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M_1 MUSCARINIC RECEPTORS INCREASE CALCIUM CURRENT AND PHOSPHOINOSITIDE TURNOVER IN GUINEA-PIG VENTRICULAR CARDIOCYTES

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SUMMARY

1. Physiological and molecular evidence for the presence and functional role of M_1 muscarinic cholinergic receptors (mAChRs) in adult guinea-pig ventricular cells is presented.

2. Whole-cell clamp measurements of the L-type calcium current (I_{Ca}) in isolated myocytes were performed. Caesium was used to suppress potassium currents. I_{Ca} was increased by the muscarinic agonist carbachol in cells pretreated with pertussis toxin which blocked the M_2 mAChR-triggered cascade of intracellular signalling, while it was not changed in untreated cells.

3. If the M_2-mediated regulation of I_{Ca} was blocked by directly saturating the cell with cyclic adenosine monophosphate (cAMP) through the patch pipette, application of carbachol induced a further small increase of the current above the level reached after cAMP perfusion. This increase was more pronounced in cells pretreated with pertussis toxin.

4. The carbachol-induced increase of I_{Ca} was blocked by the selective M_1 mAChR antagonist pirenzepine.

5. The application of high concentrations of carbachol increased the accumulation of [^{3}H]inositol monophosphate up to 240% above control levels. This increase was reduced by application of pirenzepine.

6. The expression of M_1 receptor mRNA in ventricular cardiocytes was shown by reverse transcriptase–polymerase chain reaction.

7. These results suggest that M_1 mAChR regulation of I_{Ca} can be a component of the paradoxical positive inotropism induced by high concentrations of muscarinic agonists.

INTRODUCTION

Physiological responses to muscarinic cholinergic receptor (mAChR) stimulation in the cardiac muscle have been widely investigated for over 80 years. Major effects of muscarinic agonists in the heart are species specific and depend on the cardiac

region. They include negative inotropism and chronotropism and several modifications of the action potential parameters and ionic currents (Hartzell, 1988; Pappano, 1991). This variety of responses to muscarinic agonists is triggered by the activation of a cascade of intracellular signals that regulate several cellular functions. The main signal transduction pathway activated by mAChRs in the heart involves the inhibition of adenylate cyclase. Stimulation of cardiac mAChRs inhibits adenylate cyclase through a GTP-binding protein that is sensitive to ADP ribosylation by pertussis toxin (PTX) (Hartzell, 1988; Schimerlik, 1989). The intracellular decrease of cAMP content is associated with the reduction of I_{Ca} (Hescheler, Kameyama & Trautwein, 1986) and the slowing of pacemaker cell rhythm due to the regulation of the pacemaker current, I_f (DiFrancesco & Tromba, 1987). Moreover, mainly in non-ventricular cells, muscarinic agonists activate potassium channels through the coupling to a GTP-dependent protein that can also be blocked by PTX treatment (Christie & North, 1988).

Pharmacological and molecular cloning studies have demonstrated that mAChRs comprise a family of at least five genetically distinct subtypes (M_1–M_5) that are characterized by different structure, tissue distribution and functional properties (Nathanson, 1987; Bonner, 1989; Ehlert, Delen, Yun, Friedman & Self, 1989). Major responses to muscarinic receptor agonists in the cardiac muscle appear to be mediated by the activation of a well-characterized M_2 mAChR that may couple to different signal transduction pathways (Schimerlik, 1989).

However, other cardiac effects of muscarinic receptor stimulation are not yet fully understood and appear to be partly paradoxical. Since the report of Brown & Eccles (1934), it has been clearly indicated in several experiments that higher concentrations of carbachol or other muscarinic agonists than those implied in the negative inotropism can induce opposite effects, including positive chronotropism, increase of contractility and of action potential duration (reviewed by Pappano, 1991).

In the present study we investigated whether mAChRs other than M_2 may exist and play a functional role in the ventricle of the guinea-pig. We present evidence for the presence of pertussis toxin-insensitive M_1 mAChRs in guinea-pig ventricular cells. We show that these receptors can stimulate membrane phospholipid metabolism and we suggest that their activation might contribute to the positive inotropic effect by increasing the amplitude of the L-type calcium current.

METHODS

Isolation of ventricular cardiocytes

Cells were enzymatically dispersed from young adult guinea-pig ventricles with methods modified from those previously described (Levi & Alloatti, 1988; Alloatti, Serazzi & Levi, 1991). Young adult guinea-pigs (200–350 g) of either sex were killed by stunning and cervical dislocation. The hearts were explanted, washed in modified Tyrode solution (for this and other solutions see the following section) and cannulated via the aorta. All the following operations were carried out under a laminar flow hood. The heart was perfused with Tyrode solution at a constant flux of 10 ml/min via a peristaltic pump for approximately 10 min to wash away the blood and until restoration of a regular beating rate. Afterwards the heart was perfused for 3 min with a low-calcium–low-sodium solution and with 50 ml of the first enzymatic medium (collagenase and trypsin). The heart was detached from the cannula and the ventricles cut away and minced into small fragments. The fragments were collected in the second enzymatic medium (pronase) and gently stirred with a glass blade connected to an electric motor. The medium was collected and replaced with a fresh batch

every 10 min for up to nine cycles. After the second cycle the collected supernatant contained viable cells (more than 50% rod-shaped cells) and was diluted in Tyrode solution supplemented with antibiotic (200 μl/l, Spectrum1, Sigma Tau, Rome, Italy). Isolated cells were kept at room temperature (22–25 °C) and used for up to 3 days. When indicated, pertussis toxin (PTX, 0·5 μg/ml) was added to the cells 14–16 h before starting the experiments.

Solutions

The control Tyrode solution contained (mM): 154 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5·5 D-glucose, 5 Hepes; pH 7·35 adjusted with NaOH. The low-calcium–low-sodium medium contained (mM): 33·6 NaCl, 22 glucose, 132 sucrose, 10 KCl, 1·1 KH_2PO_4, 5 $MgSO_4$, 50 taurine, 10 Hepes; pH 7·3 adjusted with KOH. Caesium Tyrode solution contained (mM): 138 NaCl, 20 CsCl, 2 $CaCl_2$, 1 $MgCl_2$, 5·5 D-glucose, 5 Hepes; pH 7·35 adjusted with NaOH. The Krebs–Henseleit solution contained (mM): 118 NaCl, 4·7 KCl, 1·3 $CaCl_2$, 1·2 $MgSO_4$, 1·2 KH_2PO_4, 25 $NaHCO_3$, 11·7 D-glucose.

The first enzymatic solution was the low-calcium–low-sodium solution with 7 mg/50 ml collagenase (type V), 10 mg/50 ml trypsin (type III) and 50 mg/50 ml bovine serum albumin (fraction V, Boehringer Mannheim, Mannheim, Germany) added. The second enzymatic solution was the same as the first which, in replacement for collagenase and trypsin, contained 2·5 mg pronase (Boehringer).

The whole-cell patch clamp standard solution contained (mM): 133 KCl, 5 EGTA free acid, 5 Na_2ATP, 5 sodium phosphocreatinine, 5 Hepes, 3 $MgCl_2$, 0·4 Na_2GTP; pH 7·3 adjusted with KOH. In the caesium intracellular solution, potassium was replaced by caesium in equimolar amounts. Pirenzepine, carbachol, isoprenaline and cAMP-containing solutions were freshly prepared before the experiments. If not specified all chemicals and drugs used in the experiments were purchased from Sigma Chemical Co., St Louis, MO, USA.

Electrophysiological measurements

The methods used for whole-cell patch clamp recording and data analysis have previously been described in detail (Levi & Alloatti, 1988; Alloatti, Serazzi & Levi, 1991). Briefly, for routine monitoring of L-type Ca^{2+} currents (I_{Ca}), the cell was depolarized from −100 to −40 mV every 5 s for 50 ms, to inactivate both fast Na^+ currents and T-type I_{Ca}s; then at different potentials of −90 to +50 mV for 300 ms.

A calcium current was measured either at its peak (approximately 0 to +10 mV in our conditions) throughout the experiment, with brief interruptions to measure the current–voltage relationship (as in Figs 1 and 4), or by repeatedly stimulating the cell throughout the range of potentials of the current–voltage relationship (as in Figs 2, 5 and 6). All experiments were performed at approximately 35 °C under thermostatic control.

During the experiments, prewarmed Tyrode solution was constantly perfused into the bath with a peristaltic pump. Each cell was used for a single experiment. Each determination was tested with cells coming from at least three independent dissociations. Calcium current was measured as the difference between the peak of the inward current and the current at the end of the 300 ms pulse, either blocking potassium currents using internal and external caesium solutions, or in normal potassium solutions. No major difference was observed between potassium and caesium conditions because the relatively slow time course and small amplitude of the delayed potassium current introduce only a small error. It was possible to measure a calcium current clearly separated from other components for times of up to 1 h. This current showed a moderate decrease with time described as 'run-down'. The steady-state current at −100 mV, the current at the end of the pulse and the fast sodium current (I_{Na}) were monitored to control the stability of the preparation and the validity of the voltage clamp. Experiments where sudden changes of one of these currents occurred, or with an excessive increase in the holding current were discarded. After the electrode tip sealed to the cell membrane and the patch was broken to achieve whole-cell clamp, we performed at least 3 min of control measurement before applying any substance to be tested. To exchange external solutions the pipette with the cell attached was moved into the tip of one of a series of six silicon plastic tubes (250 μm diameter) connected to gravity fed reservoirs of the desired test solutions. To validate the pertussis toxin treatment, experiments were conducted on each batch of toxin-treated cells to test the block of the M_2 response. In some cells it was possible to observe the block of the M_2 response and the presence of M_1-induced increase in the same experiment.

All the experiments were controlled by a stimulating computer and analysed on line with a Hewlett Packard 9816 (Hewlett Packard, Fort Collins, CO, USA) computer after analog-to-digital (16 bit linear) conversion (22 kHz sampling rate) with a modified PCM system (Sony, Japan). Analog data were filtered at 3 kHz (5-pole Bessel filter). Digitized data were stored for further analysis on videocassette. If not indicated, data are expressed as means ± S.E.M.

Accumulation of phosphoinositide breakdown products

The rate of inositol phospholipid hydrolysis was estimated by measuring the accumulation of [^{3}H]inositol monophosphate ([^{3}H]Ins*P*) in the presence of Li^+, which blocks the conversion of InsP_1 into free inositol (Berridge, Downes & Hanley, 1982). Chopped ventricular slices (500 × 500 μm) were prepared from guinea-pig hearts previously perfused with standard Tyrode solution. Slices were incubated in a shaking water bath for 30 min at 37 °C in Krebs–Henseleit bicarbonate buffer (equilibrated with 95% O_2 and 5% CO_2 to a final pH of 7·4 and prewarmed to 37 °C). Then, 50 μl of the gently packed slice suspension were pipetted into plastic vials containing 3 μCi of *myo*-2-[^{3}H]inositol (16·5 Ci/mmol, New England Nuclear, Boston, MA, USA) in 250 μl of buffer, to label membrane phospholipids. Vials were incubated at 37 °C in a shaking water bath under a constant stream of 95% O_2–5% CO_2 for 90 min, at which time 10 mM LiCl was added. After 20 min the stimulation was started by the addition of carbachol. When pirenzepine was used, it was added 10 min prior to addition of carbachol. The incubation was stopped after 30 min by washing the vials three times with 3 ml of ice-cold buffer, followed by the addition of 910 μl chloroform–methanol (1:2 v/v). Phases were then separated by further addition of 300 μl water and 300 μl chloroform and by centrifugation at 500 *g* for 10 min. The amount of [^{3}H]Ins*P* present in the aqueous phase was estimated by anion exchange chromatography, as previously described (Berridge *et al.* 1982). The aqueous phase was applied to columns containing 1 ml AG 1 × 8 (100–200 mesh, formate form, Bio-Rad Laboratories, Richmond, VA, USA). The columns were washed with 20 ml water to elute phosphate esters. Glycerophosphoinositol and inositol-1,2-cyclic monophosphate were eluted with 10 ml of 5 mM sodium tetraborate plus 60 mM sodium formate. *Myo*-[^{3}H]inositol monophosphate was eluted with 4 ml of 0·1 M formic acid plus 0·2 M ammonium formate. This eluate was collected and the radioactivity measured by scintillation spectrometry after addition of 15 ml Aquassure scintillation fluid (New England Nuclear, Boston, MA, USA). The elution profile of inositol monophosphate from the anion exchange column was monitored using [^{3}H]InsP_1 (New England Nuclear) as a standard.

Reverse transcriptase polymerase chain reaction

Expression of mRNAs encoding M_1, M_3 and M_4 mAChRs was assayed according to the reverse transcriptase–polymerase chain reaction (RT–PCR) procedure as previously described (Saiki *et al.* 1988; Eva, Bovolin, Balzac, Botta, Ricci Gamalero & Vaccarino, 1990; Ito *et al.* 1991). Total RNA was extracted from guinea-pig ventricles using the phenol–guanidine thiocyanate procedure (Chomszynsky & Sacchi, 1987). RNAs were digested with 18 units of DNAseI, RNAse-free (Boehringer Mannheim, Mannheim, Germany), then transcribed into cDNA using oligo(dT) to prime the RT as follows. Three micrograms total RNA were first incubated for 15 min at 65 °C with 1 μg oligo dT_{15} (Boehringer Mannheim, Germany) in a final volume of 10 μl, to allow RNA denaturation and annealing of the oligonucleotide primer. The annealing step was followed by 1 h incubation at 37 °C after the addition of the reverse transcriptase buffer (50 mM Tris HCl, pH 8·3; 75 mM KCl; 10 mM dithiothreitol; 3 mM $MgCl_2$), 1 mM each dATP, dCTP, dGTP, dTTP (all from Sigma) and 35 units (approximately 1 μl) of avian myeloblastosis reverse transcriptase (Life Sciences, St Petersburg, FL, USA) in a total volume of 20 μl. Synthesized cDNA was then amplified by polymerase chain reaction in a 50 μl reaction mixture containing: 1 μl of the cDNA reaction, 5 units of TAQ polymerase (Promega Co., Madison, WI, USA), 10 pmol of each primer, 500 μM of each dNTP together with 10 mM Tris HCl, pH 8·7, 50 mM KCl and 2 mM $MgCl_2$. RT–PCR reactions were run in a thermocycle (Techne Corp., Princeton, NJ, USA) for forty-five cycles. Each cycle included the following three steps: 0·6 min at 95 °C to denature double strand DNA, 0·3 min at 55 °C for primer annealing and 0·6 min at 72 °C for primer extension.

Aliquots (20 μl) of RT–PCR reactions were resolved on 1·2% agarose gel (Sigma) and gel alkali-blotted onto Hybond N membrane (Amersham) then hybridized with [^{32}P]5′-end-labelled oligonucleotide probes containing complementary DNA sequences to rat M_1, M_3 and M_4 receptors. Filters were prehybridized in 50% formamide, 5 × SSC (20 × SSC is 3 M NaCl, 0·3 M sodium citrate,

pH 7) for 5 h at 42 °C. Hybridization with the mAChR subtype-specific probes was carried out in the same buffer for 18 h at 25 °C and excess probe was washed from membranes twice in 2 × SSC for 15 min at 55 °C and twice in 0·2 × SSC for 15 min at 55 °C. Membranes were then exposed to X-ray film (Kodak) for autoradiography at −70 °C.

Oligonucleotide probes were 5′-end-labelled with [t-^{32}P]ATP by T4 polynucleotide kinase (New England Biolabs, Inc.) as described below; 10 pmol of each oligonucleotide were incubated in kinase buffer (50 mM Tris HCl, pH 7·6; 1 mM dithiothreitol; 0·1 mM EDTA, pH 7·6; 10 mM $MgCl_2$), together with 10 pmol of [t-^{32}P]ATP (Amersham, 3000 Ci/mmol; 10 mCi/ml) and 8 units (~ 1 μl) of bacteriophage T4 polynucleotide kinase in a final volume of 30 μl. Reaction was carried out for at least 30 min and stopped by the addition of 50 μl of 20 mM EDTA. ^{32}P-labelled oligonucleotides were separated from the bulk of unincorporated radioactivity by chromatography through Sephadex G-50 (Pharmacia P-L Biochemicals Inc., Milwaukee, WI, USA) columns. Specific activity of probes was approximately 10^9 c.p.m./μg of oligonucleotide. Although the mAChR cDNAs from guinea-pig have not been cloned yet, high sequence homology among different mammalian species has been found for each muscarinic receptor subtype. Oligonucleotides used in the amplification or as 5′-end-labelled probes were designed from the sequence of rat mAChR cDNAs as follows: M_1: 860–878 sense, 1180–1198 antisense, 964–999 sense; M_3: 1247–1264 sense, 1517–1534 antisense, 1403–1438 sense; M_4: 868–885 sense, 1154–1171 antisense, 1042–1077 sense.

RESULTS

Muscarinic stimulation of I_{Ca} in ventricular cardiocytes

The amplitude of I_{Ca} showed no appreciable difference in the normal Tyrode or caesium solution and the experiments were thus pooled together, the average being 842·3 ± 109·6 pA (mean ± S.E.M.; $n = 22$). Criteria to measure I_{Ca} and to exclude excessive deterioration of a cell or variation of other currents are indicated in the Methods section. As previously reported by several laboratories including ours (Hescheler *et al.* 1986; Hartzell, 1988; Levi & Alloatti, 1988), muscarinic agonists inhibit the I_{Ca} enhanced by isoprenaline or histamine, but fail to modify I_{Ca} in the absence of a previous stimulation of adenylate cyclase. In the present experiments, stimulation with 100 μM carbachol had no significant effect on I_{Ca} (−4·69 ± 2·53 %; 9 cells from 5 hearts) (Fig. 1). We performed a series of experiments aimed at unmasking other putative intracellular mechanisms triggered by muscarinic receptor agonists. For this purpose, we functionally inactivated the inhibitory effect of M_2 receptor subtypes on I_{Ca} by means of two different strategies. Firstly, the M_2-mediated inhibition of adenylate cyclase was blocked by treating dissociated ventricular cardiocytes with pertussis toxin. After this treatment the application of 1 μM carbachol after the stimulation of I_{Ca} with 1 μM isoprenaline did not produce a significant inhibition of I_{Ca}. The application of 100 μM carbachol enhanced I_{Ca} by 31·8 ± 14·2 % in ventricular cells treated with pertussis toxin (13 cells from 9 hearts).

In the experiment shown in Fig. 2, it was possible on the same cell to observe the isoprenaline (1 μM) stimulation, the failure of carbachol (1 μM) to inhibit it and the stimulation of the current by carbachol at the higher concentration (100 μM). In Fig. 3 the current–voltage (I–V) relationships presented are from the same experiment as in Fig. 2. These show that, even if some deterioration of the cell in the course of the experiment induced a slight increase of a background leak, in the range of potentials of interest for the peak I_{Ca} the increase is not an artifact due to a background current. In our experiments we have quantitatively analysed the time course of the I_{Ca}, but no marked change in the time course of the I_{Ca} was observed in the presence of high concentrations of carbachol.

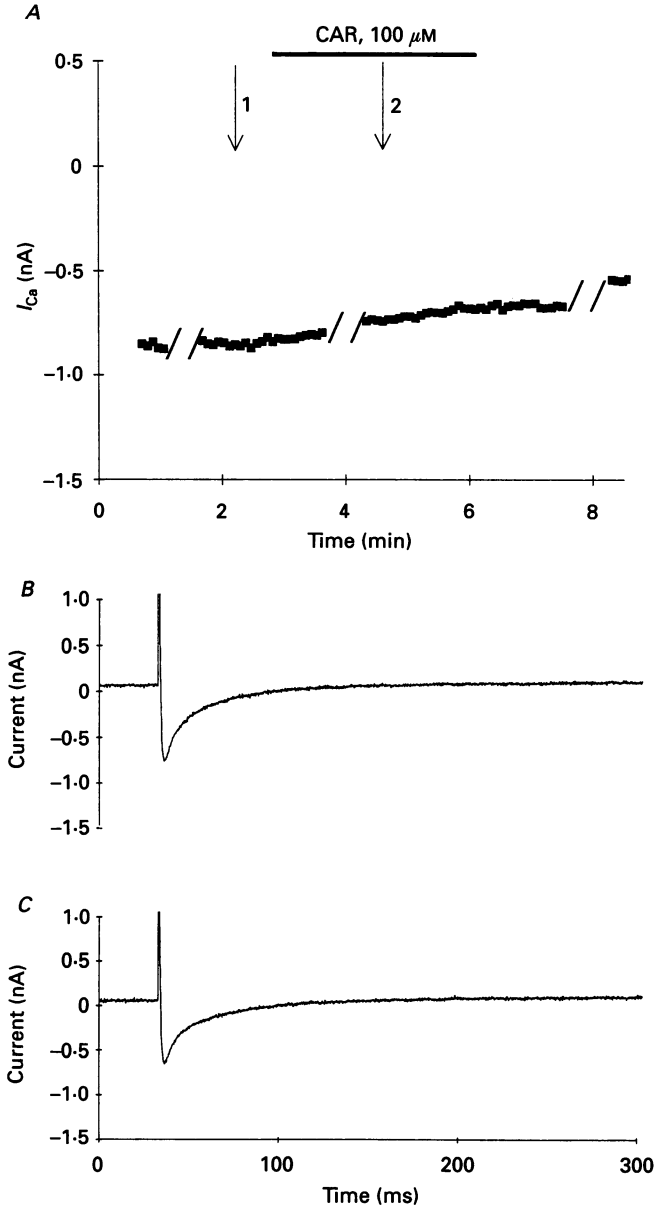

Fig. 1. Lack of effect of carbachol (CAR, 100 μM) on basal I_{Ca} in caesium internal and external solutions. Each point in *A* represents I_{Ca} measured at +10 mV. The period of application of carbachol is indicated by the horizontal bar. At the time indicated by the breaks, *I–V* relationships were measured. Single traces recorded (step to +10 mV) at the times indicated by arrows 1 and 2 are shown in *B* (control) and *C* (CAR, 100 μM), respectively.

As a second strategy, we directly saturated the intracellular content of cAMP by perfusing ventricular cells with a 50 μM cAMP-containing solution. This concentration of cAMP is sufficient to saturate the response of I_{Ca} (Kameyama,

Hofmann & Trautwein, 1985) and it is close to the maximal cAMP intracellular content reached following stimulation of adenylate cyclase (Buxton & Brunton, 1983). To test the saturation we applied isoprenaline (1 μM) to six cells and we observed very little (4·2±2·4%, 6 cells from 3 hearts) or no effect at all, as in the

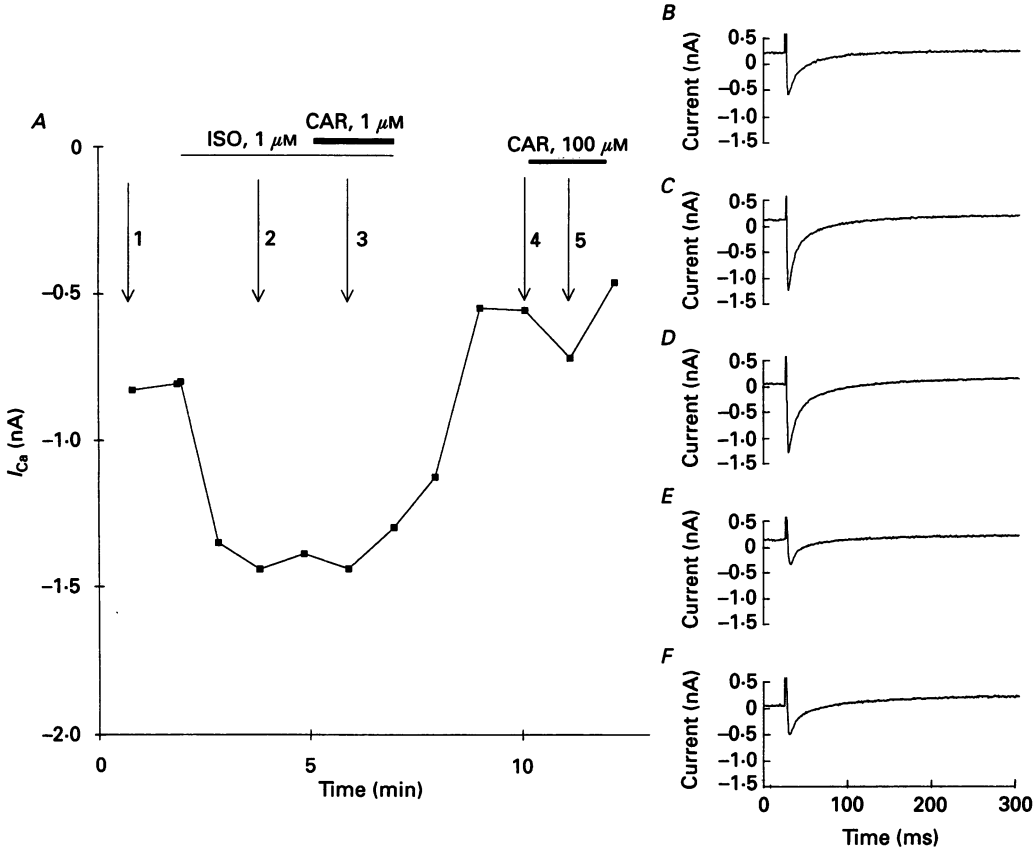

Fig. 2. In a cell treated with pertussis toxin (PTX, 0·5 μg/ml), 1 μM carbachol does not reduce I_{Ca} increased by 1 μM isoprenaline (ISO); after removal of ISO, the current was increased by 100 μM carbachol (typical experiment performed with internal and external caesium solutions). Each point in *A* represents the current at +10 mV during an *I–V* relationship measurement. The period of application of the different substances is indicated by the bars. Single traces recorded at the times indicated by arrows 1–5 are shown in the right panels: *B* (control), *C* (ISO, 1 μM), *D* (ISO and CAR, 1 μM), *E* (return to control) and *F* (CAR, 100 μM), respectively.

experiment shown in Fig. 4. During the first minutes after the membrane patch was broken and the cAMP-containing pipette solution started to perfuse the cell, the calcium current increased until it reached a maximal level, and then started to decline slowly. Carbachol (100 μM) was applied after a steady value was attained or a slow decrease started (3–5 min after the beginning of the experiment). In twenty-two cells internally perfused with cAMP, I_{Ca} further increased to 18·3±4·2% over the control value after carbachol (100 μM) application (Fig. 5). A significant

enhancement of I_{Ca} (37·6 ± 14·9%; 8 cells from 6 hearts) was also induced by carbachol in ventricular cells treated with pertussis toxin and perfused with intracellular solution containing 50 μM cAMP. In the experiment shown in Fig. 6 the cAMP-induced increase is not shown as it was complete before the start of the stimulation protocol.

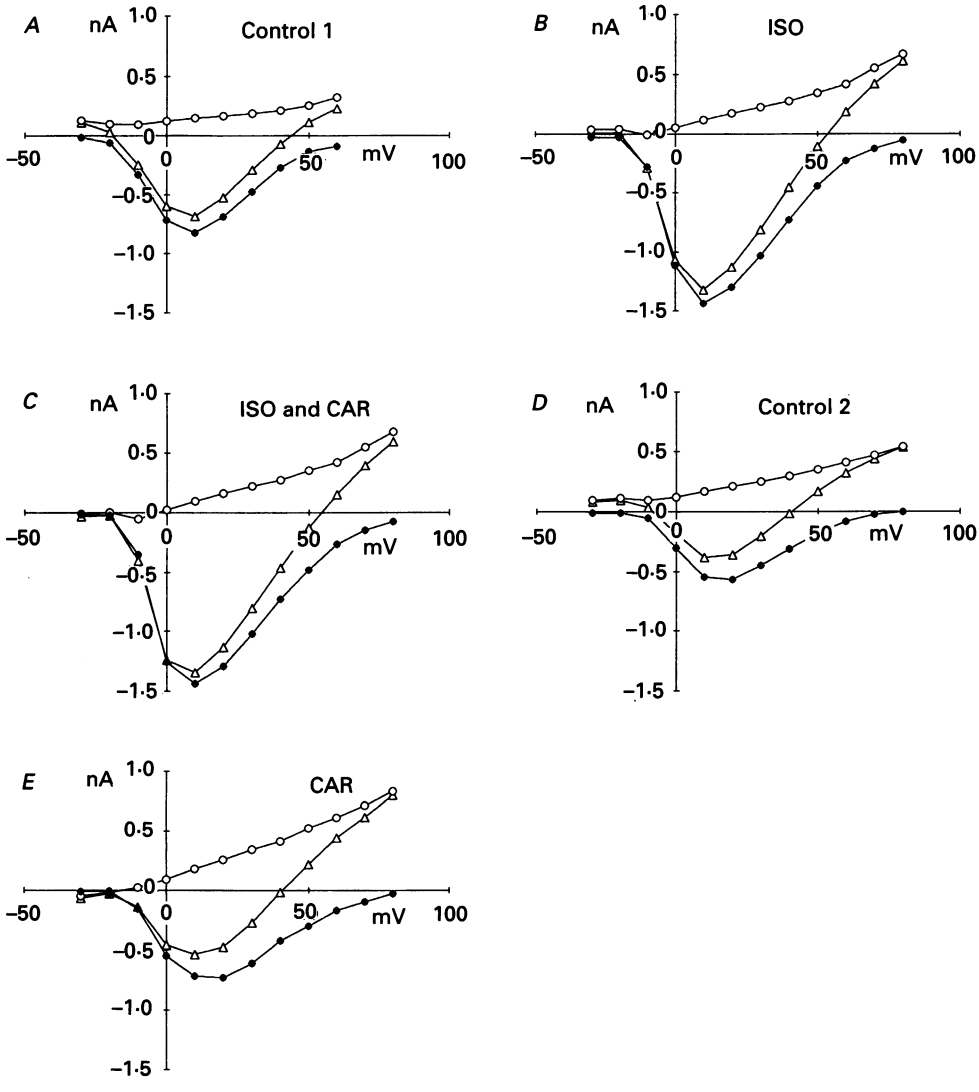

Fig. 3. Current–voltage relationships from the same experiment shown in Fig. 2. The different symbols indicate the current at the peak of I_{Ca} (△), after 280 ms (○) and the difference (●). *I–V* relationships recorded at the times indicated by arrows 1–5 in Fig. 2 are shown in panels *A* (control), *B* (ISO, 1 μM), *C* (ISO and CAR, 1 μM), *D* (return to control) and *E* (CAR, 100 μM), respectively.

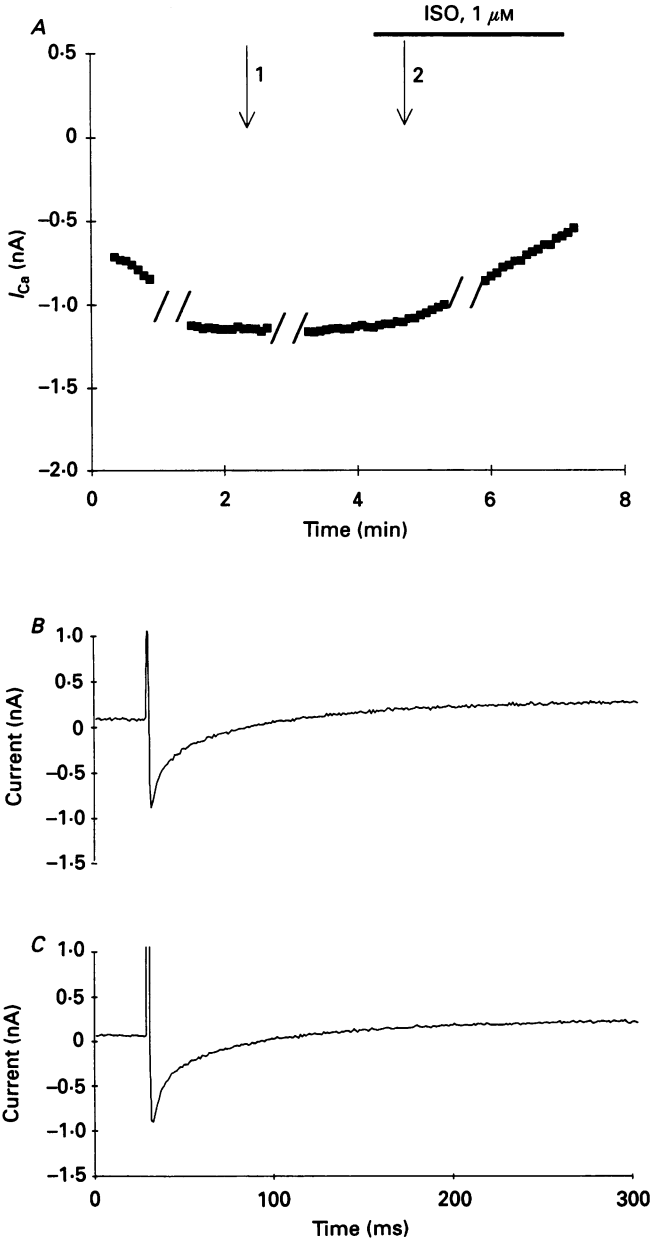

Fig. 4. Lack of effect of ISO (1 μM) in a cell perfused with 50 μM cAMP (internal and external caesium solutions). Sample protocol as in Fig. 1. *A*, the period of application of ISO is indicated by the horizontal bar. Single traces recorded at the times indicated by arrows 1 and 2 are shown in *B* (cAMP) and *C* (cAMP and ISO).

Pharmacological identification of M_1 mAChRs

To characterize pharmacologically the muscarinic receptor subtype that triggers the enhancement of I_{Ca}, the effect of carbachol in the presence of 10 nM pirenzepine was tested. At this concentration pirenzepine acts as a selective antagonist of M_1

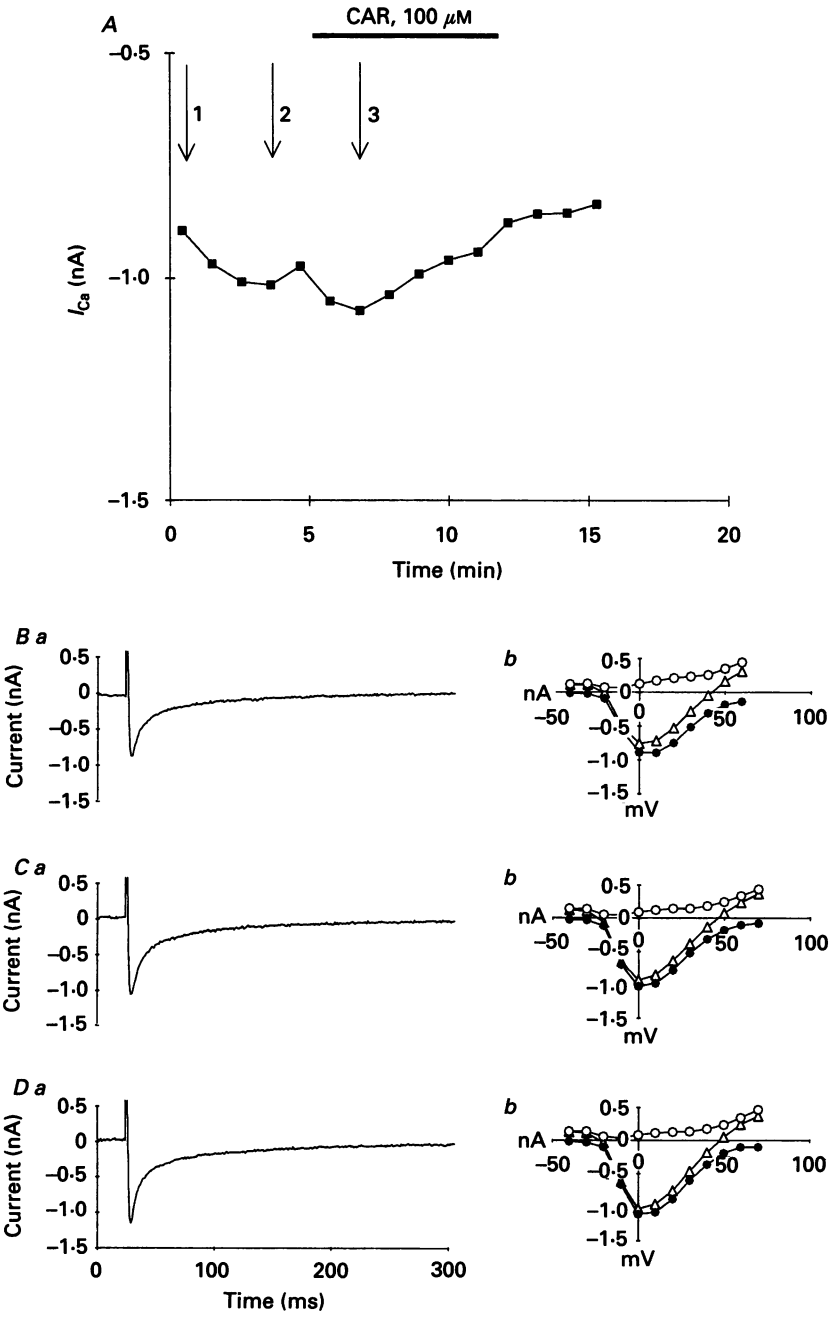

Fig. 5. *A*, increase of I_{Ca} induced by 100 μM carbachol in a cell internally perfused with 50 μM cAMP (external and internal caesium solutions). Each point represents the current at +10 mV during an *I–V* relationship measurement. The period of application of carbachol is indicated by the bar. Single traces at +10 mV (*Ba*, *Ca* and *Da*) and *I–V* relationships (*Bb*, *Cb* and *Db*) recorded at the times indicated by arrows 1–3 are shown at the beginning of cAMP perfusion (*Ba* and *Bb*), before CAR (*Ca* and *Cb*) and in the presence of CAR (*Da* and *Db*). Symbols as in Fig. 3.

receptor subtype (Hammer, Berrie, Birdsall, Burgen & Hulme, 1980) and does not modify I_{Ca} stimulated by isoprenaline as shown in Fig. 7 (4 cells from 3 hearts). The experiments were performed as follows. Cells were internally perfused with cAMP (50 μM), then treated with pirenzepine (10 nM) and carbachol (100 μM) and afterwards with carbachol alone. In this condition pirenzepine almost completely prevented (8·3 ± 2·8 %) the increase of I_{Ca} induced by carbachol (7 cells from 3 hearts). In the experiment shown in Fig. 8 it is possible to observe the carbachol-induced increase of I_{Ca} after the removal of pirenzepine. Conversely, the same pirenzepine concentration failed to block the carbachol (1–10 μM)-induced decrease of I_{Ca} stimulated by isoprenaline (1 μM). After a control period the cells were challenged with isoprenaline, which led to a rapid increase of I_{Ca} (118·0 ± 16·3 % over the control value), then with isoprenaline, pirenzepine (10 nM) and carbachol (1 μM). In these experiments, despite the presence of pirenzepine, carbachol was always able to reduce I_{Ca} to 34·3 ± 20·3 % over the control value (4 cells from 3 hearts; Fig. 9). This result is comparable to that obtained when ventricular cells stimulated with isoprenaline were challenged with carbachol in the absence of pirenzepine. In these conditions, I_{Ca} was increased to 95·5 ± 24·0 % over the control by isoprenaline (1 μM), and reduced to 49·1 ± 10·8 % over the control by carbachol (1 μM; 5 cells from 3 hearts, data not shown).

Muscarinic stimulation of [^{3}H]inositol monophosphate accumulation

Several lines of evidence have demonstrated that M_1 mAChRs are functionally coupled to the stimulation of phosphoinositide (PI) hydrolysis in many tissues (Nathanson, 1987). We therefore investigated whether carbachol stimulates [^{3}H]inositol monophosphate formation in guinea-pig ventricular muscle. Incubation of ventricular slices with carbachol induced a concentration-dependent stimulation of [^{3}H]inositol monophosphate accumulation when LiCl was present in the incubation medium (Fig. 10*A*). The dose-dependent curve tended to saturation and the carbachol concentration that elicited 50 % of the maximal response (EC_{50}) was approximately 5 μM. The maximal stimulation of [^{3}H]inositol monophosphate accumulation (240 % over control values) was observed when the carbachol concentration was raised up to 100 μM.

Figure 10*B* shows that the selective M_1 antagonist pirenzepine inhibited the carbachol stimulation of phosphoinositide breakdown in a dose-related manner. Pretreatment of ventricular slices with 1 or 10 nM pirenzepine decreased the stimulation of PI hydrolysis induced by 100 μM carbachol by 30 and 60 %, respectively.

Identification of messenger RNA encoding the M_1 mAChR subtype

Expression of mRNAs encoding muscarinic receptor subtypes was then studied. Since we failed to identify mAChR mRNAs other than M_2 by Northern blot analysis of (poly A$^+$)RNA prepared from ventricular cells, we used the RT–PCR procedure to amplify transcripts of specific genes encoding mAChR subtypes. The amplified fragments were generated within cDNA sequences which correspond to the unique large cytoplasmic domain of the receptor mRNAs. Products of the combined RT–PCR were validated by matching the actual size of the amplified transcripts

with the predicted size and by Southern blot hybridization with the 5′-^{32}P-labelled receptor-specific oligonucleotide probes. In order to exclude the possibility that PCR products resulted from amplification of DNA contaminants, PCR-amplification of not reverse-transcribed RNA from cardiac ventricles was used as a negative control (data not shown).

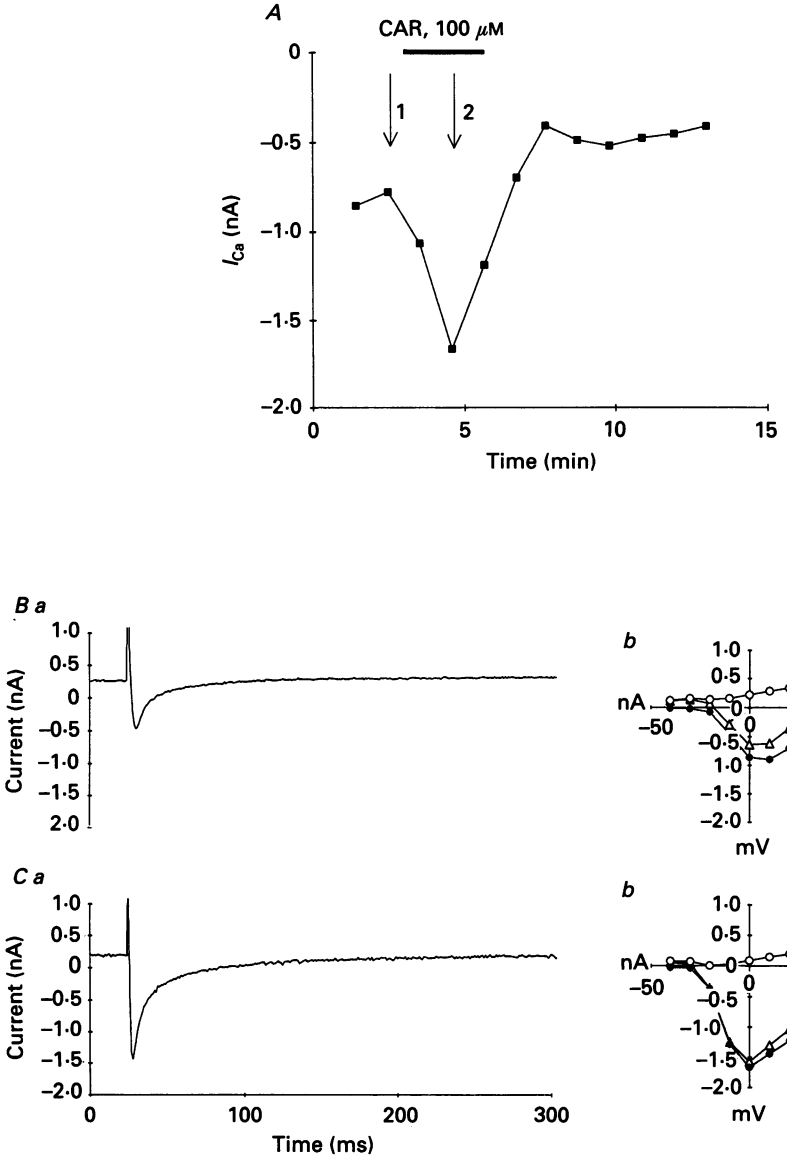

Fig. 6. Increase of I_{Ca} induced by 100 μM carbachol in a PTX-treated cell internally perfused with 50 μM cAMP (internal and external caesium solutions). Each point represents the current at 0 mV during an *I–V* relationship measurement. The period of application of carbachol is indicated by the bar. Single traces at 0 mV (*Ba* and *Ca*) and *I–V* relationships (*Bb* and *Cb*) recorded at the times indicated by arrows 1 and 2 are shown in *Ba* and *Bb* (before CAR) and *Ca* and *Cb* (CAR). Symbols as in Fig. 3.

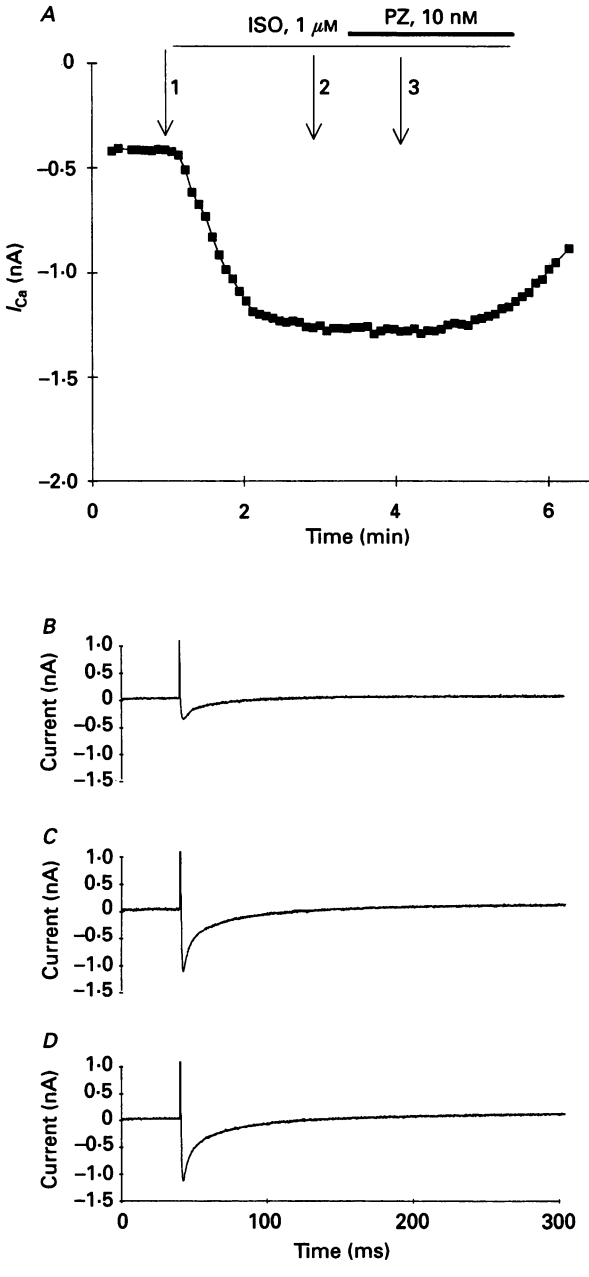

Fig. 7. Pirenzepine (PZ, 10 nM) does not modify the effect of ISO on I_{Ca} (internal and external caesium solutions). The bars indicate the period of application of the different substances. Single traces recorded at the times indicated by arrows 1–3 are shown in *B* (control), *C* (ISO) and *D* (ISO and PZ).

PCR-amplified products from the M_1 receptor RNA-derived cDNA produced visible DNA fragments of the expected size (approximately 340 base pairs) by ethidium bromide fluorescence. Southern hybridization of PCR amplified transcripts

with the specific 5′-^{32}P-labelled oligonucleotide probes demonstrated the presence of the M_1 receptor mRNAs in dissociated ventricular cardiocytes (Fig. 11). Conversely, the M_3 and M_4 mAChR subtype transcripts were not identified in this cell preparation using the sensitive RT–PCR technique.

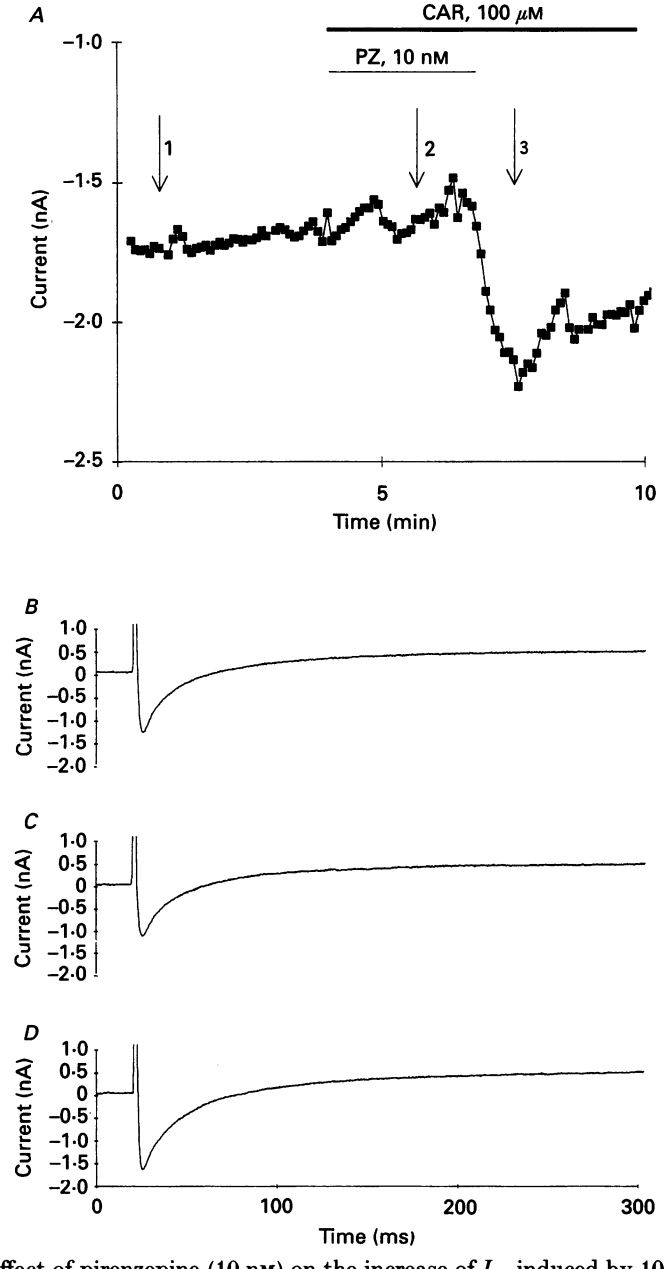

Fig. 8. Effect of pirenzepine (10 nM) on the increase of I_{Ca} induced by 10 μM carbachol in a cell perfused with 50 μM cAMP (internal and external potassium solutions). The bars indicate the period of application of the different substances (*A*). Single traces recorded at the times indicated by arrows 1–3 are shown in *B* (control), *C* (CAR + PZ) and *D* (CAR).

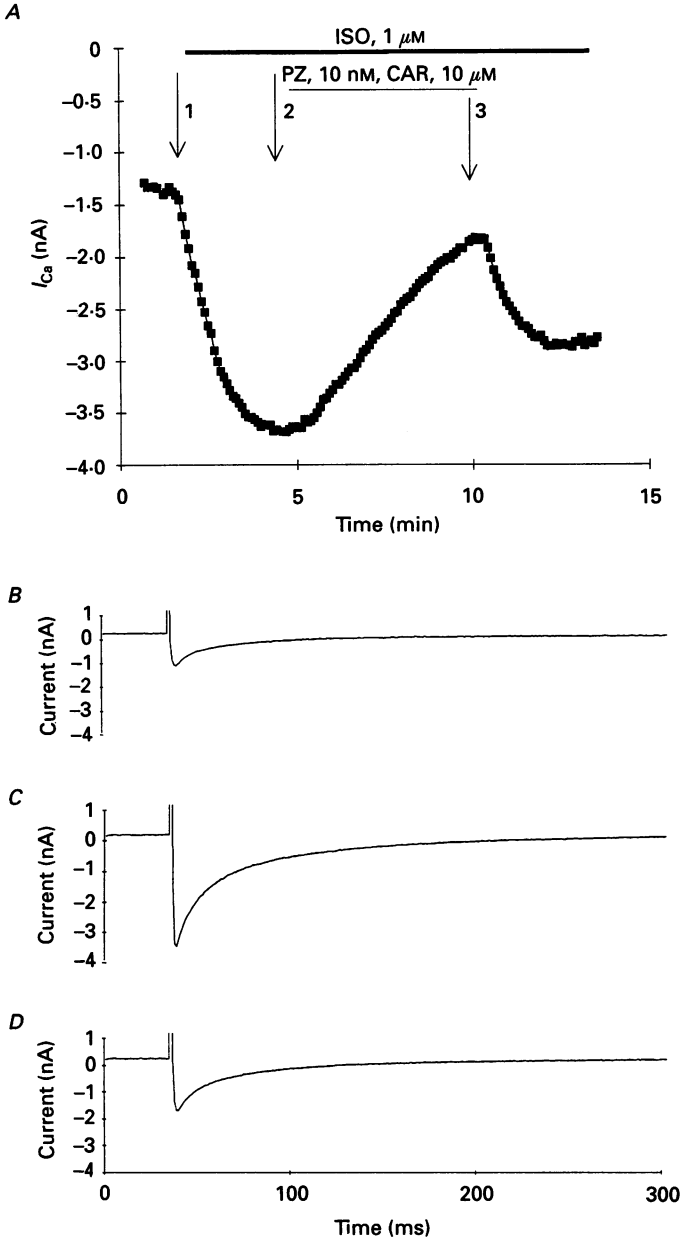

Fig. 9. Failure of pirenzepine (10 nM) to block carbachol (10 μM) inhibition of the increase of I_{Ca} induced by ISO (10 μM) (internal and external potassium solutions). In *A*, the bars indicate the period of application of the different substances. Single traces recorded at the time indicated by arrows 1–3 are shown in *B* (control), *C* (ISO) and *D* (ISO, PZ and CAR).

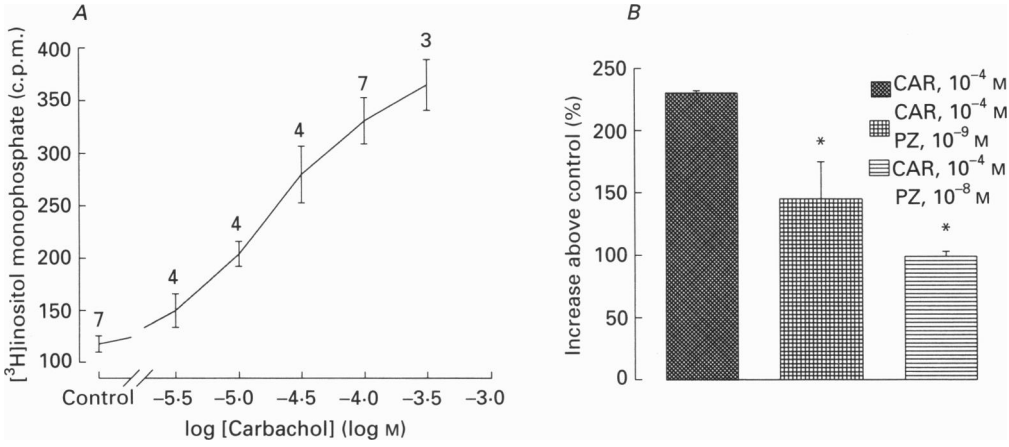

Fig. 10. *A*, dose-dependent stimulation of [^{3}H]inositol monophosphate accumulation induced by carbachol in guinea-pig ventricular slices. Data are the means ± S.E.M. The number of measurements for unstimulated control and for each concentration of carbachol tested is reported in the figure. *B*, effect of two different concentrations of pirenzepine on stimulation of [^{3}H]inositol monophosphate induced by 100 μM carbachol in guinea-pig ventricular slices. Slices were treated with carbachol (CAR), CAR and 1 nM pirenzepine (PZ) and CAR and 10 nM PZ as described in Methods. Data are the means ± S.E.M. percentage increase above control from 3 independent experiments (* $P <$ 0·05 *vs.* carbachol).

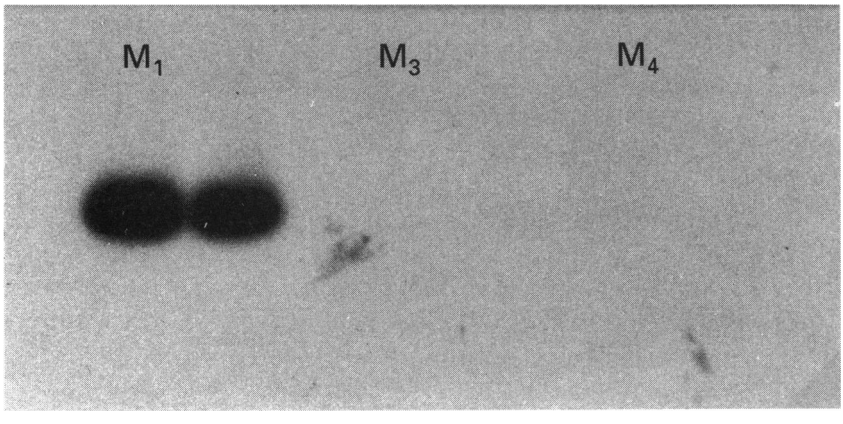

Fig. 11. Southern analysis with ^{32}P-labelled mAChR-specific probes of the RT–PCR amplified reaction products obtained after 45 cycles of PCR. Each PCR mixture contained cDNA prepared from 120 ng total RNA from guinea-pig ventricular cardiocytes and oligonucleotide primers specific for M_1, M_3 and M_4 receptors (see Methods). Each lane was hybridized with the corresponding receptor-specific ^{32}P-labelled probe. The autoradiograms were exposed with two enhancing screens at −70 °C for 2 h.

DISCUSSION

The results presented in this article demonstrate that the muscarinic agonist carbachol induces a paradoxical positive modulation of the cardiac calcium current. This effect is small, and is elicited only by high concentrations of carbachol. The

increase of I_{Ca} elicited by the muscarinic agonist can be unmasked by functional inactivation of M_2 receptors and appears to be triggered by the activation of a muscarinic receptor subtype that is selectively blocked by pirenzepine.

A number of studies have demonstrated heterogeneity among muscarinic receptors on the basis of agonist and antagonist selectivity and have ultimately led to the widely accepted classification based upon the selective muscarinic antagonist, pirenzepine, which blocks the M_1 muscarinic receptor subtype (Hammer *et al.* 1980). Indeed, molecular cloning has demonstrated that muscarinic receptors comprise a family of at least five genes which are differentially expressed in peripheral tissues and in the central nervous system and can be functionally separated into two classes: M_1, M_3 and M_5 which couple strongly to phosphoinositide hydrolysis; M_2 and M_4 which inhibit adenylate cyclase activity (Kubo *et al.* 1986; Bonner, Buckley, Young & Brann, 1987; Peralta, Winslow, Ashkenazi, Smith, Ramachandran & Capon, 1988).

Several lines of evidence have pointed out that in atrial and ventricular cardiocytes of different species the major effects of acetylcholine are mediated by the activation of M_2 receptors that are negatively coupled to adenylate cyclase through a pertussis toxin-sensitive G protein (Hartzell, 1988). The inhibition of the adenylate cyclase would then result in reduced phosphorylation of L-type calcium channels, consequent decrease of calcium current and in the regulation of the pacemaker current, I_f, in nodal and Purkinje cells.

Here we provide evidence from different sets of experiments that functionally active M_1 muscarinic receptors are present in guinea-pig ventricular cardiocytes and that their activation triggers an increase of L-type calcium current and stimulates PI hydrolysis. To avoid possible errors due to modifications of potassium currents, all the experiments were conducted both in caesium-containing solutions and in normal potassium solutions and no difference can be reported. Firstly, in our electrophysiological experiments, we have shown that carbachol stimulation of calcium current can be detected in ventricular cardiocytes under experimental conditions that unmask this effect from the major muscarinic inhibitory effect. High concentrations of carbachol induced a small increase of I_{Ca} in ventricular cardiocytes treated with PTX. Moreover, if the adenylate cyclase mechanism was bypassed by directly saturating the cell interior with cAMP, challenging the cells with carbachol produced in most cells a further increase of the current, indicating that a separate pathway of regulation of I_{Ca} must exist.

Pharmacological characterization of this effect indicates that the carbachol-induced positive modulation of calcium current is triggered by the activation of M_1 receptor subtype. Indeed, the carbachol-elicited increase of I_{Ca} is blocked by the M_1-selective muscarinic antagonist pirenzepine at concentrations that fail to affect the M_2-mediated inhibitory effect. Moreover we have shown that carbachol stimulates the hydrolysis of PI in guinea-pig ventricular slices and that this effect is blocked by low concentrations of pirenzepine. Thus M_1 receptor subtypes appear to be functionally coupled to this transduction mechanism in the guinea-pig heart as well as in many other tissues.

Finally, our data demonstrate that ventricular cardiocytes express muscarinic receptor of the M_1 subtype. Previous evidence has shown that high levels of M_2

mAChR mRNA can be detected in heart tissue from many species, and that this receptor subtype triggers major responses to vagal input (Bonner *et al.* 1987; Peralta *et al.* 1988). By using the sensitive technique of RT–PCR, we now report that muscarinic cholinergic receptors in the adult mammalian heart also comprise the M_1 receptor subtype, while the mRNAs encoding the M_3 and M_4 receptors were not found. Our data are, to our knowledge, the first direct evidence that in heart mAChR other receptors than M_2 are expressed, although in much lower amounts. The above observations strongly suggest that M_1 mAChRs are present in guinea-pig ventricles and are functionally coupled to signal transduction pathways. These include activation of calcium current and stimulation of PI breakdown.

Several reports have shown that concentrations of carbachol or acetylcholine similar to those used in our experiments can induce positive chronotropism and inotropism in mammalian cardiac preparations. However, the cellular mechanisms that trigger the positive chronotropism and inotropism still remain, at least in part, unclear. Two hypotheses have been recently proposed (see Pappano, Matsumoto, Tajima, Agnarsson & Webb, 1988; Matsumoto & Pappano, 1989; and Pappano, 1991 for a review). The former involves the entry of Na^+ which would induce an increase of cytosolic free Ca^{2+} via the Na^+–Ca^{2+} exchange. The latter involves stimulation of membrane phospholipid hydrolysis and protein kinase C activation leading to mobilization of Ca^{2+} from intracellular stores. We now propose that the stimulation of Ca^{2+} currents induced by M_1 mAChRs may directly contribute to the regulation of cardiac force. This possibility is in line with the observation that stimulation of purinergic receptors in frog cardiac cells (Alvarez, Mongo, Scamps & Vassort, 1990) and of P_2 purinergic receptors in rat ventricle also induce a cAMP-independent increase of I_{Ca} that is associated with PI-turnover activation and an increase in inotropy (Leggsyer, Poggioli, Renard & Vassort, 1988; Scamps, Leggsyer, Mayoux & Vassort, 1990). Moreover, Rosen, Steinberg & Danilo (1990) showed that in Purkinje fibres of young dogs pirenzepine selectively blocks the muscarinic stimulation of the heart rate, suggesting an involvement of the M_1 mAChR. Our results, on the other hand, do not demonstrate which mechanism couples M_1 receptors to the increase of I_{Ca}. In ventricular cardiocytes activation of M_1 mAChRs also triggers the membrane phosphoinositide breakdown. It remains to be determined whether the two phenomena are related to each other or whether they may independently contribute to regulation of the cell activity. Recent experiments published by Bourinet, Fournier, Lory, Charnet & Nargeot (1992) have shown that cardiac calcium channels expressed in *Xenopus* oocytes increase their current after stimulation of protein kinase C, thus suggesting a possible mechanism.

In conclusion, data presented in this paper suggest that functionally active M_1 mAChRs are present in ventricular cardiocytes and may mediate positive inotropism induced by muscarinic agents. It has been asked what the physiological role for the paradoxical positive inotropic effect of the stimulation of mAChRs can be. The positive inotropic effect might act as a limiting mechanism to prevent excessive suppression of cardiac activity (Pappano, 1991).

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