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# Muscarinic receptor stimulation and cyclic AMP-dependent effects in guinea-pig ventricular myocardium

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**1** The effect of carbachol on force of contraction, contraction duration, intracellular $Na^+$ activity and cyclic AMP content was studied in papillary muscles of the guinea-pig exposed to isoprenaline or the phosphodiesterase inhibitor 3-isobutyl, 1-methyl xanthine (IBMX). The preparations were obtained from reserpine-pretreated animals and were electrically driven at a frequency of 0.2 Hz.

**2** Isoprenaline (10 nM) and IBMX (100 $\mu$M) produced comparable positive inotropic effects of 9.8 and 9.7 mN, respectively. Carbachol (3 $\mu$M) attenuated the inotropic effects by 82% (isoprenaline) and by 79% (IBMX). The shortening of contraction duration which accompanied the positive inotropic effect of isoprenaline (by 14.9%) and of IBMX (by 22.4%) was not significantly affected by 3 $\mu$M carbachol.

**3** The positive inotropic effect of 10 nM isoprenaline and of 100 $\mu$M IBMX was accompanied by an increase in cellular cyclic AMP content of 58 and 114%, respectively. Carbachol (3 $\mu$M) failed to reduce significantly the elevated cyclic AMP content of muscles exposed to either isoprenaline or IBMX.

**4** In the quiescent papillary muscle, isoprenaline (10 nM) and IBMX (100 $\mu$M) reduced the intracellular $Na^+$ activity by 28 and 17%, respectively. This decline was not influenced by the additional application of 3 $\mu$M carbachol.

**5** The results demonstrate that muscarinic antagonism in guinea-pig ventricular myocardium exposed to cyclic AMP-elevating drugs is restricted to force of contraction. The underlying mechanism does not apparently involve the cytosolic signal molecule cyclic AMP.

## Introduction

The parasympathetic and sympathetic divisions of the autonomic nervous system control cardiac function by their opposing effects (for review see Levy, 1971; Higgins *et al.*, 1973). The parasympathetic modulation of sympathetic effects on the heart occurs at both the presynaptic and postsynaptic level. In the presynaptic interaction, neuronally released acetylcholine inhibits, via stimulation of muscarinic cholinoceptors, noradrenaline release from sympathetic nerve terminals. In the postsynaptic interaction, activation of atrial muscarinic cholinoceptors results in a negative inotropic effect by a mechanism that induces conductance changes of $K^+$ and $Ca^{2+}$ channels. In ventricular myocardium, acetylcholine has been demonstrated to have no direct negative inotropic effect but to attenuate force of contraction elevated by enhanced sympathetic activity (Levy *et al.*, 1981). In isolated ventricular tissues, acetylcholine antagonizes not only the positive inotropic effect of $\beta$-adrenoceptor agonists, but also that of phosphodiesterase inhibitors and of forskolin, indicating that intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) plays an important role in postsynaptic muscarinic inhibition (for review see Löffelholz & Pappano, 1985). However, the biochemical mechanisms by which activation of muscarinic cholinoceptors interferes with the cyclic AMP-modulated cell function is still a matter of controversy. Depending on the relationship between acetylcholine-induced changes in cellular cyclic AMP content and force of contraction, inhibition of adenylate cyclase and/or inhibition of some reaction beyond cyclic AMP accumulation has been proposed as the underlying mechanism (for review see Löffelholz & Pappano, 1985). In a recent study, acetylcholine was shown to reduce catecholamine- and forskolin-induced increase in $Ca^{2+}$ inward current ($I_{Ca}$) in guinea-pig isolated ventricular myocytes, but to be ineffective when the cell was dialysed directly with cyclic AMP or the catalytic subunit of protein kinase (Hescheler *et al.*, 1986). Although this observation strongly indicates that inhibition of adenylate cyclase is the underlying mechanism, the inability of acetylcholine to decrease intracellular cyclic AMP content in guinea-pig heart is not in line with this conclusion (Watanabe & Besch, 1975).

With the exception of force of contraction and $I_{Ca}$, surprisingly little is known of how muscarinic receptor agonists influence other cell functions altered by an elevated level of cyclic AMP. It was the aim of the present study to investigate whether activation of muscarinic receptors by carbachol antagonizes cyclic AMP-dependent shortening of contraction duration and $Na^+$–$K^+$ pump stimulation in guinea-pig papillary muscle. The latter was of particular interest since the $Na^+$–$K^+$ pump, besides voltage-dependent $Ca^{2+}$ channels, constitutes another cyclic AMP-modulated transsarcolemmal ion transfer system (Pecker *et al.*, 1986). In order to complement the functional experiments, a possible effect on adenylate cyclase was also directly assessed by measuring the influence of carbachol on tissue cyclic AMP levels elevated by phosphodiesterase inhibition and by $\beta$-adrenoceptor activation. Some results of this study have been presented in preliminary form (Schmied & Korth, 1988).

## Methods

### *Preparations*

Guinea-pigs of either sex weighing 250–350 g were killed by cervical dislocation. If not stated otherwise, the animals were pretreated with reserpine (5 mg kg$^{-1}$ body weight, injected intraperitoneally 24 h before the experiment) to avoid the release of endogeneous noradrenaline. Right ventricular papillary muscles (diameter 0.5–0.8 mm) were rapidly excised from the isolated heart and mounted in a two-chambered organ bath with internal circulation of the bath solution (volume 50 ml) as described by Reiter (1967). The bath solution was constantly gassed and kept in circulation by 95% $O_2$ and 5% $CO_2$; the temperature was maintained at 35°C, pH 7.5. The composition of the bath solution was (in mM): NaCl 115, KCl

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4.7, $MgSO_4$ 1.2, $CaCl_2$ 3.2, $NaHCO_3$ 25, $KH_2PO_4$ 1.2 and glucose 10.

### *Isometric contraction recording*

The muscles were stimulated at their base through two punctate platinum electrodes with square wave pulses of 1 ms in duration and an intensity slightly above threshold. Force of contraction was recorded isometrically by means of an inductive force transducer (Q-11, 10p; Hottinger Baldwin Meßtechnik, Darmstadt, F.R.G.) connected to an oscilloscope and a pen recorder. The resting force was kept constant at 4 mN throughout the experiment. An equilibration period of at least 1 h at a stimulation frequency of 1 Hz preceded each experiment. Subsequently, the frequency of stimulation was lowered to 0.2 Hz and the drug intervention was started as soon as force of contraction had reached a steady state. The following parameters of the isometric contraction were evaluated: peak force of contraction ($F_c$); positive inotropic effect ($\Delta F_c$), i.e., increase in force over its basal value; contraction duration determined at 10% of $F_c$.

### *Cyclic AMP assay*

Catecholamine-depleted papillary muscles were obtained and mounted as described above. The muscles were exposed to isoprenaline (10 nM) for 5 or 10 min and to 3-isobutyl, 1-methyl xanthine (100 $\mu$M) for 20 min. In some experiments carbachol (3 $\mu$M) was added in the presence of the catecholamine or IBMX for 5 min. Thereafter, the muscles were removed from the organ bath and immediately frozen in liquid nitrogen. Cyclic AMP was measured by radioimmunoassay by the methods of Harper & Brooker (1975), as described by Korth *et al.* (1987). Recoveries, run with each experiment, were found to be 100% and were not altered by the substances under investigation. All measurable material was destroyed by treatment with phosphodiesterase.

### *Electrophysiological measurements*

In order to measure intracellular $Na^+$ activity ($a^i_{Na}$), papillary muscles were 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 20 MΩ and small tip potentials when filled with 3 M KCl acidified to a pH of 2 with HCl. The construction and calibration of the $Na^+$-sensitive microelectrodes with 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

$$E^i_{Na} - E_m = E_0 + S \log (a^i_{Na} + k_{Na,K}\, a^i_K), \qquad (1)$$

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, $E_0$ 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 mM 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 mM NaCl/140 mM KCl. The $a^i_K$ is the intracellular $K^+$ activity which was 110 mM, as 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 the sum ($Na^+ + K^+$) kept constant at 150 mM. Any change in calibration meant that the experiments were discarded. Conventional and $Na^+$-sensitive microelectrodes were connected to a dual channel high impedance electrometer (model 773, WP Instruments, New Haven, Conn., U.S.A.). The signals were displayed separately and electronically subtracted on a pen recorder and on digital panel meters. The panel meter readings were used for calculating $a^i_{Na}$.

### *Materials*

The drugs used were: carbamylcholine chloride (carbachol), 3-isobutyl, 1-methyl xanthine, (±)-propranolol hydrochloride, reserpine (dissolved in 5% ascorbic acid) from Sigma (München, F.R.G.); (±)-isoprenaline hydrochloride from Serva (Heidelberg, F.R.G.); sodium ionophore I (ETH 227), N,N-dimethyltrimethylsilylamine from Fluka (Buchs, Switzerland); cyclic AMP radioimmunoassay ($^{125}I$) from Becton Dickinson (Heidelberg, F.R.G.).

### *Statistics*

The data are presented as arithmetic means ± s.e.mean. Significance tests were performed by Student's *t* test for paired or unpaired observations. Differences between means were regarded as statistically significant when $P < 0.05$.

## Results

### *Inotropic effects*

Figure 1a and b shows superimposed isometric contractions of a catecholamine-depleted papillary muscle and the effect of isoprenaline (a) or the phosphodiesterase inhibitor 3-isobutyl, 1-methyl xanthine (IBMX; b) in the absence and presence of 3 $\mu$M carbachol. As expected, 10 nM isoprenaline and 100 $\mu$M IBMX produced a positive inotropic effect accompanied by a reduction in contraction duration. Additional application of 3 $\mu$M carbachol strongly attenuated the positive inotropic effect but did not affect the shortening of contraction duration. In contrast to its postsynaptic action, 3 $\mu$M carbachol almost completely abolished the positive inotropic effect and shortening of contraction duration evoked by noradrenaline, released neuronally by means of field stimulation (Figure 1c). Figure 2 summarizes the concentration-dependent effects of carbachol on the isometric contraction in the presence of isoprenaline or IBMX. Positive inotropic effects of six muscles, respectively, were plotted versus contraction dura-

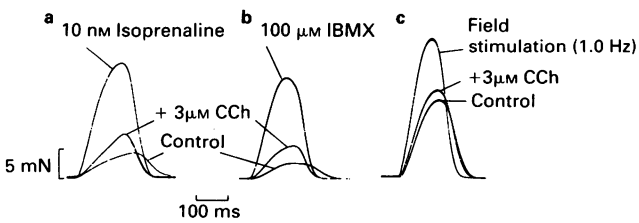


**Figure 1** Superimposed isometric contraction curves of the guinea-pig papillary muscle showing the effects of isoprenaline (a) and 3-isobutyl, 1-methyl xanthine (IBMX; b) and field stimulation (c) in the absence and presence of 3 $\mu$M carbachol (CCh). In (a) and (b) the same noradrenaline-depleted papillary muscle was used. Records were taken when steady-state effects were achieved. Contraction frequency 0.2 Hz in (a) and (b), 1.0 Hz in (c).

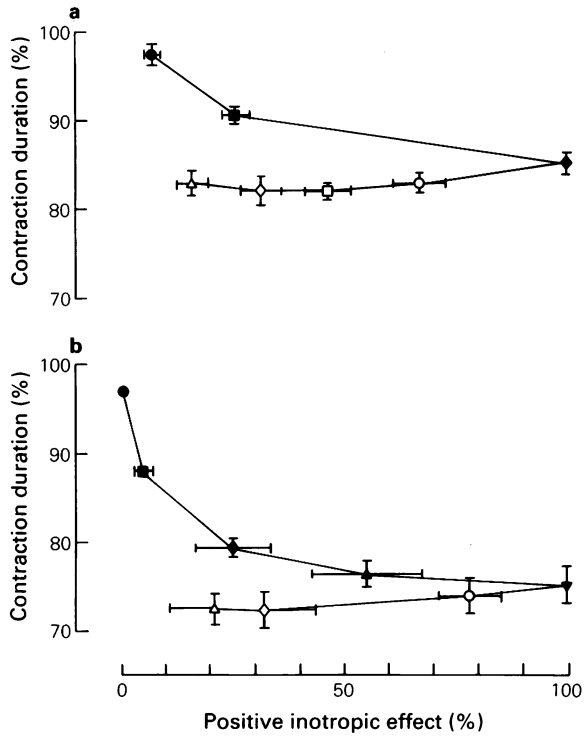


**Figure 2** The effects of different concentrations of carbachol (open symbols) on the relationship between contraction duration and positive inotropic effect in the presence of (a) isoprenaline and (b) the phosphodiesterase inhibitor 3-isobutyl, 1-methyl xanthine (IBMX). Values of contraction duration are correlated at identical concentrations with the corresponding values of the positive inotropic effect. Symbols represent the arithmetic means, with s.e.mean shown as horizontal and vertical bars, of 6 muscles. Cumulative drug application: isoprenaline (●) 1, (■) 3 and (◆) 10 nM; IBMX (●) 3, (■) 20, (◆) 60, (▲) 80, and (▼) 100 μM; carbachol (○) 0.1, (□) 0.3, (◇) 1 and (△) 3 μM. Ordinate scales: contraction duration, expressed as % of the control value (100% = 240.6 ± 10.7 ms in (a) and 231 ± 9.8 ms in (b)). Abscissa scales: positive inotropic effect, expressed as % of the value observed with 10 nM isoprenaline (100% = 9.8 ± 0.4 mN) or with 100 μM IBMX (100% = 9.7 ± 0.4 mN). Pre-drug control contraction force was 3.4 ± 0.7 mN and 3.1 ± 0.5 mN in the isoprenaline and IBMX group, respectively. Reserpine-pretreated guinea-pigs were used in all experiments depicted in (a) and (b). Contraction frequency 0.2 Hz.

tion. The data are presented before (closed symbols) and after the cumulative addition of various carbachol concentrations. In order to allow comparison of data, only those muscles which developed nearly identical positive inotropic effects at 10 nM isoprenaline ($\Delta F_c$ 100% = 9.80 ± 0.35 mN) and 100 μM IBMX ($\Delta F_c$ 100% = 9.72 ± 0.42 mN), were selected for normalization. As can be seen from Figure 2, isoprenaline (a) and IBMX (b) shortened contraction duration in a concentration-dependent fashion. However, at a given positive inotropic effect, there was a stronger effect on contraction duration in the presence of IBMX. For example, contraction was shortened with 20 μM IBMX by nearly 15% while force of contraction had barely increased. In contrast, with the same percentage of shortening, isoprenaline produced a positive inotropic effect which was 10 times stronger than that induced by 20 μM IBMX. As further shown in Figure 2, carbachol (3 μM) did not significantly affect shortening of contraction duration, although, at the same time, it attenuated the positive inotropic effect of 10 nM isoprenaline by 82 ± 3.5% ($n = 6$) and that of 100 μM IBMX by 79 ± 10.2% ($n = 6$).

### *The effect of carbachol on cellular cyclic AMP content*

In order to determine whether a decrease in cellular cyclic AMP content may be involved in the negative inotropic action of carbachol, the effect of 3 μM carbachol on force of contraction and cyclic AMP level was investigated in the presence of 10 nM isoprenaline and 100 μM IBMX. Cyclic AMP content and force of contraction were always determined in the same muscle at the same time. From Table 1 it is evident that the positive inotropic effect of isoprenaline (10 nM) 5 and 10 min after drug addition was accompanied by a significant increase in cyclic AMP content (by 58 ± 4 and 55 ± 5%, respectively; $n = 7$). Additional application of carbachol (3 μM) for 5 min, reduced the positive inotropic effect of isoprenaline by 85.4 ± 6% (catecholamine present for 10 min). However, the cyclic AMP content remained elevated, i.e., the cyclic AMP content in the presence of carbachol did not differ significantly from the corresponding values obtained with isoprenaline alone. Control experiments revealed that carbachol (3 μM) alone produced a small positive inotropic effect (see also Korth & Kühlkamp, 1985) which was not accompanied by a change in cyclic AMP content. Results similar to those observed with isoprenaline were also obtained with carbachol in the presence of 100 μM IBMX. Carbachol (3 μM) reduced the

**Table 1** Effects of carbachol on isoprenaline- and 3-isobutyl, 1-methyl xanthine (IBMX)-induced increase in cyclic AMP content and force of contraction

| | *Cyclic AMP* (pmol mg$^{-1}$ wet weight) | *Positive inotropic effect* (mN) |
|---|---|---|
| Control | 0.62 ± 0.03 (23) | — |
| Carbachol (3 μM) | 0.61 ± 0.04 (6) | 0.53 ± 0.1 (6) |
| Isoprenaline (10 nM) 5 min | 0.98 ± 0.03 (7) | 10.3 ± 0.8 (7) |
| Isoprenaline (10 nM) 10 min | 0.96 ± 0.05 (7) | 9.6 ± 0.6 (7) |
| Isoprenaline (10 nM) + carbachol (3 μM) 10 min[a] | 0.95 ± 0.04 (8)[NS] | 1.8 ± 0.2 (8)* |
| Isoprenaline (10 nM) + carbachol (3 μM) 5 min[b] | 0.93 ± 0.04 (6)[NS] | 1.5 ± 0.3 (6)* |
| IBMX (100 μM) 20 min | 1.33 ± 0.06 (7) | 11.2 ± 0.9 (7) |
| IBMX (100 μM) + carbachol (3 μM) 20 min[a] | 1.31 ± 0.05 (6)[NS] | 1.7 ± 0.3 (6)* |

Results are expressed as means ± s.e.mean. Numbers in parentheses denote numbers of experiments.
[a] Carbachol present during the last 5 min.
[b] Simultaneous addition of carbachol and isoprenaline.
* $P < 0.01$ vs isoprenaline or IBMX alone.
[NS] Not significant vs isoprenaline or IBMX alone.

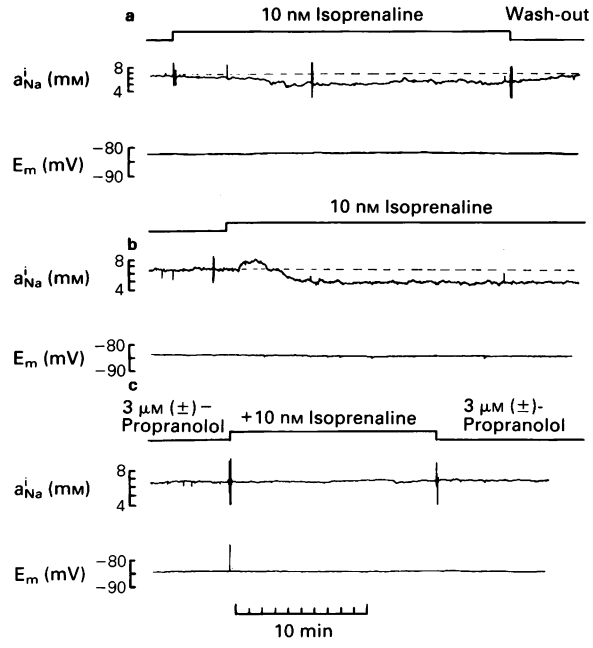


**Figure 3** (a) Isoprenaline-induced decrease in intracellular $Na^+$ activity ($a^i_{Na}$) and its reversal by washing with drug-free solution. (b) Transient increase in $a^i_{Na}$ upon superfusion of a muscle with 10 nM isoprenaline. Note that this effect was only occasionally seen. (c) Abolition of the isoprenaline-induced decrease in $a^i_{Na}$ in the presence of 3 μM (±)-propranolol. Propranolol was added 60 min before the superfusion with the catecholamine. Resting papillary muscles from reserpine-pretreated guinea-pigs were used in the experiments depicted in (a), (b) and (c).

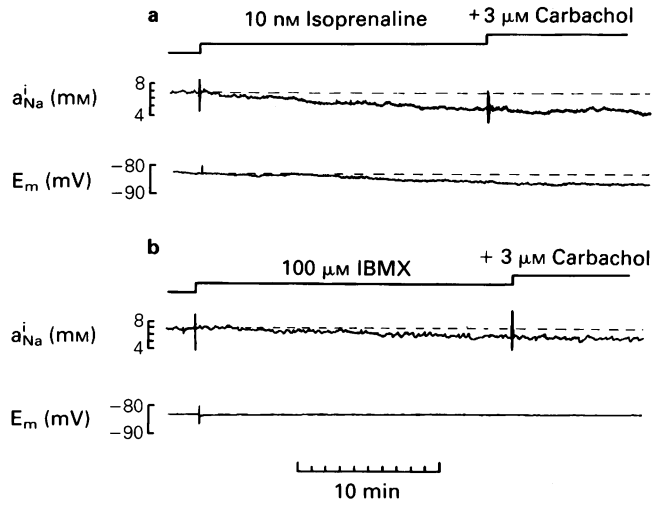


**Figure 4** Effect of the cyclic AMP-elevating drugs isoprenaline and 3-isobutyl, 1-methyl xanthine (IBMX) on intracellular $Na^+$ activity ($a^i_{Na}$) and resting membrane potential ($E_m$) in the absence and presence of carbachol. (a) Decrease in $a^i_{Na}$ and hyperpolarization of $E_m$ upon superfusion of 10 nM isoprenaline. Failure of 3 μM carbachol to attenuate both isoprenaline effects. Note that hyperpolarization of $E_m$ was very pronounced in this particular experiment. (b) Decrease in $a^i_{Na}$ during superfusion with 100 μM IBMX. This effect remains unchanged in the presence of 3 μM carbachol. Resting papillary muscles from reserpine-pretreated guinea-pigs were used in the experiments depicted in (a) and (b).

positive inotropic effect of the phosphodiesterase inhibitor by 84.8 ± 8% ($n = 7$) but failed to decrease significantly the elevated cyclic AMP content. Interestingly, IBMX evoked a positive inotropic effect within 20 min which was comparable to that of 10 nM isoprenaline, but at the same time induced an elevation of the cyclic AMP level which exceeded that of the catecholamine by 36%. The possibility that the sequence of drug addition might have influenced the effects was considered. Isoprenaline was therefore added simultaneously with carbachol instead of consecutively. As can be seen from Table 1, force of contraction rose within 5 min to 15% of the effect obtained with 10 nM isoprenaline alone, while the cyclic AMP level was increased to about the same amount as with the catecholamine alone. Thus, the results obtained with the simultaneous application of isoprenaline and carbachol were similar to those obtained after the sequential addition of the drugs.

### *The effect of carbachol on $Na^+$ activity*

The effect of 10 nM isoprenaline on $a^i_{Na}$ and resting membrane potential in a quiescent papillary muscle is shown in Figure 3. Application of isoprenaline to the superfusing solution caused $a^i_{Na}$ to decline from 6.5 mM to 5.0 mM within 12 min. After $a^i_{Na}$ was constant for 15 min, the muscle was again superfused with catecholamine-free solution, and $a^i_{Na}$ returned to its pre-drug control level within 6 min. In a total of 10 papillary muscles, exposure to isoprenaline (10 nM) caused a decrease in $a^i_{Na}$ (Table 2) with an average change of 1.9 ± 0.2 mM (equivalent to a decrease of 30%). In the experiment presented in Figure 3a, membrane resting potential remained stable at −82 mV. This, however, was not a regular finding, in 7 out of 10 experiments, isoprenaline evoked a 1 to 3 mV hyperpolarization which paralleled the decrease in $a^i_{Na}$ (see Figure 4a and Table 2). As can be seen from Figure 3c, the decline in $a^i_{Na}$ induced by isoprenaline was due to stimulation of β-adrenoceptors, for it was completely antagonized by 3 μM (±)-propranolol. In 3 out of 10 experiments, isoprenaline (10 nM) caused a transient

**Table 2** Effects of isoprenaline on intracellular $Na^+$ activity ($a^i_{Na}$) and resting membrane potential ($E_m$) before and after the addition of carbachol or in the presence of (±)-propranolol

| *Condition* | $E_m$ (mV) | $\Delta E_m$ (mV) | $a^i_{Na}$ (mM) | $\Delta a^i_{Na}$ (mM) | n |
|---|---|---|---|---|---|
| Control | −82.8 ± 0.4 | | 6.8 ± 0.2 | | 10 |
| Isoprenaline (10 nM) | −84.5 ± 0.5 | −1.7 ± 0.5** | 4.9 ± 0.2 | −1.9 ± 0.2*** | |
| Isoprenaline (10 nM) + carbachol (3 μM) | −84.4 ± 0.4 | | 4.9 ± 0.2 | | |
| After wash-out | −82.5 ± 1.0 | | 6.6 ± 0.9 | | |
| (±)-Propranolol (3 μM) | −83.8 ± 0.6 | | 6.9 ± 0.3 | | 4 |
| (±)-Propranolol (3 μM) + isoprenaline (10 nM) | −83.7 ± 0.6 | | 6.9 ± 0.3 | | |

Results are expressed as means ± s.e.mean. Preparations obtained from reserpine-pretreated guinea-pigs (5 mg kg$^{-1}$ body weight reserpine injected i.p. 24 h before the experiment).
$n$ = number of muscles.
**$P < 0.01$, ***$P < 0.001$; Student's paired $t$ test.

**Table 3** Influence of carbachol on 3-isobutyl, 1-methyl xanthine (IBMX)-induced decrease in intracellular $Na^+$-activity ($a^i_{Na}$)

| *Condition* | *Resting potential* (mV) | $a^i_{Na}$ (mM) | $\Delta a^i_{Na}$ (mM) | n |
|---|---|---|---|---|
| Control | $-82.7 \pm 0.4$ | $7.1 \pm 0.3$ | | 3 |
| IBMX (100 $\mu$M) | $-83.1 \pm 1.1$ | $6.0 \pm 0.3$ | $-1.1 \pm 0.3$* | |
| IBMX (100 $\mu$M) + carbachol (3 $\mu$M) | $-83.2 \pm 1.0$ | $6.0 \pm 0.3$ | | |

Results are expressed as means ± s.e.mean. Preparations obtained from reserpine-pretreated guinea-pigs (5 mg kg$^{-1}$ body weight reserpine injected i.p. 24 h before the experiment).
$n$ = number of muscles.
*$P < 0.05$; Student's paired $t$ test.

increase in $a^i_{Na}$ which was then followed by a sustained decrease. Figure 3b shows such an experiment in which $a^i_{Na}$ rose within 2 min from 6.7 mM to 8.2 mM and then declined over the following 5 min to a steady-state level of 4.9 mM. Since a temperature effect upon switching to the catecholamine-containing solution could be ruled out, the reason for the transient increase in $a^i_{Na}$ is not apparent. Figure 4a shows a typical experiment in which the effect of carbachol (3 $\mu$M) on isoprenaline-induced decrease in $a^i_{Na}$ was investigated. Isoprenaline (10 nM) caused $a^i_{Na}$ to decline from 6.8 mM to 4.1 mM and membrane resting potential to hyperpolarize from $-83.0$ to $-85.5$ mV. Superfusion of the preparation with isoprenaline plus carbachol for 10 min was without further effect on $a^i_{Na}$ and membrane resting potential. On switching to drug-free solution, both $a^i_{Na}$ and resting potential returned towards control levels. Table 2 summarizes the effects of isoprenaline, isoprenaline plus carbachol and of washout on $a^i_{Na}$ and resting membrane potential, determined in 10 catecholamine-depleted papillary muscles. Figure 4b shows that IBMX (100 $\mu$M) resembled the catecholamine by causing $a^i_{Na}$ to decrease from 6.8 mM to 5.3 mM within 20 min. This effect was not influenced by additional application of 3 $\mu$M carbachol. As summarized in Table 3, exposure of 2 other preparations to 100 $\mu$M IBMX in the absence and in the presence of carbachol produced changes in $a^i_{Na}$ which were identical to those shown in Figure 4b.

## Discussion

The present study shows that force of contraction when enhanced by isoprenaline or the phosphodiesterase inhibitor IBMX, was strongly reduced by carbachol in a concentration-dependent manner. Such an antagonism has been previously described in ventricular preparations exposed to drugs that either stimulate adenylate cyclase or inhibit phosphodiesterase activity (Meester & Hardman, 1967; Endoh, 1980; Inui *et al.*, 1982). Muscarinic receptor agonists have been demonstrated to decrease the transsarcolemmal $Ca^{2+}$ current, $I_{Ca}$, in guinea-pig ventricular myocytes exposed to catecholamines or phosphodiesterase inhibitors (Hescheler *et al.*, 1986) and consequently to reduce the myoplasmic $Ca^{2+}$ transient recorded in aequorin-injected contracting papillary muscles (McIvor *et al.*, 1988). Thus, reduction of cyclic AMP-enhanced $I_{Ca}$ seems to be directly responsible for the negative inotropic effect of muscarinic receptor agonists.

Because most if not all cardiac effects of elevated cyclic AMP are mediated by the cyclic AMP-dependent protein kinase (Robinson-Steiner & Corbin, 1986; Kameyama *et al.*, 1986; Solaro, 1986), it has been postulated that muscarinic receptor agonists interfere at some point in this system. The discovery that cardiac muscarinic receptors are coupled to adenylate cyclase via an inhibitory transducer protein ($G_i$), which couples the inhibitory signals from the receptors to the catalytic subunit of the cyclase (Gilman, 1984; Rodbell, 1980), led to the notion that the mechanism of muscarinic inhibition is due to reductions in cyclic AMP levels as a result of inhibition of adenylate cyclase. Indeed, muscarinic inhibition of catecholamine-induced elevation of cyclic AMP levels has been demonstrated in cardiac tissues. This has led some investigators to suggest that this is the major mechanism of action of muscarinic receptor agonists on ventricular tissues (Gardner & Allen, 1976; Keely *et al.*, 1978; Endoh, 1980; Inui *et al.*, 1982; Linden *et al.*, 1985). The present results, however, demonstrate that the negative inotropic effect of carbachol was not accompanied by a reduction in isoprenaline- or IBMX-enhanced cyclic AMP content. From this it must be concluded that inhibition of adenylate cyclase cannot be the mechanism for the decrease in $I_{Ca}$ and hence for the negative inotropic action of carbachol in guinea-pig papillary muscle. A similar conclusion was reached by Watanabe & Besch (1975) who showed, in the whole heart preparation of the guinea-pig, that acetylcholine did not influence the cyclic AMP content elevated by isoprenaline. However, in a more recent study Lindemann & Watanabe (1985b) found a small but significant inhibition of cyclic AMP accumulation when acetylcholine was given simultaneously with isoprenaline or IBMX to guinea-pig hearts perfused by the Langendorff technique. In the present study, neither the simultaneous nor the sequential addition of carbachol had any influence on the increase in cyclic AMP induced by 10 nM isoprenaline or 100 $\mu$M IBMX.

Besides their positive inotropic- and cyclic AMP-elevating effects, isoprenaline as well as IBMX shortened contraction duration of the guinea-pig papillary muscle. At low and moderately effective concentrations, this effect was due to a shortening of time to peak force, while at high concentrations shortening of relaxation time became the prevailing effect (see also Korth & Schmied, 1988). In accordance with the cyclic AMP measurements, carbachol did not abolish the shortening of contraction duration induced by either the catecholamine or the phosphodiesterase inhibitor. It appears, therefore, as if contraction duration is a more reliable parameter for estimating changes in cellular cyclic AMP content than force of contraction or $I_{Ca}$. In this context, it is interesting to note that isoprenaline and IBMX differed with respect to the relationship between positive inotropic effect and shortening of contraction duration (see Figure 2). This difference was no longer apparent when contraction duration was correlated with the respective cyclic AMP levels, as determined in an earlier study under otherwise identical conditions (Korth *et al.*, 1987). The present results are in accord with the recent finding that acetylcholine, despite its strong negative inotropic effect, failed to alter the isoprenaline-induced shortening of relaxation time in ferret papillary muscle (McIvor *et al.*, 1988). In the same study, it was shown that acetylcholine, by a cyclic AMP-independent mechanism, reversed the isoprenaline-induced decrease of the affinity of troponin for $Ca^{2+}$. Both observations combined seem to exclude the possibility that the myocardial relaxation is modulated by changes in myofibrillar $Ca^{2+}$ sensitivity. The finding that cyclic AMP-dependent phosphorylation of phospholamban is associated with increases in the rate of $Ca^{2+}$ accumulation by sarcoplasmic reticulum (Tada *et al.*, 1975; Hicks *et al.*, 1979) and shortening of relaxation time (Lindemann *et al.*, 1983; Lindemann & Watanabe, 1985a) is consistent with the hypothesis that the rate of $Ca^{2+}$ pumping out of the myoplasm determines the relaxation time of ventricular myocardium. The present findings, as well as those of McIvor *et al.* (1988), are in contrast to

studies on guinea-pig hearts perfused by the Langendorff technique (Lindemann *et al.*, 1983; Lindemann & Watanabe, 1985b). By use of cyclic AMP-elevating drugs such as isoprenaline, IBMX and forskolin, which activates directly the catalytic subunit of adenylate cyclase (Daly, 1984), these authors found that additional stimulation of muscarinic receptors attenuated phospholamban phosphorylation, Ca uptake into the sarcoplasmic reticulum and shortening of relaxation time. Since the present study and the experiments of Lindemann *et al.* (1983) and of Lindemann & Watanabe (1985b) were carried out on guinea-pig ventricular myocardium, species differences can hardly account for the discrepancy in results. Moreover, the failure of carbachol to antagonize cyclic AMP-dependent shortening of contraction duration was also apparent when the muscle was exposed to isoprenaline or IBMX initially, carbachol initially, or to both drugs simultaneously. This indicates that the experimental protocol can hardly account for the disparity in findings.

In cardiac muscle cells, catecholamines increase $Na^+$ efflux and $K^+$ influx, and, under certain conditions, hyperpolarize resting membrane potential (Glitsch *et al.*, 1965; Vasalle & Barnabei, 1971; Hougen *et al.*, 1981). More recently, direct measurements of the effects of catecholamines on intracellular $Na^+$ activity were made on multicellular heart preparations (Wasserstrom *et al.*, 1982; Lee & Vasalle, 1983) and on isolated cardiac myocytes (Désilets & Baumgarten, 1986). In all three studies, catecholamines were found to decrease $a^i_{Na}$ by directly stimulating $Na^+$–$K^+$ pump activity in heart muscle. Since cyclic AMP derivatives (Pecker *et al.*, 1986) and forskolin (Zalups & Sheu, 1987) mimicked the catecholamine effect on $a^i_{Na}$, it can be concluded that stimulation of the $Na^+$–$K^+$ pump is a cyclic AMP-dependent effect. However, the nature, and role of proteins that are presumedly phosphorylated as part of the molecular mechanism causing $Na^+$–$K^+$ pump stimulation are presently unknown. The present results are in agreement with the aforementioned studies, demonstrating a persistent decrease in $a^i_{Na}$ (by 30%) when the papillary muscle was superfused with 10 nM isoprenaline. Hyperpolarization of membrane resting potential, which accompanied the decline in $a^i_{Na}$ in most of the preparations, was probably due to the electrogenicity of the stimulated $Na^+$–$K^+$ pump (Gadsby, 1984). As with shortening of contraction duration and elevated cyclic AMP level, additional application of carbachol failed to attenuate the decline in $a^i_{Na}$ and the hyperpolarization of membrane resting potential. The phosphodiesterase inhibitor IBMX mimicked the catecholamine effect by producing a decline in $a^i_{Na}$ which was also resistant to stimulation of muscarinic receptors.

In conclusion, the present results demonstrate that the antagonism of cardiac muscarinic receptor stimulation in the presence of cyclic AMP-elevating drugs is restricted to a negative inotropic effect, which presumedly depends on inhibition of $I_{Ca}$. Elevated cyclic AMP levels, shortening of contraction duration and $Na^+$–$K^+$ pump stimulation remains unaffected by muscarinic receptor agonists. The results are compatible with the hypothesis that stimulation of muscarinic receptors leads to inhibition of voltage-dependent $Ca^{2+}$ channels by a mechanism that does not involve directly the cytosolic signal molecule cyclic AMP.

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