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POTENTIATION BY CYCLIC GMP OF β-ADRENERGIC EFFECT ON Ca^{2+} CURRENT IN GUINEA-PIG VENTRICULAR CELLS

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SUMMARY

1. Effects of cyclic GMP on L-type Ca^{2+} current (I_{Ca}) were investigated in myocytes isolated from guinea-pig ventricles using the patch clamp method in the whole-cell configuration combined with intracellular perfusion.

2. When I_{Ca} was increased by bath application of isoprenaline (0·001–0·1 μM) or forskolin (0·5–1 μM), or by intracellular dialysis with cyclic AMP (50–100 μM), dialysis with 10 μM-cyclic GMP resulted in an additional stimulation of I_{Ca}. Without these pre-treatments, cyclic GMP (1–100 μM) had no effect on the basal I_{Ca}. 5′-GMP was without effect.

3. The stimulatory effect of cyclic GMP was observed at concentrations higher than 0·1 μM with a maximum at around 10 μM in the pipette. The dose–response relation between isoprenaline and I_{Ca} was shifted to the left by (10 μM) cyclic GMP; the half-maximum isoprenaline concentration shifted from 16 to 4·6 nM.

4. The increase of I_{Ca} on dialysing 50 μM-cyclic AMP varied from cell to cell, probably due to a difference in phosphodiesterase activity. The cells responding weakly to cyclic AMP showed a greater response to cyclic GMP, and vice versa. In cells dialysed with hydrolysis-resistant derivatives (10–50 μM-8-(4-chlorophenylthio)-cyclic AMP or 50 μM-8-bromo-cyclic AMP), additional dialysis with cyclic GMP failed to modify I_{Ca}. Dialysis with cyclic GMP abolished the stimulatory effect of milrinone, a specific inhibitor of cyclic GMP-inhibited phosphodiesterase. These findings suggested that inhibition of cyclic GMP-sensitive phosphodiesterase was responsible for the stimulatory effect of cyclic GMP.

5. In the presence of isoprenaline, direct application of an active fragment of cyclic GMP-dependent protein kinase (PKG) failed to modify I_{Ca} in most cells. Activation of native PKG by intracellular dialysis with 8-bromo-cyclic GMP, or higher concentrations of cyclic GMP (100–1000 μM), depressed I_{Ca} in about 25% of the cells. Furthermore, dialysis of cyclic GMP reversed the increase of I_{Ca} by the non-specific phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX). These findings suggested the presence of antagonistic mechanisms of cyclic GMP, which are

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independent from the above synergistic action. PKG may be involved in this antagonistic effect.

INTRODUCTION

Recent studies on cyclic GMP-regulated proteins have identified several intracellular effectors for cyclic GMP. Like its counterpart, cyclic AMP, cyclic GMP activates a specific protein kinase (cyclic GMP-dependent protein kinase, PKG) which can then phosphorylate a number of proteins (for review see Lincoln & Corbin, 1983; Waldman & Murad, 1987; Walter, 1990). On the other hand, in retinal photoreceptors cyclic GMP regulates the cation channel without participation of PKG (Fesenko, Kolesnikov & Lyubarsky, 1985). Furthermore, cyclic GMP can stimulate or inhibit specific kinds of phosphodiesterase to produce either antagonistic or synergistic effects on cyclic AMP-elevating hormones (for review see Beavo, 1988).

It is well established in frog myocytes that cyclic GMP antagonizes the stimulatory action of cyclic AMP on L-type I_{Ca} by enhancing the rate of hydrolysis of cyclic AMP by activating a class of phosphodiesterase (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987). However, in mammalian hearts the role of cyclic GMP and its mechanisms of action are controversial. Many studies failed to observe a consistent effect of cyclic GMP on the electrical activity of the cardiac muscle (for review see Hartzell, 1988; Sperelakis, 1988). In the presence of β-adrenergic stimulation, Levi, Alloatti & Fischmeister (1989) observed a depression of I_{Ca} by the intracellular application of cyclic GMP in guinea-pig ventricular cells. On the other hand, we observed an increase of I_{Ca} under essentially the same experimental conditions (Ono & Trautwein, 1990). Biochemical mechanisms different from those in the frog heart are suggested for the cyclic GMP effect in the mammalian heart. Intracellular application of PKG depressed I_{Ca} in rat ventricular cells (Mery, Lohmann & Fischmeister, 1990*a*; Mery, Lohmann, Walter & Fischmeister, 1990*b*), suggesting that cyclic GMP may affect I_{Ca} via a mechanism dependent on PKG.

In this study, our results show that, in contrast to frog myocytes, cyclic GMP potentiates the stimulation of I_{Ca} by isoprenaline in guinea-pig ventricular cells via the cyclic GMP-inhibited phosphodiesterase (Harrison, Reifsnyder, Gallis, Cadd & Beavo, 1986). Independent from this synergistic pathway, cyclic GMP may also have antagonistic effects at high concentrations, some of which may be produced through activation of PKG.

METHODS

Preparation

Young adult guinea-pigs, weighing 250–350 g, were anaesthetized with pentobarbitone (70 mg/kg). Under artificial respiration the chest was opened and the proximal aorta was cannulated *in situ* to start coronary perfusion with normal Tyrode solution. The heart was excised and mounted on a Langendorff apparatus. The perfusion was continued for an additional 5 min, followed by a perfusion with a Ca^{2+}-free medium for 5–10 min and the Ca^{2+}-free medium containing 5 mg/100 ml collagenase (Yakult, Tokyo, Japan) for 5–10 min at 37 °C. After washing out collagenase by perfusing the heart with high-K^+ stock solution ('KB' medium), the digested heart was dissected and gently agitated to dissociate the cells in KB medium. The dispersed cells were then stored in KB medium at 4 °C for later use.

Solutions

The control Tyrode solution contained (in mM): NaCl, 115; NaH_2PO_4, 1·0; KCl, 5·4; $MgCl_2$, 1·0; $CaCl_2$, 1·8; glucose, 10; and HEPES, 5. pH was adjusted to 7·4 with NaOH and then 24 mM-$NaHCO_3$ was added. During the experiments, all solutions were bubbled with 95% O_2 plus 5% CO_2. The Ca^{2+}-free medium for the isolation procedure contained (in mM): NaCl, 80; KCl, 10; KH_2PO_4, 1; $MgSO_4$, 5; taurine, 50; HEPES, 5; glucose, 20; and $NaHCO_3$, 24 (pH 7·4). The KB medium contained (in mM): KCl, 30; KH_2PO_4, 30; KOH, 85; $MgSO_4$, 3; taurine, 20; glutamic acid, 20; HEPES, 10; EGTA, 0·5; and glucose, 10. The pH was adjusted to 7·4 with KOH. The standard internal (pipette) solution contained (in mM): potassium aspartate, 80; KCl, 50; KH_2PO_4, 10; $MgSO_4$, 3; Na_2ATP, 5; HEPES, 5; and EGTA, 0·1. The pH was adjusted to 7·4 by adding KOH.

Isoprenaline (Sigma) was dissolved in distilled water to give 1 mM stock solution containing 1 mM-ascorbic acid. 3-Isobutyl-1-methyl-xanthine (IBMX, Sigma) was dissolved in distilled water to give a 1 mM stock solution. Milrinone was a kind gift of Professor R. Fischmeister and was dissolved in dimethyl sulphoxide to give a 10 mM stock solution. Agents added to the pipette solution included adenosine 3′,5′-cyclic monophosphate (cyclic AMP, Sigma), guanosine 3′-5′-cyclic monosphosphate (cyclic GMP, Boehringer, Mannheim and Sigma), 8-(4-chlorophenylthio)-cyclic AMP (8-CPT-cyclic AMP, Boehringer, Mannheim), 8-bromo-cyclic AMP (Boehringer, Mannheim), 8-bromo-cyclic GMP (Boehringer, Mannheim) and guanosine-5′-monophosphate (5′-GMP, Boehringer, Mannheim). We used altogether four different batches of cyclic GMP and got qualitatively the same effect of cyclic GMP on I_{Ca}. These agents were dissolved in the standard internal solution to give 1 mM stock solutions, which were diluted by the standard internal solution to final concentrations described in the text.

An active fragment of PKG, a kind gift of Professor F. Hofmann, was purified from bovine lung and was permanently activated by the removal of the *N*-terminal regulatory region using trypsin (Hell, Landgraf & Hofmann, 1987). The buffer solution of the enzyme was dialysed against the internal solution. ATP and Mg^{2+} were omitted from the internal solutions during the buffer exchange to avoid degradation of ATP, and were added later. The final concentration of PKG in the standard internal solution was 0·5–3·0 μM.

Whole-cell voltage clamp

A drop of cell suspension was added to the Tyrode solution in the recording chamber placed on an inverted microscope. The chamber, which had a volume of about 0·5 ml, was continuously perfused with Tyrode solution at a rate of 2–4 ml/min at 34–36 °C.

Patch electrodes were fabricated from borosilicate capillary tubes (Jencons, Bedfordshire, UK) and mounted on a pipette perfusion device. The pipette perfusion device for intracellular application of various chemicals (Soejima & Noma, 1984; Kameyama, Hofmann & Trautwein, 1985) was slightly modified. Briefly, a fine fused silica capillary tube coated with polyimide (o.d. = 170 μm, i.d. = 130 μm, Scientific Glass Engineering, Germany) was used as the inlet tubing. The tip of the tubing was tapered (about 50 μm at the end) by pulling the heated capillary and positioned within 0·3 mm from the tip opening of the patch electrode. The distal end of the inlet tubing was connected to reservoirs of different solutions. The pipette tip was perfused by applying negative pressure to the back end of the patch pipette. The dead space between the reservoir and the tip of the inlet tubing caused a delay of 1–2 min before effects of a new solution became apparent. The pipette was continuously perfused throughout the experiment except for the period of switching between the reservoirs.

The procedure to establish the configuration of whole-cell patch clamp recording was as described by Hamill, Marty, Neher, Sakmann & Sigworth (1981). To facilitate formation of giga-ohm seals, the pipette was first filled with normal Tyrode solution. When a seal was formed, the pipette solution was replaced with the standard internal solution using the perfusion device. The membrane was ruptured by briefly applying stronger suction to the pipette. To facilitate equilibration of the intracellular medium with the pipette solution, patch electrodes having low resistances (1–2·5 MΩ) were used.

The whole-cell voltage clamp was achieved by the use of a patch clamp amplifier (EPC7, List Medical Electronics, Darmstadt, Germany). The cell capacitance and series resistance were compensated. The L-type Ca^{2+} current was recorded by applying a test pulse of 300 ms duration every 5 s from a holding potential of −80 to −85 mV. In order to inactivate both fast Na^+ and T-

type Ca^{2+} currents, a pre-pulse to −40 mV of 80 ms duration preceded the test pulses (see Fig. 1*A*). Current and voltage signals were stored on magnetic tape (Hewlett–Packard 3968A) at 3·75 in/s. For computer analysis, signals were replayed from the magnetic tape, filtered by a low-pass filter (1 kHz, −3 dB), digitized at 5 kHz by a 12-bit analog-to-digital converter, and fed to a computer memory.

Statistical data were given as means ± S.D. Differences between means were tested for statistical significance by the *t* test.

RESULTS

No effect of cyclic GMP on I_{Ca} in the absence of β-adrenergic stimulation

To facilitate isolation of I_{Ca}, we used K^+-free, Cs^+-rich internal and external solutions in preliminary experiments. However, under these conditions the run-down of I_{Ca} was considerable, especially after stimulation by cyclic AMP or isoprenaline (see Levi *et al.* 1989), and interfered with the measurement of the effect of nucleotides. In the normal Tyrode solution and K^+-rich internal solution (see Methods) the amplitude of I_{Ca} was usually maintained for more than 30 min with little run-down or spontaneous decrease during the stimulation by cyclic AMP (see also Kameyama, Hescheler, Hofmann & Trautwein, 1986, Fig. 1). Therefore, all of the following experiments were done during the period of negligible run-down and in the presence of K^+ in both the external and internal solutions.

In the experiment shown in Fig. 1, the cell was superfused with normal Tyrode solution and was internally dialysed with the standard internal solution (see Methods). In Fig. 1*A*, the Na^+ current was inactivated using a preceding depolarization step from −85 to −40 mV for 80 ms. The second depolarization to 0 mV activated I_{Ca}. The amplitude of I_{Ca} was unlikely to be contaminated by Na^+ current since addition of 50 μM-TTX affected neither the peak nor the configuration of I_{Ca}. In contrast, 0·5 mM-Cd^{2+} completely blocked I_{Ca} (not illustrated). Because of the inward rectification of the background K^+ conductance, I_{K1}, the level of the late current at 0 mV was less outward than at −40 mV. In Fig. 1*B* the time courses of the change of the peak inward current (I_{peak}) and of the late current (I_{late}) are shown. During the period indicated by the open box the ventricular cell was internally dialysed with 10 μM-cyclic GMP without any effect on I_{peak} or I_{late}. In order to exclude the possibility that diffusion of cyclic GMP into the cell was severely limited, the pipette tip was perfused with 50 μM-cyclic AMP-containing internal solution (filled box), resulting in a clear increase of the peak inward current (from 0·4 to 1·7 nA). This lack of an effect of cyclic GMP in the concentration range of 1–100 μM on the basal current was confirmed in nine experiments.

Stimulation by cyclic GMP of I_{Ca} in the presence of isoprenaline or cyclic AMP

In the experiment shown in Fig. 2, I_{Ca} was enhanced by 30 nM-isoprenaline; it increased from 0·5 to 1·4 nA. During maintained bath application of isoprenaline internal dialysis with 10 μM-cyclic GMP further increased I_{Ca} to 2 nA. This effect of cyclic GMP was reversed by washing out cyclic GMP from the pipette, and was reproducible on the second application. After washing out isoprenaline, I_{Ca} returned to the basal level in spite of the presence of cyclic GMP. In Fig. 2*B* current records in response to four different test potentials under three conditions (control, 30 nM-isoprenaline, 30 nM-isoprenaline plus 10 μM-cyclic GMP) are superimposed. Isoprenaline increased the peak I_{Ca} at all four test potentials, and the internal dialysis

with cyclic GMP augmented the effect of isoprenaline (see also peak *I–V* relation in Fig. 2*C*). The current level near the end of the test pulse to −20 mV became more negative as I_{Ca} was increased by isoprenaline and cyclic GMP (Fig. 2*B* and *C*). This negative shift may be attributed to incomplete inactivation of I_{Ca}. At positive

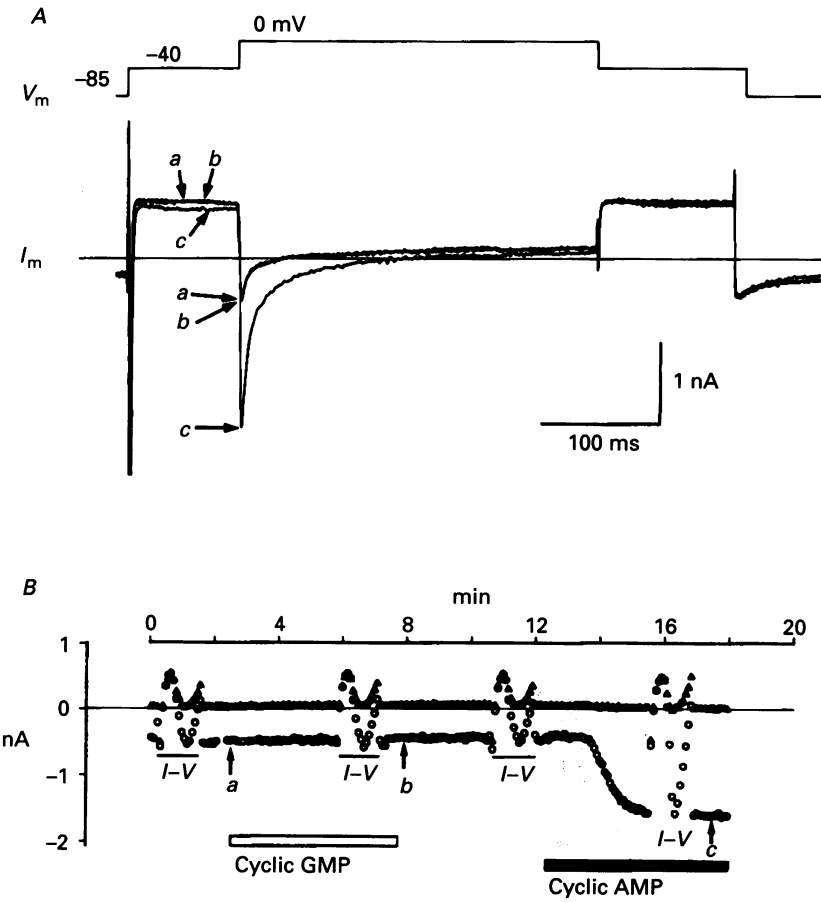

Fig. 1. Effect of intracellular dialysis with cyclic nucleotides. *A*: top, voltage clamp protocol; bottom, three representative current records obtained at times *a*, *b* and *c* in Fig. 1*B* are superimposed. Horizontal line indicates the zero-current level. Numbers indicate membrane potential in millivolts. *B*, levels of I_{peak} (○) and I_{late} (▲) measured every 5 s were plotted. The times of pipette perfusion with cyclic nucleotides, 10 μM-cyclic GMP or 50 μM-cyclic AMP, are indicated. Test pulses were 0 mV except for the period of measuring the *I–V* relationship as indicated by '*I–V*'.

potentials activation of the delayed rectifier K^+ current became predominant, and its amplitude was increased by isoprenaline and cyclic GMP. The increase of the delayed rectifier K^+ current by isoprenaline has been explained by an activation of cyclic AMP-dependent protein kinase (Yazawa & Kameyama, 1990). We assume a similar mechanism can explain the synergistic action of cyclic GMP for both I_{Ca} and the delayed rectifier K^+ current.

As a control, five cells were dialysed with 1–10 μM-5′-GMP, the metabolite of cyclic GMP, without any effect on the basal nor the isoprenaline-stimulated I_{Ca}.

In the present study, the increase of I_{Ca} by isoprenaline or cyclic AMP was usually accompanied by a shift of the peak potential in the initial I–V relations by 5–10 mV

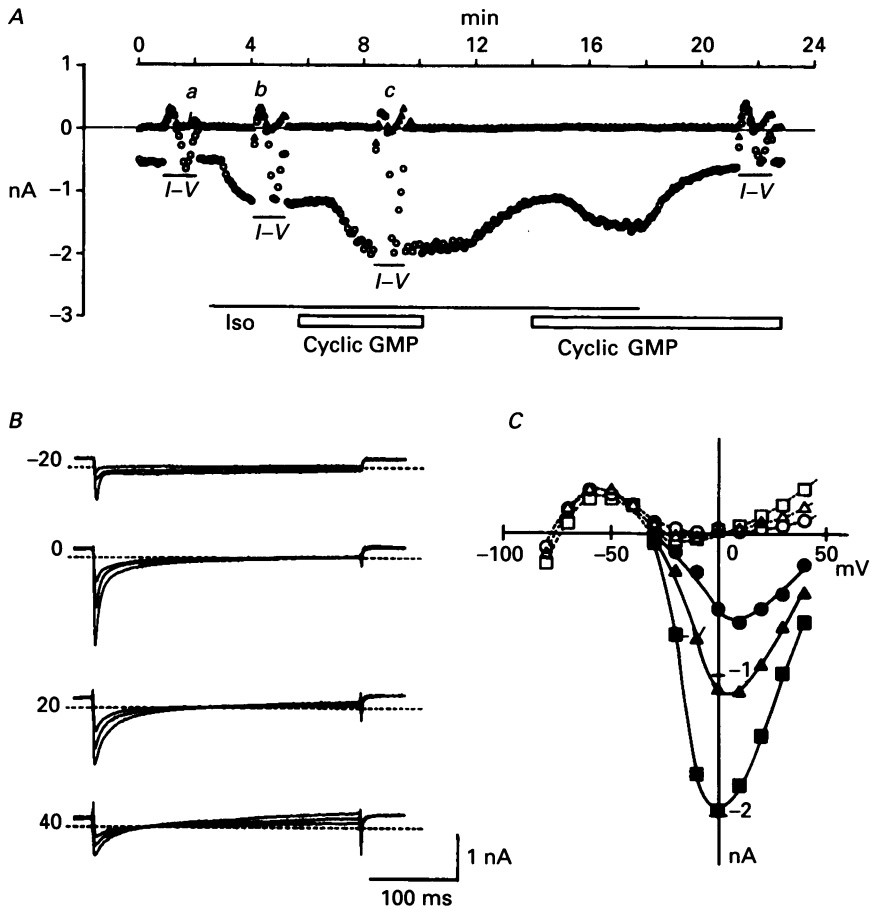

Fig. 2. Potentiation by cyclic GMP of the action of isoprenaline on I_{Ca} and the delayed rectifier K^+ current. *A*, current levels of I_{peak} (○) and I_{late} (▲) were measured by applying a clamp pulse every 5 s, and plotted against time. The test potential was 0 mV except during the measurement of the I–V relation. The period of bath application of 30 nM-isoprenaline (Iso) is indicated by a bar. Concentration of cyclic GMP was 10 μM. *B*, three superimposed currents were recorded at the times in *Aa* (control), *Ab* (in the presence of isoprenaline) and *Ac* (isoprenaline plus cyclic GMP in the pipette). Numbers indicate command potentials in millivolts and dotted lines the zero-current level. *C*, I–V relationships in control (circles), isoprenaline (triangles) and isoprenaline plus cyclic GMP (squares). Filled symbols indicate I_{peak} and open symbols I_{late}.

in the negative direction and the subsequent dialysis with cyclic GMP enhanced this shift by another 5 mV along with an increase of the peak amplitude of I_{Ca} (Fig. 2*C*, filled symbols). A shift of the peak current in a negative direction has been previously reported by Bean, Nowycky & Tsien (1984) and Shuba, Hesslinger, Trautwein,

McDonald & Pelzer (1990). The latter authors controlled the efficacy of the clamp by a second patch pipette. Therefore, this shift cannot be simply explained by a clamp artifact due to an enhanced I_{Ca} in the presence of a significant series resistance.

The stimulatory effect of cyclic GMP was also consistently observed when I_{Ca} was enhanced by 0·5–1 μM-forskolin ($n = 5$) or 50 μM-cyclic AMP ($n = 18$). An example is

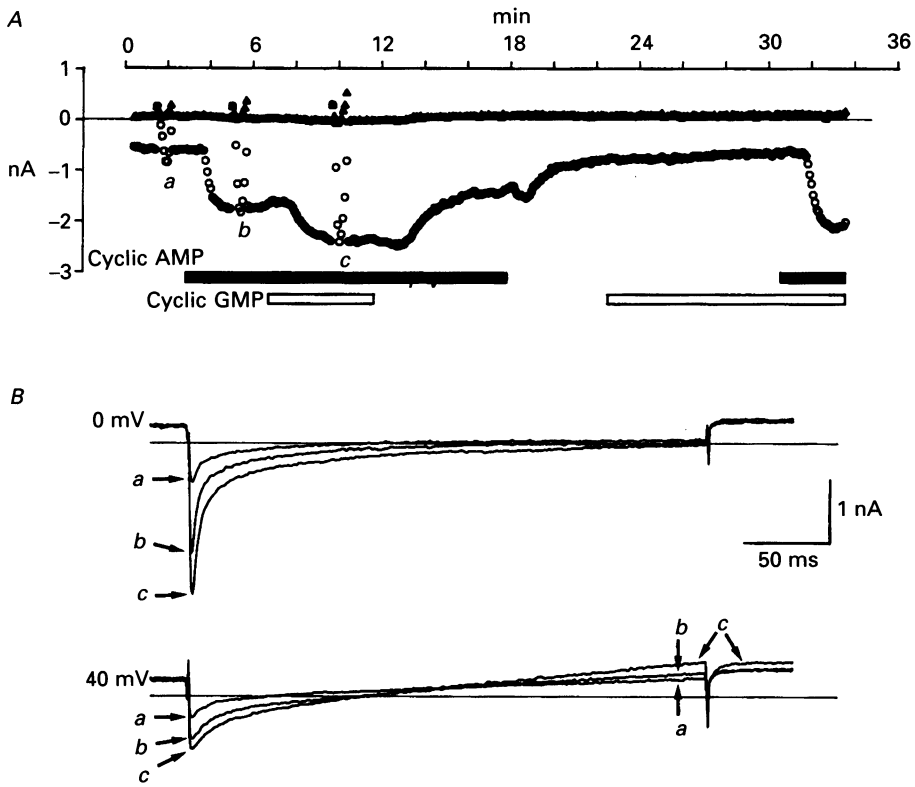

Fig. 3. Effects of cyclic GMP on cyclic AMP-stimulated I_{Ca}. *A*, time course of changes of I_{peak} (○) and I_{late} (▲). Same explanation as in Fig. 2*A*. *B*, three currents recorded at corresponding times in *A* are superimposed. The currents were in response to test pulses to 0 mv (top) and 40 mV (bottom). The concentration of cyclic AMP was 50 μM and that of cyclic GMP 10 μM. Horizontal lines indicate the zero-current level.

shown in Fig. 3, where dialysis with 50 μM-cyclic AMP increased I_{Ca} from 0·5 to 1·8 nA and the additional dialysis with 10 μM-cyclic GMP further increased the current to 2·5 nA. Once again this stimulatory effect was reversible. Subsequent dialysis of cyclic nucleotides in a reversed sequence confirmed that cyclic GMP had no effect on I_{Ca} before the stimulation by cyclic AMP.

Current recordings at 0 mV indicate that both peak and non-inactivating components of I_{Ca} increased (Fig. 3*B*). At +40 mV cumulative activation of delayed rectifier K^+ current by cyclic AMP and cyclic GMP is also evident, as shown for isoprenaline in Fig. 2.

On the whole-cell current level no significant change in the kinetics has been

documented in I_{Ca} when increased by β-adrenergic stimulation or cyclic AMP (Irisawa & Kokubun, 1983; Kameyama *et al.* 1985; see also Fig. 1). As shown by the original records in Figs 2 and 3, cyclic GMP did not significantly affect the time course of inactivation nor the time to peak of I_{Ca}. When measured by fitting two

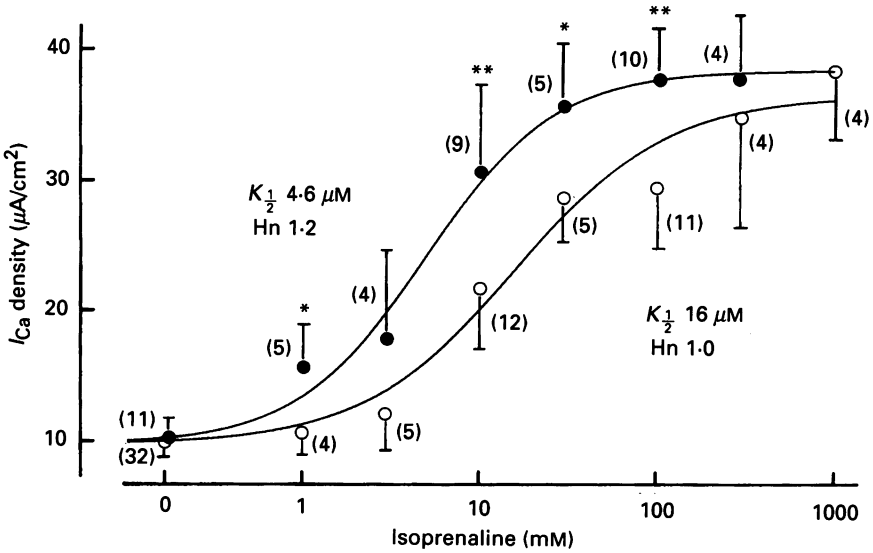

Fig. 4. Relationship between the concentration of isoprenaline and I_{Ca} density. ○, means of the control; ●, in the presence of 10 μM-cyclic GMP. The number of measurements is shown in parentheses. The smooth curves were drawn by the least-squares fit with the half-saturation concentration of isoprenaline ($K_{\frac{1}{2}}$), and the Hill coefficient (Hn) as indicated in the figure. Asterisks indicate a significant difference between points at a given concentration of isoprenaline at either the 0·05 (*) or 0·01 (**) level (*t* test).

exponentials to the inactivation time course, the time constants for the first and slow components were $9{\cdot}4 \pm 1{\cdot}9$ and 72 ± 18 ms in the control, $7{\cdot}4 \pm 1{\cdot}1$ and 77 ± 23 ms in the presence of cyclic AMP and $7{\cdot}9 \pm 1{\cdot}6$ and 91 ± 19 ms in the presence of cyclic AMP plus cyclic GMP ($n = 5$). The ratios of the magnitude of the fast component over the slow component were $1{\cdot}6 \pm 0{\cdot}7$, $2{\cdot}6 \pm 0{\cdot}8$ and $3{\cdot}0 \pm 0{\cdot}7$ in the control, in the presence of cyclic AMP and cyclic AMP plus cyclic GMP, respectively. These findings are consistent with the view that the effect of cyclic GMP is mediated by interaction with the cyclic AMP-dependent cascade.

Cyclic GMP dependence of the effect of isoprenaline on I_{Ca}

The effect of cyclic GMP was examined by measuring the dose–response relation for isoprenaline on I_{Ca} in the presence and absence of 10 μM-cyclic GMP in the pipette solution. In Fig. 4, the data points show the peak amplitude of I_{Ca} in reference to zero current and normalized by the input capacitance assuming 1 μF/cm^2 cell membrane. Fitting data points with the Hill equation gave a half-maximum concentration ($K_{\frac{1}{2}}$) of 16 nM and a Hill coefficient of 1·0. Internal dialysis of cyclic GMP shifted the relation to the lower doses; $K_{\frac{1}{2}}$ was 4·6 nM and the Hill coefficient was 1·2. It should

be noted that cyclic GMP did not affect the basal current density, and the maximum response to isoprenaline seemed not to be altered.

Dialysis of different concentrations of cyclic GMP

To establish a dose-dependent effect of cyclic GMP, the concentration in the pipette was varied. In Fig. 5*A* 30 nM-isoprenaline, which increased I_{Ca} from 0·4 to

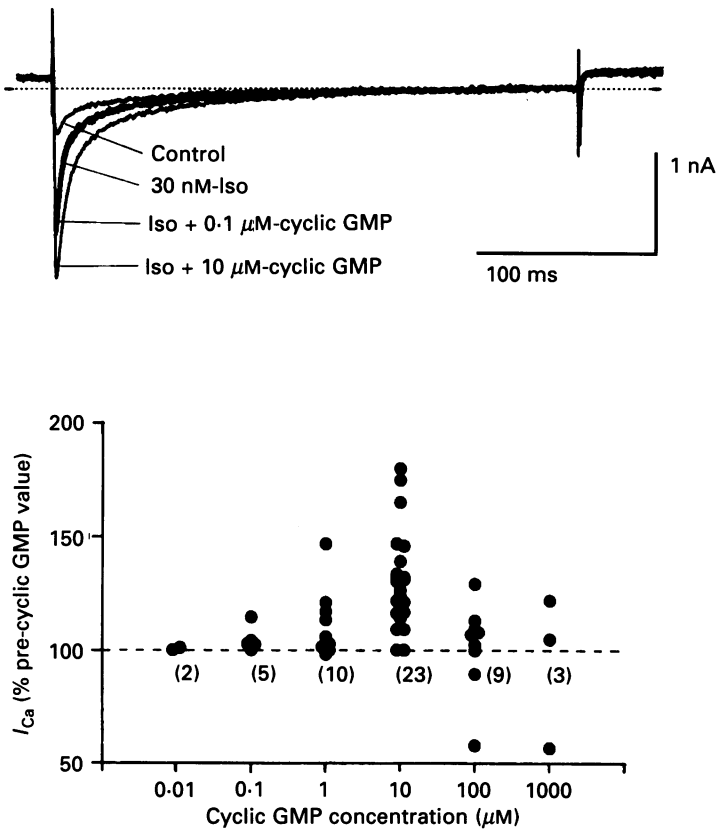

Fig. 5. Effects of various concentrations of cyclic GMP in the pipette. *A*, superimposed currents in response to test pulses to 0 mV recorded in the control, in the presence of 30 nM-isoprenaline (Iso) and isoprenaline plus increasing concentrations of cyclic GMP. *B*, changes in I_{Ca} induced by various concentrations of cyclic GMP were normalized referring to the amplitude of I_{Ca} before the application, and were plotted against cyclic GMP concentration. Numbers of measurements are indicated in parentheses.

1·1 nA, was continuously applied and the concentration of cyclic GMP was increased. The concentrations represent the minimum (0·1 μM) to observe the effect, or that (10 μM) to obtain the maximum response. The amplitudes of I_{Ca} were normalized referring to the amplitude immediately before application of cyclic GMP and were plotted against the concentration of cyclic GMP in Fig. 5*B*. Figure 5*B* summarizes

thirty-eight experiments. All data were obtained during bath perfusion with 30 nM-isoprenaline. Although there is a large scattering of the values, the dose-dependent increase of I_{Ca} is evident over the range from 0·1 to 10 μM-cyclic GMP. At higher concentrations of cyclic GMP (100–1000 μM), the effect became smaller and even reversed in some cells.

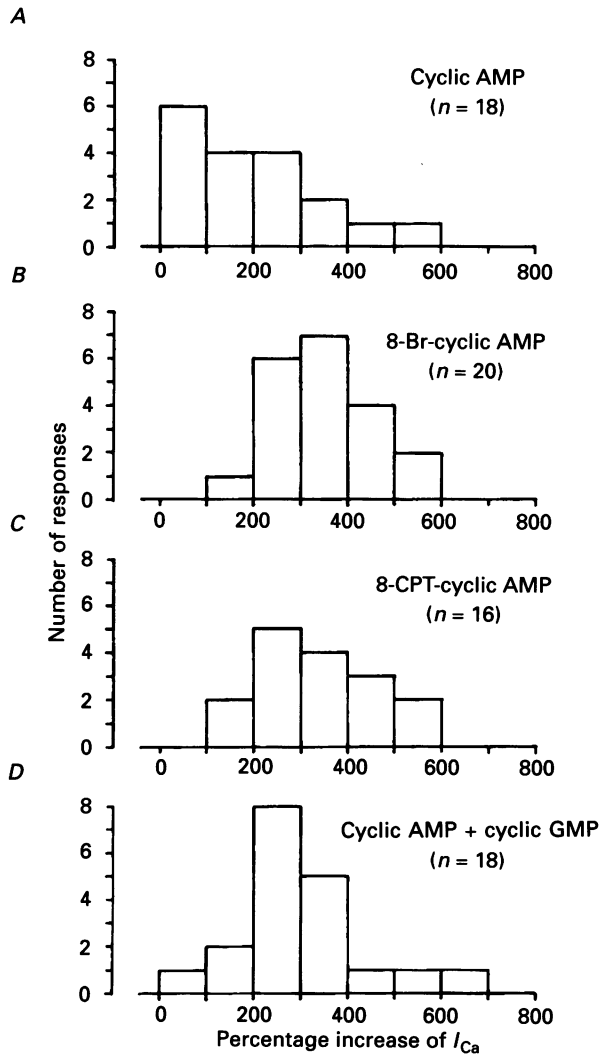

Fig. 6. Distribution of frequencies of percentage increase of I_{Ca}. *A*, during dialysis with 50 μM-cyclic AMP; *B*, 50 μM-8-Br-cyclic AMP; *C*, 50 μM-8-CPT-cyclic AMP; *D*, 50 μM-cyclic AMP plus 10 μM-cyclic GMP. Numbers of measurements are indicated.

The predominant stimulatory effects of cyclic GMP on I_{Ca} are in clear contrast to recent studies, where micro- or submicromolar concentrations of cyclic GMP regularly inhibited I_{Ca} in guinea-pig (Levi *et al.* 1989) or rat ventricular cells (Mery

et al. 1990*a*,*b*). To exclude different experimental conditions for the basis of the different results, we used similar compositions of test solutions and tissues as used in the previous studies. The synergistic effect of cyclic GMP was obtained using solutions containing 5 mM-EGTA and/or 0·4 mM-GTP, or by using a K^+-free, Cs^+-rich solution ($n = 8$) (see method by Levi *et al.* 1989). We also used single cells dissociated by Sigma collagenase instead of Yakult collagenase. We repeated the experiments using rat ventricular cells ($n = 6$) and cells isolated from human ventricular myocardium ($n = 3$). Under all conditions 10 μM-cyclic GMP additionally augmented the amplitude of I_{Ca} stimulated by isoprenaline or cyclic AMP.

Mechanism of the stimulatory action of cyclic GMP

The increment of I_{Ca} induced by dialysing with cyclic GMP in the continuous presence of cyclic AMP varied among different cells. We noticed that the cells having a smaller response to cyclic AMP generated a larger response to cyclic GMP, and vice versa. A possible explanation of this observation could be that cyclic GMP inhibits hydrolysis of cyclic AMP. In fact, a new class of phosphodiesterases was recently found in mammalian hearts to be inhibited by cyclic GMP in submicromolar concentration (cyclic GMP-inhibited phosphodiesterase; Harrison *et al.* 1986). Variation in the activity of phosphodiesterase in different cells may explain the different response to cyclic AMP, and cyclic GMP may enhance the efficacy of internal dialysis by inhibiting the activity of the phosphodiesterase. In line with this hypothesis was the observation that 10 μM-cyclic GMP did not or only slightly enhance the amplitude of the maximally stimulated I_{Ca} (factor of 4–5). The hypothesis was further supported by using the hydrolysis resistant derivatives. When I_{Ca} was increased by dialysing the cell with 8-Br-cyclic AMP ($n = 5$) or 50 μM-8-CPT-cyclic AMP ($n = 6$, not illustrated), cyclic GMP failed to produce a further increment of I_{Ca}.

The above hypothesis was supported by comparing histograms of the I_{Ca} response shown in Fig. 6, where the relative amplitude of the response was divided into a bin width of 100% increase. When the cell was dialysed with cyclic AMP, the smallest response (0–100% increase) was most frequent and the number of these responses decayed monotonically as the response became larger (Fig. 6*A*). We assume that this variation is not due to a different extent of dialysis but due to cell-to-cell variations in the phosphodiesterase activity.

The distribution tended to become normalized when the cell was dialysed with the hydrolysis-resistant derivatives (Fig. 6*B* and *C*), that is a condition in which the response does not depend on the phosphodiesterase activities. The finding that the distribution of the response to cyclic AMP plus cyclic GMP is similar to those with the hydrolysis-resistant derivatives is consistent with the hypothesis that cyclic GMP inhibits phosphodiesterase (Fig. 6*D*).

To specify the target of cyclic GMP, milrinone was used, which is known to be a specific inhibitor of cyclic GMP-inhibited phosphodiesterase (Harrison *et al.* 1986). As shown in Fig. 7*A* 10 μM-milrinone augmented the effect of isoprenaline (30 nM) on I_{Ca}, as did cyclic GMP (see Fig. 2). In four experiments, superfusing the cells with 10 μM-milrinone increased the amplitude of isoprenaline-stimulated I_{Ca} from 212 ± 45 to 345 ± 72% of the basal amplitude. On the other hand, milrinone failed to further

increase the isoprenaline-stimulated I_{Ca} (326±74%, $n = 4$), when the cell was pre-treated with 10 μM-cyclic GMP (Fig. 7*B*). These results suggested that the target of cyclic GMP is the cyclic GMP-inhibited phosphodiesterase.

In support of the above conclusion, IBMX, a non-specific phosphodiesterase inhibitor, abolished the effect of cyclic GMP. In the experiment shown in Fig. 8, the

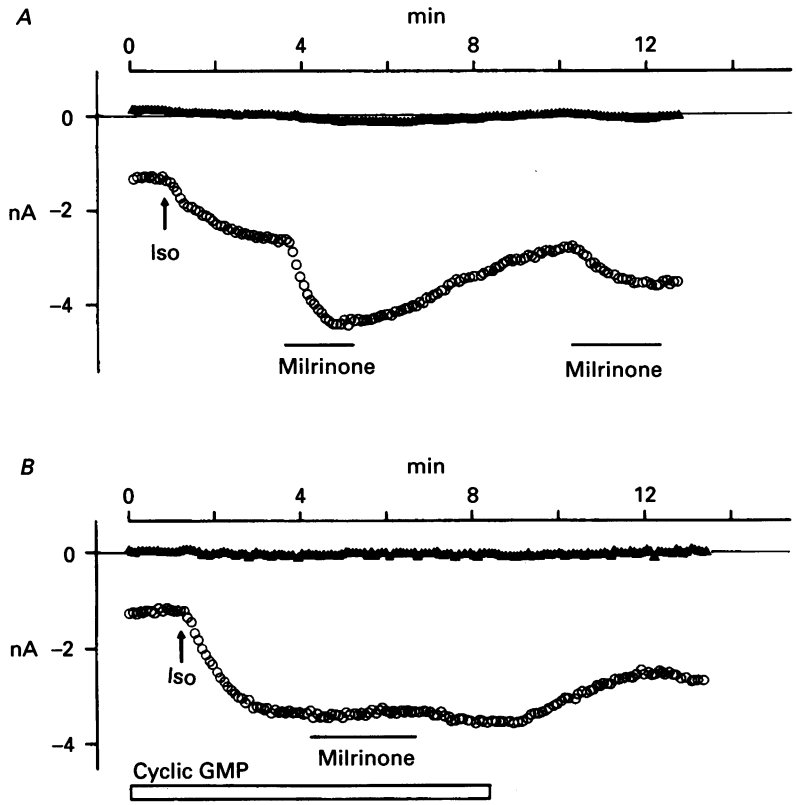

Fig. 7. Effect of milrinone on I_{Ca}. I_{peak} (○) and I_{late} (▲) were plotted against time. The test potential was 0 mv and the pulsing rate was 0·2/s. At times indicated by arrows, bath application of 30 nM-isoprenaline (Iso) was started and continued for the rest of the experiments. The periods of bath application of 10 μM-milrinone are indicated. *A*, in the absence of cyclic GMP in the pipette solution. *B*, in the presence of 10 μM-cyclic GMP.

cell was first superfused with 40 μM-IBMX (left horizontal line) and then internally dialysed with 10 μM-cyclic GMP during the period indicated (left open box). The expected increase in I_{Ca} by cyclic GMP was no longer observed. On the contrary, I_{Ca} was decreased by 10 μM-cyclic GMP from 1·6 to 1·0 nA and on washing out cyclic GMP the amplitude of I_{Ca} did not return to the control level. The depression of I_{Ca} by cyclic GMP was observed in six experiments. I_{Ca} was decreased from 205±35 to 130±24% of its control amplitude of basal I_{Ca}. It should be noted that the stimulatory effect of cyclic GMP could be demonstrated in the same cell after wash-out of IBMX.

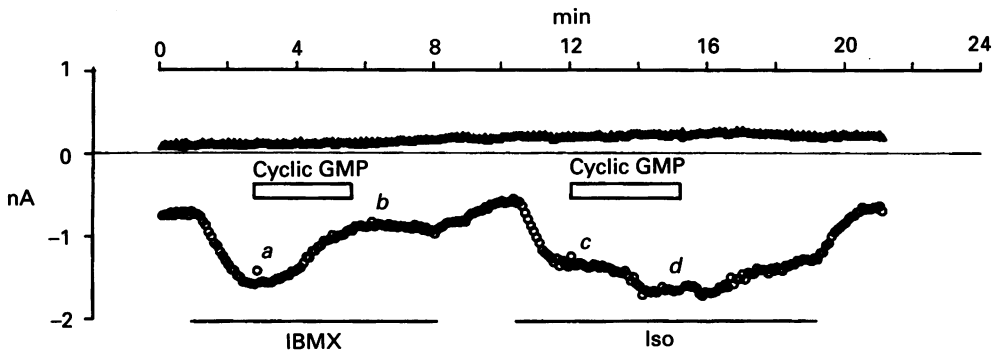

Fig. 8. Effect of cyclic GMP on I_{Ca} in the presence of IBMX. The levels of I_{peak} (○) and I_{late} (▲) were plotted against time. Bath application of 40 μM-IBMX or 30 nM-isoprenaline is indicated by bars. The concentration of cyclic GMP was 10 μM in the pipette.

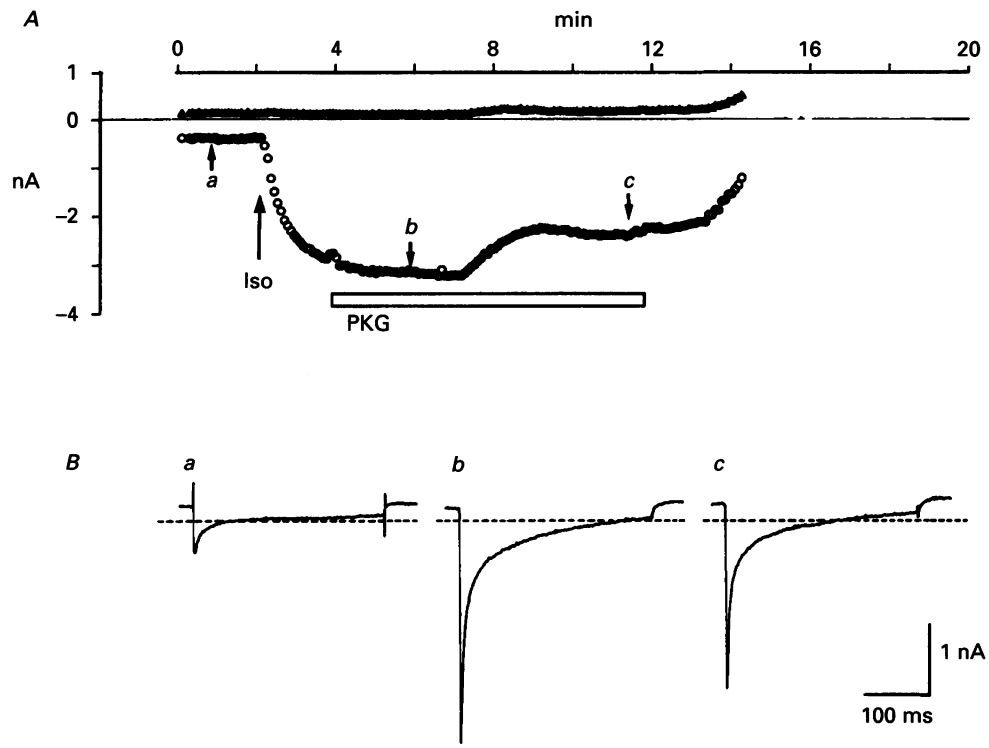

Fig. 9. Effects of dialysing the active fragment of PKG. *A*, I_{peak} (○) and I_{late} (▲) in response to test pulses to zero millivolts were plotted against time. Bath application of 0·1 μM-isoprenaline (Iso) was started at the arrow and continued throughout the experiment. Active fragment of 0·8 μM-PKG was internally applied during the period indicated by the open box. *B*, original currents recorded at the corresponding times indicated in *A*. Dashed lines indicate the zero-current level.

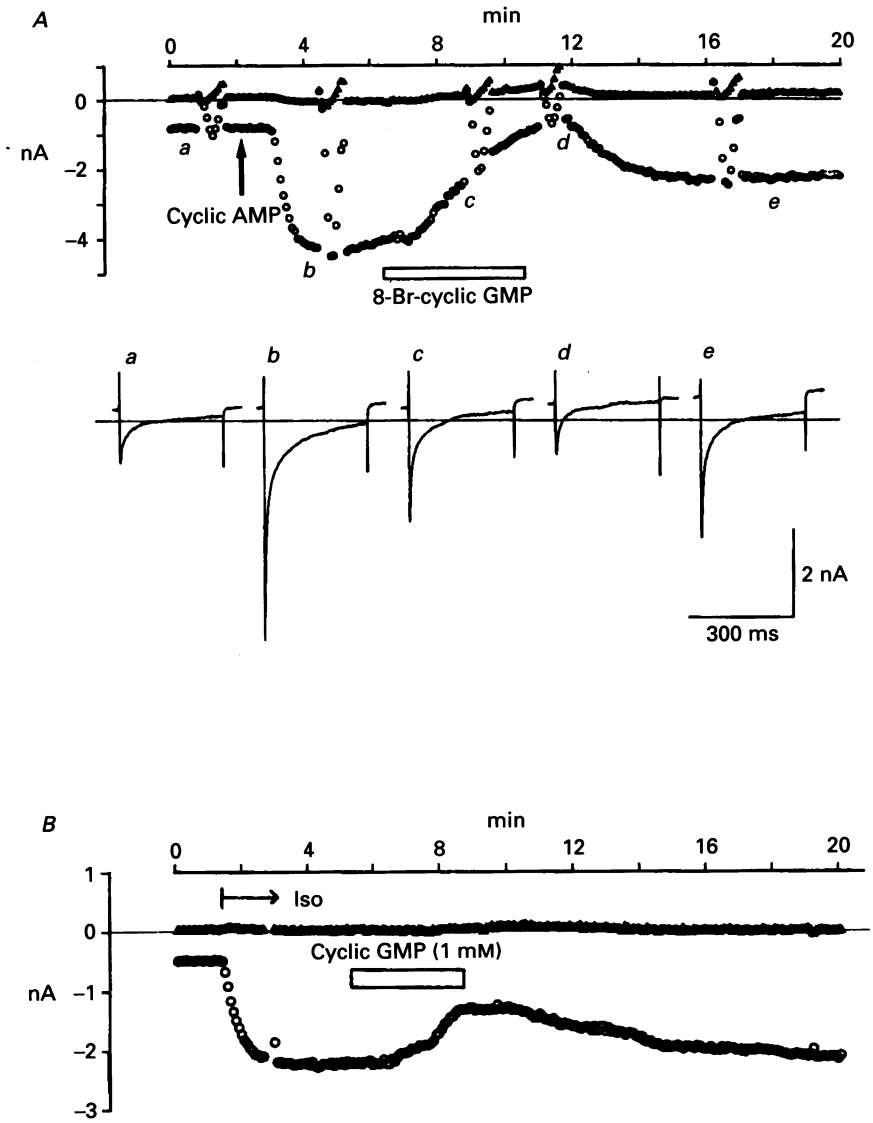

Fig. 10. *A*, effect of 8-Br-cyclic GMP on the response to cyclic AMP. I_{peak} (○) and I_{late} (▲) in response to test pulses to zero were plotted against time. Internal dialysis with 50 μM-cyclic AMP was started at the arrow and continued throughout the experiment. Dialysis of 10 μM-8-Br-cyclic GMP is indicated. Original currents recorded at corresponding times are shown underneath. *B*, inhibition of I_{Ca} by 1 mM-cyclic GMP in the presence of 30 nM-isoprenaline.

Inhibition of I_{Ca} by PKG, 8-Br-cyclic GMP and cyclic GMP

In addition to the phosphodiesterase, cyclic GMP-dependent protein kinase (PKG) is another possible target for cyclic GMP. To resolve a possible participation of PKG in the effects of cyclic GMP on I_{Ca}, the active fragment of PKG was applied intracellularly, or the intrinsic PKG was activated by 8-Br-cyclic GMP.

In the experiment shown in Fig. 9, I_{Ca} was first increased by superfusing the cell with 30 nM-isoprenaline. In the presence of isoprenaline, dialysis of 0·7 μM active fragment of PKG decreased I_{Ca} by 30%. As expected for a fragment of such large molecular mass (65 kDa), the onset of the effect was delayed as compared with the dialysis of cyclic nucleotides. Comparing the original current records of I_{Ca} suggested no significant change was induced by PKG in the inactivation time course. Reduction of I_{Ca} was observed in three out of twenty-one cells by dialysing the active fragment of PKG. In the other eighteen cells, however, I_{Ca} was not affected during more than 10 min of dialysis. In these cases positive pressure was applied to the inside of the pipette, so that injection of the pipette solution sometimes increased the cell volume. The effect of PKG was also examined in the absence of isoprenaline. Dialysis with 1–3 μM-PKG for more than 10 min did not affect basal I_{Ca}.

8-Br-cyclic GMP is 4–5 times more potent in activating PKG than cyclic GMP (Corbin, Ogreid, Miller, Suva, Jastorff & Doskeland, 1986). In the experiment shown in Fig. 10*A*, I_{Ca} was continuously stimulated by dialysing the cell with 50 μM-cyclic AMP. Additional dialysis of 10 μM-8-Br-cyclic GMP almost abolished the effect of cyclic AMP on I_{Ca} accompanied by a positive shift of the late current at 0 mV. Similar findings were obtained in four out of seven experiments. When I_{Ca} was stimulated by 30 nM-isoprenaline, depression of I_{Ca} was observed in four out of fifteen cells. In seven experiments internal dialysis with 1–100 μM-8-Br-cyclic GMP did not affect the amplitude of the basal I_{Ca} (data not shown).

Inhibitory responses were also observed on dialysis with higher concentrations of cyclic GMP (100–1000 μM) in the pipette (see Fig. 5*B*). An example is shown in Fig. 10*B*, where I_{Ca} enhanced by 30 nM-isoprenaline was inhibited from 2·2 to 1·4 nA on dialysis of 1 mM-cyclic GMP. The effect was reversible on washing out cyclic GMP from the pipette. This finding may indicate, together with the experiments with IBMX (Fig. 8) and those with PKG (Fig. 9) or 8-Br-cyclic GMP (Fig. 10*B*), the presence of an inhibitory mechanism of cyclic GMP independent from the stimulatory action described in the present study.

DISCUSSION

Stimulatory effect of cyclic GMP

In the present study internal dialysis of guinea-pig ventricular cells with micromolar concentrations of cyclic GMP enhanced the effect of isoprenaline on I_{Ca}. We propose that this enhancement is due to an inhibition of phosphodiesterase (PDE). This hypothesis is based on several experimental findings. (1) Although cyclic GMP potentiated the increase of I_{Ca} following an elevation of intracellular cyclic AMP level (β-adrenergic stimulation, forskolin and internal dialysis with cyclic AMP), the response to PDE hydrolysis-resistant derivatives, 8-Br-cyclic AMP or 8-CPT-cyclic AMP, was not enhanced. (2) The synergistic action of cyclic GMP was more evident in cells which showed smaller responses to the conditioning application of cyclic AMP, presumably due to strong hydrolytic degradation by phosphodiesterase. (3) In the presence of cyclic GMP in the pipette, the stimulation of I_{Ca} by milrinone, a specific inhibitor for cyclic GMP-inhibited phosphodiesterase, was not observed (Fig. 7). (4) Cyclic GMP did not increase I_{Ca} which was stimulated by

IBMX (Fig. 8). (5) PKG is not involved in the increase of I_{Ca} by cyclic GMP (Figs 9 and 10*A*).

Besides stimulation of I_{Ca}, the delayed rectifier K^+ current was also increased by cyclic GMP (Figs 2 and 3). This finding is in line with our present hypothesis, since the activation of PKA by increasing cyclic AMP is known to increase both this K^+ current and I_{Ca} (Yazawa & Kameyama, 1990).

Several molecular forms of phosphodiesterases are present in cardiac tissue, including cyclic GMP-stimulated phosphodiesterase (cGS-PDE) and cyclic GMP-inhibited phosphodiesterase (cGI-PDE; Weishaar, Burrows, Kobylarz, Quade & Evans, 1986; see for review, Beavo, 1988). cGI-PDE is one of the major components of phosphodiesterase in the mammalian heart and is very specific for hydrolysis of cyclic AMP (K_m = 0·2 μM, Harrison *et al.* 1986) over cyclic GMP. In bovine heart the value of half-maximal inhibition by cyclic GMP is reported to be about 0·1 μM (Harrison *et al.* 1986) and in guinea-pig ventricles 2·2 μM (Weishaar, Kobylarz-Singer & Kaplan, 1987). These concentrations may be achieved by dialysing the ventricular cells with pipette solutions containing 0·1–10 μM-cyclic GMP (Fig. 5). Recently, similar synergistic actions of cyclic AMP and cyclic GMP were found in platelets, where cyclic GMP enhanced the cyclic AMP effect on platelet function by inhibiting cGI-PDE (Maurice & Haslam, 1990). Several inhibitors of cGI-PDE, such as milrinone, amrinone and fenoximone, are known to increase the force of contractions in the whole heart and multicellular preparations (Weishaar *et al.* 1986; for review see Wetzel & Hauel, 1988). Furthermore, the findings on milrinone in the present study (Fig. 7) support the hypothesis that cGI-PDE mediates the synergistic action of cyclic GMP. We therefore conclude that cyclic GMP, as an intrinsic inhibitor for cGI-PDE, has a regulatory function under physiological conditions.

Inhibitory effect of cyclic GMP in cardiac cells

Experiments using purified antibodies showed that PKG exists in isolated guinea-pig ventricular cells used in the present study (F. Egleme & I. C. Hofmann, personal communication). Thus, the inhibitory action of PKG reported by Levi *et al.* (1989) and Mery *et al.* (1990*a*,*b*) may provide a satisfactory explanation for the antagonistic action of cyclic GMP observed in the present study. The application of cyclic GMP reversed the increase of I_{Ca}, which was caused by applying high doses of cyclic GMP (100–1000 μM; Figs 5 and 10*B*), IBMX (Fig. 8) and 8-Br-cyclic GMP (Fig. 10). Therefore we carefully examined the effect of applying the active form of PKG in many experiments. However, we failed to observe a significant inhibitory action of PKG in most cells. Insufficient dialysis of the protein molecule (PKG) is unlikely to be the reason for the lower incidence of inhibition in the present study, since similar results were obtained with dialysis of the activator (8-Br-cyclic GMP) of much smaller molecular mass. In some experiments we actually injected the pipette solution into the cell without observing any change of I_{Ca}. At present we have no convincing explanation for why we failed to observe the regular inhibition of PKG, as was the case in the experiments of the previous authors (Mery *et al.* 1990*b*).

The biphasic relationship between the concentration of cyclic GMP and the change of I_{Ca} observed in Fig. 5*B* may be explained by assuming an inhibitory action of cyclic GMP in addition to the synergistic action at lower doses. The former effect may be explained by the activation of cGS-PDE and/or PKG. For this explanation to be

plausible, it is necessary to assume a higher cyclic GMP-binding affinity for cGI-PDE than for cGS-PDE or PKG. However, the reported half-maximum concentrations for their activation overlap. The half-maximum inhibition of cGI-PDE was reported to be 0·1 μM in bovine cardiac muscle (Harrison *et al.* 1986), and 2·2 μM in guinea-pig (Weishaar *et al.* 1987). Activation of cGS-PDE was observed in the range of 0·1–5 μM (Beavo, 1988), and for activation of PKG, $K_{\frac{1}{2}} = 0{\cdot}11$ μM (Corbin *et al.* 1986). Thus, the dose dependence cannot be explained if the native enzymes behave *in vivo* as they do *in vitro*. At present we have no explanation of the mechanism underlying the antagonistic action of cyclic GMP.

In frog myocytes cyclic GMP reverses the cyclic AMP-induced increase of I_{Ca} by promoting cyclic AMP hydrolysis by activating cGS-PDE (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987). We have confirmed their experimental results; in frog ventricular cells application of cyclic GMP regularly decreased I_{Ca} stimulated by isoprenaline or cyclic AMP ($n = 12$, unpublished observation), and this inhibitory effect was blocked by IBMX. Furthermore, cyclic GMP failed to decrease I_{Ca} stimulated by 8-Br-cyclic AMP. Thus, it may be concluded that the species difference results from peculiarities in the PDEs.

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