*J. Physiol.* (1987), **387**, *pp.* 453–472
*With* 10 *text-figures*
*Printed in Great Britain*



# CYCLIC GUANOSINE 3′, 5′-MONOPHOSPHATE REGULATES THE CALCIUM CURRENT IN SINGLE CELLS FROM FROG VENTRICLE

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(*Received 14 July 1986*)

SUMMARY

1. The effect of intracellular perfusion with cyclic AMP and cyclic GMP on $Ca^{2+}$ current ($I_{Ca}$) was studied in single cells isolated from frog ventricle using the whole-cell patch-clamp technique and a perfused pipette.

2. Intracellular perfusion with cyclic GMP (0·1–20 $\mu$M) had no effect on the basal $I_{Ca}$. However, when $I_{Ca}$ was increased by isoprenaline or by intracellular perfusion with cyclic AMP, perfusion with cyclic GMP (20 $\mu$M) reduced $I_{Ca}$ by an average of 67 %. The effect of cyclic GMP on $I_{Ca}$ elevated by cyclic AMP was reversible. A half-maximal effect of cyclic GMP was observed at 0·6 $\mu$M. Cyclic GMP had no significant effect on the shape of the $I_{Ca}$ current–voltage relationship.

3. The effect of cyclic GMP was specific to the 3′, 5′ form; 2′, 3′-cyclic GMP had no effect.

4. The effect of cyclic GMP was apparently not mediated by stimulation of cyclic-GMP–dependent protein kinase because 8-bromo-cyclic GMP, a very potent activator of the protein kinase, was without effect.

5. Cyclic GMP had no effect on $I_{Ca}$ elevated by the non-hydrolysable 8-bromo-cyclic AMP. The effect of cyclic GMP on cyclic-AMP-elevated $I_{Ca}$ was partially blocked by the phosphodiesterase inhibitor, methylisobutylxanthine. Thus, it was hypothesized that the effect of cyclic GMP was mediated by hydrolysis of cyclic AMP as a result of a stimulation of a cyclic nucleotide phosphodiesterase by cyclic GMP.

6. The dose–response curve for cyclic AMP on $I_{Ca}$ was well fitted by the Michaelis equation with a $K_{50}$ (i.e. concentration of cyclic AMP at which response is 50 % of the maximum) of 0·7 $\mu$M and a maximal 11-fold stimulation of $I_{Ca}$. Cyclic GMP shifted the curve one log unit to the right and decreased the maximal stimulation to 8·6-fold. Thus, the effect of cyclic GMP appeared uncompetitive.

7. The products of cyclic AMP and cyclic GMP hydrolysis, 5′-AMP and 5′-GMP, had no effect on $I_{Ca}$. Furthermore, strong buffering of intracellular pH did not reduce the effect of cyclic GMP.

8. It is proposed that cyclic-GMP-stimulation of a cyclic nucleotide phosphodiesterase may be one of several mechanisms by which acetylcholine regulates $I_{Ca}$.

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## INTRODUCTION

The force of cardiac contraction is regulated by noradrenaline and acetylcholine (ACh). Noradrenaline increases the force of contraction by stimulating the production of cyclic AMP and activating cyclic-AMP-dependent protein kinase. The cyclic-AMP-dependent protein kinase then phosphorylates a variety of substrates involved in regulating cardiac contractility (the $Ca^{2+}$ channel, phospholamban, troponin I and C-protein: Tsien, 1977; Löffelholz & Pappano, 1985; Hartzell, 1987). Of these substrates, the $Ca^{2+}$ channel may be the most important in determining the force of contraction (Reuter, 1979, 1983; Tsien, 1983; Trautwein & Cavalié, 1985).

In addition to cyclic AMP, cyclic GMP has also been proposed to regulate cardiac contractility, but its importance remains obscure and controversial (Golberg & Haddox, 1977; Lincoln & Corbin, 1983). The once-popular idea that cyclic GMP opposes the effects of cyclic AMP (the 'Yin–Yan hypothesis' proposed by Goldberg, Haddox, Nicol, Glass, Sanford, Kuehl & Estensen (1975) has been weakened by numerous experimental inconsistencies and an inability to elucidate the molecular mechanisms of cyclic GMP action. It seems probable that cyclic GMP does have some regulatory role, however, because numerous agents and conditions, including cholinergic agonists, hypoxia, spontaneous and neurogenic hypertension, and myocardial infarction alter the steady-state levels of cyclic GMP in heart (Goldberg & Haddox, 1977; Dobson, 1981).

A role for cyclic GMP is best implicated in the negative inotropic response of the heart to ACh. The first hormonal effect on cyclic GMP levels to be discovered was that produced by ACh in rat ventricle (George, Polson, O'Toole & Goldberg, 1970) and since then many investigators have demonstrated increases in cyclic GMP levels in response to cholinergic agents in a variety of cardiac preparations (Kuo, Lee, Reyes, Walton, Donnelly & Greengard, 1972; George, Ignarro, Paddock, White & Kadowitz, 1975; Watanabe & Besch, 1975; Gardner & Allen, 1976; Ghanbari & McCarl, 1976; Brown, Polson, Krzanowski & Wiggins, 1980; Lincoln & Keely, 1981; Dobson, 1981; Flitney & Singh, 1981; Endoh, Maruyama & Iijima, 1985). Furthermore, extracellular application of cyclic GMP or membrane-permeable derivatives of cyclic GMP often decrease contractile force (Wilkerson, Paddock & George, 1976; Trautwein & Trube, 1976; Nawarth, 1977; Kohlhardt & Haap, 1978; Linden & Brooker, 1979; Endoh & Yamashita, 1981; Singh & Flitney, 1981). It has been proposed that the negative inotropic effect of cyclic GMP, like that of ACh, is mediated by a decrease in $I_{Ca}$ because cyclic GMP decreases $^{45}Ca$ flux (Nawrath, 1977), shortens action potential duration (Trautwein, Taniguchi & Noma, 1982), and inhibits $Ca^{2+}$-dependent action potentials (Kohlhardt & Haap, 1978; Bkaily & Sperelakis, 1985; Wahler & Sperelakis, 1985).

The hypothesis that cyclic GMP is involved in the negative inotropic response of the heart to ACh has been challenged on several grounds. A variety of experiments have demonstrated a dissociation between cyclic GMP levels and contractile state. It has been reported that low concentrations of ACh depress contractility without changing cyclic GMP levels (Watanabe & Besch, 1975; Diamond, Ten Eick & Trapani, 1977; Brooker, 1977; Linden & Brooker, 1979; Dobson, 1981), and that increases in cyclic GMP produced by chemicals other than ACh do not correlate with

changes in contractility (Diamond *et al.* 1977; Katsuki, Arnold & Murad, 1977). Furthermore, some investigators have failed to find a significant effect of exogenous cyclic GMP on contractile force (Endoh & Shimizu, 1979; Linden & Brooker, 1979) and elevation of intracellular cyclic GMP by a photo-activated derivative of cyclic GMP had no effect on $I_{Ca}$ (Nargeot, Nerbonne, Engels & Lester, 1983). The negative inotropic effect of ACh is not blocked by drugs that prevent the increase in cyclic GMP levels (Diamond & Chu, 1985).

In order to clarify the possible role and mechanism of action of cyclic GMP, we have undertaken a series of experiments to examine the effect of cyclic GMP on the slow inward $Ca^{2+}$ current ($I_{Ca}$) in single, isolated cardiac myocytes. We have recently reported that intracellular perfusion of low concentrations of cyclic GMP can decrease $I_{Ca}$ that has been elevated by $\beta$-adrenergic receptor stimulation or by intracellular perfusion with cyclic AMP (Hartzell & Fischmeister, 1986). In contrast, cyclic GMP has negligible effects on the basal $I_{Ca}$. We have proposed that the cyclic GMP effect is mediated by stimulation of a cyclic nucleotide phosphodiesterase which is plentiful in cardiac cells. Our purpose here is to present in more detail the evidence that we have accumulated to support this hypothesis.

## METHODS

### *Solutions and drugs*

$Ca^{2+}$-free Ringer solution contained 88·4 mM-NaCl, 2·5 mM-KCl, 23·8 mM-$NaHCO_3$, 0·6 mM-$NaH_2PO_4$, 1·8 mM-$MgCl_2$, 5 mM-D-glucose and 5 mM-sodium pyruvate. 1·8 mM-$CaCl_2$ was added to $Ca^{2+}$-free Ringer solution to make normal Ringer solution. Dissociation medium was composed of $Ca^{2+}$-free Ringer solution containing creatine (5 mM), Minimal Essential Medium amino acids and vitamins (10% v/v; Boehringer-Mannheim, F.R.G.), fatty-acid-free bovine serum albumin (1 mg/ml; Sigma, U.S.A.), penicillin (50 i.u./ml) and streptomycin (50 $\mu$g/ml). The $K^+$-free, 20 mM-$Cs^+$-Ringer solution contained 88·4 mM-NaCl, 20 mM-CsCl, 22·9 mM-$NaHCO_3$, 0·6 mM-$NaH_2PO_4$, 1·8 mM-$MgCl_2$, 5 mM-D-glucose, 5 mM-sodium pyruvate and $3 \times 10^{-7}$ M-tetrodotoxin (TTX; Sankyo, Japan). The pH of all external solutions was maintained at 7·4 by gassing with 95% $O_2$–5% $CO_2$. The standard internal solution in the patch electrode contained 120 mM-CsCl, 5 mM-$K_2$EGTA, 4 mM-$MgCl_2$, 5 mM-phosphocreatine disodium, 3 mM-$Na_2$ATP, 0·4 mM-$Na_2$GTP and 10 mM-HEPES; pH 7·1 (adjusted with KOH). The 75 mM-PIPES (piperazine-*N*,*N'*-bis(2-ethanesulphonic acid)) intracellular medium (used only in Fig. 10) contained 12 mM-CsCl, 75 mM-PIPES, 5 mM-$K_2$EGTA, 4 mM-$MgCl_2$, 5 mM-phosphocreatine disodium, 3 mM-$Na_2$ATP and 0·4 mM-$Na_2$GTP, adjusted to pH 7·1 with CsOH. ACh chloride, (±) isoprenaline, 1-methyl-3-isobutylxanthine, insulin and cyclic nucleotides were obtained from Sigma Chemical Co. (U.S.A.). Several experiments utilized cyclic AMP and cyclic GMP from Boehringer–Mannheim (F.R.G.) with identical results. M & B 22948 (2-*o*-propoxyphenyl-8-azapurin-6-one) was a gift of May & Baker, Dagenham, U.K.

### *Preparations*

Cells were enzymatically dispersed from frog (*Rana esculenta*) (Arrio-Dupont & De Nay, 1985; Fischmeister & Hartzell, 1986). The isolated heart was first perfused through the aorta at 28 °C at a rate of 3 ml/min with $Ca^{2+}$-free Ringer solution for 5 min or with dissociation medium containing porcine insulin (1 $\mu$g/ml) and isoprenaline (2 $\mu$M) for 30 min. The cells produced with insulin and isoprenaline seemed more robust, but this was not studied in a quantitative manner. The heart was then perfused with 20 ml of a recirculating dissociation medium containing trypsin (0·4 mg/ml; Boehringer–Mannheim No. 109 819) and collagenase (3 mg/ml; Boehringer–Mannheim No. 103 586) for 60–90 min. The ventricle was dissected, lacerated with forceps, and stirred under gassed atmosphere for 30 min in 5 ml of dissociation medium containing no collagenase or trypsin. The cell suspension was filtered and the cells were centrifuged, resuspended and centrifuged in dissociation medium. The cells were stored in dissociation medium containing 0·9 mM-$CaCl_2$.

*Voltage clamp*

Cells were voltage clamped with a List-7 Patch Clamp Amplifier as previously described (Fischmeister & Hartzell, 1986) using the whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch electrodes made from capillary tubing (100 $\mu$l 'Microcaps'; Drummond, U.S.A.) had resistances of 1–3 MΩ. After disruption of the membrane patch, membrane capacitance of the whole cell and series resistance were measured and compensated when necessary. Series resistance was usually $< 5$ MΩ of which 30–90% could be compensated. Membrane capacitance ranged from 30 to 90 pF. Since the membrane capacitance is proportional to the total surface membrane of the cell, its value was used in scaling $I_{Ca}$ obtained in different cells to allow for comparison between different experimental conditions (Table 1). For routine monitoring of $I_{Ca}$, the cell was depolarized every 8 s from $-80$ mV holding potential to 0 mV for 200 ms. At various times during the course of the experiment, current–voltage relationships were determined by applying the appropriate voltage-clamp protocol to the interior of the patch electrode. $I_{Ca}$ was measured as the difference between peak inward current and the current at the end of the 200 ms pulse. Adequate voltage homogeneity of these cells has been demonstrated (Fischmeister & Hartzell, 1986).

*Superfusion*

For electrophysiological recording, the cells in a 35 mm tissue culture dish were superfused by gravity (0·67 ml/min) at room temperature (18·5–23 °C) with $K^+$-free, 20 mM-$Cs^+$ Ringer solution. During recording individual cells were exposed to different extracellular solutions by positioning the cell at the extremity of one of six capillaries (Tygon microbore tubing: i.d., 250 $\mu$m; o.d., 750 $\mu$m; Norton, U.S.A.). Each capillary was connected to a 10 ml syringe containing a test solution which was gassed with 95% $O_2$–5% $CO_2$. The flow of solution from the capillaries was controlled by gravity (16 $\mu$l/min). The drain for the bath perfusion was glued 3 mm to the rear of the openings of the capillaries to avoid contamination of the dish with test solutions. One capillary contained control bath solution so that control recordings were obtained under perfusion to eliminate a possible perturbation in the recordings induced by the perfusion itself.

*Internal perfusion*

A pipette holder was constructed that permitted the solution inside the patch pipette to be changed during an experiment. This holder was very similar to that described by others (Soejima & Noma, 1984; Kameyama, Hofmann & Trautwein, 1985). A fine capillary, pulled from 0·3 mm i.d, 0·7 mm o.d. polyethylene catheter, was inserted into the patch pipette to within 0·3–0·6 mm of the tip. The distal end of the capillary was placed into reservoirs of different solutions. The pipette solution was changed by applying negative pressure to the pipette, which aspirated the solution from the capillary. The negative pressure, monitored by a 'U' tube filled with water, was 20–30 cm of water. This negative pressure was maintained throughout the experiment except for 30 s while changing solutions. With most capillaries, this negative pressure produced a flow rate of 5 $\mu$l/min. The diffusion of solution from the tip of the capillary to the tip of the electrode was monitored using various dyes and opaque solutions. When the capillary tip was within 400 $\mu$m of the tip of the pipette, the new solution could be seen to reach the pipette tip 4 min after changing reservoirs. Our standard procedure was to fill the pipette and the perfusion system with standard intracellular medium, form the gigaseal, and break the membrane patch by negative pressure. After the $Ca^{2+}$ current stabilized and control measurements were made, the capillary was moved to a reservoir containing a different solution. The time to onset of the effect of the test solution depended upon the distance of the capillary from the tip of the pipette and the total series resistance. Thus, we tried to use the lowest possible resistance pipettes. Pipettes with resistances between 0·8 and 1·6 MΩ gave the best results, but we used pipettes with resistances up to 2·5 MΩ.

Fig. 1*C* shows the typical kinetics of onset of the effect of cyclic AMP. The lag time corresponds to the time required for the cyclic AMP to reach the pipette tip. Once $I_{Ca}$ began to increase, however, the time course of the increase was very similar to that observed when cyclic AMP was included in the intracellular medium used to fill the pipette before making a gigaseal (Fischmeister & Hartzell, 1986).

*Data analysis*

Current recordings were low-pass filtered through a 3·3 kHz three-pole Bessel-response filter and recorded on FM tape (Hewlett-Packard, U.S.A., 3964A) at $3\frac{3}{4}$ in/s (band width, d.c. to 1·25 kHz). Records were simultaneously displayed on a digitizing oscilloscope (Gould, U.S.A., Type 1421) and sampled at 5 kHz by a 12-bit analog-to-digital converter (Data Translation, type 2801A), and analysed by a Compaq 286 Desk-Pro Computer. On-line analysis of the recordings was made possible by programming the microcomputer in Pascal language to determine, for each membrane depolarization, peak and steady-state current values.

## RESULTS

### *Effects of intracellular cyclic AMP and cyclic GMP*

*Experiments with non-perfused pipettes.* $Ca^{2+}$ currents were elicited by 200 ms duration depolarizations from a holding potential of −80 mV to 0 mV in cells in

TABLE 1. Effects of cyclic GMP on $I_{Ca}$ in frog ventricular cells

| Ringer solution | Intracellular medium | $I_{Ca}$ density (A/F) | $E_{max}$ (mV) |
|---|---|---|---|
| Control | Control | 3·6±0·2 (127) | 0·5±0·8 (21) |
| Control | 2 μM-cyclic GMP | 2·7±0·6 (5) | −0·5±10 (4) |
| Control | 20 μM-cyclic GMP | 3·6±0·4 (15) | 0·7±0·7 (7) |
| 2 μM-isoprenaline | Control | 27·1±2·6 (47) | −1·7±1·0 (13) |
| 2 μM-isoprenaline | 2 μM-cyclic GMP | 9·2±1·3 (3) | −1·7±1·7 (3) |
| 2 μM-isoprenaline | 20 μM-cyclic GMP | 15·9±1·8 (14) | −1·7±1·7 (6) |
| Isoprenaline + ACh | Control | 6·6±1·4 (19) | 0·0±1·3 (6) |
| Isoprenaline + ACh | 2 μM-cyclic GMP | 4·5±0·8 (3) | 0·0±0·0 (3) |
| Isoprenaline + ACh | 20 μM-cyclic GMP | 5·7±1·2 (7) | 0·0±0·0 (4) |

$I_{Ca}$ density, net inward $Ca^{2+}$ current scaled by cell membrane capacitance in amperes/farad (A/F). $E_{max}$, peak of the current–voltage relationship for $I_{Ca}$. Experiments using non-perfused pipettes only. Isoprenaline, 2 μM; ACh, 1 μM. Values are mean±S.E. Number of cells shown in parentheses.

which $K^+$ currents were blocked with intracellular and extracellular $Cs^+$ and the fast $Na^+$ current blocked with TTX. In the first series of experiments, the effects of isoprenaline and ACh on $I_{ca}$ were studied in one group of cells that were perfused with normal intracellular medium and in another group of cells that were perfused with intracellular medium containing 20 μM-cyclic GMP (Table 1). As we have previously reported (Fischmeister & Hartzell, 1986), with control intracellular medium, ACh alone had no effect on $I_{Ca}$, isoprenaline ($2 \times 10^{-6}$ M) increased $I_{Ca}$ an average of 6·6-fold, and ACh reduced the isoprenaline-stimulated $I_{Ca}$ to near the control level (a 6·6-fold reduction after approximately 5 min). When 20 μM-cyclic GMP was included in the pipette, the basal level of $I_{Ca}$ was not different from cells with control intracellular medium. However, isoprenaline increased $I_{Ca}$ only 3·5-fold. The reduction of isoprenaline-stimulated $I_{Ca}$ by ACh was not apparently affected by cyclic GMP.

To verify that cyclic GMP reduced the isoprenaline-stimulated $I_{Ca}$, we examined the change of $I_{Ca}$ with time after breaking the membrane patch under the electrode. When the cells were superfused with control Ringer solution, the membrane current remained stable or increased slightly over the first 2 or 3 min after patch break in

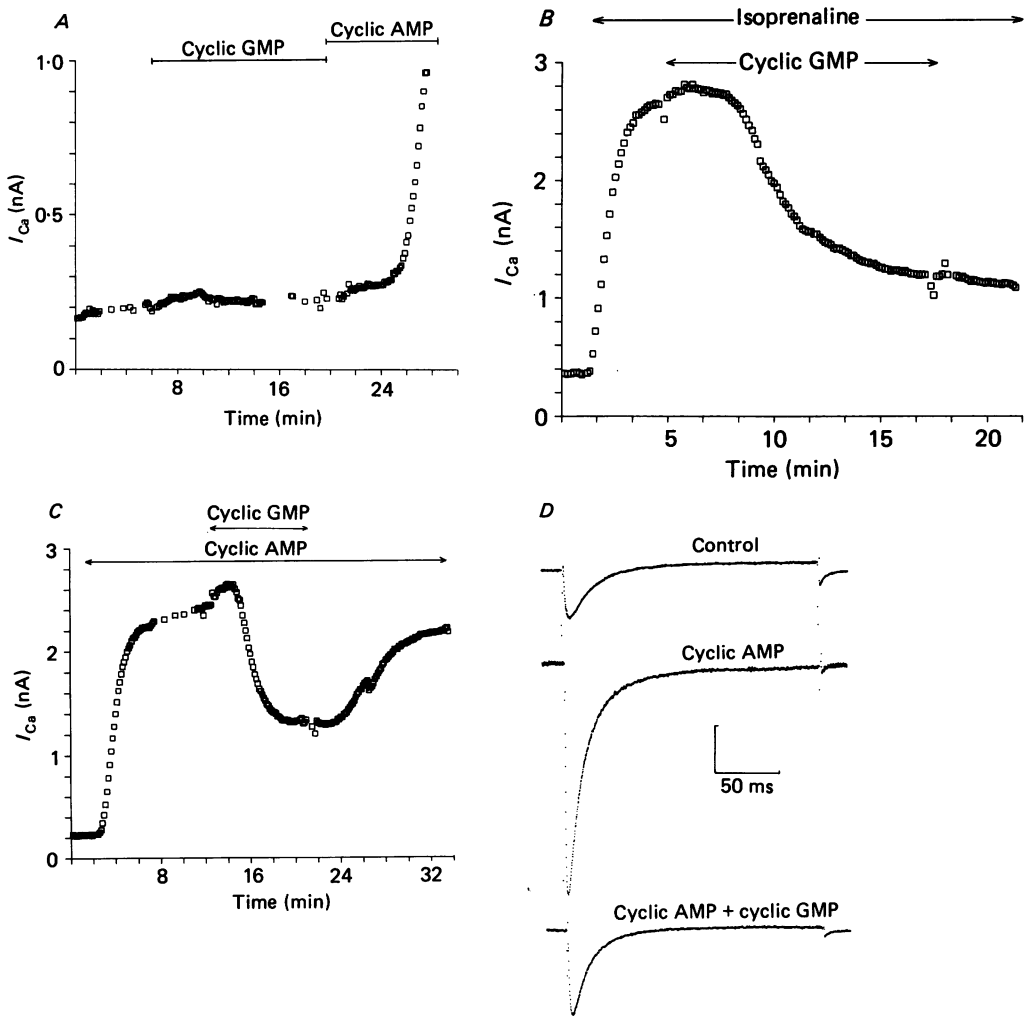


Fig. 1. Effects of intracellular perfusion of cyclic GMP on $I_{Ca}$. $I_{Ca}$ was recorded from an isolated ventricular cell using a perfusable patch pipette as described in Methods. Each square is the net $Ca^{2+}$ current measured by a depolarizing pulse every 8 s (see Methods). *A*, effects of intracellular cyclic GMP on basal $I_{Ca}$. The cell was exposed to control Ringer solution for the entire experiment. The cell was internally perfused with control intracellular medium except for the periods indicated, when it was perfused with cyclic GMP (20 $\mu$M) or cyclic AMP (5 $\mu$M). Cyclic GMP had no effect on $I_{Ca}$. Subsequent perfusion with cyclic AMP, to show that the perfusion was working properly, significantly elevated $I_{Ca}$. *B*, effects of intracellular perfusion with cyclic GMP on $I_{Ca}$ stimulated by isoprenaline. The cell was exposed to control Ringer solution for the first 1·5 min of the experiment and with Ringer solution containing 0·3 $\mu$M-isoprenaline during the period indicated. The control intracellular medium was changed to one containing 5 $\mu$M-cyclic GMP for the period indicated. *C*, effect of intracellular perfusion of cyclic GMP on cyclic-AMP-elevated $I_{Ca}$. The cell was exposed to control Ringer solution for the entire experiment. The cell was internally perfused with control intracellular medium except for the periods indicated, when it was perfused with cyclic AMP (30 $\mu$M) or cyclic AMP (30 $\mu$M) + cyclic GMP (20 $\mu$M). *D*, traces showing $I_{Ca}$ recorded in the presence and absence of cyclic AMP and cyclic GMP. $Ca^{2+}$ currents were recorded on magnetic tape at $3\frac{3}{4}$ in/s and replayed for analysis. The records were digitized at 5 kHz. Calibration: control, 400 pA; cyclic AMP, 480 pA; cyclic AMP + cyclic GMP, 540 pA.

both control cells and cyclic GMP-perfused cells. In contrast, when the cells were superfused with $10^{-7}$ M-isoprenaline beginning several minutes prior to breaking the patch, $I_{Ca}$ in cells perfused with cyclic GMP decreased from an initial value of $11{\cdot}5 \pm 0{\cdot}8$ A/F (current density in amperes/farad) to $5{\cdot}0 \pm 0{\cdot}3$ A/F (mean ± S.E., three cells) after 2 min whereas $I_{Ca}$ in control cells increased over the same time from $11{\cdot}3 \pm 2{\cdot}1$ A/F to $15{\cdot}9 \pm 3{\cdot}7$ A/F (three cells).

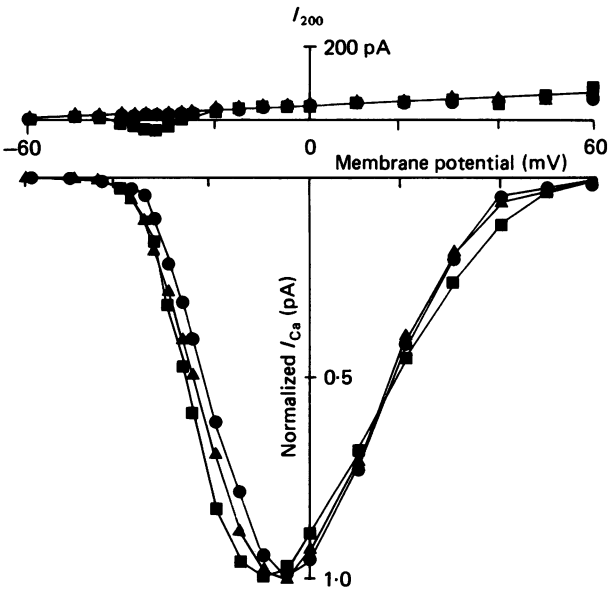


Fig. 2. Effects of cyclic AMP and cyclic GMP on $I_{Ca}$ current–voltage relationship. Triangles, control; squares, 5 μM-cyclic AMP; circles, 5 μM-cyclic AMP + 20 μM-cyclic GMP. The current–voltage relationships were determined as we have previously described (Fischmeister & Hartzell, 1986). Net $I_{Ca}$ (bottom part of Figure) was measured as the difference between the peak current and the current at the end of a 200 ms pulse. The current–voltage curves are normalized to a maximum $I_{Ca}$ of 1·0. Steady-state current ($I_{200}$; top part of Figure) was measured as the current at the end of the 200 ms pulse and is not normalized.

*Perfused pipette.* To examine further the effect of cyclic GMP on $I_{Ca}$, we constructed a perfused pipette that permitted us to change the solution in the patch electrode during an experiment. Intracellular perfusion of the cell with cyclic GMP had no effect on control $I_{Ca}$ in the absence of isoprenaline or cyclic AMP (Fig. 1*A*). In eight experiments using a perfused pipette, 20 μM-cyclic GMP had no effect on basal $I_{Ca}$ ($I_{Ca}$ in cyclic GMP was $104 \pm 6{\cdot}8$% of control $I_{Ca}$, mean ± S.E.). However, when $I_{Ca}$ was elevated either by isoprenaline or by intracellular perfusion with cyclic AMP, intracellular perfusion with cyclic GMP reduced $I_{Ca}$ (Fig. 1*B* and *C*).

The effect of cyclic GMP on the cyclic-AMP-elevated $I_{Ca}$ was usually reversible. Upon washing out the cyclic GMP, $I_{Ca}$ slowly returned to the pre-cyclic GMP level (Figs. 1*C*, 3, 5 and 10). In contrast, however, it was usually difficult to wash out the cyclic GMP effect on isoprenaline-elevated $I_{ca}$. This suggests that cyclic GMP might have a second effect on the isoprenaline-stimulated current, for example, by stimulating desensitization of the β-receptor.

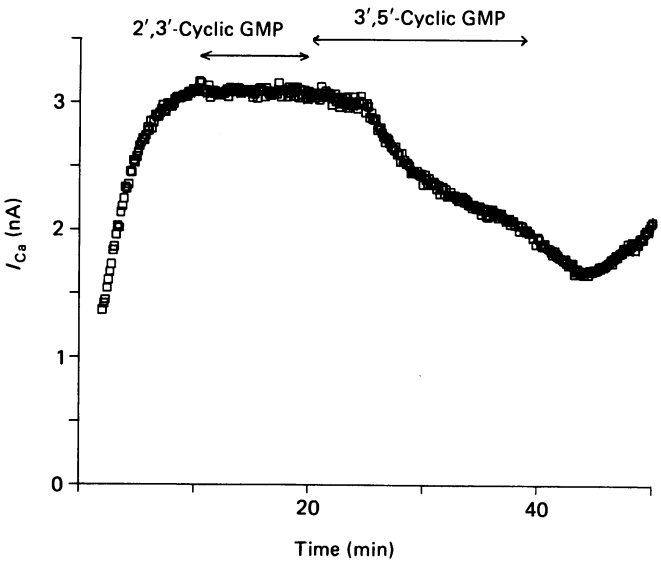


Fig. 3. Effect of 2',3'-cyclic GMP on $I_{Ca}$. At time zero, perfusion of the cell with intracellular medium containing 5 $\mu$M-cyclic AMP started. 20 $\mu$M-2',3'-cyclic GMP or 20 $\mu$M-3',5'-cyclic GMP was added to this cyclic-AMP-containing medium for the periods indicated.

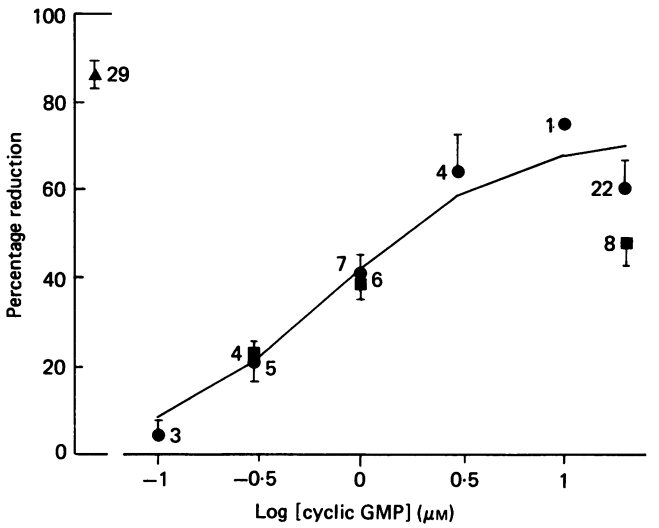


Fig. 4. Dose–response curve for effect of cyclic GMP on $I_{Ca}$. $I_{Ca}$ was elevated either with superfusion with 2 $\mu$M-isoprenaline (squares) or internal perfusion with 5 $\mu$M-cyclic AMP (circles). After the current stabilized, the cell was internally perfused with different concentrations of cyclic GMP. In some cells, cyclic GMP was perfused sequentially in increasing concentrations. The effect of one concentration of cyclic GMP was allowed to plateau for several minutes before beginning perfusion with a higher concentration. In other cells, only single concentrations of cyclic GMP were tested. Similar results were obtained with the two methods. Data points are mean values (number of cells indicated beside each point). Error bars are $\pm$S.E. The continuous line is a non-linear least-squares regression of the cyclic AMP data to the Michaelis equation. The equation of the best-fit line was: percentage reduction = 67 % $\times$ [cyclic GMP]/(0·63 $\mu$M + [cyclic GMP]). The triangle in the upper left-hand corner represents the value of basal $I_{Ca}$ relative to the cyclic-AMP-stimulated current.

$Ca^{2+}$ currents recorded in the absence and presence of intracellular perfusion with cyclic AMP and cyclic GMP are shown in Fig. 1*D*. A complete analysis of the effects of cyclic AMP and cyclic GMP on the kinetics of $I_{Ca}$ is under way, but from the records shown here, it is apparent that whatever changes cyclic AMP and cyclic GMP produce in the kinetics are not likely to influence the analysis in the present study.

The shape of the peak $I_{Ca}$ current–voltage relationship was usually not significantly affected by ACh, isoprenaline, cyclic AMP, or cyclic GMP (Table 1; Fig. 2). In some cells, however, perfusion with cyclic AMP produced a leftward shift of 5–10 mV in the peak of the current–voltage relationship. This shift was not reversible upon washing out the cyclic AMP and was not reversed by cyclic GMP. This shift requires further study in order to determine whether it is physiologically important (Bean, Nowycky & Tsien, 1984) or whether it is a series resistance artifact (Fischmeister & Hartzell, 1986). Neither cyclic AMP or cyclic GMP significantly affected the slope of the steady-state current–voltage relationship (Fig. 2) and had no consistent effect on the holding current at −80 mV. In two representative experiments, shown in Figs. 6 and 8*B*, the holding current is plotted to show that it does not change in response to cyclic AMP or cyclic GMP.

The effect of cyclic GMP on isoprenaline- or cyclic-AMP-stimulated $I_{Ca}$ was specific to 3′,5′-cyclic GMP. 2′,3′-cyclic GMP (20 and 220 μM), which is physiologically inactive, had no effect on $I_{Ca}$ amplitude (Fig. 3).

*Dose–response curve.* The response to cyclic GMP was dose dependent with a threshold in the submicromolar range. Fig. 4 shows the effect of different concentrations of cyclic GMP on $I_{Ca}$ which had been elevated with intracellular 5 μM-cyclic AMP (circles) or with extracellular 2 μM-isoprenaline (squares). The smooth curve is a non-linear least-squares fit of the cyclic AMP data to the Michaelis equation. Small, but significant decreases in $I_{Ca}$ were observed with 0·1 μM-cyclic GMP and a half-maximal effect was observed at 0·6 μM-cyclic GMP. Maximal concentrations of cyclic GMP decreases the cyclic-AMP-elevated $I_{Ca}$ by an average of 67 %. The cyclic GMP dose–response curve for isoprenaline-stimulated $I_{Ca}$ was similar to that for the cyclic-AMP-elevated $I_{Ca}$.

*Molecular mechanism of cyclic GMP action*

*Role of protein kinase.* To determine whether the effect of cyclic GMP was mediated by cyclic-GMP-dependent protein kinase, the effectiveness of analogues of cyclic GMP in reducing $I_{Ca}$ was studied. The most useful of these analogues was 8-bromo-cyclic GMP because it is about five times more potent than cyclic GMP in activating the protein kinase (Corbin, Ogreid, Miller, Suva, Jastorff & Doskeland, 1986). 8-bromo-cyclic GMP was completely ineffective in reducing $I_{Ca}$ (Fig. 5). In ten cells, 20 μM-8-bromo-cyclic GMP reduced cyclic AMP-elevated $I_{Ca}$ by less than 10 % (8 ± 4 %, mean ± S.E.). For this reason, we concluded that cyclic GMP was not acting through cyclic-GMP-dependent protein kinase.

*Hydrolysis of cyclic GMP.* It seems unlikely that hydrolysis of cyclic GMP is necessary for its effect for several reasons. First, hydrolysis of cyclic GMP should be minimal in our experiments because the $Ca^{2+}$-stimulated cyclic-GMP phosphodiesterase (PDE I) should be inhibited by the EGTA in the intracellular medium (Weishaar, Burrows, Kobylarz, Quade & Evans, 1986). To block any residual PDE

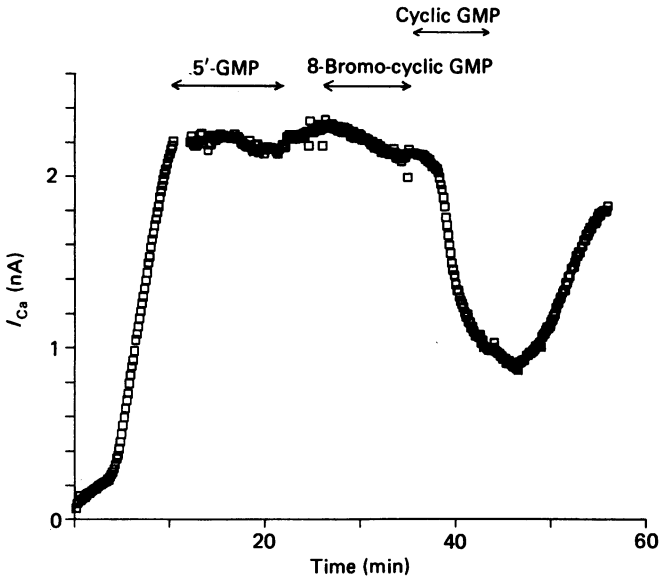


Fig. 5. Effects of 5'-GMP and 8-bromo-cyclic GMP on $I_{Ca}$. At time zero, perfusion with intracellular medium containing 5 $\mu$M-cyclic AMP was begun and continued throughout the experiment. The cell was perfused with 5'-GMP (200 $\mu$M), 8-bromo-cyclic GMP (20 $\mu$M) or cyclic GMP (20 $\mu$M) during the periods indicated.

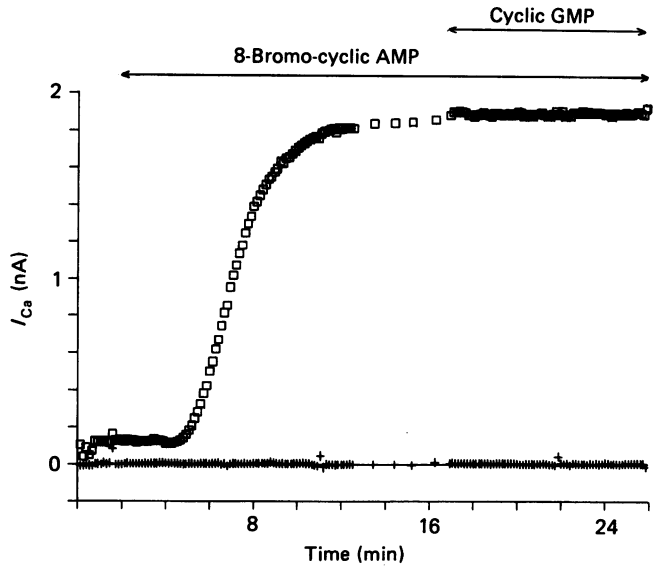


Fig. 6. Effects of cyclic GMP on $I_{Ca}$ elevated by 8-bromo-cyclic AMP. The cell was perfused with 5 $\mu$M-8-bromo-cyclic AMP or with 5 $\mu$M-8-bromo-cyclic AMP + 20 $\mu$M-cyclic GMP during the periods indicated.

I activity, we used 100 $\mu$M-M & B 22948, a selective inhibitor of this phosphodiesterase (Weishaar, Cain & Bristol, 1985). M & B 22948 had no effect on the response to cyclic GMP: in the presence of M & B 22948, 20 $\mu$M-cyclic GMP reduced $I_{Ca}$ 67 $\pm$ 9 % ($n = 5$), which was the same reduction as that found in the absence of the inhibitor. We also examined the effect of the hydrolysis product of cyclic GMP, 5′-GMP, on cyclic-AMP-elevated $I_{Ca}$. 5′-GMP was at least several hundredfold less effective than

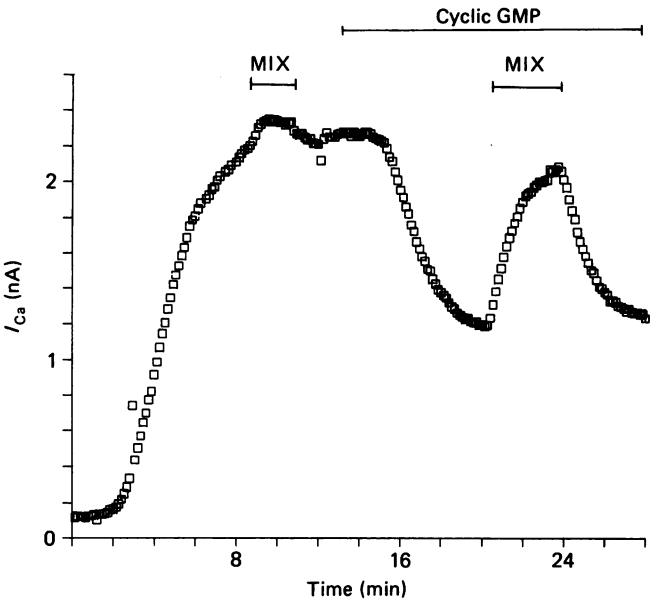


Fig. 7. Effect of 3-isobutyl-1-methylxanthine (MIX) on $I_{Ca}$. At time zero, perfusion with 5 $\mu$M-cyclic AMP was begun and continued throughout the experiment. During the periods indicated, 20 $\mu$M-cyclic GMP was added to the cyclic-AMP-containing medium, or the cell was superfused with normal Ringer solution containing 100 $\mu$M-MIX.

cyclic GMP in reducing cyclic-AMP-elevated $I_{Ca}$: 20 $\mu$M-5′-GMP had no effect ($I_{Ca}$ was reduced an average of only 3 $\pm$ 6 % ($n = 3$; Fig. 5) and even 200 $\mu$M-5′-GMP reduced $I_{Ca}$ only an average of 16 $\pm$ 4 % ($n = 4$)).

*Role of phosphodiesterase II.* One possible site of action for cyclic GMP was the cyclic-GMP-stimulated cyclic nucleotide phosphodiesterase (PDE II) (Beavo, Hardman & Sutherland, 1971; Martins, Mumby & Beavo, 1982). Two experiments suggest that the effect of cyclic GMP on $I_{Ca}$ is at least partially mediated by this phosphodiesterase.

(1) Cyclic GMP had no effect on $I_{Ca}$ stimulated by the phosphodiesterase-resistant 8-bromo-cyclic AMP (Fig. 6; $I_{Ca}$ in cyclic GMP was 94 $\pm$ 6 % of the 8-bromo-cyclic-AMP-elevated $I_{Ca}$; $n = 6$). This is what we would expect if cyclic AMP hydrolysis were involved in the response to cyclic GMP.

(2) Blocking the phosphodiesterase with 3-isobutyl-1-methylxanthine (MIX), which is a potent inhibitor of the cyclic-GMP-stimulated phosphodiesterase (Weishaar *et al.* 1985), reduced the effect of cyclic GMP (Fig. 7). In this experiment, MIX was applied to the cell by superfusion. 100 $\mu$M-MIX itself had no effect on control

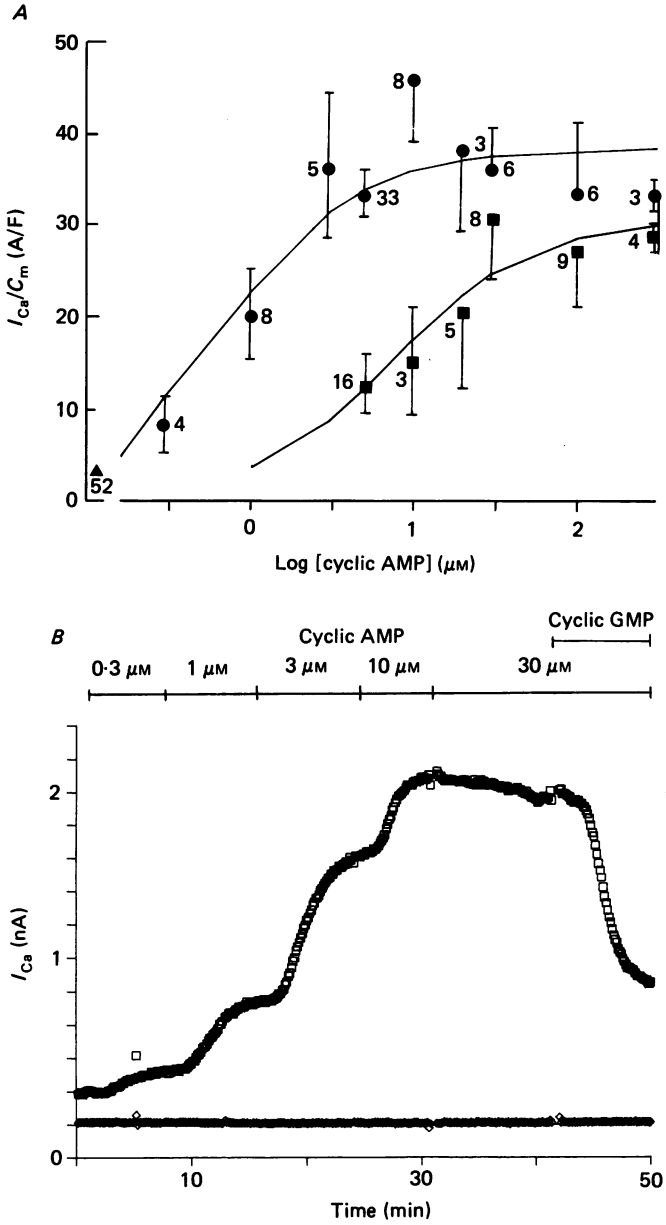


Fig. 8. Dose–response curves for cyclic AMP in the presence and absence of cyclic GMP. *A*, dose–response curves. Circles: cyclic AMP alone. Cells were perfused internally with different concentrations of cyclic AMP. $I_{Ca}$ was normalized to cell membrane capacitance ($C_m$). In some cells, an entire dose–response curve was obtained by sequential perfusion with increasing cyclic AMP concentrations (for example, the cell in *B*). In other cells, only single cyclic AMP concentrations were tested. Squares: cyclic AMP dose–response curve in the presence of 20 μM-cyclic GMP. Cells were first perfused with a certain concentration of cyclic AMP alone and then 20 μM-cyclic GMP was added. The experiment was terminated at this point with most cells. With some cells, however, the cyclic AMP concentration was increased sequentially (in the continuous presence of 20 μM-cyclic

$I_{Ca}$ ($I_{Ca}$ in MIX was $100 \pm 14$ % of control $I_{Ca}$; $n = 3$) and had only a small effect on $I_{Ca}$ stimulated by cyclic AMP. This suggested that cyclic AMP hydrolysis occurred at a low rate under these conditions. In contrast, when cyclic-AMP-elevated $I_{Ca}$ was reduced by cyclic GMP, MIX reversibly blocked the cyclic GMP effect (Fig. 7).

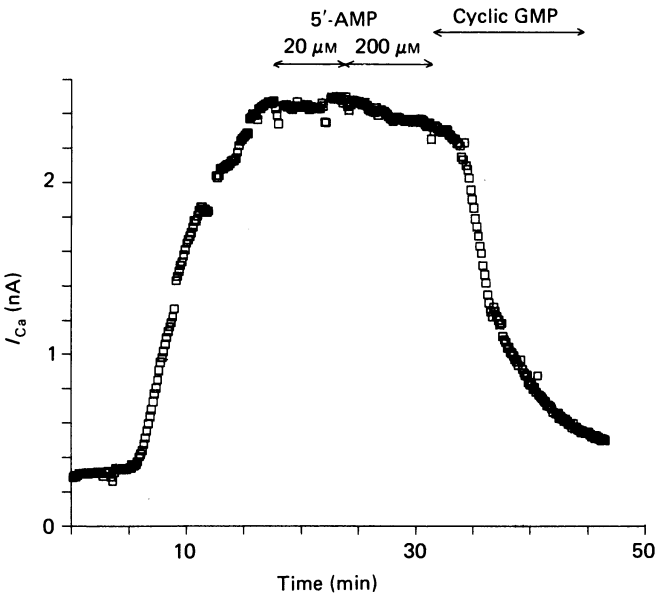


Fig. 9. Effect of 5′-AMP on $I_{Ca}$. Perfusion of the cell with 5 μM-cyclic AMP was begun near the beginning of the experiment. 20 or 200 μM-5′-AMP, or 3 μM-cyclic GMP were added during the periods indicated.

MIX was effective when applied solely by superfusion, but was somewhat more effective when it was also added to the intracellular medium. MIX in the intracellular medium alone was quite ineffective, however, in reducing the cyclic GMP effect. One possible interpretation of this finding is that the phosphodiesterase is localized close to the plasma membrane.

*Dose–response curves.* If cyclic GMP acts by activating a cyclic nucleotide phosphodiesterase, is the cyclic-GMP-stimulated reduction in $I_{Ca}$ due to a local reduction in cyclic AMP concentration or is it coupled to cyclic AMP turnover? To address this question, the effect of different concentrations of cyclic AMP on $I_{Ca}$ was examined in the presence and absence of 20 μM-cyclic GMP (Fig. 8*A*). In Fig. 8*A*, the continuous lines are the best fits of the data points to the Michaelis equation. $I_{Ca}$ was stimulated

GMP). Numbers beside data points indicate the number of cells tested. *B*, effect of cyclic GMP on $I_{Ca}$ stimulated by a maximal concentration of cyclic AMP. Near the beginning of the experiment, perfusion was started with 0·3 μM-cyclic AMP. When $I_{Ca}$ stabilized, the concentration was sequentially increased to 1, 3, 10, and finally 30 μM-cyclic AMP. 20 μM-cyclic GMP was added to this final cyclic AMP solution during the period marked. In this cell, holding current at −80 mV is also shown (diamonds).

50 % by 0·7 $\mu$M-cyclic AMP in the absence of cyclic GMP. The maximal stimulation of $I_{Ca}$ was approximately 11-fold. In the presence of cyclic GMP, the dose–response curve was shifted to the right about one log unit (a half-maximal effect of cyclic AMP was observed at 7·4 $\mu$M-cyclic AMP). In addition, although the two curves converge at high cyclic AMP concentrations, the maximal stimulation in the presence of cyclic GMP was only 8·6-fold. Thus, it appears that cyclic GMP acts in an uncompetitive

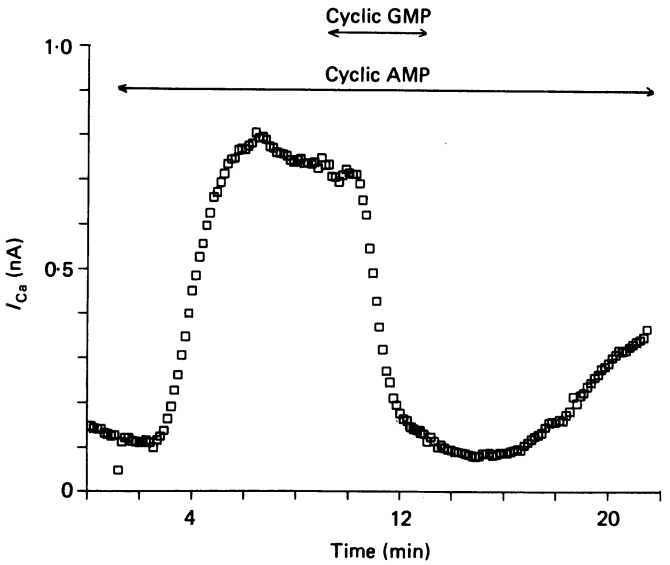


Fig. 10. Effect of high-pH buffer on response to cyclic GMP. The cell perfused with 75 mM-PIPES intracellular medium (see Methods) containing either 10 $\mu$M-cyclic AMP or 10 $\mu$M-cyclic AMP + 20 $\mu$M-cyclic GMP.

manner. In every experiment, 20 $\mu$M-cyclic GMP was capable of reducing $I_{Ca}$ a small amount even with concentrations of cyclic AMP as high as 300 $\mu$M. These results suggest that the effect of cyclic GMP is not due solely to a reduction in cyclic AMP concentration. Another experiment that supports this hypothesis is shown in Fig. 8*B*. In this experiment, the cell was perfused with increasing concentrations of cyclic AMP (from 0·3 to 30 $\mu$M) and then 20 $\mu$M-cyclic GMP was added to the final cyclic AMP concentration (30 $\mu$M). The cyclic GMP reduced $I_{Ca}$ to a level comparable to that found with 1 $\mu$M-cyclic AMP alone. Thus, to account for this effect, cyclic GMP would need to reduce cyclic AMP levels nearly 30-fold. This conclusion seems untenable, considering the large 'buffering' capacity of the cyclic AMP reservoir in the patch pipette, unless local cyclic nucleotide levels are not well buffered.

If factors other than reduction of cyclic AMP concentration in the cell are responsible for the effect of cyclic GMP, these factors could include the products of cyclic AMP hydrolysis ($H^+$ and 5′-AMP). 5′-AMP had no effect on $I_{Ca}$ at concentrations of 20 $\mu$M (three cells), but did decrease $I_{Ca}$ slightly at concentrations of 200 $\mu$M (two cells; Fig. 9). To examine the possible role of pH, intracellular pH was buffered with a concentrated buffer (75 mM-PIPES). Under these conditions, both the basal $I_{Ca}$ and the cyclic-AMP-stimulated $I_{Ca}$ were smaller than normal, for reasons we do

not yet understand. However, cyclic GMP was still capable of reducing $I_{Ca}$ elevated by cyclic AMP under these conditions (three cells; Fig. 10).

## DISCUSSION

### *Molecular mechanism*

We propose that cyclic GMP reduction of $I_{Ca}$ involves activation of a cyclic nucleotide phosphodiesterase. This hypothesis was suggested previously by Flitney & Singh (1981) on the basis of the observation that cyclic AMP levels decrease when cyclic GMP levels rise. Our evidence supporting this hypothesis comes from several kinds of experiments. (1) The effect of cyclic GMP is not mediated by the cyclic-GMP-dependent protein kinase, because 8-bromo-cyclic GMP, a very potent activator of the cyclic-GMP-dependent protein kinase, has no effect on $I_{Ca}$. (2) Cyclic GMP was unable to reduce $I_{Ca}$ elevated by non-hydrolysable derivatives of cyclic AMP. (3) Phosphodiesterase inhibitors block or reverse the effect of cyclic GMP.

The cyclic-GMP-stimulated cyclic nucleotide phosphodiesterase (PDE II) exhibits several features in common with our effect. This enzyme is not stimulated by 8-bromo-cyclic GMP (Erneux, Couchie, Dumont, Baraniak, Stec, Abbad, Petridis & Jastorff, 1981; Francis, Lincoln & Corbin, 1980; Lincoln & Corbin, 1983). The enzyme is activated by submicromolar concentrations of cyclic GMP (Lincoln & Corbin, 1983). For example, the enzyme from bovine heart is activated about 6-fold by 1 $\mu$M-cyclic-GMP and half-maximal binding of cyclic GMP occurs at about 0·3 $\mu$M (Martins *et al.* 1982). The substrate specificity of the enzyme may differ among different tissues, but in general, the cardiac enzyme exhibits similar $K_m$ and $V_{max}$ values for cyclic AMP and cyclic GMP (Martins *et al.* 1982; Weishaar *et al.* 1986). Thus, although the effect of cyclic GMP on $I_{Ca}$ does not require cyclic GMP hydrolysis (Fig. 5), under normal circumstances the enzyme may hydrolyse both cyclic AMP and cyclic GMP. Since cyclic GMP concentrations in the cell are usually about an order of magnitude lower than those of cyclic AMP (Flitney & Singh, 1981), the velocity of cyclic GMP hydrolysis would be expected to be less than that of cyclic AMP.

It remains puzzling whether cyclic GMP activation of the phosphodiesterase decreases $I_{Ca}$ because cyclic AMP concentration decreases locally or whether cyclic AMP hydrolysis is more closely coupled to channel regulation. We have presented evidence that cyclic AMP concentrations would need to fall nearly 30-fold (from 30 to 1 $\mu$M in Fig. 8*B*, for example) to explain the effect of cyclic GMP. This seems rather unlikely because the decrease in $I_{Ca}$ occurs sufficiently slowly for cyclic AMP diffusion from the pipette to maintain the cyclic AMP concentration at a constant level.

If cyclic AMP turnover, rather than cyclic AMP concentration, determines the effect of cyclic GMP on $I_{Ca}$, the signal could theoretically be secondary to increases in $H^+$ or 5′-AMP concentrations produced during cyclic AMP hydrolysis. However, 5′-AMP was ineffective even at concentrations of 200 $\mu$M. Furthermore, high concentrations of pH buffers did not diminish the effect of cyclic GMP. Goldberg, Ames, Gander & Walseth (1983) have proposed that the free energy of hydrolysis of cyclic AMP (12 kcal/mol) could be coupled to specific regulatory processes. This is an attractive idea, but our data do not provide an unambiguous answer.

*Physiological role of cyclic GMP*

There is compelling evidence from a large number of studies that cyclic GMP levels in the heart change under a variety of different physiological and pathological conditions (see references in Goldberg & Haddox, 1977; Lincoln & Corbin, 1983). The best studied of these conditions has been the stimulatory effect of ACh on cyclic GMP levels. Despite the rather massive body of evidence that cyclic GMP levels change in response to ACh (George *et al.* 1970, 1975; Kuo *et al.* 1972; Watanabe & Besch, 1975; Gardner & Allen, 1976; Ghanbari &McCarl, 1976; Brown *et al.* 1980; Lincoln & Keely, 1981; Dobson, 1981; Flitney & Singh, 1981; Endoh *et al.* 1985), the hypothesis that cyclic GMP actually plays a regulatory role has fallen into disfavour for a variety of reasons.

First, changes in contractile state can be produced in the absence of cyclic GMP changes (Brooker, 1977; Diamond *et al.* 1977; Linden & Brooker, 1978; Diamond & Chu, 1985). This discrepancy, however, does not seem to pose a serious challenge to the hypothesis that cyclic GMP plays a regulatory role in contraction, since one would not expect the contractile state to correlate with cyclic GMP levels if the force of contraction is regulated by several systems that use different second messengers. In fact, ACh is known to act on a variety of different systems: it not only decreases $I_{Ca}$, but it also increases a $K^+$ current ($I_{K(ACh)}$) and stimulates dephosphorylation of phospholamban, troponin I, and C-protein (for references see Löffelholz & Pappano, 1985; Hartzell & Simmons, 1987; Hartzell, 1987). In mammalian atrium $I_{K(ACh)}$ may be more sensitive than $I_{Ca}$ to ACh (Ten Eick, Nawrath, McDonald & Trautwein, 1976; Iijima, Irisawa & Kameyama, 1985) and $I_{K(ACh)}$ is not regulated by cyclic GMP (Nawrath, 1977; Fleming, Giles & Lederer, 1981; Trautwein *et al.* 1982; Soejima & Noma, 1984; Pfaffinger, Martin, Hunter, Nathanson & Hille, 1985). Thus, in atrial cells, in particular, one would expect decreases in contractile force to result from a shortening of action potential duration due to increased $I_{K(ACh)}$ in the absence of changes in cyclic GMP levels.

Furthermore, ACh can regulate $I_{Ca}$ by a mechanism that is different from the cyclic GMP mechanism we have described here. We have previously shown that ACh can quickly reduce $I_{Ca}$ elevated by $\beta$-adrenergic agonists but cannot reduce $I_{Ca}$ which has been elevated by intracellular perfusion with cyclic AMP (Fischmeister & Hartzell, 1986). However, cyclic GMP is capable of reducing cyclic-AMP-elevated $I_{Ca}$. Thus, cyclic GMP may not be involved in the reduction of $\beta$-agonist-elevated $I_{Ca}$ by ACh under our conditions. Indeed, we would expect that the medium in our pipette would inhibit many, if not all, guanylate cyclases. Various types of guanylate cyclase require $Ca^{2+}$ and others are inhibited by ATP (see Goldberg & Haddox, 1977). Furthermore, it is possible that soluble guanylate cyclase diffuses out of the cell during perfusion.

The second criticism of the cyclic GMP hypothesis is that elevation of cyclic GMP levels by certain drugs (notably sodium azide and nitroprusside) produces no change in contractile state (Diamond *et al.* 1977; Katsuki *et al.* 1977; Lincoln & Keely, 1981). This criticism has been addressed by a series of studies that show that there are at least two distinct pools of cyclic GMP in the heart (Lincoln & Keely, 1981). One pool, which may be synthesized by soluble guanylate cyclase, is increased by nitroprusside. Increase in the steady-state level of cyclic GMP in this pool does not stimulate

cyclic-GMP-dependent protein kinase or change contractile force. The other pool, which is increased by ACh, stimulates cyclic-GMP-dependent protein kinase and also decreases contractile force.

Another line of evidence against the cyclic GMP hypothesis is that exogenous cyclic GMP or cyclic GMP derivatives do not always decrease contractile force (Endoh & Shimizu, 1979; Linden & Brooker, 1979) or affect $I_{Ca}$ (Nargeot *et al.* 1983). Other studies, however, have shown the opposite (Wilkerson *et al.* 1976; Trautwein & Trube, 1976; Nawrath, 1977; Kohlhardt & Haap, 1978; Linden & Brooker, 1979; Endoh & Yamashita, 1981; Singh & Flitney, 1981). In fact, it is not entirely clear how derivatives such as 8-bromo-cyclic GMP produce their effect. In our experiments and those of Linden & Brooker (1979), 8-bromo-cyclic GMP was ineffective. This is consistent with the hypothesis that the effect of cyclic GMP is mediated by the phosphodiesterase. It is possible that the negative inotropic effects of 8-bromo-cyclic GMP and dibutyryl cyclic GMP found by others are actually due to increases in endogenous cyclic GMP due to inhibition of cyclic GMP phosphodiesterase(s) by these derivatives. If this is the case, variable responses to these derivatives would be expected, depending on the activity of the phosphodiesterases in the preparation examined. The experiments by Nargeot *et al.* (1983) are more difficult to dismiss. Nargeot *et al.* (1983) have used a photo-activatable derivative of cyclic GMP to raise cyclic GMP levels in the cell, but no effects on $I_{Ca}$ were observed. We have no explanation for the difference between these results and ours, unless the photo-activated cyclic GMP, unlike the photo-activated derivative of cyclic AMP, was not active.

The hypothesis that cyclic GMP is involved in regulation of cardiac contractility has lost favour also because of difficulties demonstrating specific cyclic-GMP-dependent phosphorylation in heart (Lincoln & Corbin, 1983). Although a specific substrate has been found in cell homogenates (Wrenn & Kuo, 1981), it has not been identified and it is unknown whether its phosphorylation correlates with contractility. The discovery of cyclic GMP binding proteins other than the protein kinase suggests that cyclic GMP may not always act through a cyclic-GMP-dependent phosphorylation reaction (Lincoln & Corbin, 1983). For example, in photoreceptors cyclic GMP has been shown clearly to regulate cation conductance through a phosphorylation-independent mechanism (Fesenko, Kolesnikov & Lyubarsky, 1985; Koch & Kaupp, 1985). Since the cyclic-GMP-stimulated cyclic nucleotide phosphodiesterase is found in a wide variety of tissues (Martins *et al.* 1982; Lincoln & Corbin, 1983), a common mechanism of cyclic GMP action may be the stimulation of cyclic AMP hydrolysis. If so, this would provide a simple molecular explanation for the Yin–Yang hypothesis and the common observation that cyclic GMP and cyclic AMP levels are inversely correlated.

Thus, although it is clear that cyclic GMP is not required for the negative inotropic effects of ACh, it is likely that cyclic GMP has a regulatory function. For example, cyclic GMP may play an important role in regulating the rate of change in $I_{Ca}$ in response to ACh by modulating phosphodiesterase activity or, alternatively, may mediate responses to other hormones.

We thank Dr Guy Vassort for stimulating discussions, Patrick Lechêne for skilful computer programming, Adama Diarra and Mireille Brocas-Randolph for preparing the cells, and May & Baker Ltd. for M & B 22948. Supported by INSERM. and NIH grants HL-21195 and HL-27385 to C.H.

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