidodiphosphate), all from Sigma Chemical Co. Forskolin was prepared as a stock solution of 10 mM in anhydrous ethanol and an appropriate amount of ethanol was added to each solution so that the same ethanol concentration, corresponding to that present in the solution containing the highest concentration of forskolin, was present in all solutions tested. AF-DX 116 was a generous gift of Boehringer Ingelheim.

Preparations

Frog ventricular and atrial cells were isolated as described in Fischmeister & Hartzell (1986) and Breitweiser & Szabo (1988), respectively.

Briefly, ventricular cells were enzymatically dispersed from frog (*Rana esculenta*) ventricle. The frog was killed by decapitation and pithing; the heart was removed and washed in, and then perfused at 28 °C for 5 min with Ca^{2+}-free Ringer solution, which had been thoroughly oxygenated by gassing for 10 min with 95% O_2–5% CO_2. Subsequently, the heart was perfused for approximately 90 min with 20 ml recirculating dissociation medium. After this time the heart became soft and was placed in 5 ml storage Ringer solution. Auricle and bulbus arteriosis were discarded. The ventricle was then gently stirred and the cell suspension was filtered before the addition of 40 ml of storage Ringer solution. The cells were then stored at 4 °C for 1–48 h prior to the experiments.

Preparation of frog atrial cells followed a similar procedure except that the temperature was 30 °C. Solutions were buffered with Hepes and therefore oxygenated by bubbling with 100% O_2. The heart was perfused with Ca^{2+}-free Ringer solution for 25 min and with dissociation medium for approximately 30 min. The atrium was removed and suspended in low-Ca^{2+} Ringer (Ca^{2+}-free solution supplemented with 200 μM $CaCl_2$). The tissue was stored in the refrigerator for up to 2 days. Myocytes were obtained by gentle agitation of the tissue in the suspension medium.

Rat ventricular myocytes were dissociated from hearts of urethane-anaesthetized (1 ml of a stock urethane solution (0·2 g ml^{-1}) per 100 g rat weight) male Wistar rats (200–250 g) using the collagenase method as described earlier (Méry, Lohmann, Walter & Fischmeister, 1991). Using this procedure, the yield of rod-shaped viable cardiomyocytes is routinely ≈ 80%.

Experimental arrangements

The method used for whole-cell patch clamp recording, superfusion of the cells and data analysis have been extensively described in previous papers (Fischmeister & Hartzell, 1986; Méry *et al.* 1991) and were used with no major modification in the present study.

I_{Ca} was recorded in frog or rat ventricular cells by depolarizing the cell every 8 s to 0 mV during 200 or 400 ms from −80 mV holding potential. Solutions were applied to the exterior of the cell by placing the cell at the opening of 250 μm inner diameter capillary tubing from which solution was flowing at a rate of 10 μl min^{-1}. Solutions were applied to the interior of the cell via the patch electrode and could be modified by a system that permitted perfusion of the patch electrode with different solutions (see e.g. Méry *et al.* 1991). Currents were digitized at 10 kHz (16 bit A/D

converter) and analysed on-line by a Compaq 386 System-Pro computer using programs written in PASCAL language. For each cell, membrane capacitance (C_m) was recorded to give an estimate of the total calcium current density.

To record $I_{K(ACh)}$ in frog atrial cells, the membrane potential was stepped for 250 ms successively to −5 and −135 mV from a holding potential of −85 mV. Currents were measured as the average of the last 10 ms of the response to −5 mV pulses and plotted as a function of time.

Mean values ± S.E.M. are given in the text. Statistical analysis was performed with the computer program STATGRAPH (STSC Inc. Rockville, MD, USA). Differences between means were tested for statistical significance by Student's t test. Experiments on $I_{K(ACh)}$ were performed in Charlottesville and on I_{Ca} in Orsay. All experiments were carried out at room temperature (19–25 °C).

RESULTS

Stimulatory effect of atropine on I_{Ca}

Experiments such as the one illustrated in Fig. 1 prompted our interest in the study of the intrinsic action of atropine on cardiac cells. As shown earlier, exposure of a frog ventricular cell to the β-adrenergic agonist isoprenaline (Iso) strongly enhances I_{Ca} and this effect is suppressed by nanomolar concentrations of ACh (Fischmeister & Hartzell, 1986). A 1 nM concentration of ACh produced a 38 ± 8 % (mean ± S.E.M., $n = 9$) significant ($P < 0.01$) inhibition of I_{Ca} elevated by 2 μM Iso. Surprisingly, when as low as 0·1 nM atropine was applied to the cell in the presence of Iso (2 μM) and ACh (1 nM), the amplitude of I_{Ca} reached a level which was substantially larger than in Iso alone (Fig. 1). A further increase in atropine concentration to 1 nM additionally increased I_{Ca}. At a saturating concentration of 1 μM in the presence of both Iso and ACh, atropine increased I_{Ca} by 56 ± 18 % ($n = 4$) over its level in Iso alone (see e.g. Fig. 5). These experiments provide a single cell parallel to the findings of Giles & Noble (1976) obtained in multicellular frog atrial preparations.

These initial results could imply that nanomolar concentrations of atropine induce an over-recovery of I_{Ca} from ACh inhibition. To examine whether this novel phenomenon required the previous stimulation of mAChRs, subsequent experiments were performed in the absence of ACh. As shown in Fig. 2*A*, atropine (100 nM) had no effect on basal I_{Ca}. On average, basal I_{Ca} density was 2·6 ± 0·4 pA pF^{-1} ($n = 9$), and was not significantly affected by 1 μM atropine (Table 1). This absence of effect of large concentrations of atropine under basal conditions contrasted with the strong stimulatory effect of nanomolar concentrations of atropine on Iso-stimulated I_{Ca} (Fig. 2*B*). Moreover, when atropine (100 nM) was applied to a given cell successively under basal and stimulated conditions, atropine increased I_{Ca} only when the current had been previously elevated by Iso (Fig. 2*A*).

The stimulatory effect of various concentrations of atropine was examined on I_{Ca} stimulated by 2 μM Iso. As summarized by the results of several experiments (Fig. 2*B*), the effects of atropine were dose dependent. The dose–response curve was fitted using a non-linear least-mean-squares regression of the means to the Michaelis equation. Maximal stimulation of I_{Ca}, (E_{max}) and concentration of atropine required for half-maximal stimulation of I_{Ca}, (EC_{50}) were derived from this analysis. EC_{50} and E_{max} were respectively 0·6 ± 0·5 nM and 35 ± 8 %. When a submaximal concentration of Iso (0·1 μM, see Table 1) was used to stimulate I_{Ca} prior to atropine application, the stimulatory effect of 1 μM atropine on I_{Ca} was not significantly different from the

degree of stimulation induced by atropine in the presence of a twentyfold higher concentration of Iso (Table 1). Therefore, like the inhibitory effect of ACh (Fischmeister & Shrier, 1989), the stimulatory effect of atropine on Iso-stimulated I_{Ca} did not depend on the degree of Iso stimulation.

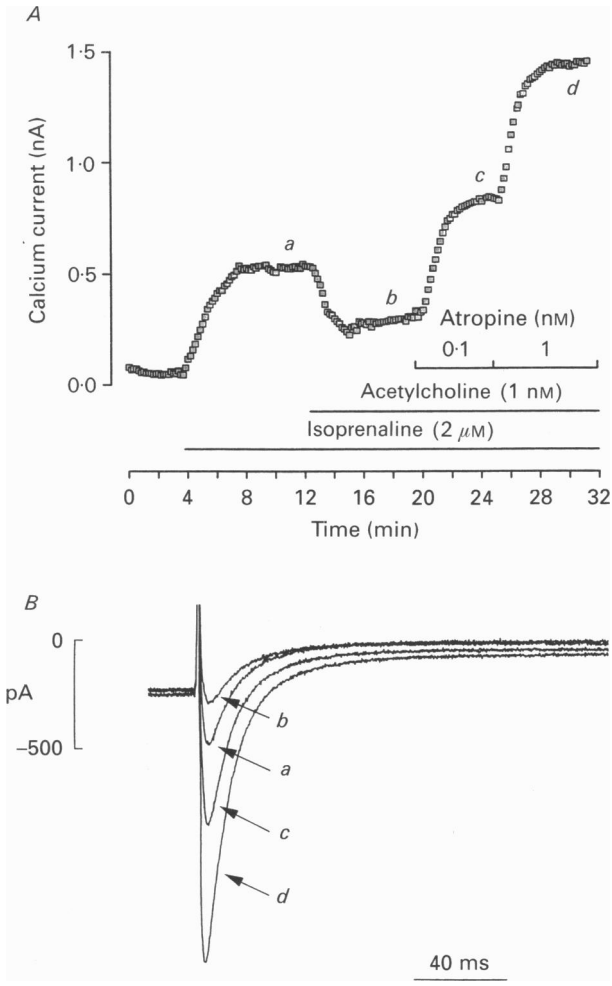

Fig. 1. Atropine induces an 'over-recovery' from ACh inhibition of I_{Ca} in frog ventricular cells. The cell was initially superfused with control caesium Ringer solution. During the periods indicated, the cell was successively exposed to isoprenaline (Iso, 2 μM), acetylcholine (ACh, 1 nM), and to successively increasing concentrations of atropine (0·1 and 1 nM) in the presence of Iso and ACh. I_{Ca} was the difference between the peak current and the current at the end of the 400 ms pulse. Each square corresponds to a measure of I_{Ca} at 0 mV, obtained every 8 s (for more details see Methods). *B*, current traces were obtained at the times indicated by the corresponding letters in *A*.

Involvement of adenylyl cyclase activity in the stimulatory effect of atropine on I_{Ca}

To examine whether atropine stimulation simply required an elevated I_{Ca} in order for the stimulatory effect of atropine to be resolved, we used means other than β-adrenergic agonists to produce a strong elevation of I_{Ca}. In a first set of experiments I_{Ca} was increased by an application of 1 μM of the dihydropyridine agonist (−)-Bay

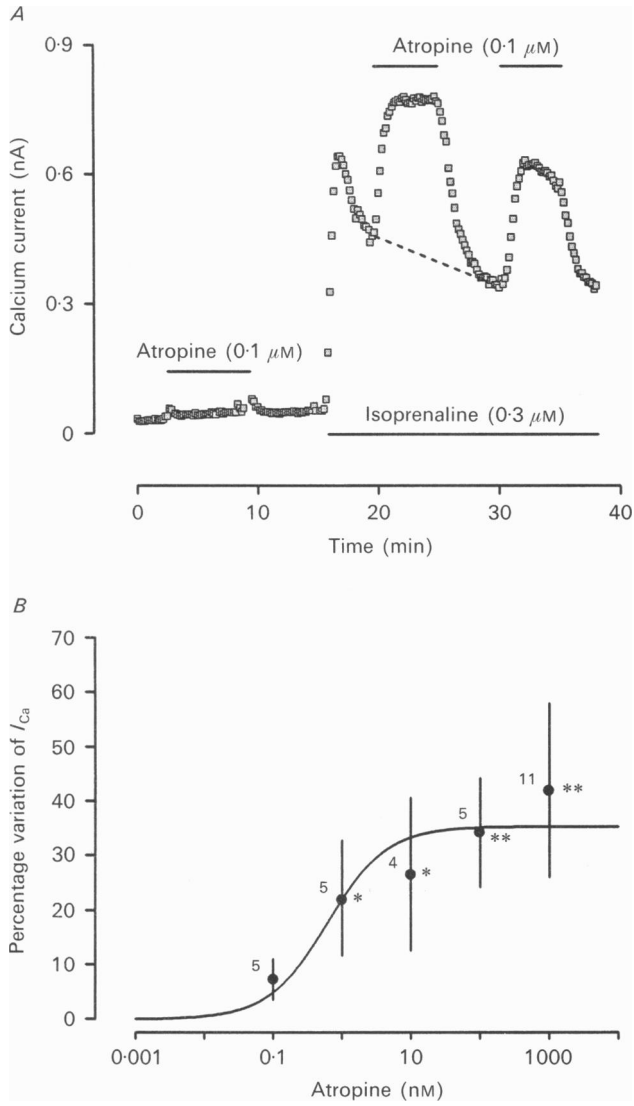

Fig. 2. Effects of atropine on basal I_{Ca} and I_{Ca} elevated by isoprenaline. *A*, the frog ventricular cell was initially superfused with control caesium Ringer solution. During the periods indicated, the cell was successively exposed to atropine (0·1 μM), isoprenaline (0·3 μM), or Iso + atropine. The dashed line extrapolates I_{Ca} run-down in Iso before first atropine exposure. *B*, dose–response curve for the effect of atropine on I_{Ca} in the presence of 2 μM isoprenaline. The points show the means ± S.E.M. of the number of cells indicated near the symbols. Each determination involved the measurement of the amplitude of I_{Ca} in Iso (2 μM) and then in the presence of a concentration of atropine indicated on the *X*-axis. In some cells, an entire dose–response curve was obtained by sequential superfusion with increasing concentrations of atropine, whereas in other cells only one or several atropine concentrations were tested. The response to atropine is expressed as a percentage variation of I_{Ca} with respect to the level in Iso alone. The continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation: effect $= E_{max}$ [atropine]/([atropine] + EC_{50}). The numerical values derived for E_{max} and EC_{50} are given in the text. Asterisks indicate a significant difference between points and the zero value at the 0·1 (*) or 0·05 (**) levels (*t* test).

K 8644. Although the stimulation of I_{Ca} induced by (−)-Bay K 8644 was on average 66% of the level reached by a stimulation by 0·1 μM Iso, atropine (1 μM) had no effect in the presence of the dihydropyridine (Table 1). The absence of effect of atropine on basal I_{Ca} or I_{Ca} elevated by a dihydropyridine agonist suggests that the stimulatory effect of atropine does not involve a direct interaction with the calcium channel.

TABLE 1. Effect of atropine on I_{Ca} in frog ventricular cells

Initial conditions	Number of cells	dI_{Ca} (pA pF^{-1}) (no atropine)	Percentage increase by 1 μM atropine
Control	9	2·6±0·4	3·1±11
(−)-Bay K 8644 (1 μM)	5	11·4±1·7	1·7±0·9
Cyclic AMP (3 μM inside)	5	15·9±3·3	−3·0±0·9
Isoprenaline (0·1 μM)	11	17·3±2·5	35±9*
Isoprenaline (2 μM)	11	25·3±3·9	42±16*
Forskolin (0·3 μM)	6	15·7±3·5	76±15**
Forskolin (3 μM)	4	31·0±2·9	13·6±11

Data are means±S.E.M. For each condition, the effect of atropine (1 μM) is expressed as percentage change with respect to I_{Ca} in the initial condition. The I_{Ca} density (dI_{Ca}) is given prior to atropine application. Statistical significance was determined using a one-sample analysis t test when comparing the mean percentage change values to zero: *, $P < 0·05$; **, $P < 0·01$.

More likely, stimulation of adenylyl cyclase activity seemed to be required for atropine to stimulate I_{Ca}. This hypothesis was further supported by the absence of effect of atropine on I_{Ca} elevated by intracellular perfusion with cyclic AMP (3 μM), when adenylyl cyclase activity was bypassed (Table 1). These experiments rule out the action of atropine on other mechanisms, downstream from the locus of cyclic AMP production, such as phosphodiesterase or phosphatase activities, which might be involved in the cyclic AMP regulation of I_{Ca}.

Atropine effect on forsokolin-stimulated I_{Ca}

The diterpene forskolin, when used at concentrations > 100 nM, is known to directly activate adenylyl cyclase activity in a variety of tissues, and thus, to stimulate I_{Ca} in a number of cardiac preparations, including frog ventricular cells (Fischmeister & Shrier, 1989). Consistent with an action on adenylyl cyclase activity, atropine also reversibly stimulated forskolin elevated I_{Ca} (Fig. 3). However, the stimulatory effect of 1 μM atropine on I_{Ca} was much smaller and not significant in the presence of a large concentration of forskolin (3 μM), compared to its large effect in the presence of a ten times lower forskolin concentration (Table 1). This contrasted with the effects of atropine on Iso-elevated I_{Ca} (see above), and shared some common features with the apparent competitivity of the effects of forskolin and ACh on I_{Ca} in the same preparation (Fischmeister & Shrier, 1989).

Voltage dependence of the stimulatory effect of atropine on I_{Ca}

The voltage dependence of the stimulatory effect of 1 μM atropine on Iso-elevated I_{Ca} was examined. As shown in Fig. 4*A*, atropine stimulated Iso (2 μM)-elevated I_{Ca} without causing a significant change in the shape of the current–voltage relationship

of either I_{Ca} or the current (I_{200}) measured at the end of the 200 ms depolarizing pulse. Therefore, the stimulatory effect of atropine appeared to be essentially independent of membrane potential. The lack of effect of atropine on I_{200} also suggests that stimulation of I_{Ca} was not mediated by a perturbation in membrane structure.

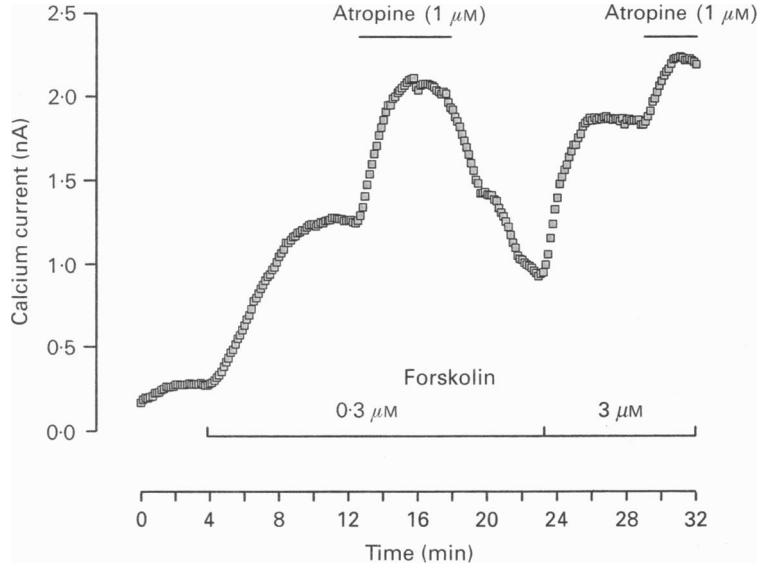

Fig. 3. Effect of atropine on I_{Ca} elevated by forskolin. The frog ventricular cell was initially superfused with control caesium Ringer solution. During the periods indicated, the cell was successively exposed to forskolin (0·3 or 3 μM), and atropine (1 μM) in the presence of forskolin.

Inactivation of I_{Ca} was determined by examination of the effect of 200 ms-duration prepulses to various potentials on the response to a subsequent test pulse to 0 mV (Fischmeister & Hartzell, 1986). The shape of the inactivation curve (Fig. 4*B*) was similar to that which has been previously described (Fischmeister & Hartzell, 1986). As shown in Fig. 4*B*, atropine did not significantly modify the inactivation curve of I_{Ca}. Moreover, atropine did not modify the time course of I_{Ca} at 0 mV (see e.g. individual current traces in Fig. 1). These results indicate that atropine mainly modifies the number of functional Ca^{2+} channels without modifying the voltage dependence and inactivation properties of the functioning channels.

Effects of various selective muscarinic antagonists on I_{Ca}

Several subtypes of mAChRs have been previously described. With the exception of chick heart (Tietje, Goldman & Nathanson, 1990), essentially M2 receptors are involved in the cardiac negative chronotropic and inotropic effects of ACh (Hulme *et al.* 1990). However, to our knowledge, the subtype(s) of mAChR(s) has(ve) not been characterized yet in frog heart. For this reason, we examined the antagonistic effects of two different 'selective' mAChR antagonists, pirenzepine (an 'M1-selective' antagonist) and AF-DX 116 (an 'M2-selective' antagonist) (Hulme *et al.* 1990), in comparison with the effects of atropine, in an attempt to characterize the

pharmacological profile of the mAChR involved in the regulation of I_{Ca} by ACh in frog myocytes. The potencies of the three mAChR antagonists, used at a 1 μM concentration, to reverse the inhibitory effect of 10 nM ACh on Iso (2 μM)-elevated I_{Ca} are compared in Fig. 5. It appears that the order of potency for the inhibition of the

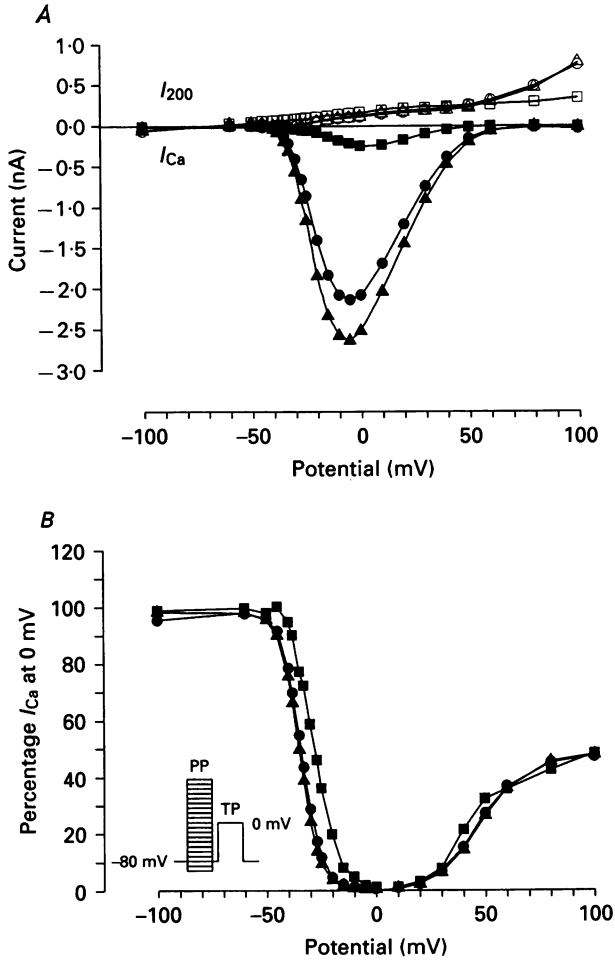

Fig. 4. Voltage dependence of the stimulatory effect of atropine on I_{Ca}. *A*, current–voltage relationship of I_{Ca} (filled symbols) and steady-state leak current (I_{200}) (open symbols) measured during 200 ms depolarizations to various potentials in a frog ventricular cell extracellularly superfused with control solution (squares), 2 μM isoprenaline (circles) and 2 μM isoprenaline + 1 μM atropine (triangles). I_{Ca} and I_{200} were measured as previously described (Fischmeister & Hartzell, 1986). *B*, inactivation curve of I_{Ca}. A double-pulse protocol was used as indicated in the inset. I_{Ca}, measured during a 200 ms test pulse (TP) at 0 mV, is plotted as a function of prepulse (PP) potential (200 ms duration) and expressed as a percentage of I_{Ca} at 0 mV in the absence of prepulse. Squares, control; circles, 2 μM isoprenaline; triangles, 2 μM isoprenaline + 1 μM atropine.

effect of ACh on I_{Ca} clearly was atropine > AF-DX 116 ≫ pirenzepine. Thus, the inhibitory effect of ACh on I_{Ca} is probably due to an activation of the M2 muscarinic receptor subtype.

Figure 5 also shows that, of the three mAChR antagonists used, only atropine was capable of inducing an 'over-recovery' from ACh inhibition at 1 μM concentration. However, when used in the absence of ACh, AF-DX 116, like atropine, potentiated the response of I_{Ca} to 2 μM Iso (Fig. 6*A*). The results of several similar experiments

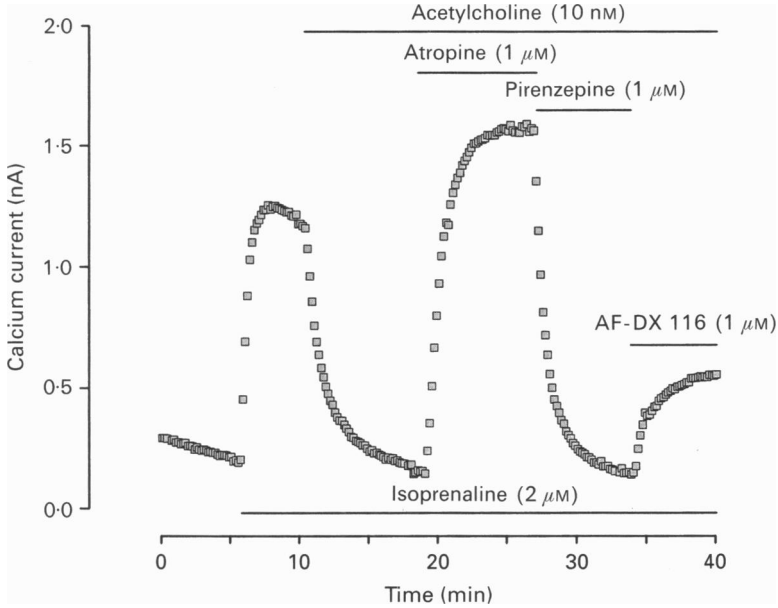

Fig. 5. Comparison of the potencies of AF-DX 116, pirenzepine, and atropine to reverse the inhibitory effect of ACh on I_{Ca}. The frog ventricular cell was initially superfused with control caesium Ringer solution. During the periods indicated, the cell was successively exposed to Iso (2 μM), Iso + ACh (10 nM), Iso + ACh + atropine (1 μM), Iso + ACh + pirenzepine (1 μM), and Iso + ACh + AF-DX 116 (1 μM).

are summarized in Fig. 6*B*. AF-DX 116 induced a dose-dependent stimulation of I_{Ca} previously elevated by 2 μM Iso. I_{Ca} density obtained when 10 μM AF-DX 116 and 2 μM Iso were applied in combination was about 50% higher than that obtained in the presence of Iso alone (31 ± 5 pA pF^{-1}, $n = 3$ *vs.* 20 ± 1 pA pF^{-1}, $n = 17$). The dose–response curve for the effects of AF-DX 116 on Iso-elevated I_{Ca} was fitted using a non-linear least-mean-squares regression of the means to the Michaelis equation. EC_{50} and E_{max} were respectively 732 ± 576 nM and 54 ± 10%. Therefore, AF-DX 116 was about 1000 times less potent than atropine in stimulating Iso-elevated I_{Ca}, but the maximal stimulations induced by both mAChR antagonists were comparable. On the contrary, pirenzepine had no effect on Iso-elevated I_{Ca}, even when used at 10 μM concentration (not shown). Therefore, the order of potency for mAChR antagonists to stimulate Iso-elevated I_{Ca} was atropine > AF-DX 116 $\gg$ pirenzepine, i.e. identical to the order of potency of these compounds to inhibit the effects of ACh. This strongly suggests that the stimulatory effect of atropine on I_{Ca} is due to its interaction with the mAChR responsible for the inhibitory effect of ACh.

Effects of atropine on Gpp(NH)p-induced inhibition of Iso-elevated I_{Ca}

Our results so far suggest that the stimulatory effect of atropine on I_{Ca} is due to binding of atropine to the mAChR and stimulation of adenylyl cyclase activity. Since ACh inhibition of I_{Ca} is due to inhibition of adenylyl cyclase caused by mAChR-

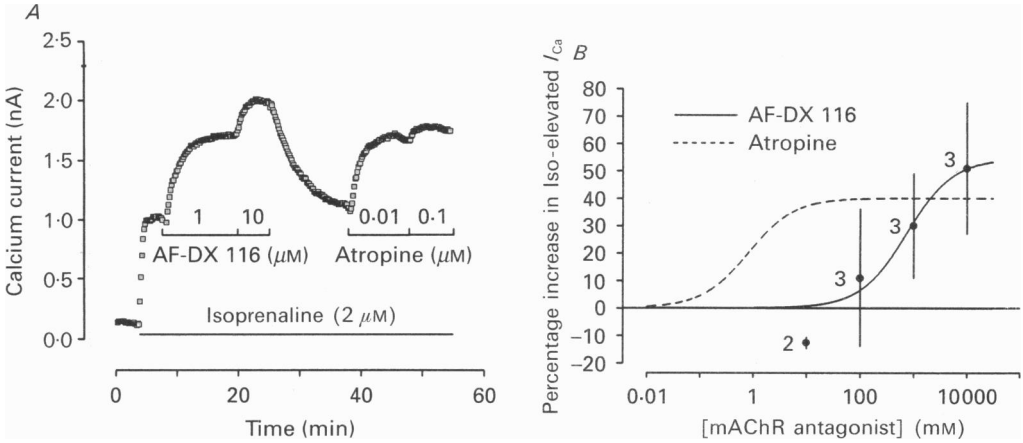

Fig. 6. Comparative potencies of AF-DX 116 and atropine to stimulate I_{Ca}. *A*, the frog ventricular cell was initially superfused with control caesium Ringer solution. During the periods indicated, the cell was exposed to isoprenaline (2 μM), and then to increasing concentrations of AF-DX 116 (1 and 10 μM) or atropine (10 and 100 nM) in the presence of isoprenaline. *B*, dose–response curve for the effect of AF-DX 116 on I_{Ca} in the presence of 2 μM isoprenaline. The points show the means ± S.E.M. of the number of cells indicated near the symbols. The response to AF-DX 116 is expressed as a percentage of variation of I_{Ca} with respect to the level with isoprenaline alone. The continuous line was derived from a non-linear least-mean-squares regression of the means to the Michaelis equation: effect = E_{max} [atropine]/([atropine] + EC_{50}). The numerical values derived for E_{max} and EC_{50} are given in the text. The dashed line represents the fit to the dose–response curve for the effect of atropine on Iso-elevated I_{Ca}, and was redrawn from Fig. 2*B* for comparison.

induced activation of G protein, G_i, it is tempting to assume that the mechanism of atropine action is a mirror image of that of ACh. The following experiments were aimed at examining the possible participation of G_i in the stimulatory effect of atropine on I_{Ca}.

The hydrolysis-resistant GTP analogue, Gpp(NH)p, was used to amplify the agonist-independent activity of G_i. Indeed, it was recently demonstrated that intracellular perfusion of a frog ventricular cell with Gpp(NH)p induces a strong inhibition of I_{Ca} previously elevated by Iso or forskolin, and that this effect is due to the irreversible activation of G_i and inhibition of adenylyl cyclase (Parsons *et al.* 1991). In the experiment shown in Fig. 7, all intracellular GTP (420 μM) was substituted by Gpp(NH)p after I_{Ca} had been increased by 0·1 μM Iso and started to decline slowly (spontaneous run-down: 17·8 ± 6·2 pA min^{-1} from a maximal I_{Ca} of 1344 ± 164 pA, n = 9). About 3 min after its introduction in the intracellular perfusion system, Gpp(NH)p started to inhibit I_{Ca} as evidenced by the accelerated decline of I_{Ca}

(average: 168 ± 33 pA min^{-1}, $n = 4$, measured 6 ± 4 min after Gpp(NH)p application). External application of 1 μM atropine in the early phase (7 ± 4 min) of Gpp(NH)p action increased I_{Ca} and significantly ($P < 0{\cdot}01$) slowed down, by a factor of two, the rate of inhibition of I_{Ca} (average: 74 ± 18 pA min^{-1}, $n = 4$, measured

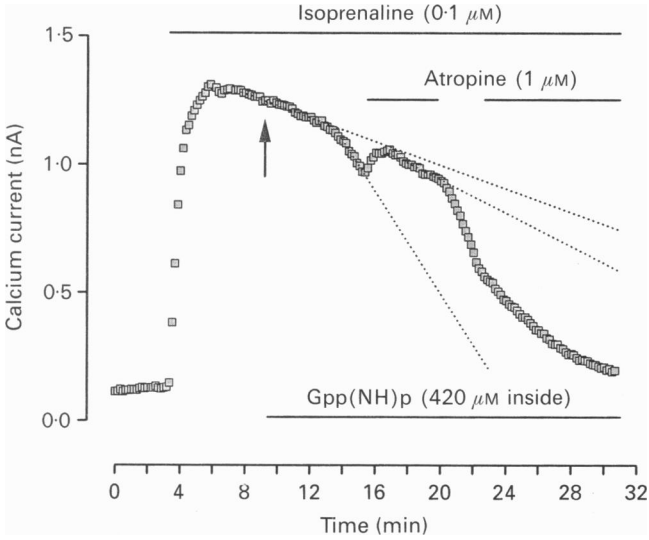

Fig. 7. Effect of atropine on Gpp(NH)p-mediated inhibition of Iso elevated I_{Ca}. The frog ventricular cell was initially superfused with control caesium Ringer solution and intracellularly perfused with control (GTP-containing) solution. During the periods indicated the cells were superfused with Iso (0·1 μM) and at the various times indicated atropine (1 μM) was added to the Iso-containing solution. At the arrow, all intracellular GTP (420 μM) was substituted by Gpp(NH)p which perfused the cell for the rest of the experiment. The dotted lines illustrate the rate of I_{Ca} run-down or Gpp(NH)p-mediated inhibition of I_{Ca}, either in the presence or absence of atropine.

3 ± 4 min after atropine application) which remained about four times faster than in GTP. The effect of atropine depended on the timing of application. Indeed, when atropine was applied first, a subsequent substitution of internal Gpp(NH)p for GTP had a much less pronounced inhibitory effect on I_{Ca}. In seven such experiments where Gpp(NH)p was applied in the presence of atropine, I_{Ca} declined at a rate (32 ± 16 pA min^{-1}, measured 11 ± 2 min after application of Gpp(NH)p) which was not significantly different from the spontaneous run-down seen with internal GTP (Iso alone: 14 ± 7 pA min^{-1}; Iso + atropine: 19 ± 9 pA min^{-1}). On the contrary, when applied in the late phase of Gpp(NH)p perfusion, atropine had a negligible effect on I_{Ca} (Fig. 7; see Discussion).

Effects of atropine on Gpp(NH)p-induced activation of $I_{K(ACh)}$

Intracellular application of the hydrolysis-resistant GTP analogue Gpp(NH)p leads to the gradual appearance of $I_{K(ACh)}$ in atrial myocytes. The extent of the channel activation reflects the degree of persistent G_K activation in the cell membrane (Breitwieser & Szabo, 1988). Atropine decreased the rate of this basal, agonist-

independent activation process. Figure 8 illustrates that the decrease is seen both when atropine is applied continually in the bathing solution and also when it is applied during the course of $I_{K(ACh)}$ activation.

Figure 8*A* shows representative current traces normalized to the maximal, steady-state response. Trace *b*, obtained in the presence of 1 μM atropine rises more slowly

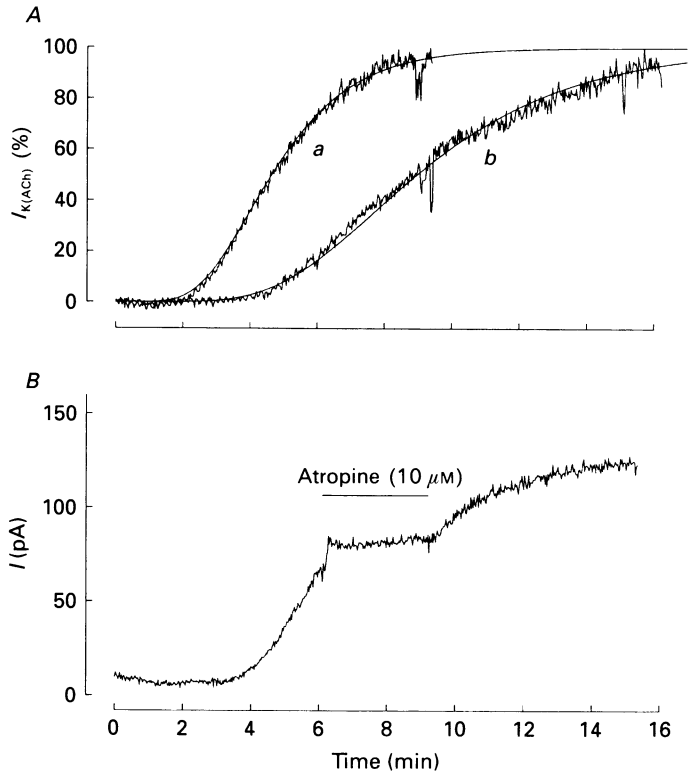

Fig. 8. Effect of atropine on the rate of Gpp(NH)p-induced, agonist-independent activation of $I_{K(ACh)}$ in frog atrial cells. Currents measured at 1·5 s intervals at the end of a 250 ms pulse to −5 mV are plotted as a function of time, starting from the moment whole-cell configuration was obtained. The pipette solution contained 1 mM Gpp(NH)p. *A*, the traces represent the time course of $I_{K(ACh)}$ for two different cells, one bathed in normal potassium Ringer solution and the other in Ringer solution containing 1 μM atropine. *B*, upon application of 10 μM atropine at 6 min in a different cell, the rate of $I_{K(ACh)}$ activation decreased significantly but resumed near its original value following atropine wash-out.

than the control trace (*a*). These differences could be summarized quantitatively by noting that the time course of $I_{K(ACh)}$ activation is well fitted by the equation:

$$I_{K(ACh)} = (1-\exp(1-t/\tau-\exp(-t/\tau)))^4, \quad (1)$$

where t is the time following Gpp(NH)p application and τ is a parameter that measures the speed of the persistent $I_{K(ACh)}$ activation (K. Manivanan & G. Szabo, unpublished observations). The smooth lines in Fig. 8*A*, drawn using eqn (1) with τ = 1·7 and 3·2 min respectively for traces *a* and *b*, illustrate the validity of this

procedure. While the speed of agonist-independent activation varied among experimental animals, for a given preparation it was always slower when atropine was present in the bathing solution. Thus in one set of experiments atropine slowed the time course of spontaneous activation by a factor of 3·1 ($\tau = 0{\cdot}70 \pm 0{\cdot}14$ min (n

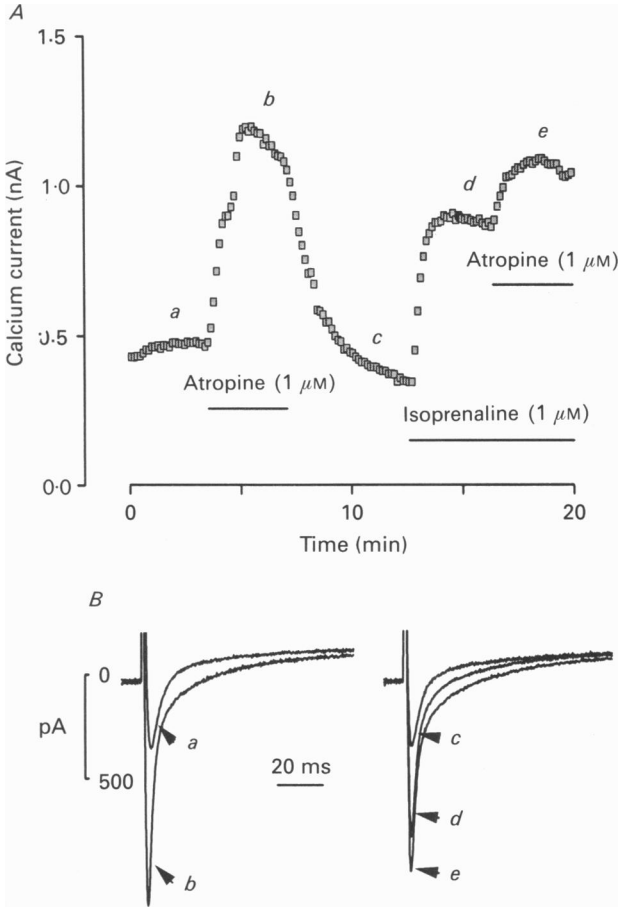

Fig. 9. Effect of atropine on I_{Ca} in rat. The rat ventricular cell was initially superfused with control caesium Ringer solution ('rat' composition, see Methods). During the periods indicated, the cell was successively exposed to atropine (1 μM), isoprenaline (2 μM), or Iso + atropine. Current traces shown in *B* were obtained at the times indicated by the corresponding letters in *A*.

= 3) for control cells and $\tau = 2{\cdot}17 \pm 0{\cdot}68$ min ($n = 3$) for 1 μM atropine) while in another preparation τ increased by a factor of 1·8 ($\tau = 1{\cdot}8 \pm 0{\cdot}29$ min ($n = 3$) for control cells and $\tau = 3{\cdot}33 \pm 0{\cdot}35$ min ($n = 2$) for 1 μM atropine). Application of Student's t test indicates that for both cases the effect of atropine is significant ($P < 0{\cdot}05$). A similar and significant 2·3-fold slow-down in the time course of spontaneous activation was observed with the irreversibly bound muscarinic antagonist propylbenzyl choline mustard ($\tau = 1{\cdot}17 \pm 0{\cdot}15$ min ($n = 2$) for control cells and $\tau = 2{\cdot}74 \pm 0{\cdot}84$ min ($n = 3$) for treated cells, $P < 0{\cdot}05$).

Figure 8*B* demonstrates that atropine slows down spontaneous activation even when it is applied during the course of the activation process, following intracellular dialysis with Gpp(NH)p. Again, while there was variability among preparations, atropine always slowed the activation process. Taken together, these data demonstrate that atropine significantly decreases the rate of G_K spontaneous activation in frog atrial cells.

Effect of atropine on mammalian cardiac I_{Ca}

Phophodiesterase inhibitors enhance I_{Ca} in frog cardiac myocytes only after a previous stimulation of adenylyl cyclase or intracellular perfusion with cyclic AMP (Fischmeister & Hartzell, 1990). However, in rat and guinea-pig cardiac myocytes, these compounds stimulate I_{Ca} without any preliminary increase in cyclic AMP concentration (see e.g. Méry *et al.* 1991). This could imply that the basal cellular level of cyclic AMP is larger in mammalian cardiac myocytes than in amphibian cells, possibly because of a larger basal activity of adenylyl cyclase in mammalian heart cells. Basal activity of adenylyl cyclase reflects to some extent the relative proportion of spontaneous activation of the two G proteins, G_s and G_i, which interact with this enzyme. It was, therefore, interesting to compare the effect of atropine on I_{Ca} in rat and frog cardiac cells.

Figure 9 shows the effect of atropine on basal and Iso-stimulated I_{Ca} measured in an isolated ventricular myocyte from rat. Unlike the situation in frog cells, atropine (1 μM) had a strong stimulatory effect on basal I_{Ca} ($66 \pm 23\%$, $n = 6$, $P < 0.05$). In the presence of 1 μM Iso, which stimulated rat I_{Ca} by $104 \pm 31\%$ ($n = 7$), addition of atropine induced a further increase in I_{Ca} by $11 \pm 3\%$ ($n = 7$, $P < 0.05$).

DISCUSSION

Our results demonstrate that, in the absence of mAChR agonist, muscarinic antagonists exert intrinsic action on I_{Ca} and $I_{K(ACh)}$ in cardiac myocytes. As will be outlined in the discussion below, our findings are best interpreted by the two following assumptions: (1) an agonist-independent/mAChR-dependent mechanism mediates a spontaneous activation of G_i, as has been recently suggested for G_K by Ito, Sugimoto, Kobayashi, Takahashi & Kurachi (1991) and Okabe *et al.* (1991). As a result, cardiac adenylyl cyclase is under a tonic G_i-mediated inhibition which holds back its basal and/or hormonally stimulated activity; (2) in addition to displacing muscarinic agonists from their binding site on the mAChR, atropine and 'M2-selective' antagonists exhibit intrinsic negative activity on the cardiac mAChR which leads to a reduced interaction between the receptor and the corresponding G proteins, G_i and G_K. Although the question has not been addressed directly in the present study, we will consider that G_i and G_K are separate entities. This is only for the sake of clarity, since none of our conclusions is dependent on that assumption.

Contamination by endogenous ACh?

The effects of atropine on I_{Ca} and $I_{K(ACh)}$ could be explained if the mAChR was occupied by an intrinsic ACh molecule. Indeed, addition of an antagonist would result in the displacement of the agonist from the receptor, and, thereby, remove a

tonic inhibition of I_{Ca} and a tonic activation of $I_{K(ACh)}$. However this hypothesis, which was initially proposed to account for the effects of atropine in multicellular cardiac preparations (Giles & Noble, 1976), was discarded for several reasons. (1) The stimulatory effect of atropine on I_{Ca} was reversible (see e.g. Figs 2*A* and 3). This, however, may not be a strong enough argument in case the cell would secrete its own mAChR agonist. (2) The latter seems improbable because the amount of agonist a single cell could produce is unlikely to be sufficiently high to activate mAChRs, especially because the cell is constantly superfused at a relative rapid rate (10 μl min^{-1}, with a linear flow velocity of $\approx$ 0·2 cm s^{-1}). (3) In frog cardiac cells, the sensitivity of I_{Ca} to ACh inhibition is in the nanomolar range (Giles & Noble, 1976; Nargeot, Garnier & Rougier, 1981; Fischmeister & Hartzell, 1986), while concentrations of ACh needed to activate $I_{K(ACh)}$ are about one to two orders of magnitude higher (Giles & Noble, 1976; Nargeot *et al.* 1981; Breitweiser & Szabo, 1988). Therefore, if the concentration of ACh around the cell was sufficient to activate $I_{K(ACh)}$, that concentration would have to be > 10 nM, which is incompatible with the strong inhibitory effect on Iso-elevated I_{Ca} observed when adding exogenous ACh at a 1 nM concentration (Fischmeister & Hartzell, 1986; Fig. 1).

Adenylyl cyclase activity is tonically inhibited by G_i

Regulation of adenylyl cyclase activity by G protein-coupled receptors, such as β-adrenergic receptors and mAChRs, involves two different G proteins, respectively G_s and G_i, which regulate adenylyl cyclase activity in opposite manner (Gilman, 1987; Holmer & Homcy, 1991). G_s and G_i are heterotrimers which dissociate upon receptor activation into α_s-GTP and α_i-GTP and $\beta\gamma$ subunits (Gilman, 1987). It is widely accepted that α_s-GTP and/or $\beta\gamma$ directly interacts with the catalytic unit of adenylyl cyclase to stimulate cyclic AMP production. However, it is still unclear which of α_i-GTP or $\beta\gamma$ subunits conveys the inhibition of adenylyl cyclase activity *in vivo* (Gilman, 1987; Reithmann, Gierschik, Sidiropoulos, Werdan & Jakobs, 1989; Wong, Federman, Pace, Zachary, Evans, Pouyssegur & Burp, 1991). Also it remains unclear to what extent G proteins sustain a spontaneous agonist-independent activation under *in vivo* conditions. If not negligible, this basal G protein activation is likely to affect adenylyl cyclase and $I_{K(ACh)}$. Interestingly, it has been shown that single mAChR-activated K^+ channels can open spontaneously even in the absence of mAChR stimulation (Soejima & Noma, 1984; Kaibara, Nakajima, Irisawa & Giles, 1991; Okabe *et al.* 1991; Ito *et al.* 1991) and that this spontaneous activity can be totally suppressed by pertussis toxin pretreatment (but see Kaibara *et al.* 1991). Similarly, G_i may undergo a significant basal spontaneous activation since pretreatment of isolated cardiac cells with pertussis toxin increased basal I_{Ca} in guinea-pig (Hescheler, Kameyama & Trautwein, 1986) and newborn rabbit ventricles (Osaka & Joyner, 1992), and potentiated the effects of β-adrenergic agonists on cyclic AMP production in rat (Hazeki & Ui, 1981), contraction in guinea-pig (Brown, Humphrey & Harding, 1990), and I_{Ca} in guinea-pig (Hescheler *et al.* 1986) and rabbit (Osaka & Joyner, 1992) ventricular cells. The situation may be more complicated in frog cardiac myocytes. Indeed, we have recently found that intracellular application of pertussis toxin did not prevent ACh inhibition of Iso-elevated I_{Ca}, while it completely and readily inhibited ACh activation of $I_{K(ACh)}$ (Li,

Otero, Hanf, Fischmeister & Szabo, 1992). Therefore, in frog heart, different G proteins with different sensitivities for pertussis toxin would mediate adenylyl cyclase inhibition and $I_{K(ACh)}$ activation. Consistent with this hypothesis is the finding that, in the continual presence of intracellular pertussis toxin, atropine still increased Iso-elevated I_{Ca} in frog cardiac myocytes (data not shown).

Stimulatory effect of atropine on cardiac I_{Ca}

The stimulatory effect of atropine and AF-DX 116 on frog I_{Ca} was not seen under basal conditions, nor upon a stimulation of I_{Ca} by the dihydropyridine agonist (−)-Bay K 8644 or by intracellular perfusion with cyclic AMP, but required a preliminary stimulation of adenylyl cyclase activity by either Iso or forskolin. Therefore, atropine probably increased adenylyl cyclase activity. Because the order of potency of mAChR antagonists to produce this effect was similar to the order of potency of mAChR antagonists to reverse the inhibitory effect of ACh on I_{Ca}, it was concluded that the stimulatory effect of atropine and AF-DX 116 on I_{Ca} was consecutive to the binding of these antagonists to the cardiac mAChR rather than to the cyclase or another protein regulating its catalytic activity.

Interestingly, atropine did not enhance I_{Ca} when stimulated by a high (3 μM) concentration of forskolin, but did have an effect upon I_{Ca} stimulated by a 0·3 μM concentration (Table 1). On the contrary, atropine enhanced I_{Ca} upon stimulation by both maximal and submaximal concentrations of Iso (Table 1). Although the reason for this difference is not clear, it should be compared with the difference in the mechanism of ACh inhibition of forskolin and Iso-stimulated I_{Ca} (Fischmeister & Shrier, 1989). Indeed, high concentrations of forskolin could overcome the inhibitory effect of ACh on I_{Ca} but high concentrations of Iso could not (Fischmeister & Shrier, 1989). The symmetry between atropine and ACh action on Iso-or forskolin-stimulated I_{Ca} reinforce our conclusion that atropine behaves as a reverse agonist on the cardiac mAChR (see below).

The stimulatory effect of atropine on I_{Ca} was also found in rat ventricular myocytes. This suggests that the mechanism outlined in the present study is not simply a 'frog phenomenon', but more likely represents a general feature of cardiac mAChR–antagonists interaction. Differences were observed, however, in the effect of atropine on basal I_{Ca} in frog and rat myocytes. While atropine had no effect on basal I_{Ca} in frog, it strongly increased basal I_{Ca} in rat. It is likely that these species differences result from the relative amount of G_s over G_i, available for spontaneous and/or agonist-dependent activation, which may vary from one animal to the other. This hypothesis is supported by the findings that in mammalian myocytes the inhibitory effect of ACh on I_{Ca} could be overcome by large concentrations of Iso (Hescheler *et al.* 1986), while, as discussed above, in frog ventricular cells this effect of Iso has not been observed (Fischmeister & Shrier, 1989). Thus, a removal of G_i inhibition of adenylyl cyclase would produce an increase in adenylyl cyclase activity only if a sufficient amount of spontaneously activated G_s protein is available for stimulation. Since, in the heart, there is a large excess of mAChRs compared to β-adrenergic receptors (see references in Fischmeister & Shrier, 1989) and a considerable excess of G_i over G_s (Gilman, 1987; Holmer & Homcy, 1991), it is understandable that spontaneous activation of G_i overcomes that of G_s.

Atropine reduces the rate of spontaneous activation of the G proteins, G_i and G_K

The effect of atropine on I_{Ca} and $I_{K(ACh)}$ during intracellular perfusion of frog cardiac myocytes with Gpp(NH)p (Figs 7 and 8) suggests that atropine slows down the rate at which GTP or Gpp(NH)p spontaneously activates G_i and G_K. However, atropine did not fully prevent spontaneous activation of the G proteins, since atropine did not stop the Gpp(NH)p-induced reduction of I_{Ca} or activation of $I_{K(ACh)}$. When GTP is present, i.e. the G proteins can undergo a complete cycle of activation/deactivation, the atropine-induced reduction in the rate of spontaneous GTP accumulation into the G proteins associated with the mAChR will eventually lead to a reduction in the total amount of activated G_i and G_K. This would explain the inhibitory effect of atropine on spontaneous $I_{K(ACh)}$ single channel activity (Soejima & Noma, 1984) as well as the increase in I_{Ca} seen here, which occurs as a consequence of a reduced G_i-mediated inhibition of adenylyl cyclase. The situation is different when Gpp(NH)p is present. The arrest of GTPase activity on the G-α subunits results in the progressive accumulation of activated G_i and G_K, and, consequently, a gradual inhibition of adenylyl cyclase activity and a progressive reduction in I_{Ca} (Parsons *et al.* 1991 and the present study), as well as a gradual activation of $I_{K(ACh)}$ (Breitweiser & Szabo, 1988; Otero *et al.* 1991 and the present study). A reduction in the rate of spontaneous incorporation of Gpp(NH)p into G_i and G_K will slow down this process. However, unless the incorporation process is completely stopped, accumulation of activated G_i and G_K will proceed, although at a reduced speed.

This may help to explain why atropine exerted different effects on I_{Ca} during Gpp(NH)p perfusion depending on the timing of application (Fig. 7). During the early phase of Gpp(NH)p substitution for intracellular GTP, a mixture of GTP/Gpp(NH)p occurs within the cell. Atropine applied in this phase both stimulates I_{Ca} amplitude and partially reverses the inhibitory effect of Gpp(NH)p (Fig. 7). However, atropine had no stimulatory effect on I_{Ca} and negligible effect on its rate of decline when applied in the late phase of Gpp(NH)p perfusion (Fig. 7), i.e. at a time when a sufficient amount of G_i protein to produce maximal inhibition of adenylyl cyclase is activated by Gpp(NH)p and no more GTP is available.

Antagonist binding to the receptor interferes with receptor-mediated G protein activation

According to classical models of drug–receptor interactions, a competitive antagonist shares with agonists the ability to bind to a common site on the receptor molecule but not to trigger the 'stimulus' that leads to biological responses (Costa & Herz, 1989). Therefore, in the case of G protein-coupled receptors, an antagonist can be considered as being 'inert' with respect to the receptor if (i) binding of the antagonist does not modify the basal receptor–G protein interaction, and (ii) guanine nucleotides do not modify the affinity of the receptor for the antagonist. However, the latter may not always be the case as biochemical evidence exists for guanine nucleotide (Gpp(NH)p)-induced increase in affinity of various receptors for antagonists: e.g. adenosine A_1 receptors (Ramkumar & Stiles, 1988; Leung & Green, 1989), dopamine D_2 receptors (De Léan, Kilpatrick & Caron, 1982), β-adrenergic receptors (Wolfe &

Harden, 1981), and δ opioid receptors (Costa & Herz, 1989). Interestingly, the cardiac mAChR belongs to this family of receptors since Gpp(NH)p has been shown to increase the affinity of the cardiac receptor for various antagonists (Berrie, Birdsall, Burgen & Hulme, 1979; Burgisser, De Léan & Lefkowitz, 1982) including

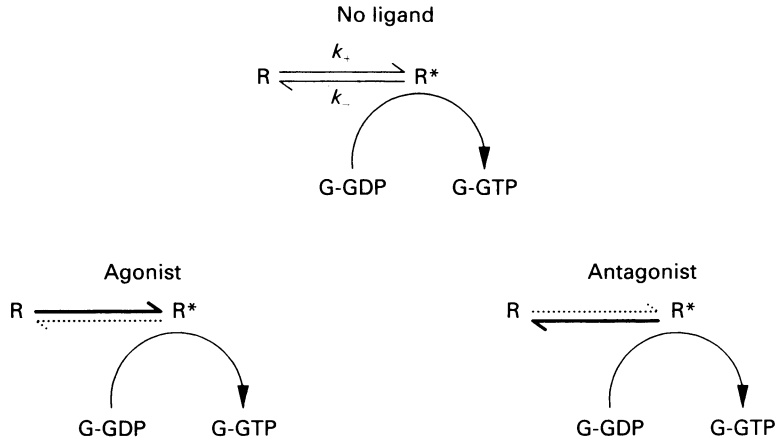

Fig. 10. A model for the agonist–antagonist interaction with the mAChR (for further details, see text).

atropine (Hosey, 1982). Most relevant to our study are the findings obtained by Burgisser *et al.* (1982) in frog heart membranes. These authors found that guanine nucleotides converted a form of mAChR with high-agonist–low-antagonist affinities to another form with high-antagonist–low-agonist affinities (Burgisser *et al.* 1982). Such a reciprocal modulatory effect of guanine nucleotides on agonist and antagonist binding led the authors to conclude that 'antagonists might be expected to exert biological actions opposite to those of agonists' (Burgisser *et al.* 1982).

In support of this assumption are the findings that in various types of receptors, including the adenosine A_1 receptor (Leung & Green, 1989), the δ opioid receptor (Costa & Herz, 1989), the β-adrenergic receptor (Götze & Jakobs, 1990) as well as the cardiac mAChR (Matesic & Luthin, 1991; Hilf, 1991), antagonists favour the dissociation of receptor–G protein complexes. Our results, together with those of others (Giles & Noble, 1976; Soejima & Noma, 1984), would fit into that general scheme and provide a biological counterpart to these *in vitro* experiments. It is also worth mentioning that recently atropine was shown to modify the tonic coupling between mAChR and phospholipase C in rat parotid acini (Horn, Baum & Ambudkar, 1991).

A proposed mechanism for the agonist–antagonist regulation of receptor-dependent activation of G proteins

Based on the present results and on *in vitro* biochemical studies, we propose the following model (Fig. 10) which is inspired from the ternary complex model (Wreggett & De Léan, 1984; Costa, Ogino, Munson, Onaran & Rodbard, 1992). mAChR may be found in two different conformational states: an 'inactive receptor' state (R) and

an 'active receptor' state (R*). G proteins may be activated by the receptor only in its active form, R*. In the absence of agonist and antagonist, the mAChR fluctuates spontaneously between the two states, R and R*, with rate constants k_+ and k_-. Binding of the agonist to the receptor increases k_+ and/or decreases k_-, so that the probability that the receptor being active is increased. Therefore, more G proteins will become activated. Symmetrically, binding of the antagonist to the receptor increases k_- and/or decreases k_+, so that the probability of the receptor being active is decreased. Consequently, less G protein will become activated. With such a model, receptor antagonists behave both as *competitive antagonists*, in that they displace the agonist from its binding site, and as *reverse agonists* (or negative antagonists according to Costa *et al.* 1992), in that they induce conformational changes at the receptor opposite to those induced by the agonists.

Although the physiological implications of our findings are yet unknown, the present study demonstrates that mAChR antagonists may exert intrinsic effects on heart function which: (i) are mediated by their binding to the mAChR and (ii) result from a reduction in basal–agonist-independent–mAChR-dependent G protein (G_i and G_K) activation. It would be interesting to study the effects of mAChR antagonists under various physiological or pathological conditions which modify the levels of G proteins (G_s and/or G_i) as well as mAChRs, e.g. during long-term exposure to various agonists (Milligan & Green, 1991) such as catecholamines (Reithmann *et al.* 1989), during development (Nathanson, 1989; Osaka & Joyner, 1992), or during heart failure (Reithmann *et al.* 1989; Holmer & Homcy, 1991; Feldman, 1991). Also, the biochemical set of evidence available from the literature would suggest that, in addition to cardiac mAChR antagonists, antagonists of other types of cardiac receptors could produce physiological effects on their own.

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