Cyclic GMP-mediated inhibition of L-type Ca^{2+} channel activity by human natriuretic peptide in rabbit heart cells

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1 Effects of atrial natriuretic peptide (ANP) on the L-type Ca^{2+} channels were examined in rabbit isolated ventricular cells by use of whole-cell and cell-attached configurations of the patch clamp methods. ANP produced a concentration-dependent decrease (10–100 nM) in amplitude of a basal Ca^{2+} channel current.

2 The inactive ANP (methionine-oxidized ANP, 30 nM) failed to decrease the current.

3 8-Bromo-cyclic GMP (300 μM), a potent activator of cyclic GMP-dependent protein kinase (PKG), produced the same effects on the basal Ca^{2+} channel current as those produced by ANP. The cyclic GMP-induced inhibition of the Ca^{2+} channel current was still evoked in the presence of 1-isobutyl-3-methyl-xanthine, an inhibitor of phosphodiesterase. ANP failed to produce inhibition of the Ca^{2+} channel current in the presence of 8-bromo-cyclic GMP.

4 In the single channel recording, ANP and 8-bromo-cyclic GMP also inhibited the activities of the L-type Ca^{2+} channels. Both agents decreased the open probability (NP_o) without affecting the unit amplitude.

5 The present results suggest that ANP inhibits the cardiac L-type Ca^{2+} channel activity through the intracellular production of cyclic GMP and then activation of PKG.

Keywords: Atrial natriuretic peptide; cyclic GMP; L-type Ca^{2+} channel; cardiomyocyte; patch clamp

Introduction

Atrial natriuretic peptide (ANP) is well known as a potent vasodilator. The underlying mechanism of its vasodilator action is related to an increase in the intracellular concentration of guanosine 3': 5'-cyclic monophosphate (cyclic GMP) in the vascular smooth muscles (Needleman *et al.*, 1989). However, it remains unsettled whether ANP is able to influence directly cardiac function. Some reports suggested that ANP directly affected cardiac performance *in vivo* (Lappe *et al.*, 1985; Seymour *et al.*, 1987), while others denied such direct cardiac actions of ANP (Berger & Kotler, 1985; Wangler *et al.*, 1985; Criscione *et al.*, 1987). In such a controversy, recent studies on isolated cardiomyocytes clearly showed a directly depressive effect of ANP on contractility (Neyses & Vetter, 1989; Vaxelaire *et al.*, 1989; McCall & Fried, 1990).

The effect of ANP on cardiac Ca^{2+} channels is also controversial. In frog ventricular myocytes, ANP decreased a Ca^{2+} channel current that had been increased by β -adrenoceptor stimulation, but did not affect the basal Ca^{2+} channel current (Gisbert & Fischmeister, 1986). It was suggested that this reduction by ANP of the increased Ca^{2+} channel current may be mediated by activation of cyclic GMP-dependent phosphodiesterase (PDE II) (Hartzell & Fischmeister, 1986; Fischmeister & Hartzell, 1987). However, later work showed that activation of PDE II by cyclic GMP might play only a minor role in cyclic GMP-induced decrease in Ca^{2+} channel current in mammalian (guinea-pig and rat) heart cells (Levi *et al.*, 1989; Méry *et al.*, 1991; Ono & Trautwein, 1991). Furthermore, cardiomyocytes of rat (Sorbera & Morad, 1990), chick embryos (Bkaily *et al.*, 1993) and human atrial cells (LeGrand *et al.*, 1992) were reported to respond to ANP with a decrease in the basal Ca^{2+} channel current.

Sperelakis and his colleagues (Wahler *et al.*, 1990; Tohse &

Sperelakis, 1991) reported that basal activities of L-type Ca^{2+} channels in embryonic chick cardiomyocytes were inhibited by 8-bromo-cyclic GMP, a potent activator of cyclic GMP-dependent protein kinase (PKG) (Lincoln & Corbin, 1983). It is very possible that ANP inhibits the basal activity of L-type Ca^{2+} channels through activation of PKG. However, no investigations have been made into a causal relation between ANP, cyclic GMP, and the Ca^{2+} channels in mammalian cardiomyocytes.

In the present study, we examined effects of α -human natriuretic peptide (α -hANP, 1–28) on the whole-cell current and single-channel activities of L-type Ca^{2+} channels in rabbit ventricular cells. The present study suggests that activation of PKG mediates the inhibition by ANP of basal activities of Ca^{2+} channels.

Methods

Cell preparation

Single ventricular cells were enzymatically isolated from hearts of New Zealand White rabbits (1.5–2.0 kg), according to essentially the same methods as previously described (Tohse *et al.*, 1990). In brief, collagenase (0.01% wt./vol.; Wako Chemicals, Osaka Japan) dissolved in nominally Ca^{2+} -free (<60 μM) Tyrode solution was retrogradely perfused into the aorta of isolated hearts suspended in a Langendorff apparatus. The single cells obtained were suspended in a high K^+ , low Cl^- solution [Kraftbrühe (KB) solution] (Isenberg & Klöckner, 1982) and stored in a refrigerator (4°C) until later use.

Patch clamp recording

Whole-cell and cell-attached (single channel) patch clamp recordings were made with a patch clamp amplifier (EPC-7, List, Germany, or CEZ 2300, Nihon Kohden, Japan) according to the standard techniques. The patch pipettes had tip resistances of 2–4 M Ω (whole-cell recording) and 5–10 M Ω

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(cell-attached recording). The cells were placed in a perfusion chamber attached to an inverted microscope (IMT-2, Olympus, Japan), and constantly superfused with Tyrode solution or one of the external solutions (their compositions are described later) at 35–37°C. Current signals were filtered at 1 kHz (four-pole Bessel low-pass filter), digitized at 2.5 (whole-cell recording) and 5 kHz (single channel recording), and analysed on a personal computer (PC-98 RL, NEC, Japan) having an AD converter (ADX-98E, Canopus Electronics, Japan).

Calcium channel current recording

In whole-cell recording, L-type Ca^{2+} -channel current was measured in an external solution containing 2 mM Ba^{2+} . The Ca^{2+} channel current carried by Ba^{2+} was stable, and did not exhibit the 'run-down' phenomenon during 30 min after a start of voltage clamp. The Ba^{2+} current was suitable for observing an inhibitory effect of ANP and cyclic GMP derivatives on the Ca^{2+} channel activity.

The liquid junction potential between intra-pipette solution and external normal Tyrode solution was -8 mV. Therefore, all values of potentials given in whole-cell experiments were corrected.

Single-channel recording

In cell-attached recording of single-channel activities, the cells were perfused with an external depolarizing solution containing 150 mM K^{+} . The membrane potential of the cells should be approximately 0 mV in this solution. This allowed us to approximate the potential of the patch membrane from the value of the pipette potential.

Activities of a single Ca^{2+} channel were measured as Ba^{2+} current through the channel, by use of a pipette solution containing 110 mM Ba^{2+} . However, the basal activity was too low to observe any inhibitory effect on the Ca^{2+} channel activity of ANP and cyclic GMP derivatives. Therefore, Bay K 8644 (1 μM), a Ca^{2+} channel activator, was used to enhance the channel activities in almost all experiments. In this condition, the inhibitory effect of tested compounds was consistently observed.

Single-channel currents were elicited by depolarization test pulses to 0 mV from a holding potential of -80 mV. The duration of the test pulse was 150 ms. The test pulses were applied to cells at a rate of 0.5 Hz.

Channel activity is represented by the product NP_0 , i.e. number of channels in the patch (N) times a proportion of channel open time to the total time (150 ms) of sampled test-depolarization (P_0). An ensemble-averaged current was digitally calculated from sampled current traces (60–120 traces).

Experimental solutions and drugs

The composition of Tyrode solution was (mM): NaCl 143, KCl 5.4, CaCl_2 1.8, MgCl_2 0.5, NaH_2PO_4 0.33, glucose 5.5 and HEPES-NaOH buffer 5. The composition of KB solution was (mM): KCl 40, KOH 70, KH_2PO_4 20, L-glutamic acid 50, taurine 20, MgCl_2 3, EGTA 1, glucose 10, and HEPES-KOH buffer 10. The external solution for the whole-cell recording contained (mM): choline Cl 143, CsCl_2 5.4, BaCl_2 2, MgCl_2 0.5, 4-aminopyridine 3, glucose 5.5, and HEPES-HCl buffer 5. In order to eliminate a weak cholinergic effect of choline Cl, atropine (5 μM) was added to the external solution for whole-cell recording. Recently, atropine was shown to exert an inhibitory effect on activation of inhibitory G-proteins, which was observed even in the absence of agonists to muscarinic receptors (Hanf *et al.*, 1993). Therefore, it is possible that, in the presence of atropine, adenylyl cyclase activity is altered from the basal state and thus the measured Ca^{2+} currents are not accurately indicative of its basal activity. However, having confirmed

the essentially similar effect of ANP on the Ca^{2+} channel current in the absence of atropine ($n = 2$, data not shown), we assumed that the influence of atropine would be minimum in our present study. The composition of the pipette solution for whole-cell recording was (mM): L-glutamic acid 110, CsCl 20, CsOH 110, MgCl_2 3, Na_2ATP 5, Na_2 creatine phosphate 5, EGTA 10 and HEPES- CsOH buffer 5. For the single-channel recording, the external depolarizing solution containing (mM): KCl 140, MgCl_2 0.5, glucose 5.5 and EGTA 10; HEPES-KOH buffer 5, was perfused extracellularly. The pipette solution for the single-channel recording contained (mM): BaCl_2 110, Bay K 8644 0.001, and HEPES-Tris base buffer 5. The pH of all experimental solutions used in the present study was adjusted to 7.4.

α -hANP (1–28) and methionine-oxidized ANP were obtained from Peptide Institute Inc. (Osaka, Japan), and were dissolved in 10 mM Na-phosphate buffer (pH 7.0) to provide a 10 μM stock solution. Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer, Germany) was dissolved in ethanol to provide a 10 mM stock solution. 8-Bromo-cyclic GMP and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Sigma (St. Louis, U.S.A.). These two compounds were directly dissolved in the external solutions to provide solutions at desired concentration.

Statistics

All values are presented as mean $\pm$ s.e.mean. Statistical analyses were performed with Student's paired t test; $P < 0.05$ was defined as significant. Time courses of inactivation of the Ca^{2+} channel current were fitted by the method of least squares.

Results

Effects of human natriuretic peptide (ANP)

Whole-cell Ba^{2+} currents carried through L-type Ca^{2+} channels were elicited by depolarizing steps of 300 ms duration from a holding potential of -78 mV in rabbit ventricular cells. In these cells, K^{+} currents were blocked with intra- and extracellular Cs^{+} , and fast Na^{+} current was blocked by a substitution of extracellular Na^{+} with choline. ANP at a concentration of 30 nM decreased the Ca^{2+} channel currents elicited by the depolarizations above -28 mV (Figure 1a). Figure 1b shows relations between the peak current and the potential of depolarising pulse. ANP did not shift the potentials for the threshold (-40 mV) and the maximum peak current (-10 mV). Figure 2 shows concentration-response relation between ANP and the Ca^{2+} channel current. ANP at concentrations above 10 nM decreased the peak current at the depolarization steps to -8 mV. We could not observe a maximal response to ANP even at concentrations of 100 nM. Methionine-oxidized ANP (Met(O)¹²-ANP, 30 nM), an inactive analogue of ANP (Watanabe *et al.*, 1988), did not decrease the current ($-1.5 \pm 4.1\%$, $n = 4$). This finding strongly indicates that the ANP-induced decrease in the Ca^{2+} channel current may be mediated by ANP receptors (A- or B-receptor) (Koller & Goeddel, 1992).

Effects of 8-bromo-cyclic GMP

ANP increases intracellular production of cyclic GMP in rabbit isolated ventricular cells (Cramb *et al.*, 1987). Therefore, it is possible that the ANP-induced inhibition of the Ca^{2+} channel current is mediated by intracellular cyclic GMP. Figure 3 shows the effects of 8-bromo-cyclic GMP, a membrane-permeable derivative of cyclic GMP, on the Ca^{2+} channel current of rabbit ventricular cells. 8-Bromo-cyclic GMP at a concentration of 300 μM decreased the Ca^{2+} channel current elicited by 300 ms depolarizations from the hold-

ing potential of -78 mV. Current-voltage relations of the Ca^{2+} channel current (Figure 3b) indicate that 8-bromo-cyclic GMP decreased the current without affecting the threshold potential and the potential for the maximum current. In 10 cells, 8-bromo-cyclic GMP decreased the peak current at the depolarization steps to -8 mV by $23.8 \pm 3.1\%$. Increasing its concentration to 1 mM did not result in a further increase of its suppressive effect on the Ca^{2+} channel current ($15.1 \pm 3.5\%$, $n = 3$). Therefore, $300 \mu\text{M}$ 8-bromo-cyclic GMP exerted the maximal suppressive effect on the Ca^{2+} channel current in the rabbit ventricular cells. This finding was different from the data obtained in chick embryonic heart cells (Tohse & Sperelakis, 1991) in which 1 mM 8-bromo-cyclic GMP abolished Ca^{2+} channel activity.

In order to exclude a possible contribution of phosphodiesterase (PDE) to the inhibition of the Ca^{2+} channel current by 8-bromo-cyclic GMP, we examined the effect of

8-bromo-cyclic GMP on the Ca^{2+} channel current in the cells pretreated with 3-isobutyl-1-methyl-xanthine (IBMX). IBMX is well known to inhibit non-selectively all types of PDE (Simmons & Hartzell, 1988). IBMX ($100 \mu\text{M}$) alone markedly increased the Ca^{2+} channel current (Figure 4a), indicating that IBMX inhibited PDE, and then increased cyclic AMP. In the presence of IBMX, 8-bromo-cyclic GMP ($300 \mu\text{M}$) still decreased the Ca^{2+} channel current (Figure 4a). In 4 cells, 8-bromo-cyclic GMP decreased the peak current by $29.8 \pm 8.1\%$ of the IBMX-stimulated current. These findings suggest that increase in intracellular cyclic GMP inhibits the Ca^{2+} channel current without involving activation of PDE. 8-Bromo-cyclic GMP is reported to be a potent activator of cyclic GMP-dependent protein kinase (PKG) (Lincoln & Corbin, 1983). It is thus quite possible that 8-bromo-cyclic GMP inhibits the Ca^{2+} channel current through activation of PKG.

Figure 1 Effects of 30 nM atrial natriuretic peptide (ANP) on whole-cell Ba^{2+} current through the L-type Ca^{2+} channels of freshly isolated rabbit ventricular myocytes. (a) Superimposed current tracings elicited by 300 ms depolarizing test pulses to -28 , -18 , 2 and 22 mV from a holding potential (HP) of -78 mV. (○) Control currents at each potential; (●) current after external application of 30 nM ANP. (b) Current-voltage relation of the Ca^{2+} channel current (Ba^{2+} current). Amplitude of peak currents at each potential were plotted in absence (○) and presence (●) of 30 nM ANP.

Figure 2 Concentration-response relation between atrial natriuretic peptide (ANP) and the decrease in Ca^{2+} channel current. Numbers in parentheses give the number of cells. Data are mean $\pm$ s.e.mean. *Difference from control is statistically significant at $P < 0.05$.

Figure 3 Effects of $300 \mu\text{M}$ 8-bromo-cyclic GMP on whole-cell Ca^{2+} channel current. (a) Superimposed current tracings elicited by 300 ms depolarizing test pulses from a holding potential (HP) of -78 mV. (○) Control current at each potential; (●) current after external application of $300 \mu\text{M}$ 8-bromo-cyclic GMP. (b) Current-voltage relation of the Ca^{2+} channel current. Amplitude of peak currents at each potential were plotted in absence (○) and presence (●) of $300 \mu\text{M}$ 8-bromo-cyclic GMP.

Figure 4 (a) Effects of 8-bromo-cyclic GMP ($300 \mu\text{M}$) on the Ca^{2+} channel current in the presence of $100 \mu\text{M}$ 3-isobutyl-1-methylxanthine (IBMX). Current tracings (1), (2) and (3) indicate control, IBMX, and 8-bromo-cyclic GMP with IBMX, respectively. 8-Bromo-cyclic GMP decreased the Ca^{2+} channel current even in the presence of the inhibitor of phosphodiesterase (IBMX). (b) Effects of ANP (30 nM) on the Ca^{2+} channel current decreased by $300 \mu\text{M}$ 8-bromo-cyclic GMP, and atrial natriuretic peptide (ANP) with 8-bromo-cyclic GMP, respectively. ANP did not produce further decrease in the decreased Ca^{2+} channel current. The current was elicited by 300 ms depolarizing pulse to -8 mV from the holding potential of -78 mV, at a rate of 0.1 Hz.

Relationship between ANP and 8-bromo-cyclic GMP

To confirm the involvement of cyclic GMP in the ANP-induced inhibition of the Ca^{2+} channel current, we examined whether ANP was able to exert its suppressive effect on the Ca^{2+} channel current which was maximally inhibited by 8-bromo-cyclic GMP (Figure 4b). ANP at a concentration of 30 nM failed to suppress further the Ca^{2+} channel current inhibited by 300 μM 8-bromo-cyclic GMP ($3.2 \pm 3.1\%$ decrement of the cyclic GMP-decreased current, $n = 6$). This finding indicated that ANP and 8-bromo-cyclic GMP share a common pathway for the inhibition of the Ca^{2+} channel current, and that the ANP-induced inhibition of the Ca^{2+} channel current is likely to be mediated by cyclic GMP production and resulting PKG activation.

Effects of ANP on single-channel activity

In order to examine the effects of ANP and cyclic GMP on single-channel activities of the L-type Ca^{2+} channels, the cell-attached patch clamp recording was performed by use of patch pipettes containing 110 mM Ba^{2+} and 1 μM Bay K 8644. Unitary Ba^{2+} currents through the channels were elicited by 150 ms-depolarization to 0 mV from a holding

potential of -80 mV, at a rate of 0.5 Hz (Figure 5). Extracellular perfusion with 30 nM ANP markedly inhibited the activities of the Ca^{2+} channel without affecting the unit amplitude of channels. Figure 5 also shows that the channel openings were rarely observed in the later period during the depolarizations. The lowest traces show the ensemble-averaged current of the Ca^{2+} channels. ANP decreased the peak current and accelerated decay of the current. Similar effects were observed in another 4 patches (Table 1). On the other hand, the inactive ANP (Met(O)¹²-ANP, 30 nM) did not inhibit the channel openings, and did not change the ensemble-averaged current ($n = 5$, data not shown). Figure 6a shows a time course of the NP_0 value during each depolarizing pulse. The NP_0 values decreased within 2 min of application of 30 nM ANP, and recovered gradually after washout of ANP. Therefore, the decrease by ANP in NP_0 was not caused by the 'run-down' phenomenon.

Effects of 8-bromo-cyclic GMP on single-channel activity

Figure 7 shows effects of 300 μM 8-bromo-cyclic GMP on the single-channel activities of the Ca^{2+} channels. 8-Bromo-cyclic GMP also inhibited the channel openings, as observed in the

Figure 5 Effects of atrial natriuretic peptide (ANP) on single Ca^{2+} channel activity in rabbit cardiomyocytes. The channel activities were elicited by 150 ms-depolarizing pulses to 0 mV (from a holding potential of -80 mV) every 2 s in the cell-attached patch configuration. Patch pipettes contained 110 mM Ba^{2+} and 1 μM Bay K 8644. Pulse protocol is shown above the set of tracings. Representative current tracings were chosen. Linear leak and capacity currents have been subtracted. The ensemble-averaged Ca^{2+} current from 90 tracings (including tracings shown in this figure) are shown at the bottom of the tracings. The thin horizontal line indicates the zero current level.

Table 1 Effects of atrial natriuretic peptide (ANP) and 8-bromo-cyclic GMP on characteristics of the single channel activities of the Ca^{2+} channel

	Averaged NP_0 (%decrease)	Peak Amp. (%decrease)	τ (ms)	
			Before drug	After drug
ANP (30 nM)	$57.9 \pm 11.1^*$	$49.5 \pm 6.4^*$	41.8 ± 11.7	$21.3 \pm 2.9^{**}$
8-Bromo-cyclic GMP (300 μM)	$54.1 \pm 9.7^*$	$36.7 \pm 9.4^*$	77.9 ± 7.7	$48.2 \pm 7.3^{**}$

NP_0 : averaged from 60–120 depolarizations, Peak Amp.: peak amplitude of the ensemble-averaged current; τ : time constant of decay of the ensemble-averaged current. $n = 5$, mean $\pm$ s.e. $^*P < 0.05$ vs. control, $^{**}P < 0.05$ vs. before drug.

presence of ANP. The channel openings were rarely observed in the later period of the depolarizations in the presence of 8-bromo-cyclic GMP. 8-Bromo-cyclic GMP decreased the peak amplitude of ensemble-averaged current shown in the lowest panels. A decay of the ensemble-averaged current was accelerated by 8-bromo-cyclic GMP. Similar effects were observed in another 4 patches (Table 1). All the changes produced by 8-bromo-cyclic GMP were very similar to those produced by ANP (Figure 5). Figure 6b shows the time course of the 8-bromo-cyclic GMP-induced suppressive effects on the NP_0 value during each depolarizing pulse. The effect of 8-bromo-cyclic GMP was also reversible after washing of the drug.

Figure 6 Time course of effects of atrial natriuretic peptide (ANP) and 8-bromo-cyclic GMP on NP_0 value of each depolarization pulse. The depolarization pulses were elicited at a rate of 0.5 Hz.

Comparison of effects of ANP and cyclic GMP on single-channel activity

Table 1 summarizes the effects of ANP and 8-bromo-cyclic GMP on averaged NP_0 from 60–120 depolarization pulses, the peak amplitude of the ensemble-averaged current, and the time constant of decay of the ensemble-average current. ANP and 8-bromo-cyclic GMP decreased the NP_0 value. The peak amplitude of the ensemble-averaged current was also decreased by ANP and 8-bromo-cyclic GMP. The decay of the ensemble-averaged current was well-fitted by a single exponential curve. ANP and 8-bromo-cyclic GMP decreased the time constant of decay of the ensemble-averaged current. However, ANP and 8-bromo-cyclic GMP did not affect the time constant of the whole-cell Ca^{2+} channel current (data not shown). At the present time, we cannot explain this discrepancy.

Discussion

In the present study, we have examined the effects of ANP on the cardiac L-type Ca^{2+} channels, and compared them with those of 8-bromo-cyclic GMP. The results obtained clearly showed that ANP inhibited the Ca^{2+} channel activities in both the whole-cell voltage-clamp experiments and the single-channel current recordings, and that the ANP-induced inhibition of the Ca^{2+} channel activity had similar characteristics to those of the cyclic GMP-induced one. Furthermore, the inhibitory effects of ANP and cyclic GMP were not additive, suggesting that the ANP-induced inhibition is mediated by an increase in intracellular cyclic GMP.

Relationship between ANP and cyclic GMP

ANP is well known to increase the intracellular concentration of cyclic GMP in various types of cells (Needleman *et al.*, 1989) including cardiomyocytes of rabbits (Cramb *et al.*, 1987), chick embryos (Vaxelaire *et al.*, 1989), and rats (Neyses & Vetter, 1989; McCall & Fried, 1990). Detection of

Figure 7 Effects of 8-bromo-cyclic GMP on single Ca^{2+} channel activities by use of a pipette containing $1 \mu\text{M}$ Bay K 8644. Pulse protocol is shown above the set of tracing. The representative current tracings are chosen. Linear leak and capacity currents have been subtracted. The ensemble-averaged Ca^{2+} currents from 60 tracings (including tracings shown in this figure) are shown at the bottom of the tracings.

guanylate cyclase activities in molecular form of a subtype of the ANP receptor (Leitman *et al.*, 1986; Chinkers *et al.*, 1989; Koller & Goeddel, 1992) implied that the ANP-induced increase in cyclic GMP occurs through activation of its specific receptor mechanism.

The present study provides pharmacological evidence demonstrating a causal relation between ANP and cyclic GMP in rabbit ventricular cells. Firstly, both ANP and 8-bromo-cyclic GMP inhibited the Ca^{2+} channel activity. In addition, both agents produced similar effects on the kinetics of the Ca^{2+} channels in the macroscopic and microscopic current levels. Secondly, Met(O)^{12} -ANP, which is less potent in increasing the cyclic GMP content (Watanabe *et al.*, 1988), failed to inhibit the channel activity. Finally, in the presence of 8-bromo-cyclic GMP, ANP failed to decrease further the Ca^{2+} channel current. All these findings indicate that ANP inhibits the Ca^{2+} channel activity through an intracellular increase in cyclic GMP.

Target molecule for ANP-increased cyclic GMP

Hartzell & Fischmeister (1986), using frog ventricular cells, reported that intrapipette cyclic GMP suppressed the β -agonist-stimulated Ca^{2+} current. This suppression was not observed in the cells pretreated with IBMX. In addition, the Ca^{2+} current amplified by IBMX was not suppressed by intracellular perfusion of 8-bromo-cyclic GMP, which is reported to be ineffective in activating PDE II (Lincoln & Corbin, 1983). From these findings, they concluded that intracellular cyclic GMP might decrease the stimulated Ca^{2+} current through activation of PDE II. However, the situation is quite different in mammalian cardiac cells. It seems unlikely that reduction of cyclic AMP through the activation of PDE II is the major pathway for regulation of the Ca^{2+} channel by cyclic AMP. Levi *et al.* (1989), Méry *et al.* (1991) and Ono & Trautwein (1991) observed that intracellular application of 8-bromo-cyclic GMP decreased the stimulated Ca^{2+} current, and that pretreatment with IBMX did not inhibit the cyclic GMP-induced decrement of Ca^{2+} current. These findings and ours are inconsistent with those of Hartzell & Fischmeister (1986), and deny the possibility of involvement of PDE II in cyclic GMP-induced suppression of Ca^{2+} current.

8-Bromo-cyclic GMP has been reported to be a potent activator of PKG (Lincoln & Corbin, 1983; White *et al.*, 1993). Ono & Trautwein (1991) and Méry *et al.* (1991) pointed out a similarity between suppressive effects induced by 8-bromo-cyclic GMP and PKG on the β -receptor-stimulated Ca^{2+} channel current. Therefore, it is possible that 8-bromo-cyclic GMP activates PKG and inhibits the Ca^{2+} channel activity in rabbit ventricular cells. In the present study, we found that pretreatment of rabbit cells with 8-bromo-cyclic GMP completely abolished the ANP-induced inhibition of the Ca^{2+} channel current. This finding strongly suggests that ANP inhibits the Ca^{2+} channel activity through the activation of PKG.

PKG-induced inhibition of basal activity

Méry *et al.* (1991) and Ono & Trautwein (1991) reported that intracellular administration of PKG did not affect the basal activity of Ca^{2+} channels, but was effective in inhibiting the β -stimulated Ca^{2+} channel current. In contrast with these

reports, it was found that the basal Ca^{2+} channel activity was inhibited by 8-bromo-cyclic GMP in chick embryonic heart cells (Wahler *et al.*, 1990; Tohse & Sperelakis, 1991). The present study revealed that ANP and 8-bromo-cyclic GMP inhibited the basal Ca^{2+} channel current in rabbit ventricular cells. The inhibition of the basal Ca^{2+} channel activity by cyclic GMP-PKG system was also observed in pituitary tumour cells (White *et al.*, 1993) and vascular smooth muscular cells (Clapp & Gurney, 1991).

One possible explanation of this discrepancy between the present study and those of Méry *et al.* (1991) and Ono & Trautwein (1991) is that the rabbit and chick cardiomyocytes may have very high basal (non-stimulated) cyclic AMP activity. With high activity of cyclic AMP, the Ca^{2+} channels may be already activated. Actually, the chick cell was reported to have a high level of intracellular cyclic AMP (Thakkar & Sperelakis, 1987). It is noteworthy that inhibition of the basal Ca^{2+} channel activity was clearly observed in the cell-attached patch clamp, in which the intracellular environment was not modified, as shown in the present study and by Tohse & Sperelakis (1991).

Another explanation is that native PKG may be very effective in inhibiting the basal Ca^{2+} channel activity. As mentioned above, ANP inhibited the basal Ca^{2+} channel current in mammalian cells (Sorbera & Morad, 1990; Le-Grand *et al.*, 1992). Therefore, the native PKG activated by ANP may be more potent in inhibiting basal activity than the PKG perfused intracellularly through the patches pipettes (Méry *et al.*, 1991; Ono & Trautwein, 1991). Further experiments are required to clarify the discrepancy concerning the cyclic GMP-induced inhibition of the basal Ca^{2+} channel activity. Recently, Han *et al.* (1993) reported that cyclic GMP and 8-bromo-cyclic GMP increased the basal Ca^{2+} current in rabbit ventricular myocytes. At the present time, we cannot explain the discrepancy between their results and ours.

Significance of ANP-induced inhibition of Ca^{2+} channels

Recently, several studies showed that ANP directly decreased contractility in isolated cardiomyocytes (Neynes *et al.*, 1989; Vaxelaire *et al.*, 1989; McCall & Fried, 1990). This negative inotropic action of ANP was also reported *in vivo* (Lappe *et al.*, 1985; Seymour *et al.*, 1987). Therefore, it is possible that the ANP-induced inhibition of Ca^{2+} channel activity contributes to the negative inotropic action of ANP. The negative inotropic action of ANP may be important for pathogenesis of chronic heart failure (CHF) in addition to the physiological regulation of cardiac function. Plasma ANP was reported to be increased in the dog with experimental heart failure (Perrella *et al.*, 1992) and patients with CHF (Shenker *et al.*, 1985; Burnett *et al.*, 1986). Furthermore, CHF patients with high levels of ANP had a significantly low rate of survival (Gottlieb *et al.*, 1989). The ANP-induced inhibition of the Ca^{2+} channel activity should be investigated in some model animals with CHF.

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References

- BERGEY, J.L. & KOTLER, D. (1985). Effects of atriopeptins I, II and III on atrial contractility, sinus nodal rate (guinea pig) and agonist-induced tension in rabbit aortic strips. *Eur. J. Pharmacol.*, **110**, 227–281.
- BKAILY, G., PERRON, N., WANG, S., SCULPTOREANU, A., JACQUES, D. & MÉNARD, D. (1993). Atrial natriuretic factor blocks the high-threshold Ca^{2+} current and increases K^{+} current in fetal single ventricular cells. *J. Mol. Cell. Cardiol.*, **25**, 1305–1316.
- BURNETT, Jr, J.C., KAO, P.C., HU, D.C., HESE, D.W., HEUBLEIN, D., GRANGER, J.P., OPGENORTH, T.J. & REEDER, G.S. (1986). Atrial natriuretic peptide elevation in congestive heart failure in the human. *Science*, **231**, 1145–1147.
- CHINKERS, M., GARBERS, D.L., CHANG, M.-S., LOWE, D.G., CHIN, H., GOEDDEL, D.V. & SCHULZ, S. (1989). A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature*, **338**, 78–83.

- CLAPP, L.H. & GURNEY, A.M. (1991). Modulation of calcium movements by nitroprusside in isolated vascular smooth muscle cells. *Pflügers Arch.*, **418**, 462–470.
- CRAMB, G., BANKS, R., RUGG, E.L. & AITON, J.F. (1987). Action of atrial natriuretic peptide (ANP) on cyclic nucleotide concentrations and phosphatidylinositol turnover in ventricular myocytes. *Biochem. Biophys. Res. Commun.*, **148**, 962–970.
- CRISCOINE, L., BURDET, R., HANNI, H., KAMBER, B., TRUOG, A. & HOFBAUER, K.G. (1987). Systemic and regional hemodynamic effects of atriopeptin II in anaesthetized rats. *J. Cardiovasc. Pharmacol.*, **9**, 135–141.
- FISCHMEISTER, R. & HARTZELL, H.C. (1987). Cyclic guanosine 3',5'-monophosphate regulates the calcium current in single cells from frog ventricle. *J. Physiol.*, **387**, 453–472.
- GISBERT, M.-P. & FISCHMEISTER, R. (1988). Atrial natriuretic factor regulates the calcium current in frog isolated cardiac cells. *Circ. Res.*, **62**, 660–667.
- GOTTLIEB, S.S., KUKIN, M.L., AHERN, D. & PACKER, M. (1989). Prognostic importance of atrial natriuretic peptide in patients with chronic heart failure. *J. Am. Coll. Cardiol.*, **13**, 1534–1539.
- HAN, J., LEEM, C., AHN, C., SO, I., KIM, E., HO, W. & EARM, Y.E. (1993). Effect of cyclic GMP on the calcium current in rabbit ventricular myocytes. *Korean J. Physiol.*, **27**, 51–62.
- HANF, R., LI, Y., SZABO, G. & FISCHMEISTER, R. (1993). Agonist-independent effects of muscarinic antagonists on Ca^{2+} and K^{+} currents in frog and rat cardiac cells. *J. Physiol.*, **461**, 743–765.
- HARTZELL, H.C. & FISCHMEISTER, R. (1986). Opposite effects of cyclic GMP and cyclic AMP on Ca^{2+} current in single heart cells. *Nature*, **323**, 273–275.
- ISENBERG, G. & KLÖCKNER, U. (1982). Calcium tolerant ventricular myocytes prepared by preincubation in a 'KB medium'. *Pflügers Arch.*, **395**, 6–18.
- KOLLER, K.J. & GOEDEL, D.V. (1992). Molecular biology of the natriuretic peptides and their receptors. *Circulation*, **86**, 1081–1088.
- LAPPE, R.W., SMITS, J.F.M., TODT, J.A., DEBETS, J.J.M. & WENDT, R.L. (1985). Failure of atriopeptin II to cause arterial vasodilation in the conscious rat. *Circ. Res.*, **56**, 606–612.
- LEGRAND, B., DEROUBAIX, E., COUTETIL, J.-P. & CORABOEUF, E. (1992). Effects of atrionatriuretic factor on Ca^{2+} current and Ca_i -independent transient outward K^{+} current in human atrial cells. *Pflügers Arch.*, **421**, 486–491.
- LEITMAN, D.C., ANDRESEN, J.W., KUNO, T., KAMISAKI, Y., CHANG, J.-K. & MURAD, F. (1986). Identification of multiple binding sites for atrial natriuretic factor by affinity cross-linking in cultured endothelial cells. *J. Biol. Chem.*, **261**, 11650–11655.
- LEVI, R.C., ALLOATTI, G. & FISCHMEISTER, R. (1989). Cyclic GMP regulates the Ca -channel current in guinea pig ventricular myocytes. *Pflügers Arch.*, **413**, 685–687.
- LINCOLN, T.M. & CORBIN, J.D. (1983). Characterization and biological role of the cGMP-dependent protein kinase. *Adv. Cyclic Nucleotide Res.*, **15**, 139–192.
- MCCALL, D. & FRIED, T.A. (1990). Effects of atriopeptin II on Ca influx, contractile behaviour and cyclic nucleotide content of cultured neonatal rat myocardial cells. *J. Mol. Cell. Cardiol.*, **22**, 201–212.
- MÉRY, P.-F., LOHMANN, S.M., WALTER, U. & FISCHMEISTER, R. (1991). Ca^{2+} current is regulated by cyclic GMP-dependent protein kinase in mammalian cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 1197–1201.
- NEEDLEMAN, P., BLAINE, E.H., GREENWALD, J.E., MICHENER, M.L., SAPER, C.B., STOCKMANN, P.T. & TOLUNARY, H.E. (1989). The biochemical pharmacology of atrial peptides. *Annu. Rev. Pharmacol. Toxicol.*, **29**, 23–54.
- NEYSES, L. & VETTER, H. (1989). Action of atrial natriuretic peptide and angiotensin II on the myocardium: studies in isolated rat ventricular cardiomyocytes. *Biochem. Biophys. Res. Commun.*, **163**, 1435–1443.
- ONO, K. & TRAUTWEIN, W. (1991). Potentiation by cyclic GMP of β -adrenergic effect on Ca^{2+} current in guinea-pig ventricular cells. *J. Physiol.*, **443**, 387–404.
- PERRELLA, M.A., SCHWAB, T.R., O'MURCHUM B., REDFIELD, M.M., WEI, C.-M., EDWARDS, B.S. & BURNETT, Jr, J.C. (1992). Cardiac atrial natriuretic factor during evolution of congestive heart failure. *Am. J. Physiol.*, **262**, H1248–H1255.
- SEYMOUR, A.A., SWEET, C.S., STABILITO, I.I. & EMMERT, S.E. (1987). Cardiac and hemodynamic responses to synthetic atrial natriuretic factor in rats. *Life Sci.*, **40**, 511–519.
- SHENKER, Y., SIDER, R.S., OSTAFIN, E.A. & GREKIN, R.J. (1985). Plasma levels of immunoreactive atrial natriuretic factor in healthy subjects and in patients with edema. *J. Clin. Invest.*, **76**, 1684–1687.
- SIMMONS, M.A. & HARTZELL, H.C. (1988). Role of phosphodiesterase in regulation of calcium current in isolated cardiac myocytes. *Mol. Pharmacol.*, **33**, 664–671.
- SORBERA, L.A. & MORAD, M. (1990). Atrionatriuretic peptide transforms cardiac sodium channels into calcium-conducting channels. *Science*, **247**, 969–973.
- THAKKAR, J.K. & SPERELAKIS, N. (1987). Changes in cyclic nucleotide levels during embryonic development of chick hearts. *J. Dev. Physiol.*, **9**, 497–505.
- TOHSE, N., NAKAYA, H., HATTORI, Y., ENDOU, M. & KANNO, M. (1990). Inhibitory effect mediated by α_1 -adrenoceptors on transient outward current in isolated rat ventricular cells. *Pflügers Arch.*, **415**, 575–581.
- TOHSE, N. & SPERELAKIS, N. (1991). cGMP inhibits the activity of single calcium channels in embryonic chick heart cells. *Circ. Res.*, **69**, 325–331.
- VAXELAIRE, J.-F., LAURENT, S., LACOLLEY, P., BRIAND, V., SCHMITT, H. & MICHEL, J.B. (1989). Atrial natriuretic peptide decreases contractility of cultured chick ventricular cells. *Life Sci.*, **45**, 41–48.
- WAHLER, G.M., RUSCH, N.J. & SPERELAKIS, N. (1990). 8-Bromo-cyclic GMP inhibits the calcium channel current in embryonic chick ventricular myocytes. *Can. J. Physiol. Pharmacol.*, **68**, 531–534.
- WANGLER, R.D., BREUHAUS, B.A., OTERO, H.O., HASTINGS, D.A., HOLZMAN, M.D., SANEI, H.H., SPARKS, Jr, H.V. & CHIMOSKEY, J.E. (1985). Coronary vasoconstrictor effects of atriopeptin II. *Science*, **230**, 558–561.
- WATANABE, T.X., NODA, Y., CHINO, N., NISHIUCHI, Y., KIMURA, T., SAKAKIBARA, S. & IMAI, M. (1988). Structure-activity relationships of α -human atrial natriuretic peptide. *Eur. J. Pharmacol.*, **147**, 49–57.
- WHITE, R.E., LEE, A.B., SHCHERBATKO, A.D., LINCOLN, T.M., SCHONBRUNN, A. & ARMSTRONG, D.L. (1993). Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation. *Nature*, **361**, 263–266.

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