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SYNERGISTIC ACTION OF CYCLIC GMP ON CATECHOLAMINE-INDUCED CHLORIDE CURRENT IN GUINEA-PIG VENTRICULAR CELLS

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SUMMARY

1. Effects of cyclic GMP on the catecholamine-induced chloride current (I_{Cl}) were studied using the whole-cell patch-clamp technique combined with internal perfusion in single ventricular myocytes dispersed from guinea-pig heart.

2. When I_{Cl} was activated by submaximal doses of isoprenaline (0·01–0·1 μM), adrenaline (0·5–1 μM) and histamine (0·2–0·5 μM), intracellular dialysis with cyclic GMP (10–100 μM) induced an extra increase of I_{Cl}. No further increase of I_{Cl} was induced by cyclic GMP when I_{Cl} was maximally activated. In the absence of agonists, cyclic GMP failed to induce I_{Cl}.

3. The enhancement by cyclic GMP was also observed when I_{Cl} was activated by external application of 0·2–1·0 μM-forskolin or by internal dialysis with a pipette solution containing 50–200 μM-cyclic AMP.

4. In contrast to cyclic GMP, 10–1000 μM-dibutyryl cyclic GMP and 8-bromo-cyclic GMP were ineffective in modifying I_{Cl}.

5. Milrinone (1–10 μM), a specific inhibitor of a kind of phosphodiesterase which is inhibited by cyclic GMP, also enhanced I_{Cl} activated by submaximal doses of isoprenaline. Milrinone itself did not activate I_{Cl}.

6. When I_{Cl} was enhanced by 5 μM-milrinone, an additional application of cyclic GMP failed to increase I_{Cl}. In the presence of cyclic GMP, milrinone failed to enhance I_{Cl}.

7. The above findings on I_{Cl} are analogous to the enhancement by cyclic GMP of the β-adrenergic stimulation of the Ca^{2+} current reported in the same preparation, and support the hypothesis that in mammalian cardiac cells cyclic GMP potentiates elevation of cyclic AMP induced by β-adrenergic agents, and thereby increases the amplitudes of ionic currents.

INTRODUCTION

The cyclic GMP level increases when cardiac muscles are stimulated by various agonists, such as acetylcholine and atrial natriuretic peptide (George, Polson,

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O'Toole & Goldberg, 1979; Cramb, Banks, Rugg & Aiton, 1987). To examine the role of cyclic GMP, effects of direct application of cyclic GMP were studied in single cardiac cell preparations, and several mechanisms have been considered underlying the modulation of ionic currents by cyclic GMP (for review see Hartzell, 1988). In the frog heart the intracellular application of cyclic GMP reverses the increase of the Ca^{2+} current (I_{Ca}) caused by the β-adrenergic stimulation (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987). It was concluded that cyclic GMP activated a class of phosphodiesterases, and thereby nullified the increase of cyclic AMP caused by the β-adrenergic stimulation. A similar inhibitory action of cyclic GMP was described in mammalian ventricular cells, and an activation of cyclic GMP-dependent protein kinase was suggested in these cases (in guinea-pig, Levi, Alloatti & Fischmeister, 1989; in rat, Mery, Lohmann & Fischmeister, 1990; Mery, Lohmann, Walter & Fischmeister, 1991). However, different experimental results were recently reported in mammalian cardiac cells. The intracellular dialysis of guinea-pig ventricular cells with cyclic GMP enhanced the β-adrenergic increase of I_{Ca} (Ono & Trautwein, 1990; Ono & Trautwein, 1991). The authors suggested an involvement of a different kind of phosphodiesterase, which is biochemically shown to be inhibited by cyclic GMP (Harrison, Reifsnyder, Gallis, Chad & Beavo, 1986). Thus, in mammalian heart the action of cyclic GMP is still a matter of debate.

We recently found that the catecholamine-induced chloride current (I_{Cl}) was also enhanced by intracellular application of cyclic GMP (Tareen, Ono, Noma & Ehara, 1991), but the detailed mechanisms were not thoroughly examined. Since the activation of both I_{Cl} and I_{Ca} is regulated by a common intracellular cyclic AMP system (for I_{Cl}, Bahinski, Nairn, Greengard & Gadsby, 1989; Harvey & Hume, 1989; Harvey, Clark & Hume, 1990; for I_{Ca}, Kameyama, Hofmann & Trautwein, 1985), the determination of the effects of cyclic GMP on I_{Cl} may provide decisive information to resolve the above question. We have therefore conducted a systematic study on I_{Cl} to clarify mechanisms underlying the action of cyclic GMP. The present results show that I_{Cl}, when activated by isoprenaline, histamine, forskolin and cyclic AMP, is enhanced by internal dialysis with cyclic GMP, and the action of cyclic GMP is mimicked by milrinone, a selective inhibitor of the cyclic GMP-inhibited phosphodiesterase. The findings support the hypothesis that cyclic GMP potentiates the β-adrenergic response of ionic currents by inhibiting the cyclic GMP-inhibited phosphodiesterase in mammalian heart.

Some of these results have been communicated to the Physiological Society (Ono, Tareen, Yoshida, Noma & Trautwein, 1991).

METHODS

Single-cell preparation

Single ventricular cells were obtained by treating guinea-pig hearts with collagenase as previously described (Powell, Terrar & Twist, 1980; Isenberg & Klöckner, 1982). In brief, guinea-pigs (300–400 g) were anaesthetized with sodium pentobarbitone (50–70 mg/kg). Under artificial respiration, the chest was opened and the aorta was cannulated *in situ* to start perfusion of control Tyrode solution. The heart was excised and the perfusate was switched to a Ca^{2+}-free Tyrode solution. The relaxed heart was then perfused with the Ca^{2+}-free Tyrode solution containing 0·04 % collagenase (Yakult, Tokyo, Japan) for 5–10 min. After the enzyme treatment, the ventricle was cut into pieces in high-K^+, low-Cl^- solution. The dispersed cells were filtrated through a 105 μm

mesh and centrifuged at 300–500 r.p.m. for 3 min. The cells were then stored in a culture medium (Minimum Essential Medium, Flow Laboratory, UK) at 30 °C for later use.

Solutions

The control Tyrode solution contained (in mM): NaCl, 140; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·33; glucose, 5·5; and the pH was adjusted to 7·4 with 5·0 mM-HEPES–NaOH. The external solution used to isolate I_{Cl} contained (in mM): NaCl, 140; $MgCl_2$, 2; $BaCl_2$, 2; nicardipine, 0·001; and ouabain, 0·02. The pH was adjusted to 7·4 with 5 mM-HEPES–CsOH.

The composition of the internal solution was (in mM): CsOH, 100; CsCl, 15; aspartate, 90; $MgCl_2$, 5; tetraethylammonium chloride (TEA-Cl), 20; the tris-(hydroxymethyl)-aminomethane salt of ATP, 5; and EGTA, 5. The pH was adjusted to 7·4 by 5 mM-HEPES–CsOH.

Drugs

Isoprenaline, histamine, cyclic AMP, cyclic GMP, dibutyryl cyclic GMP and 8-bromo-cyclic GMP were purchased from Sigma Co. (USA). Adrenaline was purchased from Daiichi Pharmaceutical Co. (Japan) and forskolin from Calbiochem Corporation (USA). Milrinone was kindly supplied to us by Professor R. Fischmeister.

Voltage-clamp and recording technique

Single ventricular cells were voltage clamped using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The command potential and current were recorded through a glass suction pipette having a tip internal diameter of about 3 μm and a resistance of 1–3 MΩ when filled with the internal solution. To avoid the liquid junction potential between the pipette solution and the control Tyrode solution, and also to facilitate formation of a gigaohm seal, the pipette was first filled with the control Tyrode solution. After formation of the gigaohm seal the pipette solution was replaced with the internal solution by using a pipette perfusion device (Soejima & Noma, 1984). A brief strong negative pressure was applied to the pipette interior to rupture the patch membrane. Ramp voltage-clamp pulses (triangular wave; $dV/dt = \pm 0{\cdot}46$ volts/second) were applied from the holding potential of −40 mV. The I–V relations were measured from the hyperpolarizing portion of the ramp pulses (Fig. 1). Current and voltage signals were stored on magnetic tape (PCM data recorder, RD101, TEAC, Japan) for later computer analysis (NEC, PC98, Tokyo, Japan).

Numerical data are given as means ± S.D. All experiments were carried out at 35 ± 0·5 °C.

RESULTS

Enhancement of the isoprenaline- and histamine-induced Cl^- current by cyclic GMP

Activation of I_{Cl} by isoprenaline and subsequent modulation of the response by intracellular cyclic GMP is demonstrated in the experiment shown in Fig. 1. The top trace (*A*) indicates a continuous chart recording of the membrane current, and *B* shows representative records of current in response to ramp pulses, which were applied every 6 s from the holding potential of −40 mV. The voltage relations of the isoprenaline-induced current (I–V curve) determined from the negative slope of the ramp pulse are indicated in *C*. After switching the superfusate to the one containing 30 nM-isoprenaline, the membrane conductance gradually increased and reached a steady response within 30 s. The current recordings on a faster time scale (*B*) or the current–voltage relations (*C*) indicate that this increase in the membrane conductance is due to I_{Cl}. The I–V curves showed outward rectification because of asymmetrical Cl^- concentrations across the membrane, and crossed at a potential which is close to the expected equilibrium potential for Cl^-. During the maintained superfusion of isoprenaline, addition of 20 μM-cyclic GMP in the pipette solution further increased the amplitude of the current deflection in response to ramp pulses,

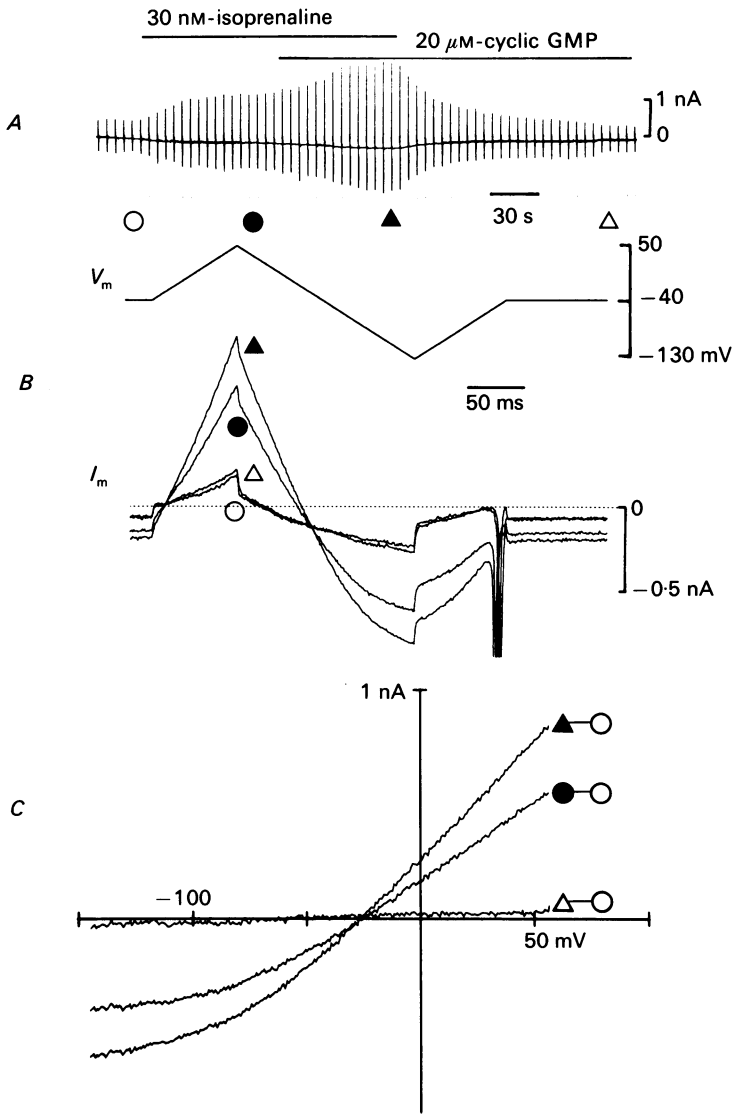

Fig. 1. Enhancement of β-adrenergic activation of I_{Cl} by cyclic GMP. *A*, a chart recording of the membrane current. The vertical deflections indicate changes induced by the ramp pulse, which repeated every 6 s. The times for superfusion of 30 nM-isoprenaline and internal perfusion of 20 μM-cyclic GMP are indicated above the record. The holding potential was −40 mV. *B*, command ramp pulse (V_m) and corresponding current changes (I_m), which were averaged for five consecutive records. Symbols indicate corresponding ramps on the chart recordings: ○, control; ●, response to isoprenaline; ▲, response to isoprenaline in the presence of cyclic GMP; △, cyclic GMP after washing out isoprenaline. *C*, the isoprenaline-induced current–voltage relations are obtained by subtracting the control (○ in *B*) from those in the presence of isoprenaline and/or cyclic GMP, indicated by corresponding symbols in *A* and *B*. The negative limb of the ramp pulse was used to plot the *I–V* curves.

keeping the reversal potential unchanged. Washing out isoprenaline, while maintaining the dialysis with cyclic GMP, resulted in a decrease of the current towards the control level. This finding indicates that cyclic GMP modulates the activation of I_{Cl} by isoprenaline, but cyclic GMP alone has no influence on the background membrane conductance.

TABLE 1. Drug-induced conductance (G_m) in the absence and presence of cyclic GMP

		Drug-induced G_m (nS)			
Drug	Concentration	Drug only	In the presence of cyclic GMP	E_{rev}(mV)	n
Isoprenaline	10–30 nM	4·5±2·2	7·1±3·8**	−27±2·0	12
Isoprenaline	1000 nM	5·1±2·8	5·3±2·7	−26±1·7	7
Adrenaline	100 nM	1·3±0·8	3·9±1·6*	−25±1·2	3
Histamine	0·2–0·5 μM	4·9±4·8	11·3±8·0*	−28±1·3	4
Forskolin	0·5–0·7 μM	5·4±5·9	7·2±6·7*	−29±2·0	4
Cyclic AMP	100, 200 μM	1·6±0·1	5·1±0·5**	−28±2·0	3

E_{rev} is the reversal potential. Values are means ±S.D. n is the number of experiments.
* Statistically significant (Student's paired t test, $P = 0·025$).
** Statistically significant ($P = 0·005$).

The increase in the membrane conductance was observed in all twelve experiments when cyclic GMP was applied in the presence of 10–30 nM-isoprenaline. For statistics we measured the slope conductance by calculating the regression line near the reversal potential of the I_{Cl}–V relation, which was 4·5±2·2 nS ($n = 12$) with 10–30 nM-isoprenaline (Table 1), and 7·1±3·8 nS in the presence of 10–20 μM-cyclic GMP in the pipette solution. When I_{Cl} was activated by the less selective agonist, adrenaline at a concentration of 0·1 μM, cyclic GMP increased the Cl^- conductance from 1·3±0·8 to 3·9±1·6 nS ($n = 3$). The ineffectiveness of sole application of cyclic GMP in activating I_{Cl} was confirmed in four experiments.

Cyclic GMP failed to increase I_{Cl} when I_{Cl} was previously activated maximally. In the experiment shown in Fig. 2, the cell was first exposed to 1 μM-isoprenaline, which induced the maximal activation of I_{Cl} (Ono *et al.* 1991; Tareen *et al.* 1991). During the continuous stimulation by isoprenaline, internal perfusion of 20 μM-cyclic GMP did not enhance I_{Cl}. Similar results were obtained in six other experiments (Table 1). The measurement of the dose–response relation for isoprenaline and I_{Cl} (Ono *et al.* 1991) showed a shift to the left of the relation in the presence of 10 μM-cyclic GMP in the pipette solution when compared with the control (a half-maximum concentration, $K_{\frac{1}{2}} = 21$ nM in the control and $K_{\frac{1}{2}} = 5·5$ nM in the presence of 10 μM-cyclic GMP; Ono *et al.* 1991). This effect of cyclic GMP on the dose–response relation is quite similar to the leftward shift of the dose–response curve for the enhancement of I_{Ca} by isoprenaline reported elsewhere (Ono & Trautwein, 1991).

Effects of cyclic GMP on histamine-induced Cl^- current

Although the receptor (H_2-receptor) is different, histamine shares a common cascade of intracellular reactions with the β-adrenergic agonists in activating ion channels (Hescheler, Tang, Jastorff & Trautwein, 1987; Harvey & Hume, 1990). In

the experiment shown in Fig. 3, the cell was first exposed to 0·2 μM-histamine to activate I_{Cl}, and subsequently dialysed with 20 μM-cyclic GMP. The increase of the membrane conductance by histamine was enhanced by cyclic GMP. When histamine was washed out leaving cyclic GMP in the pipette, the membrane conductance

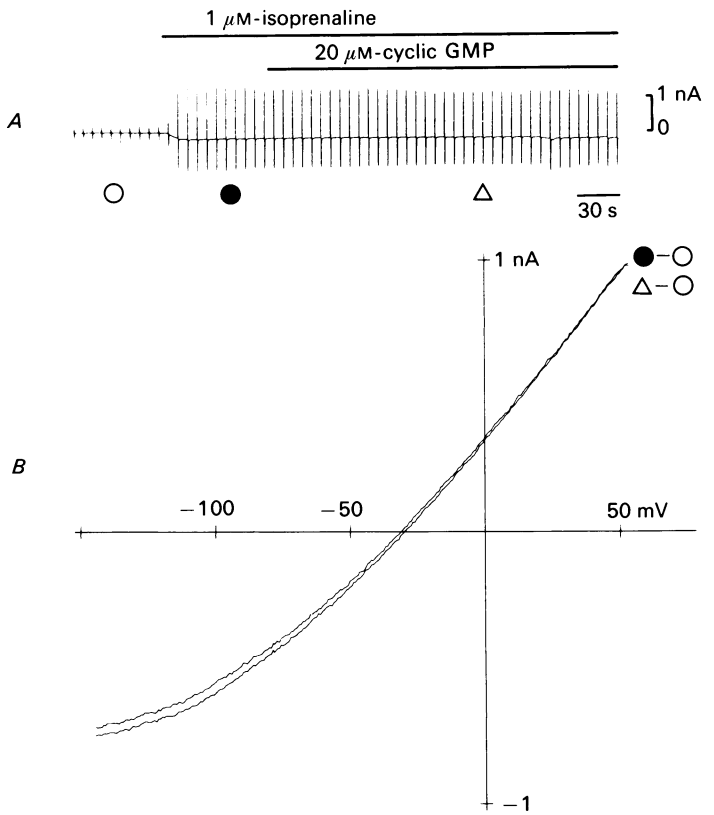

Fig. 2. Effect of cyclic GMP on I_{Cl} maximally activated by isoprenaline. *A*, a chart recording of the membrane current. The times for superfusion of 1 μM-isoprenaline and internal perfusion of 20 μM-cyclic GMP are indicated above the record. *B*, the isoprenaline-induced current–voltage relations are obtained by subtracting the control current (○ in *A*) from those in the presence of isoprenaline and/or cyclic GMP, indicated by corresponding symbols in *A*.

returned to the control level. The changes in the membrane conductance are attributable to I_{Cl}, since the drug-induced current–voltage relations showed similar shape and a common reversal potential which is near the chloride equilibrium potential (E_{Cl}). The finding is essentially the same as obtained with isoprenaline (Fig. 1). In four experiments, the slope conductance of I_{Cl} was 4·9 ± 4·8 nS with 0·2–0·5 μM-histamine. The presence of 10–20 μM-cyclic GMP further increased the conductance to 11·3 ± 8·0 μM (Table 1). When I_{Cl} was maximally activated by 5 μM-histamine, no further increase was observed by dialysis with 20 μM-cyclic GMP ($n = 2$, not shown). We conclude that cyclic GMP interacts with the intracellular cascade of reactions.

Effect of cyclic GMP on forskolin- and cyclic AMP-induced Cl^- current

Attempts to specify the site of action of cyclic GMP were made by activating I_{Cl} with forskolin or internal dialysis of cyclic AMP (Fig. 4). Since the forskolin response varied from cell to cell, we applied several concentrations of forskolin to find the

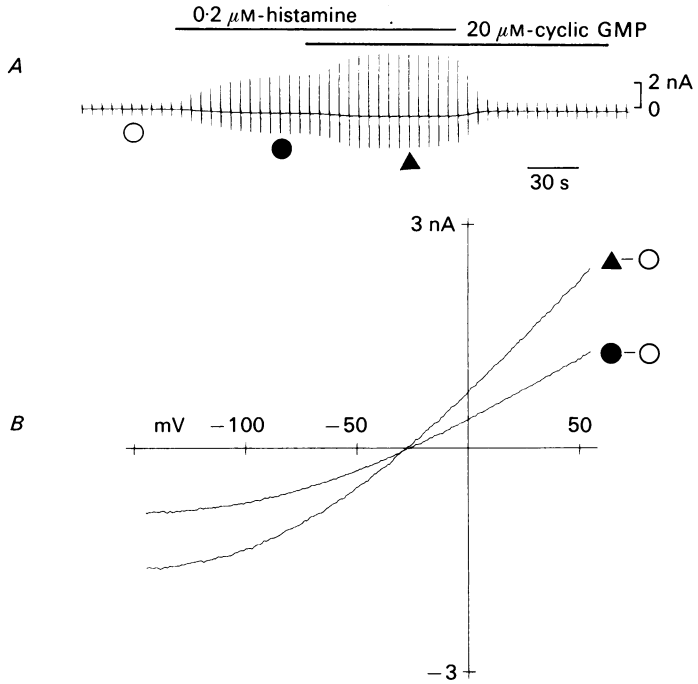

Fig. 3. Enhancement of histamine response of I_{Cl} by cyclic GMP. *A*, current trace on the chart recorder. The times for superfusion of 0·2 μM-histamine and intrapipette perfusion of 20 μM-cyclic GMP are indicated above the record. *B*, the *I–V* curves of the drug-induced current were measured by subtracting the control current (○ in *A*) from those recorded with 0·2 μM-histamine (● in *A*) and histamine in the presence of 20 μM-cyclic GMP (▲ in *A*). Averages of five ramp pulses at the corresponding times in the above chart recordings were illustrated.

saturating drug concentration in each cell prior to the application of cyclic GMP. Subsequent internal dialysis of 10–20 μM-cyclic GMP only enhanced I_{Cl} when I_{Cl} was submaximally activated by forskolin, as shown in Fig. 4*A* and Table 1.

I_{Cl}, when activated moderately by the internal dialysis of cyclic AMP, was also enhanced by the additional application of cyclic GMP. Essentially the same finding as in Fig. 4*B* was obtained in three experiments (Table 1). Usually quite high concentrations (> 100 μM) of cyclic AMP were required in the pipette solution to activate I_{Cl}, probably due to the relatively high activity of phosphodiesterases (see also Tareen *et al.* 1991). The response to cyclic AMP also varied from cell to cell. When I_{Cl} was markedly activated by cyclic AMP, enhancement by cyclic GMP was not significant (data not shown). These findings suggest that cyclic GMP enhances

the activation of I_{Cl} by interacting with the reaction cascade during β-adrenergic stimulation following the production of cyclic AMP.

Effects of milrinone on I_{Cl}

During the β-adrenergic increase of I_{Ca}, inhibition of a class of phosphodiesterases is considered to underlie the stimulatory effect of cyclic GMP (Ono & Trautwein,

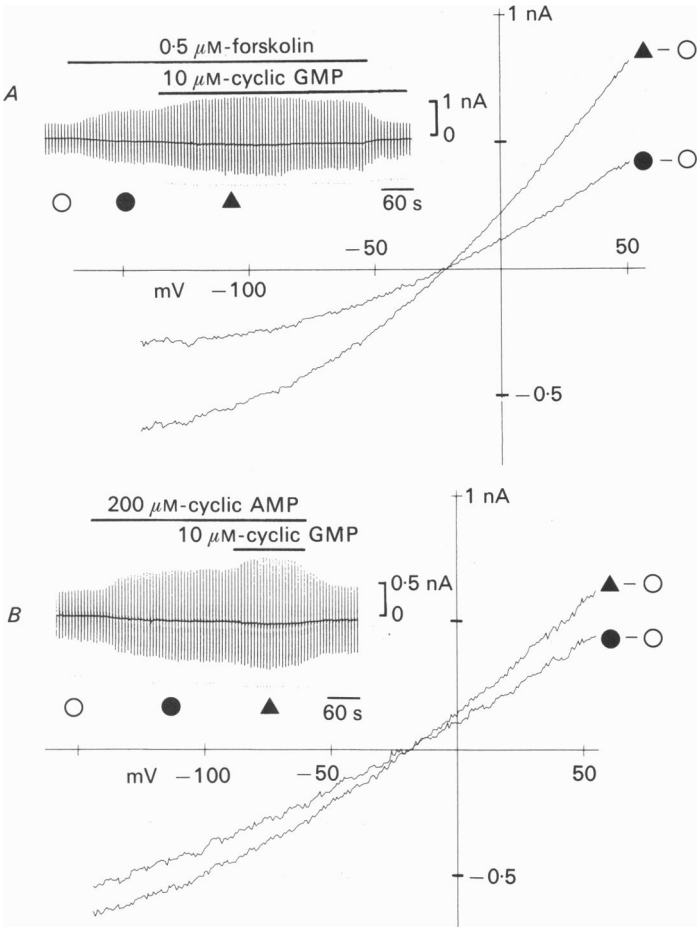

Fig. 4. Enhancement of response to forskolin and cyclic AMP. *A*, forskolin (0·5 μM) was applied to the cell, and then cyclic GMP (10 μM) was internally perfused (see inset). Average difference current–voltage relations obtained at times indicated by symbols in the inset are shown in the graph. *B* shows the result of an experiment similar to that in *A*, but instead of forskolin intrapipette perfusion of cyclic AMP (200 μM) was used in this case. *A* and *B* show data from different cells.

1991). It is known that this class of phosphodiesterases, which is inhibited by micromolar concentrations of cyclic GMP, is selectively inhibited by milrinone (Harrison *et al.* 1986; for review see Beavo, 1988). We first tested whether milrinone

can mimic the effect of cyclic GMP (Fig. 5). When I_{Cl} was activated by 20 nM-isoprenaline, additional application of 5 μM-milrinone enhanced I_{Cl}. This effect was reversible on wash-out of milrinone. The *I–V* curves of the isoprenaline-induced current obtained before (● – ○) and during (▲ – ○) the superfusion of milrinone in

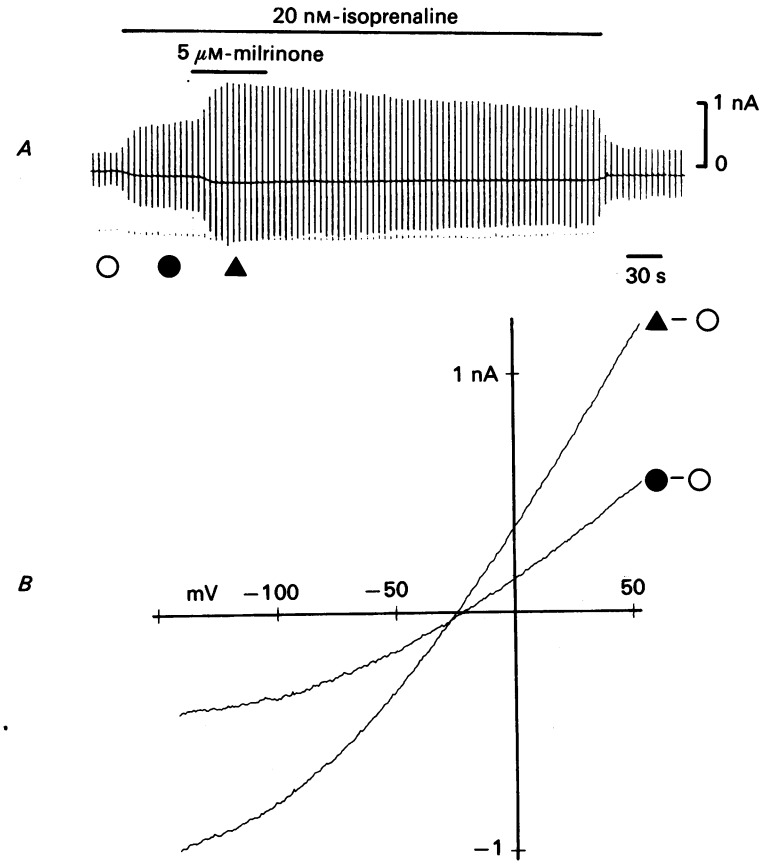

Fig. 5. Effects of milrinone on the Cl^- current. *A*, current trace on the chart recorder. The times for superfusion of 20 nM-isoprenaline and 5 μM-milrinone are indicated above the record. *B*, the average difference current–voltage relations obtained at times indicated by the corresponding symbols in *A*.

Fig. 5*B* confirmed that milrinone enhanced the isoprenaline-induced I_{Cl}. In six experiments, 1–10 μM-milrinone increased the isoprenaline (20 nM)-induced Cl^- conductance from $4{\cdot}1 \pm 3{\cdot}9$ to $9{\cdot}3 \pm 8{\cdot}3$ nS. By contrast, when I_{Cl} was activated maximally by 1 μM-isoprenaline, milrinone did not significantly affect I_{Cl} ($n = 4$, not shown). Milrinone itself failed to induce I_{Cl} ($n = 4$, not shown). Thus, the action of milrinone resembles the stimulatory effect of cyclic GMP.

If milrinone shares a common target of reaction with cyclic GMP, the maximal response to one of these chemicals should not be further modified by the following application of the other. This was tested in the experiment shown in Fig. 6. In

Fig. 6*A*, the cell was internally perfused with 10 μM-cyclic GMP solution throughout the experiment, and I_{Cl} was activated by the extracellular application of 20 nM-isoprenaline. Under these conditions, subsequent extracellular application of 10 μM-milrinone caused little change in the amplitude of the current (Fig. 6*A*). Then the

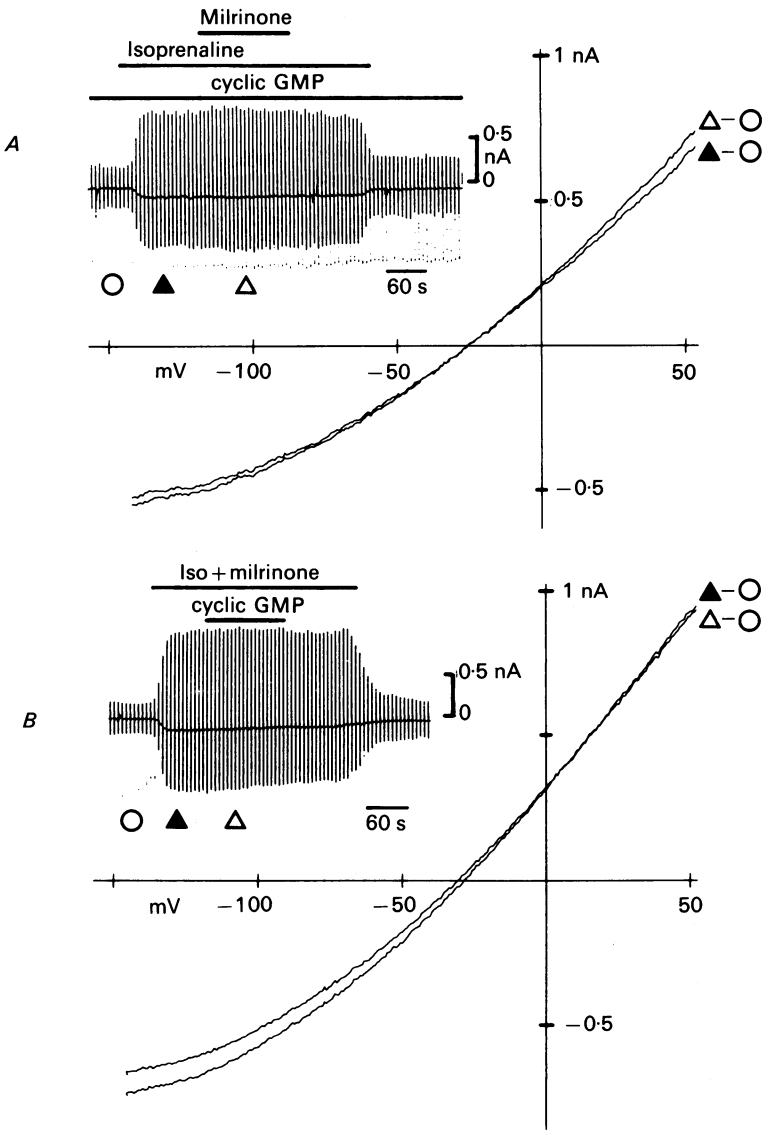

Fig. 6. *A*, the recording electrode contained 10 μM-cyclic GMP throughout the experiment. Isoprenaline (20 nM) was applied to the cell, and then milrinone (10 μM) was added to the bath solution (see inset). Average difference current–voltage relations obtained at times indicated by symbols in the inset are shown in the graph. *B* shows the result of an experiment similar to that in *A*, but the sequence of application of cyclic GMP and milrinone was reversed in this case. *A* and *B* show data from different cells.

sequence of the drug application was reversed in the experiment in Fig. 6*B*. The internal perfusion of cyclic GMP failed to modify the I_{Cl}, which was previously activated by isoprenaline in the presence of 10 μM-milrinone. Similar results were obtained in eight other experiments.

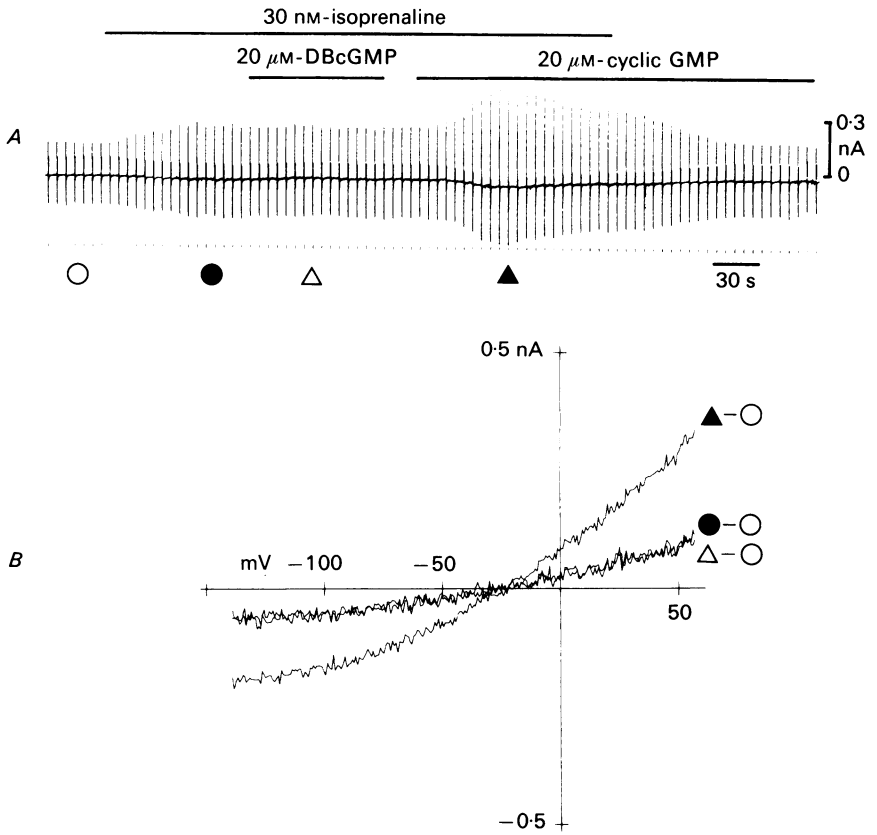

Fig. 7. Effects of dibutyryl cyclic GMP on the Cl^- current. *A*, current trace on the chart recorder. The times for superfusion of 20 nM-isoprenaline and intrapipette perfusion of dibutyryl cyclic GMP (20 μM-DBcGMP) or cyclic GMP (20 μM) are indicated above the record. *B*, the average difference current–voltage relations obtained at times indicated by the corresponding symbols in *A*.

Effects of membrane-permeable derivatives of cyclic GMP

Membrane-permeable derivatives have often been used to study the effect of cyclic GMP (for review see Hartzell, 1988). The intracellular action of cyclic GMP described above, however, was not mimicked by dibutyryl cyclic GMP or 8-bromo-cyclic GMP. In the experiment shown in Fig. 7, I_{Cl} was activated by 30 nM-isoprenaline. During the continuous presence of isoprenaline in the bath solution, 20 μM-dibutyryl cyclic GMP was internally perfused without affecting I_{Cl}. This is in contrast to the result that the subsequent application of 10 μM-cyclic GMP did enhance I_{Cl}. No significant

effect was consistently observed in three other experiments. Essentially the same results were obtained with 8-bromo-cyclic GMP (10–100 μM, four experiments). Also, the results were the same if the derivatives (100–1000 μM) were applied extracellularly (not shown). If derivatives of cyclic GMP act through the cyclic GMP-dependent protein kinase (Corbin, Ogreid, Miller, Suva, Jastorff & Doskeland, 1986; Sperelakis, 1988), these findings may exclude a contribution of the cyclic GMP-dependent protein kinase to the effect of cyclic GMP on I_{Cl}.

DISCUSSION

The experimental conditions used in the present study are essentially the same as used in previous studies to isolate I_{Cl} (Matsuoka, Ehara & Noma, 1990; Tareen *et al.* 1991). Furthermore, we always monitored the reversal potential of the drug-induced current to confirm that the current changes were attributable to the catecholamine-induced Cl^- current (Bahinski *et al.* 1989; Harvey & Hume, 1989; Harvey *et al.* 1990; Matsuoka *et al.* 1990). As far as we tested this I_{Cl} the intracellular application of cyclic GMP never inhibited the β-adrenergic activation of the current. When I_{Cl} was activated submaximally by moderate concentrations of various agonists, the amplitude of I_{Cl} increased with the subsequent application of cyclic GMP. The finding is in good agreement with the enhancement of the β-adrenergic stimulation of I_{Ca} by cyclic GMP (Ono & Trautwein, 1991). It might be generalized that an increase of the cyclic GMP level is synergistic to the β-adrenergic stimulation of ion channels in mammalian hearts.

In the frog heart the effect of cyclic GMP is antagonistic to β-adrenergic activation of I_{Ca}, and it was attributed to an activation of phosphodiesterase (a cyclic GMP-stimulated one). In contrast, inhibition of a different class of phosphodiesterase was proposed underlying the synergistic action of cyclic GMP in the β-adrenergic stimulation of I_{Ca} in the guinea-pig cardiac myocytes (Ono & Trautwein, 1991). The cyclic GMP-inhibited phosphodiesterase was first identified by Harrison *et al.* (1986) in mammalian heart. The main features of this phosphodiesterase are its high affinity for cyclic AMP as substrate (Michaelis–Menten constant, $K_m < 0{\cdot}2$ μM) and its inhibition by cyclic GMP (K_i, 0·2 μM in bovine heart; 2·2 μM in guinea-pig heart, Weishaar, Kobylarz-Singer & Kaplan, 1987). Several cardiotonic agents such as milrinone, amrinone and fenoximone are known to inhibit this class of phosphodiesterase (for review see Beavo, 1988; Fischmeister & Hartzell, 1991).

The findings obtained on both I_{Ca} (Ono & Trautwein, 1991) and I_{Cl} (the present study) are summarized as follows to support the hypothesis that in mammalian heart cyclic GMP enhances the cyclic AMP response of ionic currents via the inhibition of cyclic GMP-inhibited phosphodiesterase (Ono & Trautwein, 1991). (1) The application of cyclic GMP did not affect the currents without previous stimulation with various agonists. (2) The effect of cyclic GMP was observed when currents were enhanced through elevation of the cyclic AMP level, irrespective of whether the cyclic AMP level was raised by stimulating β-adrenergic receptors or H_2-receptors, by stimulating adenylate cyclase directly by forskolin, or by applying extrinsic cyclic AMP. (3) The enhancement by cyclic GMP was prominent when the currents were activated moderately by cyclic AMP elevating agents. (4) Milrinone mimicked the

action of cyclic GMP, and one of these chemicals could not further increase the maximal effect of the others.

The involvement of cyclic GMP-dependent protein kinase (kinase G) was suggested in the antagonistic action of cyclic GMP against the β-adrenergic stimulation of I_{Ca} (Levi *et al.* 1989; Mery *et al.* 1990, 1991). The internal dialysis of kinase G (in the presence of isoprenaline) slightly inhibited I_{Ca} in some experiments (Ono & Trautwein, 1991). The application of 8-bromo-cyclic GMP or higher doses of cyclic GMP partially reversed the activation of I_{Ca} (Ono & Trautwein, 1991). In the present study we never observed inhibition of I_{Cl} by cyclic GMP or the derivatives of cyclic GMP. Thus, the involvement of kinase G is unlikely for I_{Cl}.

Application of the non-specific phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX) activates I_{Cl} (Egan, Noble, Noble, Powell, Twist & Yamaoka, 1988) and also increases the amplitude of I_{Ca} (Trautwein, Kameyama, Hescheler & Hofmann, 1986; Ono & Trautwein, 1991). The findings are explained by assuming an accumulation of cyclic AMP due to basal production in the absence of β-agonists in the isolated cardiac myocytes. If milrinone selectively inhibits a certain class of phosphodiesterase among multiple classes of phosphodiesterases, the remaining phosphodiesterases might have counterbalanced the production of cyclic AMP, thereby neither I_{Ca} nor I_{Cl} was increased by the sole application of milrinone.

If the above hypothesis is correct, measurement of the dose–response relation (Ono *et al.* 1991; see also Table 1) indicates that the production of cyclic AMP, activated by moderate concentrations (1–30 nM) of isoprenaline, is partially catalysed by the cyclic GMP-sensitive phosphodiesterase. The stronger activation of adenylate cyclase by isoprenaline of more than 100 nM may achieve more than a saturating concentration of cyclic AMP within the cell. It should be noted that the current amplitudes do not linearly reflect the intracellular cyclic AMP level. If I_{Cl} is maximally stimulated, it may not be enhanced further in response to additional increase in cyclic AMP level.

The possible physiological roles of cyclic GMP in the heart are not yet clear. Most substances, like acetylcholine, which increase cellular cyclic GMP levels show 'accentuated antagonism' against β-adrenergic effects. It is established now that the accentuated antagonism is mediated through an inhibition of adenylate cyclase by the GTP-binding protein independently from cyclic GMP (Hescheler, Kameyama & Trautwein, 1986; Tareen *et al.* 1991). Thus, cyclic GMP is not involved in the antagonistic effect of acetylcholine in mammalian heart. However, cyclic GMP may, in part, contribute to the β-adrenergic and muscarinic interaction in a different way. Tareen *et al.* (1991) observed a transient increase of the isoprenaline response after quick removal of acetylcholine (post-acetylcholine rebound phenomenon), and they suggested that it may be attributed to the remaining cyclic GMP. This is supported by the finding that the rebound phenomenon was much less frequent when the pipette solution contained cyclic GMP (F. M. Tareen, A. Yoshida & K. Ono, unpublished observation).

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