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Effect of Cyclic GMP on the Calcium Current in Rabbit Ventricular Myocytes

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= ABSTRACT =

In order to investigate the effect of intracellular cyclic GMP on calcium current, the whole-cell patch clamp technique with internal perfusion method was used in isolated ventricular myocytes of the rabbit. Cyclic GMP, 8-bromo-cyclic GMP, cyclic AMP, isoprenaline and forskolin were perfused into cells and their effects on calcium current were analysed by applying depolarizing step pulses of + 10 mV in amplitude for 300 msec from holding potential of − 40 mV. Not only cyclic AMP (100 μM) but also cyclic GMP (100 μM) increased the basal calcium current. 8-Bromo-cyclic GMP (100 μM), a good stimulator of the cyclic GMP-dependent protein kinase, also increased the basal calcium current and its peak amplitude of calcium current was larger than that in the presence of cyclic AMP or cyclic GMP alone. In the presence of 100 μM cyclic GMP or 100 μM 8-bromo-cyclic GMP, already augmented calcium current was potentiated by intracellular application of 100 μM cyclic AMP or 1 μM isoprenaline or 1 μM forskolin. In the presence of cyclic GMP, acetylcholine reduced the calcium current only when the calcium current was increased by isoprenaline. From the above results it could be concluded that intracellular perfusion with cyclic GMP increases the basal calcium current via a mechanism involving a cyclic GMP-dependent protein kinase.

Key Words: Ventricular myocytes, Whole-cell patch clamp, Calcium current, Cyclic GMP, 8-Bromo-cyclic GMP, Cyclic GMP-dependent protein kinase.

INTRODUCTION

The calcium channel is fundamental in the generation of action potentials and neuro-hormonal regulation of cardiac function, as well as in the initiation and development of myocardial contraction. It is well established that cyclic AMP is an important modulator of the calcium channel current in the heart (Robison et al, 1971; Tsien, 1977; Stull, 1980; Drummond & Severson, 1979; Tsien et al, 1986; Trautwein et al, 1987). Cyclic AMP, increased by activation of the sympathetic nervous system or by β-adrenergic agonists, activates cyclic AMP-dependent protein kinase which then phosphorylates the calcium channel (Tsien et al, 1986; Trautwein et al, 1987) or other proteins involved in regulating cardiac contractility such as troponin-I (Solaro & Shiner, 1976; England, 1977; Stull, 1980), C-protein (Jeacocke & England, 1980; Harzell & Titus, 1982; Harzell & Glass, 1984) and phospholamban (Kranias & Solaro, 1982; Lindemann & Watanabe, 1985). In addition to cyclic AMP, it seems probable that cyclic GMP has some possible role in regulating the calcium current (Goldberg & Haddox, 1977; Dobson, 1981; Lincoln & Corbin, 1983). Early studies in the whole heart indicated that cyclic GMP antagonized the positive inotropic effects of cyclic AMP or β-adrenergic

agonists (Watanabe & Besch, 1975; Endoh & Yamashita, 1981; Singh & Flitney, 1981). The regulation of cyclic GMP levels by various agents, however, was not always consistent with their effects on contractility (Diamond et al, 1977; Katsuki et al, 1977; Lincoln & Keeley, 1981). At present the role of cyclic GMP and its mechanisms of actions are still controversial. For example, it was demonstrated that cyclic GMP depressed cardiac action potentials (Trautwein et al, 1982; Kohlhardt & Haap, 1978; Bkaily & Sperelakis, 1985) and, after β-adrenergic stimulation or in the presence of intracellular cyclic AMP, inhibited the calcium current both in frog (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987) and rat (Hatem & Morad, 1992) ventricular myocytes. In the presence of β-adrenergic stimulation Levi et al (1989) also observed a depression of calcium current by the intracellular application of cyclic GMP in guinea-pig ventricular myocytes. In contrast, Ono & Trautwein (1991) observed an increase of calcium current under essentially the same experimental conditions as Levi et al (1989). In these studies, however, basal calcium current was unaffected by cyclic GMP. On the other hand, in our previous experiments (Han et al, 1992), we observed thát intracellular application of cyclic GMP increased the basal calcium current in rabbit ventricular myocytes. In order to determine the possible mechanism of action of cyclic GMP, we have performed a series of experiments to examine the effect of cyclic GMP on the calcium current in rabbit ventricular myocytes. In this study, our results show that, in contrast to frog, rat and guinea-pig ventricular myocytes, cyclic GMP increases the basal calcium current in rabbit ventricular myocytes throught the cyclic GMP-dependent protein kinase which exists in cardiac tissues (Flockerzi et al, 1978; Kuo, 1974).

METHODS

Preparation of single ventricular myocytes

Single ventricular myocytes were isolated from rabbit hearts by enzymatic dissociation, as discribed previously (Earm et al, 1990). Rabbits (600~800 g) of either sex were anaesthetized with intravenously administered sodium pentobarbitone (40~50 mg/kg) after heparin administration (300 IU/kg). The thorax and pericardium were opened under artificial respiration and the aorta was cannulated before removal of the heart. The excised heart was connected to a Langendorff perfusion apparatus and perfused with 80 cmH_2O pressure. Normal Tyrode solution was first perfused until the blood was washed out completely. And then Ca^{2+}-free Tyrode solution was perfused for 5 min and Ca^{2+}-free Tyrode solution containing 0.01% collagenase (5 mg/50cc, Yakult, Japan) was perfused for 15~25 min. After enzymatic treatment, Krafts-Brühe (KB) solution (Isenberg & Klöcknor, 1982) was perfused to wash out the remaining enzyme in the heart. The KB solution contained (in mM): 50 glutamate, 20 HEPES, 20 taurine, 10 glucose, 3 $MgSO_4$, 0.5 EGTA, 30 KCl, 30 KH_2PO_4, the pH was adjusted to 7.4 with KOH. The temperature of the Langendorff column was kept at 37°C during all previous steps. The heart was then placed in 50 ml KB solution at room temperature. The ventricles were cut and small pieces of ventricular tissue were gently agitated. Isolated ventricular cells were equilibrated with 100 % oxygen. The rod shape of the cells and the clear striations of sarcomere were important criteria used for selecting viable cells.

Solutions

The bath solution used to superfuse was normal Tyrode solution, the composition of which was (in mM): 143 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 glucose, 5-N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), the pH was adjusted to 7.4 with NaOH. The composition of the standard internal solution in the patch electrode was (in mM): 110 CsCl, 5 Mg-ATP, 2.5 di-Tris creatine phosphate, 2.5 disodium creatine phosphate, 1 $MgCl_2$, 5 ethyleneglycol-bis-(b-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 5 HEPES, 20 tetra-

ethylammonium chloride (TEA-Cl), the pH was adjusted to 7.4 with CsOH. Isoprenaline, forskolin, cyclic AMP (cAMP), cyclic GMP (cGMP) and 8-bromo-cGMP (8-Br-cGMP) were obtained from Sigma chemical Co. (St. Louis, MO, U.S.A.). Other drugs and chemicals were also obtained from Sigma. The bath solution could be replaced within 10 sec by switching from one solution to another. Experiments were performed at 37℃.

Electrophysiological measurements

Calcium current and action potential recordings were obtained through the whole-cell patch method (Hamill et al, 1981) by using a patch-clamp amplifier (EPC-7, LIST, Darmstadt, FRG). Patch electrodes were pulled with a vertical puller (model PP-83, Narishige, Tokyo, Japan) from borosilicate capillary tubes (Mercer glass NO. MX-999, N.Y, U.S.A.) and had tip resistances of 2~3 MΩ when filled with internal solution. Neither the capacitance current nor series resistance with the cell membrane were compensated. Membrane current and potential signals were digitized at a sampling rate of 48 kHz, and recorded on a chart recorder (Recorder 220, Gould) or digital tape recorder (DTR-1200, Biologic, Grenoble, France) for later analysis. In current clamp mode, action potentials were elicited by constant current pulses of 500-800 pA amplitude and 4~10 msec duration. Calcium currents were evoked by depolarizing step pulses (300 msec) from a holding potential of −40 mV to various potentials (from −80 mV to +40 mV) delivered at a frequency 0.1 or 0.5 Hz. Usually the holding potential was −40 mV to inactivate the fast sodium current. The amplitude of calcium current was measured as the difference between peak current and holding current measured at the start of 300 msec pulse. The current amplitude was then standardized to a membrane capacitance of 100 pF.

Statistical analysis

The values were expressed as the mean plus or minus the standard dveiation of the mean (SD). Statistical significance was determined by an analysis of variance with simultaneous multiple-comparison procedures. A P value less than 0.05 was assumed significant.

RESULTS

The cells used for the experiments were cylindrical in shape and their striation was regular and fine. Such cells had membrane capacitances of 108.5 ± 6.9 pF (n = 52) and displayed resting potentials of −77.4 ± 2.8 mV (n = 52). We first examined the effect of cyclic GMP (cGMP) on the action potential configuration. In Fig. 1A, the application of 10 μM cyclic AMP (cAMP) caused an increase in the action

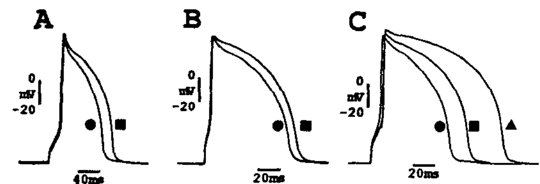

Fig. 1. Effects of intracellular perfusion with cyclic GMP on the action potential of rabbit ventricular myocytes. A. Superimposed records of action potentials in control (●) and with intracellular perfusion of cyclic AMP (■). Resting membrane potential was slightly depolarized from −73 mV to −71 mV and the action potential duration (95% repolarization) was increased from 80 msec in control to 100 msec by intracellular perfusion of 10 μM cyclic AMP. B. Superimposed records of action potentials in control (●) and in the presence of isoprenaline (■). Isoprenaline (0.2 μM) also caused an increase in the duration (from 75 msec to 86 msec) and plateau level of the action potential. C. Superimposed records of action potentials in control (●), when dialyzed with cyclic AMP (■) and when dialyzed with cyclic AMP plus cyclic GMP (▲). As in A, intracellular perfusion of 10 μM cyclic AMP increased the duration and plateau level of action potential compared with control. Moreover, addition of 10 μM cyclic GMP increased the overshoot, duration and the plateau level of the action potential even more.

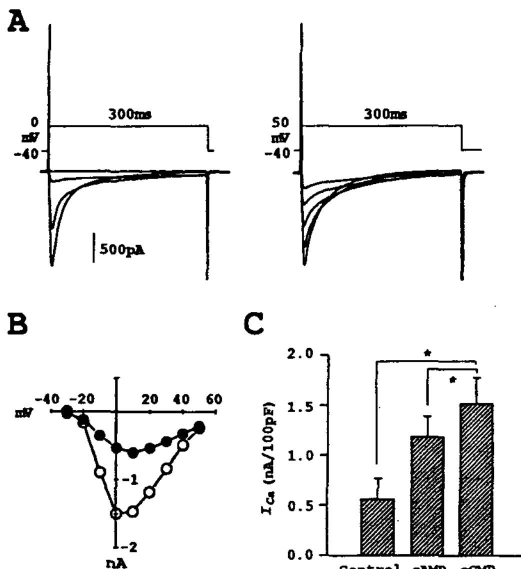

*Fig. 2. Effect of cyclic GMP (100 μM) was applied by internal perfusion of the patch pipette. A. Calcium current traces activated by cyclic GMP at the membrane potentials of −30 to 0 mV (left panel) and at +10 to +50 mV (right panel). B. Current-voltage relationships in control and in the presence of cyclic GMP were plotted on a current scale normalized to a membrane capacitance of 100 pF. C. Comparison of effect of cyclic AMP and cyclic GMP on the peak amplitude of calcium currents evoked by a depolarizing pulse to +10 mV from a holding potential of −40 mV. Data are means ± SD of the peak amplitude values of calcium currents (n = 13 for control, n = 8 for cyclic AMP, n = 9 for cyclic GMP). * P>0.05.*

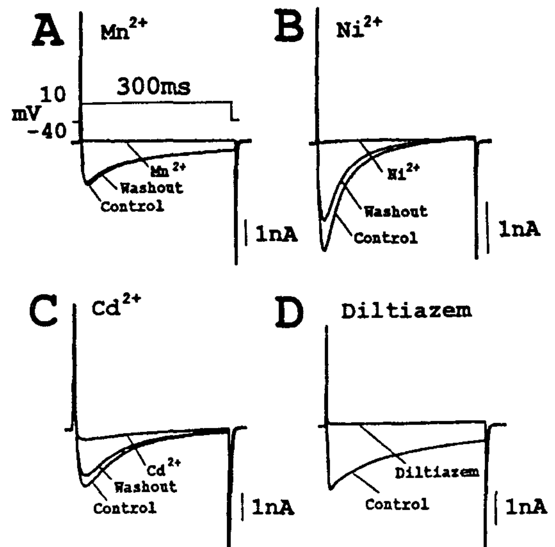

Fig. 3. Block of the cyclic GMP-induced inward current by inogranic or organic calcium channel blockers. A. Effect of manganese (Mn^{2+}). Superimposed current traces from the same ventricular cell (membrane capacitance, 107.1 pF) elicited by 300 msec depolarizing step pulses from −40 mV to +10 mV under control conditions, in the presence of manganese (3 mM) and after washout. B. Effect of nickel (Ni^{2+}). Superimposed current traces were elicited in rabbit ventricular myocyte (membrane capacitance = 121.9 pF) by 300 msec depolarizaing pulses from −40 mV to +10 mV under control conditions, in the presence of nickel (5 mM) and after washout. C. Effect of cadmium (Cd^{2+}). Superimposed current traces from the same ventricular cell (membrane capacitance=75.3 pF) elicited by 300 msec depolarizing step pulses from the −40 mV to +10 mV under control conditions, in the presence of cadmium (1 mM) and after washout. D. Effect of diltiazem. Superimposed current traces from the same ventricular cell (membrane capacitance, 111.2 pF) elicited by 300 msec depolarizing step pulses from −40 mV to +10 mV under control conditions, and in the presence of diltiazem (5 μM).

potential duration at 95% repolarization from 80 msec to 100 msec. When the action potential lengthened, there was also a more positive plateau region. Isoprenaline (0.2 μM) also displayed the same effects as 10 μM cyclic AMP (Fig. 1B). When the action potential duration was increased by intracellular application of 10 μM cyclic AMP from 71 msec to 99 msec, internal perfusion with 10 μM cyclic GMP resulted in an additional increase in the action potential duration (121 msec) and also increased the plateau level (Fig. 1C). The changes of the plateau level by cyclic AMP, isoprenaline and cyclic GMP suggest that calcium current is affected by these agents. The increase in the action potential duration and more positive plateau with cGMP, therefore, could be caused

by an increase in calcium current. In order to investigate the effect of cyclic GMP on calcium current, a whole-cell voltage-clamp technique was used. We applied the depolarizing step pulses from a holding potential of −40 mV to various potentials for 300 msec to inactivate the fast sodium current. Potassium currents were blocked with intracellular cesium (100 mM). Our prior experiments showed that various calcium channel blockers abolished the current activated by this protocol, and that any remaining time-dependent current was negligible (data not shown). This indicated that our protocol activated calcium current with little contamination from other currents, so that it could be used to investigate changes of calcium current with cyclic GMP. In control cells, calcium currents usually activated from −30 mV and then reached a peak at +10 mV (Fig. 2B, ●). The peak amplitude of the calcium current at +10 mV was 0.56 ± 0.20 nA/100pF (n = 13). Fig. 2A shows a representative set of membrane currents obtained with a holding potential of −40 mV in the presence of intracellular cyclic GMP (100 μM). The current-voltage relationship from the same experiment (Fig. 2B, ○) shows for calcium current a threshold potential at about −30 mV, a maximum at 0 mV and an apparant reversal potential at about + 60 mV. As we expected from the change of the plateau level in Fig. 1C, calcium current was significantly increased by intracellular application of 100 μM cyclic GMP. The peak amplitudes of the calcium currents by intracellular application of cyclic AMP and cyclic GMP are summarized in Fig. 2C. In the presence of 100 μM cyclic GMP, the peak amplitude of the calcium current at +10 mV was 1.52 ± 0.25 nA/100pF (n = 9). This peak amplitude was also larger than that in the presence of cyclic AMP (1.19 ± 0.20 nA/100 pF, n = 8)($P<0.05$). The shape of the current-voltage relationship was usually not singificantly affected by cyclic GMP. In some cases, however, perfusion with isoprenaline, cyclic AMP or cyclic GMP produced a leftward shift of about 10 mV in the peak of the current-voltage relationship. This phenomenon was also osberved by Bean et al. (1984) and Fischmeister & Harzell (1986). The former suggested that it was physiologically important. The latter, however, insisted that it was a series resistance artifact. Further studies, therefore, were needed in regard to this shift. In order to confirm the nature of cyclic GMP-induced inward current we performed the experiments using various calcium channel blockers. Fig. 3 shows the effects of inorganic and organic calcium channel blockers on calcium current

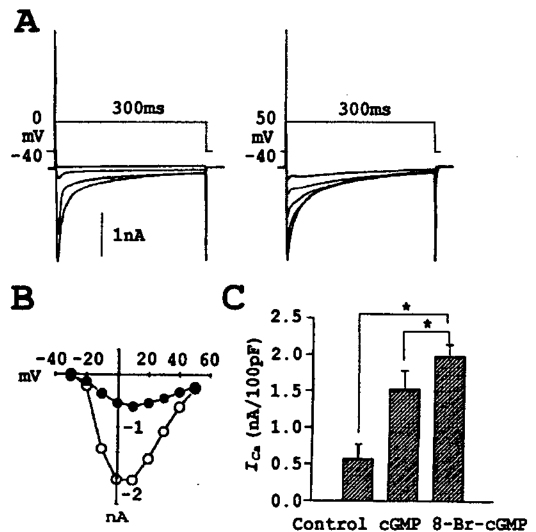

*Fig. 4. Effect of 8-bromo-cyclic GMP on the activation of calcium current. 8-bromo-cyclic GMP (100 μM), a specific stimulator of cyclic GMP-dependent protein kinase, was applied internal perfusion of the patch pipette. A. Calcium current traces activated by 8-bromo-cyclic GMP at the membrane potentials of −30 to 0 mV (left panel) and at +10 to +50 mV (right panel). B. Current-voltage relationships in control and in the presence of 8-bromo-cyclic GMP were plotted on a current scale normalized to a membrane capacitance of 100 pF. C. Comparison of effect of cyclic GMP and 8-bromo-cyclic GMP on the peak amplitude of calcium currents evoked by a depolarizing pulse to +10 mV from a holding potential −40 mV. Data are means±SD of the peak amplitude values of calcium currents (n = 13 for control, n = 9 for cyclic GMP, n = 8 for 8-bromo-cyclic GMP). * $P<0.05$.*

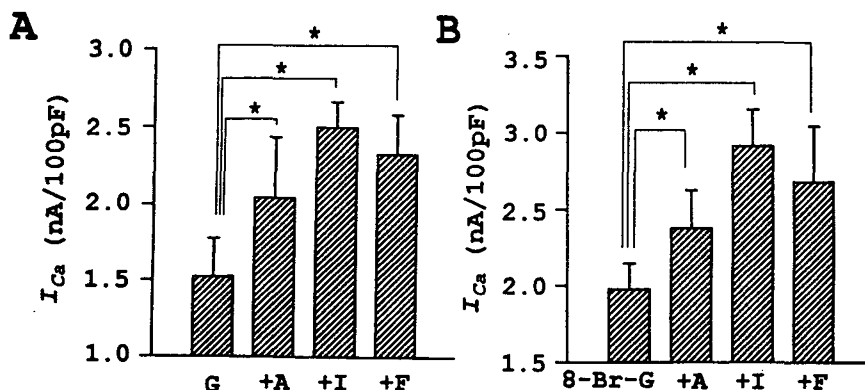

*Fig. 5. Effects of cyclic AMP, isoprenaline and forskolin on peak calcium current evoked by a depolarizing pulse to +10 mV from a holding potential −40 mV in the presence of cyclic GMP (A) or 8-bromo-cyclic GMP (B). G, 100μM cyclic GMP; 8-Br-G, 100 μM 8-bromo-cyclic GMP; A, 100 μM cyclic AMP; I, 1 μM isoprenaline; F, 1 μM forskolin. Note that the additional increases in the amplitude of the calcium current by cyclic AMP, isoprenaline and forskolin were significant. Vertical bars are mean ± SD(n = 9 for cyclic GMP only, n = 6 for cyclic GMP plus cyclic AMP, n=7 for cyclic GMP plus isoprenaline, n = 6 for cyclic GMP plus forskolin, n = 8 for 8-bromo-cyclic GMP only, n = 6 for 8-bromo-cyclic GMP plus cyclic AMP, n = 8 for 8-bromo-cyclic GMP plus isoprenaline, n = 6 for 8-bromo-cyclic GMP plus forskolin). * P<0.05.*

induced by cyclic GMP. Depolarizing step pulses were applied from −40 mV to +10 mV, eliciting the maximum inward peak current. Manganese (3 mM) blocked cyclic GMP-induced inward current (Fig. 3A). Nickel (5 mM) (Fig. 3B) and 1 mM cadmium (Fig. 3C) resulted in a complete block of cyclic GMP-induced inward current. The blocking effects of these divalent cation were reversed after washout. Diltiazem (5 μM) also resulted in block of the cyclic GMP-induced inward current. In contrast to inorganic divalent cations, prolonged washout for 20 min could not reverse the blocking effect of this drug. From these tesults, we confirmed that cyclic GMP increased the basal calcium current. Since our results suggest that cyclic GMP may increase basal calcium currents, we investigated the effect of 8-bromo-cyclic GMP (8-Br-cGMP) on basal calcium current. 8-Br-cGMP is one of the most useful cyclic GMP analogues to determine the mechanism of cyclic GMP because it is five times more potent than cyclic GMP in activating the protein kinase (Corbin et al,

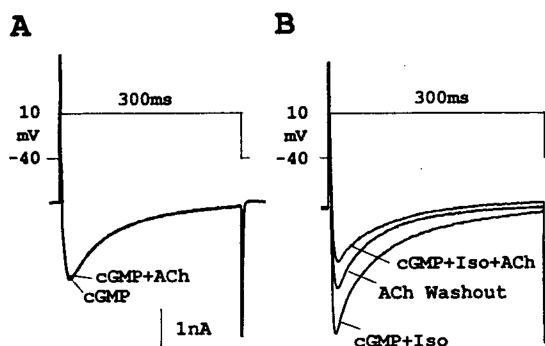

Fig. 6. Effect of acetylcholine on the calcium current in the presence of cyclic GMP. A. Superimposed current traces from the same ventricular cell (membrane capacitance = 89.9 pF) elicited by 300 msec depolarizing step pulses from −40 mV to +10 mV. Additional application of 1 μM acetylcholine was without effect on calcium current in the presence of cyclic GMP alone. B. Superimposed current traces from the same ventricular cell (membrane capacitance = 114.4 pF) elicited by 300 msec depolarizing step pulses from −40 mV to +10 mV. Isoprenaline (1 μM) enhanced calcium current and addition of 1 μM acetylcholine reduced the amplitude.

1986). Fig. 4A is an example of the calcium currents obtained with a holding potential of −40 mV in the presence of 100 μM 8-bromecyclic GMP. The shape of the current-voltage cure for calcium current was only slightly altered by 8-br-cyclic GMP, with the threshold for calcium current activation and the command potential at which maximal calcium current occurred being similar in the absence and presence of 8-bromo-cyclic GMP (Fig. 4B, ○). 8-Bromo-cyclic GMP increased the calcium current significantly. Its peak amplitude at +10 mV was 1.97 ± 0.16 nA/100pF(n = 8) and was larger than that in cyclic GMP ($P<0.05$) (Fig. 4C). One possible interpretation of this result, therefore, is that cyclic GMP increases basal calcium current in rabbit ventricular myocytes throught stimulation of the cyclic GMP-dependent protein kinase, and subsequent phosphorylation of some protein involved in calcium channel function. When calcium current was increased by intracellular application of 100 μM cyclic GMP (Fig. 5A) or 8-bromocyclic GMP (Fig. 5B), intracellular application of cyclic AMP (100 μM) or bath application of isoprenaline (1 μM) or forskolin (1 μM) resulted in an additional stimulation of calcium current. Increases in calcium current by cyclic AMP, isoprenaline or forskolin were significant in all cases ($P<0.05$). These results also indicated that cyclic GMP increased calcium current by a cyclic AMP independent phosphorylation mechanism in rabbit ventricular cells, most likely through a cyclic GMP-dependent protein kinase mediated phosphorylation. Lastly, we investigated the effect of 1 μM acetylcholine (ACh) on the calcium current in the presence of cyclic GMP because many investigators have demonstrated increases in cyclic GMP levels in response to cholinergic agents in a variety of cardiac preparation (Dobson, 1981; Endoh et al, 1985; Flitney & Singh, 1981; Gardner & Allen, 1976). In the presence of cyclic GMP, acetylcholine produced little or no change in the amplitude of calcium current from 1.138 nA/100pF to 1.103 nA/100 pF (Fig. 6A). Whereas, when calcium current was further increased by 1 μM isoprenaline, additional application of acetylcholine decreased the calcium current from 1.842 nA/100 pF to 0.779 nA/100pF. The effect of acetylcholine was partially reversed on washout (1.163 nA/100pF) (Fig. 6B). In spite of sufficient washout of acetylcholine, this decrease in the amplitude of calcium current was due to typical "run-down" of calcium current. The simplest interpretation of this result is that acetylcholine may regulate calcium current by a mechanism which is different from the mechanism of cyclic GMP.

DISCUSSION

The major findings of the present study are: (1) not only cyclic AMP but also cyclic GMP increased the basal calcium current; (2) 8-bromo-cyclic GMP, a potent stimulator of cyclic GMP-dependent protein kinase, also increased the basal calcium current and its peak amplitude of calcium current was larger than that in the presence of cyclic AMP or cyclic GMP; (3) in the presence of cyclic GMP or 8-bromo-cyclic GMP, already increased calcium current was potentiated by intracellular application of cyclic AMP or bath application of isoprenaline or forskolin; and (4) in the presence of cyclic GMP, acetylcholine reduced the calcium current only when the calcium current was increased by isoprenaline. Based on our results, it may be proposed that intracellular perfusion with cyclic GMP increases the basal calcium current via a mechanism involving a cyclic GMP-dependnet protein kinase.

In addition to cyclic AMP, it has been also proposed that cyclic GMP may play a role in regulating cardiac contractility as an intracellular second messenger because intracellular cyclic GMP is generated in cardiac myocytes in response to physiological agonists such as acetylcholine (George et al, 1973), atrial natriuretic peptide (Cramb et al, 1987) and histamine (Hattori et al, 1988). But the role of cyclic GMP in regulating the calcium current and its mechanisms of action are less clear and remain

controversial. Some investigators have reported that cyclic GMP inhibits the calcium current in frog (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987), rat (Hatem & Morad, 1992), embryonic chick (Gordon et al, 1989; Tohse & Sperelakis, 1991) and guinea-pig (Levi et al, 1989) cardiac myocytes. In contrast, others have reported that cyclic GMP increases the calcium current in guinea-pig (Ono & Trautwein, 1991) and rabbit (Han et al, 1991, 1992) ventricular myocytes. The former demonstrated that cyclic GMP caused a decrease in the calcium current that was increased by stimulation of β-adrenergic receptor or internal perfusion with cyclic AMP in frog (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987) and guinea-pig (Levi et al, 1989) ventricular myocytes, whereas in embryonic chick ventricular myocytes (Gordon et al, 1989; Tohse & Sperelakis, 1991), cyclic GMP inhibited basal calcium current through a cyclic AMP-independent mechanism. On the other hand, the latter displayed that only when calcium current was increased by bath application of isoprenaline or forskolin or by intracellular dialysis with cyclic AMP, did dialysis with cyclic GMP result in an additional stimulation of calcium current in guinea-pig ventricular myocytes (Ono & Trautwein, 1991) and in rabbit ventricular myocytes, cyclic GMP also increased basal calcium current (Han et al, 1992). Moreover, opinions have been also divided on the mechanism of cyclic GMP action. It has been reported that in isolated frog cardiac myocytes cyclic GMP inhibits calcium current by stimulation of cyclic AMP phosphodiesterase (cyclic GMP-stimulated cyclic nucleotide phosphodiesterase) (Harzell & Fischmeister, 1986; Fischmeister & Harzell, 1987), whereas in purified rat, embryonic chick and guinea-pig ventricular myocytes, cyclic GMP predominently inhibits calcium current via a mechanism involving cyclic GMP-dependent protein kinase (Gordon et al, 1989; Levi et al, 1989; Mery et al, 1990; Tohse & Sperelakis, 1991). In contrast, Ono and Trautwein (1991) reported that in guinea-pig ventricular myocytes, micromolar concentrations of cyclic GMP enhanced the effect of isoprenaline on calcium current due to an inhibition of cyclic GMP-inhibited cyclic nucleotide phosphodiesterase. Thus, the most likely explanation for these divergent findings is that the mechanism of action of cyclic GMP may be dependent on species or the affinity of cyclic nucleotide-dependent enzymes for cyclic GMP. We observed that intracellular application of cyclic GMP increased plateau level and prolonged action potential duration (Fig. 1). These results suggested that cyclic GMP could increase calcium current. Thus, we used the whole-cell voltage-clamp technique to measure directly the effect of cyclic GMP on calcium current and to determine the mechanism of action of cyclic GMP. Not only cyclic AMP but also cyclic GMP increased the basal calcium current. 8-bromo-cyclic GMP, a potent stimulator of the cyclic GMP-dependent protein kinase, also increased the basal calcium current and its peak amplitude of calcium current was larger than that in the presence of cyclic AMP or cyclic GMP in rabbit ventricular myocytes. Because 8-bromo-cyclic GMP does not involve the cyclic GMP-dependent phosphodiesterase, our results suggest that cyclic GMP increase calcium current independent of cyclic AMP in this preparation. Thus, while there is agreement that cyclic GMP can increase calcium current in ventricular myocytes (Ono & Trautwein, 1991), we suggest, in contrast to previous findings of Ono and Trautwein (1991) in the guinea-pig, that the mechanism involving phosphodiesterase is not the major mechanism responsible for the effect of cyclic GMP on calcium current in rabbit ventricular cells. We displayed that in the presence of cyclic GMP or 8-bromo-cyclic GMP, already increased calcium current was potentiated by intracellular application of cyclic AMP or bath application of isoprenaline or forskolin. These results also suggest that cyclic GMP increases calcium current independent of the cyclic AMP-dependent pathway. The idea that cyclic GMP opposes the effects of cyclic AMP ('Yin-Yan hypothesis') (Goldberg et al, 1975) is therefore no longer at-

tractive to explain the effect of cyclic GMP. Acetylcholine has been reported to produce a negative inotropic effect accompanied by an increase in the cyclic GMP level (Dobson, 1981; Flitney & Singh, 1981; Endoh et al, 1985). Some other reports, however, displayed that low concentrations of acetylcholine depressed contractility without changing cyclic GMP levels (Watanabe & Besch, 1975; Diamond et al, 1977; Brooker, 1977; Linden & Brooker, 1979; Dobson, 1981) and did not agree with the hypothesis that cyclic GMP is involved in the negative inotropic response of the heart to acetylcholine. Although acetylcholine elevated myocardial cyclic GMP levels, some experiments demonstrated a dissociation between cyclic GMP levels and contractile state (Diamond et al, 1977; Katsuki et al, 1977). In the present study, our results showed that in the presence of cyclic GMP, acetylcholine reduced the calcium current only when the calcium current was increased by isoprenaline. Such an antagonism between β-adrenergic stimulation and acetylcholine was previously described for the rate of beating and the force of contraction (Meester & Hardman, 1967), for the plateau and duration of action potential (Bailey et al, 1979) and the calcium current of ventricular myocytes (Hino & Ochi, 1980). In biochemical studies, acetylcholine was found to decrease cyclic AMP levels elevated by β-adrenergic agonists (Biegon et al, 1980; Linden et al, 1985). The results indicate that acetylcholine inhibits the production of cyclic AMP. Furthermore, Heschler et al (1986) suggested that the inhibition of calcium current by acetylcholine in the presence of β-adrenergic stimulation was due to an inhibition of adenylate cyclase since acetylcholine did not reduce the amplitude of calcium current enhanced by intracellular dialysis with cyclic AMP-dependent protein kinase in their experiments. Also some investigators did not find a significant lowering of the cyclic AMP-dependent protein kinase activity by acetylcholine in rat hearts (Keely et al, 1978). It should be noted that the increase in calcium current by intracellular application of cyclic GMP was not affected by acetylcholine in the present study. Therefore, it is unlikely to suggest that acetylcholine elevates myocardial cyclic GMP levels and that cyclic GMP appears to play a role in the action of acetylcholine on ventricular cells in addition to the ability of acetylcholine to inhibit adenylate cyclase. The simplest interpretation of our results and all previous results in various species is that the effect of cyclic GMP on calcium current is dependent on species and may be associated with the degree of affinity of cyclic nucleotide-dependent enzymes in myocardial cells. In the future, therefore, biochemical characterization of the endogenous cyclic GMP-dependent phosphodiesterase and protein kinase in cardiac myocytes will provide further evidence of the mechanisms of cyclic GMP action on calcium current. The latest developments in biochemical techniques provide several sophisticated tools for intracelluar signal transduction research. Thus, in the near future, further experiments should be done in order to clarify the precise mechanism of action of cyclic GMP using new tools.

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