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Muscarinic receptors mediate negative and positive inotropic effects in mammalian ventricular myocardium: differentiation by agonists

Michael Korth & Volker Kühlkamp

Institut für Pharmakologie und Toxikologie der Technischen Universität München, Biedersteiner Strasse 29, D-8000 München 40, Federal Republic of Germany

1 The concentration-dependence of the negative and positive inotropic effect of choline esters and of oxotremorine was studied in isometrically contracting papillary muscles of the guinea-pig. The preparations were obtained from reserpine-pretreated animals and were electrically driven at a frequency of 0.2 Hz.

2 In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX, 100 $\mu mol\ l^{-1}$), choline esters and oxotremorine produced concentration-dependent negative inotropic effects. Oxotremorine exhibited the highest negative inotropic potency (with a half-maximal effective concentration, EC_{50}, of 20 $nmol\ l^{-1}$) followed by carbachol (139 $nmol\ l^{-1}$), methacholine (490 $nmol\ l^{-1}$), acetylcholine in the presence of 10 $\mu mol\ l^{-1}$ physostigmine (1.36 $\mu mol\ l^{-1}$ and bethanechol (10 $\mu mol\ l^{-1}$)). Atropine was a competitive antagonist of the negative inotropic effects. Carbachol and oxotremorine decreased $\dot{V}_{max}$, overshoot and duration of slow Ca^{2+}-dependent action potentials which had been elicited in the presence of 100 $\mu mol\ l^{-1}$ IBMX.

3 Choline esters produced a concentration-dependent positive inotropic effect. With an EC_{50} of 32 $\mu mol\ l^{-1}$, carbachol was the most potent compound, followed by methacholine (35 $\mu mol\ l^{-1}$), acetylcholine in the presence of 10 $\mu mol\ l^{-1}$ physostigmine (46 $\mu mol\ l^{-1}$) and bethanechol (142 $\mu mol\ l^{-1}$). Compared to carbachol and methacholine which increased force by 100% of control, the increase induced by acetylcholine and bethanechol was only 64 and 58%, respectively. Atropine shifted the concentration-effect curves of all choline esters to higher concentrations. Choline esters caused intracellular Na^+ activity to increase in the quiescent papillary muscle. This effect was reversed by atropine.

4 Oxotremorine produced a small concentration-dependent positive inotropic effect (about 30% of the maximal effect of carbachol) which was resistant to atropine. Oxotremorine was a potent inhibitor of the positive inotropic effect of choline esters, and did not cause an increase in intracellular Na^+ activity in the quiescent papillary muscle.

5 The results show that muscarinic receptors of the ventricular myocardium mediate two inotropic effects, which are opposite in direction and differ in their concentration-dependence by a factor of 100. Although agonists differentiate between both inotropic effects, it is unknown whether the receptors involved represent receptor states or separate receptor subpopulations. The negative inotropic effect of choline esters and of oxotremorine can be best explained by adenylate cyclase inhibition. While stimulation of phosphoinositide hydrolysis might have been responsible for the positive inotropic effect of choline esters via modulation of cation-fluxes across the cell membrane, such a mechanism was not involved in the positive inotropic effect of oxotremorine.

Introduction

Activation of muscarinic receptors in heart muscle results in various biochemical effects, including increased synthesis of cyclic GMP, decreased synthesis of cyclic AMP (George *et al.*, 1973), rise in intracellular Na^+ activity (Korth & Kühlkamp, 1985) and enhanced turnover of membrane phosphoinositides (Brown & Brown, 1983). While most of the biochemical events have been linked with the inotropic

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state of the heart, either via modulation of voltage-controlled ionic channels (Trautwein *et al.*, 1982), Na^+-Ca^{2+} exchange (Korth & Kühlkamp, 1985) or by regulation of the sensitivity of cardiac myofibrils to Ca^{2+} (Horowits & Winegrad, 1983), a possible role of phosphoinositide turnover in the control of force has not yet been established. In a recent study utilizing embryonic chick heart cells, Brown & Brown (1984) have demonstrated that activation of phosphoinositide metabolism by carbachol requires full receptor occupancy, while only a fraction of muscarinic receptors need to be occupied to cause inhibition of adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) synthesis. As a consequence, the concentration-effect relationships differed 100 fold for both effects. Interestingly, the muscarinic receptor agonist oxotremorine has been found, in the same study, to be an extremely weak agonist for phosphoinositide turnover, but to be a full agonist for inhibition of cyclic AMP synthesis.

The present study was undertaken to test the hypothesis that the positive inotropic effect of high concentrations of carbachol (Korth & Kühlkamp, 1985) could be related to enhanced phosphoinositide metabolism. For this purpose, concentration-effect relationships for the negative inotropic action, due to inhibition of cyclic AMP synthesis, and for the positive inotropic action of choline esters and of oxotremorine were determined on guinea-pig papillary muscles. The accordance of the present results with those of Brown & Brown (1984), together with recent findings of elevated intracellular Ca^{2+} levels in response to phosphoinositide hydrolysis (Berridge, 1984), makes a causal relationship between enhanced phospholipid metabolism and positive inotropic effect an attractive hypothesis.

Methods

Preparations

Guinea-pigs of either sex weighing 250–350 g were killed by cervical dislocation. The animals were pretreated with reserpine ($5\,mg\,kg^{-1}$ body weight, injected intraperitoneally 24 h before the experiment) to avoid the release of endogenous noradrenaline. Right ventricular papillary muscles with a diameter of <1 mm were rapidly excised from the isolated heart and mounted in a two-chambered organ bath with an internal circulation of the bath solution (volume 50 ml) as described by Reiter (1967). The incubation medium was constantly gassed and kept in circulation by 5% CO_2 and 95% O_2; the temperature was 35°C, pH 7.5. The composition of the medium was (in $mmol^{-1}$): NaCl 115, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 10, $CaCl_2$ 3.2 or 6.2.

Force measurement

The muscles were stimulated electrically at their mural end through two punctate platinum electrodes with square wave pulses of 1 ms in duration and at an intensity slightly above stimulation threshold. Force of contraction was recorded isometrically by means of an inductive force transducer (Q11, 10p; Hottinger Baldwin Messtechnik, Darmstadt, FRG) connected to an oscilloscope and a pen recorder. The resting force was kept constant at 3.96 mN throughout the experiment. After dissection, the muscles were allowed to equilibrate in the bath solution for 1 h at a stimulation frequency of 1 Hz. Thereafter, the stimulation frequency was lowered to 0.2 Hz and the drug intervention was started as soon as force of contraction had reached a steady state.

Electrophysiological measurements

In order to measure force and transmembrane electrical activity, the papillary muscle was mounted horizontally in a perfusion chamber (volume 2 ml) perfused at a constant rate of $10\,ml\,min^{-1}$. The voltage recording electrodes had tip resistances of 15–20 MΩ and small tip potentials when filled with $3\,mol\,l^{-1}$ KCl acidified to a pH of 2 with HCl. The construction and calibration of Na^+-sensitive microelectrodes using the neutral ion exchange resin ETH 227 (Steiner *et al.*, 1979) has been described in detail elsewhere (Sheu & Fozzard, 1982). The muscles were impaled with a conventional electrode and a Na^+-sensitive microelectrode so that the impalements were as close as possible. From the potentials measured with the two microelectrodes, the a^i_{Na} of the cell was calculated by means of the following equation

$$E^i_{Na} - E_m = E_o + S \log (a^i_{Na} + k_{Na,K}\, a^i_K), \qquad (1)$$

where E^i_{Na} is the transmembrane potential measured with the Na^+-sensitive microelectrode with respect to the reference electrode in the bath, E_m the transmembrane potential measured with the conventional microelectrode, E_o a constant potential of the Na^+-sensitive microelectrode, and S the slope of the Na^+-sensitive microelectrode (ranging from 50 to 61 mV per decade) as determined in NaCl solutions containing $0.1\,mmol\,l^{-1}$ EGTA. The $k_{Na,K}$ is the selectivity coefficient of the Na^+-sensitive microelectrode which ranged from 0.01 to 0.02 for $K^+ : Na^+$ in a mixture of $10\,mmol\,l^{-1}$ NaCl/$140\,mmol\,l^{-1}$ KCl. The a^i_K is the intracellular K^+ activity which was $110\,mmol\,l^{-1}$, as experimentally determined with K^+-sensitive microelectrodes in 3 papillary muscles. Before and after each experiment the electrodes were calibrated at 35°C with pure solutions of NaCl and with mixtures of NaCl and KCl with constant total ionic strength of $300\,mmol\,l^{-1}$. Any change in calibration meant that

the experiments were discarded. Pure NaCl activity coefficients were calculated using the equations derived by Pitzer & Mayorga (1973). For the mixed solutions the activity coefficients were calculated by Guggenheim-Scatchard-Robinson generalized equations (Harned & Robinson, 1968). Ion activity coefficients were calculated using the McInnes convention (1961). Na^+-sensitive microelectrodes are affected by Ca^{2+} (Steiner *et al.*, 1979). The average selectivity coefficient, $k_{Na,Ca}$, of the Na^+-sensitive microelectrode was 2.8 in a mixture of $10\,mmol\,l^{-1}$ NaCl/ $140\,mmol\,l^{-1}$ KCl containing pCa 6. The pCa was calculated according to Bers (1982). A rise of Ca^{2+} from 0 to pCa 6 gave an apparent extra Na^+ activity of about $1\,mmol\,l^{-1}$. Since in the experiments described below contractures never developed, the contribution of intracellular Ca^{2+} to the a^i_{Na} measurements must have been negligibly small which justified the omission of $k_{Na,Ca}$ and a^i_{Ca} in calculating a^i_{Na} in Equation 1.

Conventional and Na^+-sensitive microelectrodes were connected to a dual channel high impedance electrometer (model F-223 A, WP Instruments, New Haven, Conn., U.S.A.). The signals were displayed separately and electronically subtracted on digital panel meters. The panel meter readings were used for calculating a^i_{Na}. Potential and force measurements were also displayed on a Digital Oscilloscope (Nicolet, Madison WI, U.S.A.) and a chart recorder for data recording. Electronic differentiation was used to obtain the first time derivative of the action potential upstroke, $\dot{V}_{max}$. In some experiments the K^+ concentration of the bath solution was raised to $24\,mmol\,l^{-1}$ in order to inactivate the Na^+ channels. Under this condition, a 2–3 times higher intensity of stimulation was necessary to evoke slow action potentials.

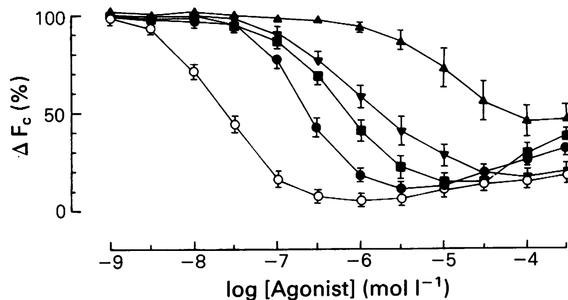

Figure 1 The negative inotropic effect of oxotremorine and of choline esters in the presence of 3-isobutyl-l-methyl xanthine (IBMX). Cumulative concentration-effect curves in the presence of $100\,\mu mol\,l^{-1}$ IBMX for oxotremorine (○), carbachol (●), methacholine (■), acetylcholine + $10\,\mu mol\,l^{-1}$ physostigmine (▼) and for bethanechol (▲). Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol, are shown (5 muscles). Ordinate scale: decrease of the positive inotropic effect of $100\,\mu mol\,l^{-1}$ IBMX (ΔFc 100% = 13.1 ± 1.8 mN (○), 15.3 ± 2.5 mN (●), 16.7 ± 3.2 mN (■), 15.9 ± 4.3 mN (▼), 14.0 ± 1.4 mN (▲). Pre-drug control of F_c before the application of IBMX was 2.8 ± 0.5 mN (○), 4.8 ± 1.2 mN (●), 2.3 ± 0.9 mN (■), 4.2 ± 1.5 mN (▼), 2.7 ± 0.6 mN (▲). Reserpine-pretreated guinea-pigs were used for all experiments. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o$ = $3.2\,mmol\,l^{-1}$.

Materials

The drugs used were: carbamylcholine chloride (carbachol), acetyl-β-methylcholine chloride (methacholine), acetylcholine chloride, carbamyl-β-methylcholine chloride (bethanechol), oxotremorine sesquifumarate and 3-isobutyl-l-methyl xanthine (Sigma München, FRG); reserpine (dissolved in 5% ascorbic acid) was obtained from Serva (Heidelberg, FRG). The ion exchanger resin ETH 227 was a gift from Prof. Simon, Zürich.

Statistics and calculations

The data are presented as arithmetic means ± s.e.mean. Significance tests were performed by Student's *t* test. Significance was assumed when $P < 0.05$. Competitive antagonism was calculated according to the proposal of Waud (1975). Calculations were performed on a PDP-11 (Digital Equipment Corporation).

Results

The negative inotropic effect of choline esters and of oxotremorine

Figure 1 shows that oxotremorine and choline esters antagonized the positive inotropic effect of the phosphodiesterase inhibitor 3-isobutyl-l-methyl xanthine (IBMX). The concentrations that half-maximally inhibited the inotropic effect of $100\,\mu mol\,l^{-1}$ IBMX (log EC_{50}) were -7.72 ± 0.05 for oxotremorine, -6.66 ± 0.06 for carbachol, -6.35 ± 0.1 for methacholine, -5.96 ± 0.15 for acetylcholine (in the presence of $10\,\mu mol\,l^{-1}$ physostigmine) and -5.01 ± 0.12 for bethanechol. Oxotremorine and carbachol were about equally efficacious, they inhibited the IBMX-induced effect by 95 and 89%, respectively. Methacholine, acetylcholine (in the presence of $10\,\mu mol\,l^{-1}$ physostigmine) and bethanechol caused an inhibition by 85, 81 and 54%, respectively. As can be seen from Figure 1, application of high concentrations of oxotremorine or choline esters

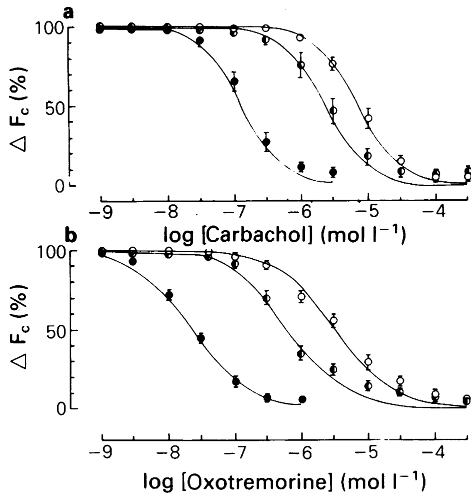

Figure 2 Competitive antagonism by atropine of the negative inotropic effect of (a) carbachol and (b) oxotremorine. Three cumulative concentration-effect curves were obtained for either oxotremorine or carbachol from each muscle, first in the absence (●), and then in the presence of 3 (◐) and 10 nmol l^{-1} atropine (○), respectively. Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol, are shown (5 muscles). The data were fitted by theoretical curves describing competitive antagonism. Ordinate scales: decrease of the positive inotropic effect of 100 μmol l^{-1} 3-isobutyl-l-methyl xanthine (IBMX) (ΔF_c 100% before application of oxotremorine = 12.8 ± 2.3 mN in the absence, 14.9 ± 2.8 mN and 15.2 ± 2.2 mN in the presence of 3 and 10 nmol l^{-1} atropine, respectively; ΔF_c 100% before application of carbachol = 17.3 ± 2.5 mN in the absence, 21.5 ± 3.1 mN and 22.0 ± 3.0 mN in the presence of 3 and 10 nmol l^{-1} atropine, respectively). Pre-drug control of F_c before application of IBMX was 2.9 ± 0.7 mN, 3.0 ± 0.8 mN and 2.9 ± 0.8 mN in the oxotremorine series and 4.3 ± 1.2 mN, 4.8 ± 1.2 mN and 4.5 ± 1.0 mN in the carbachol series. Reserpine-pretreated guinea-pigs were used for all experiments. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o$ = 3.2 mmol l^{-1}.

resulted in a decrease of the negative inotropic effect. At an agonist concentration of 300 μmol l^{-1}, this decrease was significant for oxotremorine, carbachol and methacholine. Inhibition of the negative inotropic effects of oxotremorine and of choline esters by atropine is evidence for the involvement of muscarinic receptors. Figure 2 shows that atropine (3 and 10 nmol l^{-1}) in the presence of 100 μmol l^{-1} IBMX shifted the concentration-effect curves of oxotremorine and of carbachol parallel to higher concentrations. The experimentally obtained data could be well fitted by a competitive antagonism. The curves shown in in Figure 2 were calculated according to the method proposed by Waud (1975). The dissociation constants for the atropine-receptor complex, K_B, were 0.18 ± 0.01 nmol l^{-1} in the presence of carbachol and 0.17 ± 0.01 nmol l^{-1} in the presence of oxotremorine. The stoichiometric coefficient for the binding of atropine to the receptor was close to 1 in the presence of carbachol (0.96 ± 0.02). In the presence of oxotremorine, however, a coefficient of 1.41 ± 0.02 was found indicating that the antagonism increased with increasing concentrations of atropine.

In order to determine whether the negative inotropic effects of oxotremorine and of choline esters in the presence of IBMX were due to a decrease in slow Ca^{2+} inward current, the effects of both muscarinic receptor agonists were studied on slow Ca^{2+}-dependent action potentials in potassium-depolarized preparations. As shown in Figure 3, oxotremorine and carbachol at concentrations that produced a 50% decrease of the positive inotropic effect of IBMX, attenuated $\dot{V}_{max}$, overshoot and duration of slow Ca^{2+}-dependent action potentials which had been enhanced in the presence of 100 μmol l^{-1} IBMX. As summarized in Table 1, results similar to those of Figure 3 were also obtained in 3 other catecholamine-depleted papillary muscles.

The positive inotropic effect of choline esters and of oxotremorine

In order to test whether the small increase in force of contraction induced by high concentrations of oxotremorine and choline esters in the presence of IBMX was due to the receptor-inactivation, concentration-effect relationships were obtained for muscarinic receptor agonists in the absence of cyclic AMP-mediated effects. As shown in Figure 4, acetylcholine and its congeners carbachol, methacholine and bethanechol produced a concentration-dependent positive inotropic effect in the noradrenaline-depleted papillary muscle of the guinea-pig. Half-maximal effective concentrations (log EC_{50}) were −4.55 ± 0.1 for carbachol, −4.46 ± 0.03 for methacholine, −4.35 ± 0.04 for acetylcholine and −3.96 ± 0.23 for bethanechol (n = 6 for each choline ester). As can be seen from Figure 4, carbachol and methacholine had the same inotropic efficacy, force of contraction increased by 6.87 ± 1.35 and 6.86 ± 1.4 mN, respectively. In contrast, the inotropic efficacy of acetylcholine and bethanechol was only 64 and 58% that of a maximally effective concentration of carbachol. Concentration-effect curves which were obtained for acetylcholine after the muscles had been pre-incubated with 10 μmol l^{-1} physostigmine for 40 min showed a slightly higher mean log EC_{50} (−4.38 ± 0.05) than those obtained in the absence of the esterase inhibitor. The inotropic efficacy of acetylcholine was not in-

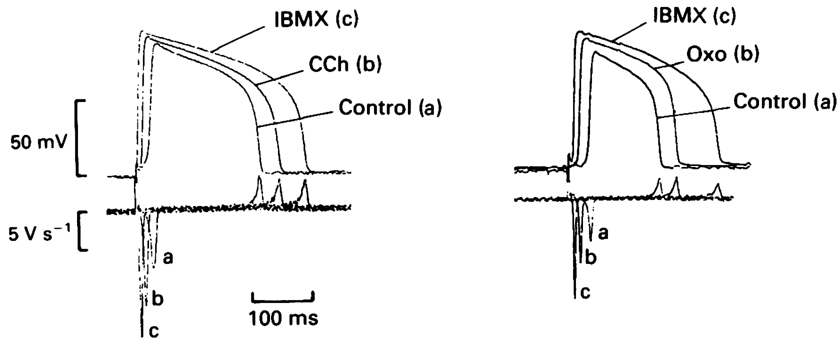

Figure 3 The effect of 100 μmol l^{-1} 3-isobutyl-l-methyl xanthine (IBMX) on the slow action potential and maximum rate of depolarization (V s^{-1}) and the attenuation of these effects by 0.3 μmol l^{-1} carbachol (CCh) or 30 nmol l^{-1} oxotremorine (Oxo). The concentrations correspond to EC_{50} values obtained from Figure 1. Different noradrenaline-depleted papillary muscles were used in the carbachol and oxotremorine experiment. Records are from one continuous microelectrode impalement. IBMX was allowed to act for 30 min before the record was taken. The effects of oxotremorine and of carbachol were recorded 5 min after drug-application. $[K^+]_o$ = 24 mmol l^{-1}. $[Ca^{2+}]_o$ = 3.2 mmol l^{-1}. Contraction frequency 0.2 Hz.

creased in the presence of physostigmine. The positive inotropic effect of choline esters started within 10 s after application, was maximal within 5 to 6 min and remained constant thereafter. The time parameters of the isometric contraction curve (time to peak force and relaxation time) were not changed by any of the choline esters up to a concentration of 1 mmol l^{-1}. The concentration-effect curves of choline esters were shifted to higher concentrations by 10 nmol l^{-1} atropine and were almost completely suppressed in the presence of 100 nmol l^{-1} atropine. This effect of atropine (100 nmol l^{-1}) is shown in Figure 4 for one representative choline ester. The muscarinic receptor agonist oxotremorine also produced a concentration-dependent positive inotropic effect (0.1–300 μmol l^{-1}) in the noradrenaline-depleted papillary muscle. As can be seen from Figure 5a, the maximum positive inotropic effect induced by 300 μmol l^{-1} oxotremorine

Table 1 Effects of carbachol and oxotremorine on slow Ca^{2+}-dependent action potentials in the presence of 100 μmol l^{-1} 3-isobutyl-l-methyl xanthine (IBMX)

Condition‡	*Resting potential* (mV)	*Overshoot* (mV)	*Max. rate of rise* (Vs^{-1})	*Duration of action potential at 90% repolarization* (ms)
Control				
24 mmol l^{-1} K^+	−49.2 ± 0.5	37.4 ± 1.5	8.9 ± 1.7	175.3 ± 30.7
+ 100 μmol l^{-1} IBMX (4)	−48.1 ± 0.3	46.5 ± 2.0	23.9 ± 6.1	257.2 ± 22.7
+ 30 nmol l^{-1} oxotremorine‡§	−49.2 ± 0.5	39.9 ± 1.5*	12.4 ± 4.1*	200.1 ± 12.9*
Control				
24 mmol l^{-1} K^+	−49.4 ± 0.9	38.7 ± 0.5	7.6 ± 0.4	181.0 ± 6.1
+ 100 μmol l^{-1} IBMX (4)	−49.4 ± 1.0	47.4 ± 0.5	21.3 ± 0.3	259.5 ± 6.0
+ 0.3 μmol l^{-1} carbachol‡§	−49.4 ± 1.0	41.6 ± 0.6*	14.2 ± 0.4*	212.6 ± 8.8*

Preparations obtained from reserpine-pretreated guinea-pigs (5 mg kg^{-1} body weight reserpine injected intraperitoneally 24 h before the experiment); stimulation frequency 0.2 Hz. Mean values ± s.e.mean are presented. Numbers in parentheses give the number of experiments.
‡ Electrode impalements were maintained during drug interventions.
§ Concentration that inhibits the positive inotropic effect by 50%.
* Significant ($P < 0.001$) vs the values in the presence of IBMX alone.

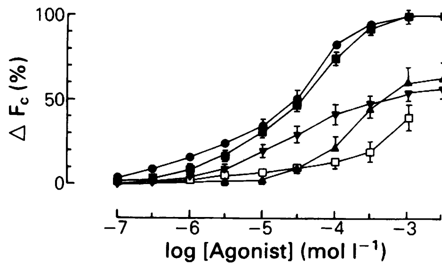

Figure 4 The positive inotropic effect of choline esters. Two cumulative concentration-effect curves were obtained from each muscle, one for carbachol ●) the other either for methacholine (■), acetylcholine (▼) or bethanechol (▲). A concentration-effect curve for methacholine in the presence of a 100 nmol l^{-1} atropine (□) is also shown. Means (with s.e.mean indicated by vertical lines if it exceeds the size of the symbol) for carbachol (18 muscles) and the other choline esters (6 muscles) are shown. Ordinate scale: positive inotropic effect, ΔF_c, expressed as % of the value observed with a maximally effective concentration of carbachol (100% = 6.9 ± 1.4 mN). Pre-drug control of F_c was 5.9 ± 0.9 mN (●), 6.6 ± 0.8 mN (▼), 9.1 ± 1.3 mN (■) 6.1 ± 0.6 mN (▲), 5.4 ± 1.6 mN (□). Reserpine-pretreated guinea-pigs were used for all experiments. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o$ = 6.2 mmol l^{-1}.

was only 30% that of a maximal effective concentration of carbachol, when determined on the same preparation. The positive inotropic effect of oxotremorine developed with a time course comparable to that of choline esters. Figure 6 shows that the shape of the isometric contraction curve was unaffected by oxotremorine up to a concentration of 300 μmol l^{-1}. Figure 6 also shows (substantiated in 5 other muscle preparations) that 300 μmol l^{-1} oxotremorine did not alter resting membrane potential (V_m), overshoot (OS) and maximum rate of depolarization ($\dot{V}_{max}$), but prolonged significantly action potential duration at 0 (APD_0) and at 90% repolarization (APD_{90}). The values (n = 6) before and 10 min after the addition of 300 μmol l^{-1} oxotremorine were −82.3 ± 1.8 and −82.0 ± 1.6 mV for V_m, 46.2 ± 1.2 and 46.0 ± 0.8 mV for OS, 209.0 ± 16.0 and 208.0 ± 15.6 V s^{-1} for $\dot{V}_{max}$, 157.5 ± 4.3 and 168.6 ± 3.8 ms for APD_0 ($P < 0.05$) and 211.8 ± 5.3 and 229.8 ± 4.3 ms for APD_{90} ($P < 0.05$), respectively. In contrast to choline esters, the positive inotropic effect of oxotremorine was not inhibited after prior incubation of the muscles with 10 or 100 nmol l^{-1} atropine (see Figure 5a). Furthermore, blockade of nicotinic receptors, histamine H_2-receptors, α- and β-adrenoceptors with 300 μmol l^{-1} hexamethonium bromide, 100 μmol l^{-1} cimetidine, 3 μmol l^{-1} phentolamine and 3 μmol l^{-1} (±)-

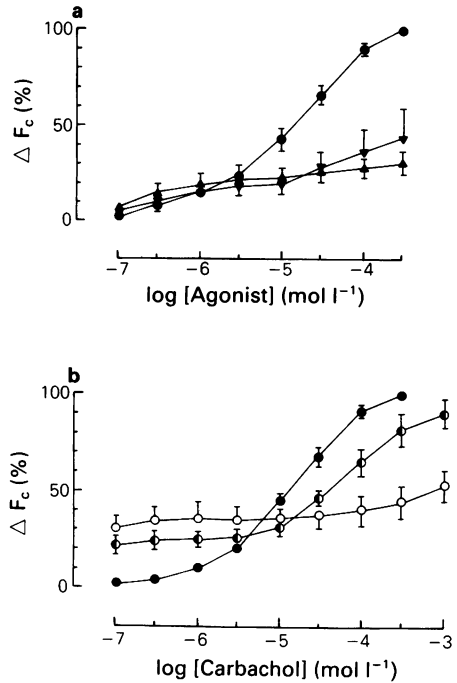

Figure 5 (a) Failure of atropine to inhibit the positive inotropic effect of oxotremorine. Three cumulative concentration-effect curves were obtained from each muscle, one for carbachol (●) the others for oxotremorine in the absence (▲) and in the presence of 100 nmol l^{-1} atropine (▼). Means, with s.e.mean indicated by vertical lines if it exceeds the size of the symbol (6 muscles) are shown. Ordinate scale: positive inotropic effect, ΔF_c, expressed as % of the value obtained with 300 μmol l^{-1} carbachol (100% = 6.5 ± 1.0 mN). Pre-drug control of F_c was 6.0 ± 0.4 mN (●), 5.3 ± 0.5 mN (▲), 6.2 ± 1.3 mN (▼). (b) Inhibition of the positive inotropic effect of carbachol by oxotremorine. Three carbachol concentration-effect curves were obtained from each muscle, one without oxotremorine (●), the two others in the presence of 1 μmol l^{-1} (◐) and 100 μmol l^{-1} oxotremorine (○), respectively. Ordinate scale: positive inotropic effect, ΔF_c, expressed as % of the value obtained with 300 μmol l^{-1} carbachol (100% = 6.4 ± 0.9 mN). Pre-drug control of F_c was 7.3 ± 2.9 mN (●), 6.2 ± 2.3 mN (◐), 6.8 ± 2.2 mN (○). Reserpine-pretreated guinea-pigs were used in all experiments depicted in (a) and (b). $[Ca^{2+}]_o$ = 6.2 mmol l^{-1}. Contraction frequency of 0.2 Hz.

propranolol, respectively, failed to antagonize the positive inotropic effect of oxotremorine. Tetrodotoxin (10 μmol l^{-1}) likewise did not attenuate the oxotremorine-induced inotropic effects. To determine whether oxotremorine interacted with mus-

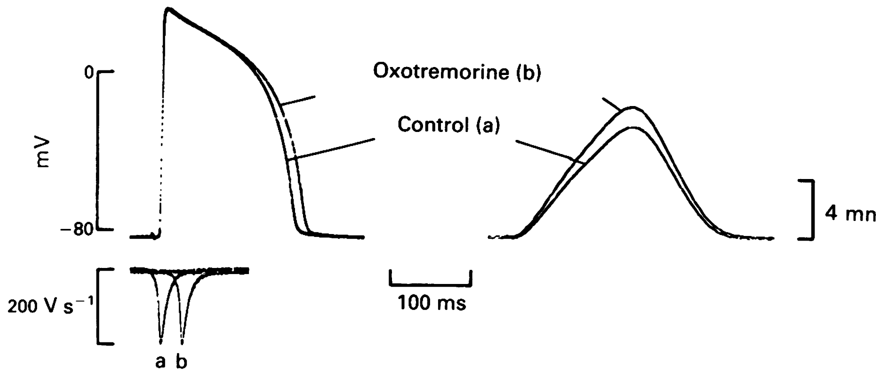

Figure 6 Effect of oxotremorine on the transmembrane action potential. Original records of the transmembrane action potential, maximum rate of depolarization ($V s^{-1}$) and isometric contraction of a noradrenaline-depleted papillary muscle before (control) and 10 min after the addition of 300 μmol l^{-1} oxotremorine. Records from the same microelectrode impalement. Contraction frequency 0.2 Hz. $[Ca^{2+}]_o = 6.2$ mmol l^{-1}.

carinic receptors responsible for the positive inotropic effect of choline esters, carbachol concentration-effect curves were obtained in the presence of 2 different oxotremorine concentrations. Figure 5b shows that oxotremorine inhibited the positive inotropic effect of carbachol and shifted the concentration-effect curves to higher carbachol concentrations. Due to the positive inotropic effect of 1 and 100 μmol l^{-1} oxotremorine, concentration-effect curves for carbachol intersected with the control curve at 2 different levels. As can be further seen from Figure 5b, the antagonism, at least in the presence of 1 μmol l^{-1} oxotremorine, was surmountable.

Intracellular Na^+ activity

Figure 7 shows the effects of 300 μmol l^{-1} acetylcholine (a) and of 300 μmol l^{-1} oxotremorine (b) on a^i_{Na} and resting membrane potential in a quiescent papillary muscle before and after the addition of 100 nmol l^{-1} atropine. Application of acetylcholine to the superfusing solution containing 6.2 mmol l^{-1} Ca^{2+} caused a^i_{Na} to increase from 5.8 mmol l^{-1} to 7.2 mmol l^{-1} within 6 min. After a^i_{Na} was stable for 15 min, 100 nmol l^{-1} atropine was added to the acetylcholine-containing solution, and a^i_{Na} returned within 15 min to the control level. Membrane resting potential remained at −82.8 mV throughout the experiment. In contrast to acetylcholine, oxotremorine did not increase a^i_{Na} significantly (Figure 7b). The values for a^i_{Na} before and 15 min after the addition of 300 μmol l^{-1} oxotremorine were 5.7 mmol l^{-1}, respectively. Additional application of 100 nmol l^{-1} atropine was also without effect on a^i_{Na}. Membrane resting potential remained stable at −83.1 mV throughout the experiment. Table 2 summarizes the effects of acetylcholine and oxotremorine on resting potential and a^i_{Na} obtained from 3 catecholamine-depleted papillary muscles bathed in 6.2 mmol l^{-1} Ca^{2+}.

Discussion

The present study demonstrates that muscarinic receptor agonists produced two distinct inotropic effects in guinea-pig ventricular myocardium which were opposite in direction and differed markedly in their concentration-dependence. The half-maximal effective concentrations needed to decrease force of contraction were about 100 fold lower than those necessary to elicit positive inotropic effect.

It is well known that the negative inotropic effect of muscarinic receptor agonists depends on the presence of drugs which increase force of contraction by augmenting cellular cyclic AMP (for review see Löffelholz & Pappano, 1985). In order to increase the cellular cyclic AMP level, the phosphodiesterase inhibitor 3-isobutyl-l-methyl xanthine (IBMX) was used in the present study. This compound has been shown to increase the cyclic AMP content in guinea-pig papillary muscles and, in contrast to other phosphodiesterase inhibitors,to mimic the mechanical and membrane electrical effects of catecholamines (Korth, 1978; Korth & Engels, 1981; Brückner *et al.*, 1985). Because of the stability of its positive inotropic effect, IBMX was preferred to catecholamines. With respect to its antagonistic effect on force of contraction and on slow Ca^{2+}-dependent action potential, oxotremorine was more potent than choline esters, probably due to

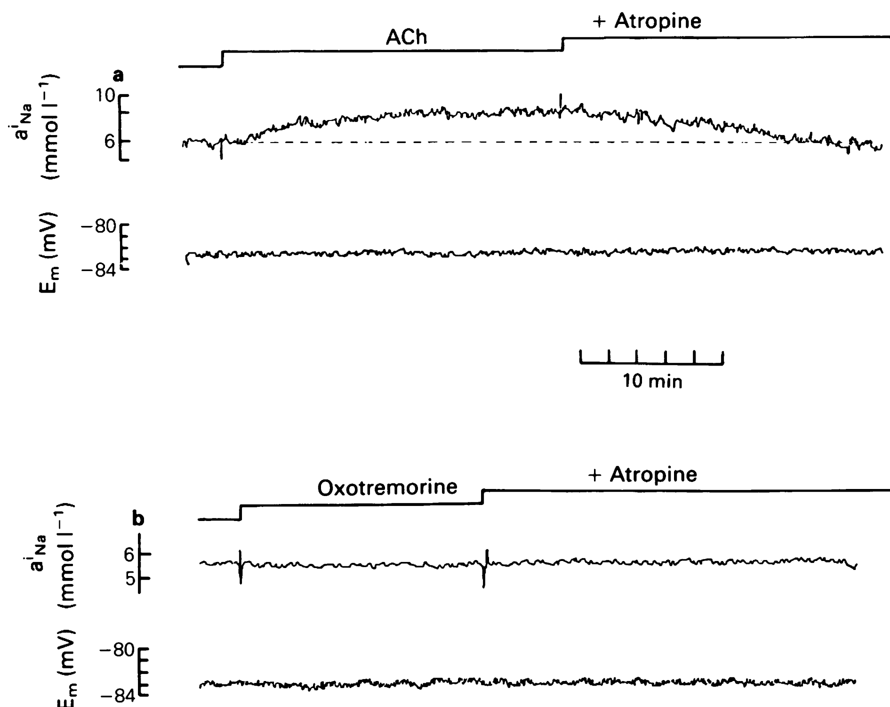

Figure 7 (a) Acetylcholine (ACh, 300 μmol l^{-1})-induced increase in intracellular Na^{+} activity. Increase in a^i_{Na} during superfusion of a resting noradrenaline-depleted papillary muscle with acetylcholine and its reversal by 100 nmol l^{-1} atropine. The resting membrane potential (E_m) remained unchanged. (b) Failure of oxotremorine (300 μmol l^{-1}) to induce an increase in intracellular Na^{+} activity. No change of resting membrane potential was observed. $[Ca^{2+}]_o$ = 6.2 mmol l^{-1} in (a) and (b).

Table 2 Effects of acetylcholine and oxotremorine on a^i_{Na} in quiescent guinea-pig papillary muscles exposed to a 6.2 mmol l^{-1} Ca^{2+}-containing solution

Condition	*Resting potential* (mV)	a^i_{Na} (mmol l^{-1})	Δa^i_{Na}‡ (mmol l^{-1})
Control	−83.4 ± 0.8	5.5 ± 0.6	
300 μmol l^{-1} acetylcholine (3)	−83.6 ± 0.8	7.6 ± 0.8*	1.6 ± 0.4
+ 100 nmol l^{-1} atropine	−83.5 ± 0.7	5.5 ± 0.6	
Control	−84.1 ± 1.2	5.3 ± 0.5	
300 μmol l^{-1} oxotremorine (3)	−84.2 ± 1.2	5.3 ± 0.5	—
+ 100 nmol l^{-1} atropine	−84.3 ± 1.2	5.3 ± 0.5	

Results are expressed as mean ± s.e.means. Preparations obtained from reserpine-pretreated animals (5 mg kg^{-1} body weight reserpine injected intraperitoneally 24 h before the experiment).
Numbers in parentheses give the number of experiments.
* $P < 0.01$ vs control.
‡ Increase in a^i_{Na} above control.

its higher potency at inhibiting cyclic AMP formation (Delhaye *et al.*, 1984; Brown & Brown, 1984). Atropine was a competitive inhibitor of the negative inotropic action of oxotremorine and of choline esters in the presence of IBMX. From the identical dissociation constants of atropine, it can be concluded that oxotremorine and choline esters interacted with the same population of muscarinic receptors. When applied at high concentrations, either in the absence or in the presence of IBMX, choline esters produced, via muscarinic receptors, a concentration-dependent positive inotropic effect. It has previously been proposed that the positive inotropic effect of high concentrations of carbachol is due to an increase in intracellular Na^+ activity, a^i_{Na}, which enhances force of contraction by stimulating Na^+-Ca^{2+} exchange (Korth & Kühlkamp, 1985). This hypothesis is strengthened by the present study; acetylcholine which was only half as effective as carbachol on force of contraction, induced an increase in a^i_{Na} which was 50% of that of a maximally effective concentration of carbachol (compare Korth & Kühlkamp, 1985). Since the carbachol-induced increase in force of contraction is strenghthened by raising the extracellular Ca^{2+} concentration (Korth & Kühlkamp, 1985), the positive inotropic effect and a^i_{Na} were determined in this study in the presence of 6.2 mmol l^{-1} Ca^{2+}. It should be noted, however, that raising or lowering Ca^{2+} in the bath solution had no influence on the EC_{50} values of the positive or the negative inotropic effect of the muscarinic receptor agonists. Among the choline esters, carbachol exhibited the highest positive inotropic potency and efficacy, followed by methacholine, acetylcholine and bethanechol. Oxotremorine produced a comparatively small increase in force of contraction, which, in contrast to the positive inotropic effect of choline esters, was not due to an increase in a^i_{Na} and was not antagonized by atropine. On the other hand, oxotremorine inhibited in a concentration-dependent manner the positive inotropic effect of carbachol and thus oxotremorine must have occupied the same muscarinic receptor as carbachol but, like an antagonist, failed to exhibit intrinsic activity. Failure of various antagonists to inhibit the oxotremorine-induced increase in force of contraction, excludes the involvement of specific membrane receptors which are known to mediate positive inotropic effects in heart muscle. On the other hand, oxotremorine prolonged the duration of the transmembrane action potential at 0 and 90% repolarization, and it may be speculated that a prolonged Ca^{2+} influx during depolarization was, at least in part, responsible for the small increase in force. The atropine-resistant positive inotropic effect of oxotremorine offers a straightforward explanation for the increase in antagonist potency of increasing concentrations of atropine in the presence of oxotremorine and IBMX. Since atropine did not prevent oxotremorine from counteracting its negative inotropic effect, the concentration-effect curves were shifted farther to the right and a stoichiometric coefficient of atropine binding greater than 1 was obtained.

In a recent study, Brown & Brown (1984) have found that carbachol elicited two biochemical effects in embryonic chick heart cells: inhibition of catecholamine-stimulated cyclic AMP formation and stimulation of phosphoinositide hydrolysis. The half-maximal effective concentrations (EC_{50}) for these biochemical effects were 0.2 and 20 μmol l^{-1}, respectively, and thus remarkably close to the EC_{50} values of 0.25 and 32 μmol l^{-1}, determined for the negative and for the positive inotropic effect of carbachol in guinea-pig heart. In accordance with the present results, Brown & Brown (1984) have also observed that oxotremorine, which was more effective than carbachol at inhibiting cyclic AMP formation, failed to stimulate phosphoinositide turnover. In the mammalian heart, muscarinic receptors are known to exhibit high and low affinity states for agonists, and it has been proposed that the high affinity state may represent receptors that can couple to and inhibit adenylate cyclase, while the low affinity state may be associated with effects on phosphoinositide turnover (Quist, 1982; Brown & Brown, 1983; 1984; McMahon & Hosey, 1985). As an alternative explanation, Brown & Brown (1984) suggested that inhibition of adenylate cyclase and activation of phosphoinositide metabolism by muscarinic receptor agonists could result from actions on the same receptor state, which is efficiently coupled to the former and inefficiently coupled to the latter effect.

While all available evidence indicates that inhibition of adenylate cyclase is the cause for the negative inotropic effect of choline esters and of oxotremorine, phosphoinositide metabolism and the positive inotropic effect of high choline ester concentrations may be merely epiphenomena. It should be noted, however, that inositol 1,4,5-trisphosphate which is one of the products of phosphoinositide hydrolysis, is known to mobilize intracellular Ca^{2+} from non-mitochondrial Ca^{2+} stores in various cell types, including the sarcoplasmic reticulum of canine heart (Hirata *et al.*, 1984; Streb *et al.*, 1983; Volpe *et al.*, 1985; Hashimoto *et al.*, 1986). Although it seems to be very unlikely that release of Ca^{2+} from cardiac sarcoplasmic reticulum was responsible for the long-lasting positive inotropic effect of choline esters, an increased Ca^{2+}-influx across the cardiac plasma membrane in response to an accumulation of inositol 1,4,5-triphosphate should be taken into consideration (see also Berridge, 1984). Whether the increase in intracellular Ca^{2+} occurs only secondary to an increase in intracellular Na^+ via Na^+-Ca^{2+} exchange (this study; Korth & Kühlkamp, 1985)

or is also due to an increased permeability of the sarcolemma for Ca^{2+} remains to be determined. Alternatively, the second product of phosphoinositide hydrolysis, 1,2-diacylglycerol, which has been shown to activate a membrane-bound pump that exchanges H^+ for Na^+ (for review, see Macara, 1985), could have increased intracellular pH and a^i_{Na} which in turn was then exchanged for extracellular Ca^{2+}.

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