Phosphorylation-independent inhibition by intracellular cyclic nucleotides of brain inwardly rectifying K^+ current expressed in *Xenopus* oocytes

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Abstract An inwardly rectifying K^+ current, which was heterologously expressed in *Xenopus* oocytes, was inhibited by isoproterenol, a β-adrenergic agonist. Poly(A)$^+$ mRNA isolated from guinea-pig brain was injected into oocytes 2–3 days before experiments. Isoproterenol inhibition of the K^+ current was time- and voltage-dependent: the inhibition became faster and more pronounced as the command voltage steps were applied to more negative potentials. This inhibition was prevented by propranolol. Dibutylyl cyclic (dB-c) AMP could mimic the effect of isoproterenol, while injection of the catalytic subunit of cAMP-dependent protein kinase into the oocytes did not affect the K^+ current. Inhibitors of the protein kinases, WIPTIDE and H-8, did not prevent the inhibition by dB-cAMP. Furthermore, dB-cGMP also inhibited the K^+ current in a similar time- and voltage-dependent manner. We propose that the phosphorylation-independent action of cyclic nucleotides mediates β-adrenergic inhibition of brain inwardly rectifying K^+ channels expressed in *Xenopus* oocytes.

Key words: Potassium channel; Brain; Isoproterenol; Dibutylyl cyclic AMP; Dibutylyl cyclic GMP; *Xenopus* oocyte

1. Introduction

Inwardly rectifying K^+ channels play a pivotal role in maintaining resting potential, in regulation of action potential duration, and in buffering extracellular K^+ in various cells including neurons and glial cells [1]. The K^+ channel activity can be modulated by various neurotransmitters and metabolites. Most well-known cases are the G protein-gated inwardly rectifying K^+ (K_G) channels and the ATP-sensitive K^+ (K_{ATP}) channels. The K_G channels are activated by the βγ subunits of pertussis toxin-sensitive GTP-binding proteins, thus its activity can be controlled by various rhodopsin-type G protein-coupled receptors [2]. Activation of K_G channels causes inhibition of excitation in various cells. Inhibition of K_{ATP} channels by glucose causes excitation and secretion of insulin in pancreatic β cells [3]. Although modulation of classical inwardly rectifying K^+ channels are less well documented, it was reported in cultured neurons from, e.g. locus ceruleus and nucleus basalis that neurotransmitters, such as substance P, inhibit inwardly rectifying K^+ channels via activation of PKC [4,5]. Inhibition of classical inwardly rectifying K^+ channels results in increase of cellular excitability and should have important physiological implications, especially in the central nervous system.

In the present study, we have found that isoproterenol, a β-adrenergic agonist, inhibits a brain inwardly rectifying K^+ current heterologously expressed in *Xenopus* oocytes. The inhibition was time- and voltage-dependent and apparently mediated by an increase of intracellular cAMP, but surprisingly which was independent of the activation of cAMP-dependent protein kinase.

2. Material and methods

2.1. Preparation of poly(A)$^-$ mRNA from the guinea pig brain

Total RNA was extracted by the acid guanidium isothiocyanate/phenol/chloroform method [6]. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose [7].

2.2. Functional expression of a brain inwardly rectifying K^+ current

The poly(A)$^-$ mRNA was dissolved in sterile water, and injected to manually defolliculated oocytes (50 nl of 100 ng/μl). After injection, oocytes were incubated in a modified Barth solution at 18°C, and electrophysiological studies were undertaken 48–96 h later after injection. Expression of inwardly rectifying K^+ channel currents by injection of total mRNA extracted from guinea pig brain and effects of cyclic nucleotides showed significant seasonal differences, due to unidentified reasons. The present data were obtained mainly between April and June, 1992, at Mayo Clinic, Rochester, MN.

2.3. Electrophysiology

Two-electrode voltage clamp experiments were performed with microelectrodes using a commercial amplifier (Axoclamp, Axon Instruments, Foster City, CA). The microelectrodes, when filled with 3 M KCl, had resistances of 0.5–1.5 MΩ. Oocytes were bathed in a solution which contained 30 mM KCl, 60 mM NaCl, 3 mM $MgCl_2$, 5 mM HEPES (pH was adjusted to 7.4 with NaOH). Oocytes were voltage-clamped at −30 mV and voltage steps of 2.0 s duration were applied from −130 to 0 mV in 10 mV increments every 7 s. Data were stored on video tapes using a PCM data recording system and subsequently replayed for computer analysis.

3. Results

Injection of the poly(A)$^+$ mRNA into *Xenopus* oocytes resulted in expression of a classical inwardly rectifying K^+ current. The inward current evoked by command voltage steps (2 s in duration) to −100 mV from a holding potential of −30 mV rapidly activated and gradually inactivated. This current was not observed in oocytes which were not injected with the poly(A)$^+$ mRNA ($n=22$). Isoproterenol (ISP, 1 μM), added to the bathing solution, inhibited the inward K^+ currents during hyperpolarizing pulses (Fig. 1A). During perfusion of ISP (1 μM), the current at the onset of the command pulse was not affected significantly (91.7 ± 3.3%, mean ± S.D., of the control, $n=5$), while the time-dependent current decay during hyperpolarizing pulses was greatly enhanced by ISP (Fig. 1Ab). At the end of the command pulse, the current was reduced to 49.7 ± 8.9% of the control steady-state current ($n=5$, Fig. 1Ab). Thus, the current inhibition proceeded time-dependently during a hyperpolarizing voltage step. The

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inhibitory effect of ISP was completely reversible after washout (Fig. 1Ac). At the end of this experiment, 2 mM Ba^{2+} added to the bathing solution inhibited the inwardly rectifying K^+ current completely (Fig. 1Ad). This inhibitory effect of ISP on the K^+ current was not observed under application of 1 μM of propranolol ($n=4$, not shown).

Fig. 1B shows the effect of ISP on the inwardly rectifying K^+ current at various membrane potentials. The oocyte was held at −30 mV, where the net membrane current was nearly zero. This was close to the expected K^+ equilibrium potential (−31 mV), assuming an intracellular K^+ concentration of 100 mM in *Xenopus* oocytes [8,9]. The reversal potential of the expressed current was -2 ± 2 mV ($n=4$) in 90 mM $[K^+]_o$, -28 ± 3 mV ($n=10$) in 30 mM $[K^+]_o$, and -60 ± 3 mV ($n=4$) in 10 mM $[K^+]_o$, indicating the expressed currents were dominantly carried by K^+ ions. The outward currents were hardly evoked during depolarizing command steps, but large inward currents were elicited by hyperpolarizing steps, indicating that the expressed K^+ current showed a classical inwardly rectifying property (see also Fig. 4). The inwardly rectifying K^+ currents at various potentials were isolated by subtracting the current under application of 2 mM Ba^{2+} from that in the absence of Ba^{2+} at each command step. ISP hardly affected the peak currents at various potentials. The current decay was more prominently accelerated as the command pulses were applied to more negative potentials under application of ISP. The current-voltage relationships of the Ba^{2+}-sensitive current in the control and under application of ISP clearly indicate that the block by ISP of the K^+ current became more pronounced at more negative potentials. Thus, the blocking effect of ISP on the expressed inwardly rectifying K^+ current was not only time-dependent but also voltage-dependent.

In Fig. 2, the possible involvement of intracellular cAMP-protein kinase A cascade in the β-adrenergic inhibition of the K^+ current was examined. Dibutylyl-cAMP (dB-cAMP, 500 μM), a membrane-permeable cAMP analogue, inhibited the K^+ current in a similar manner to ISP: it hardly affected the initial current but accelerated the current decay during hyperpolarizing pulses (Fig. 2A). This observation suggests that ISP inhibition was mediated by an increase of intracellular cAMP through activation of adenylyl cyclase. Direct intracellular injection of catalytic (C) subunit of cAMP-dependent protein kinase (A-kinase) was, however, without effect on the inward K^+ current, but rather enhanced an outward current at the holding level (Fig. 2B). Furthermore, neither WIPTIDE (1 μM), a potent and specific synthetic peptide inhibitor against A-kinase [10], nor H8 (10 μM), which blocks A-kinase as well as cGMP-dependent protein kinase [11], affected the current inhibition by dB-cAMP (Fig. 2C,D) and by ISP (not shown). During this series of experiments, we noticed that ISP and dB-cAMP sometimes caused transient activation of outward currents at the holding potential level and inward currents at negative potentials (e.g. −100 mV), as in Fig. 2D. Although we have not examined properties of this current, the current could also be evoked by C subunit of A-kinase (Fig. 2B). In Fig. 2D, H8 clearly inhibited activation of this current by dB-cAMP but did not affect the inhibition of inwardly rectifying K^+ current. The results suggest the β-adrenergic inhibition of the K^+ current was caused by an increase of intracellular cAMP, but without the involvement of protein phosphorylation by A-kinase.

In Fig. 3, the effect of another cyclic nucleotide, cGMP, on the heterologously expressed inwardly rectifying K^+ current was examined. dB-cGMP also rapidly inhibited the expressed K^+ current in a time-dependent manner (Fig. 3Ab,c). The peak current was only slightly reduced by dB-cGMP as in the case of dB-cAMP. Both dB-cGMP and dB-cAMP accel-

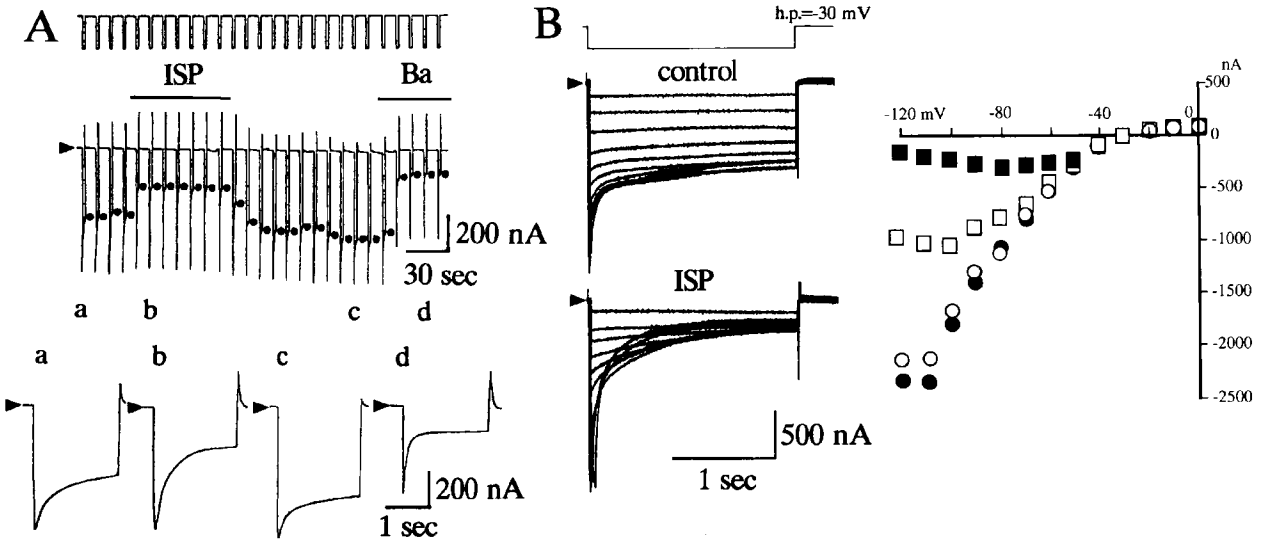

Fig. 1. Isoproterenol (ISP) inhibits brain inwardly rectifying K^+ current expressed in *Xenopus* oocytes. A: ISP (1 μM) and Ba^{2+} (2 mM) were perfused to the oocyte as indicated by the bars above the current trace. Command pulses of 2 s duration to −100 mV were applied form the holding potential of −30 mV as shown in the upper panel. The middle panel depicts the current trace with slow time scale. Arrow heads are the zero current level. ● indicates the current level at the end of the voltage pulse. Representative current traces (indicated by a, b, c, and d) were time-expanded and are shown in the bottom panel. B: Voltage-dependent effect of ISP on the Ba^{2+}-sensitive component of the oocyte membrane current. Command pulses were applied from −130 mV to 0 mV in 10 mV steps from a holding potential of −30 mV before and during ISP application. Then Ba^{2+} (2 mM) was perfused and residual current was subtracted from the record at each voltage. In the left panel, only the inward currents are shown. Right panel: current-voltage relations before (open symbols) and during (closed symbols) ISP application. Circles indicate the peak current, squares the steady-state current at the end of the clamp pulse.

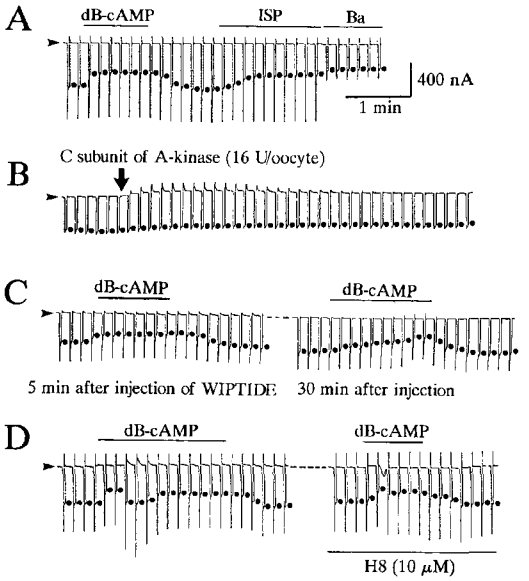

Fig. 2. The dibutylyl (dB) cAMP inhibition of the expressed K^+ current does not involve protein kinase A. K^+ currents were activated by hyperpolarizing pulses to −100 mV from −30 mV. ●, the current level at the end of each pulse. A: dB-cAMP (500 μM), ISP (1 μM) or Ba^{2+} (2 mM) was perfused to the oocyte as indicated by the bars above the current trace. B: The catalytic (C) subunit of cAMP-dependent protein kinase (A-kinase) (16 U) was injected into the oocyte at the arrow. C: The effect of dB-cAMP was examined 5 and 30 min after injection of WIPTIDE (1 μM) into the oocyte. D: The effect of dB-cAMP was examined in the absence and presence of H8 (10 μM) as indicated by the bars above and below the trace.

erated the current decay during the hyperpolarizing voltage steps and blocked the steady-state K^+ current by ~80% of the Ba^{2+}-sensitive component (Fig. 3B,C). Thus, it is likely that cAMP and cGMP inhibit the K^+ current by utilizing a similar mechanism. As in this experiment, *Xenopus* oocytes injected with total mRNAs from brain sometimes expressed a Ba^{2+}-sensitive current of unknown origin as indicated by the outward current tails at −30 mV after hyperpolarizing pulses in Figs. 4 and 5. Because the inhibition of the inwardly flowing K^+ current by cyclic nucleotides were so prominent and because the similar time- and voltage-dependent inhibition were also observed in oocytes which did not express the unidentified currents, we ignored contamination of this type of currents from the current analyses.

Fig. 4 compares the voltage dependence of blocking action of cGMP and cAMP. The currents were recorded in the absence and presence of dB-cAMP and dB-cGMP. The measurement was made on the Ba^{2+} (2 mM)-sensitive component of inward currents. The families of current traces of the Ba^{2+}-sensitive component at various potentials were shown in the left of Fig. 4A. Both of dB-cGMP and dB-cAMP only slightly inhibited the initial currents, accelerated the current decay. The block of the currents at the end of voltage steps became more pronounced as the command potential became more negative (the current-voltage relationship of dB-cGMP effect in the right panel of Fig. 4). In the experiment presented in Fig. 5, inhibition of the unidentified current components at depolarized potentials by cyclic nucleotides contaminated the current traces, which was more evident in the case of dB-cAMP. This current appeared in oocytes during some period of experiments and was difficult to be avoided. However, because the time- and voltage-dependent nature of inhibition of the inwardly rectifying K^+ current was evident, the analyses were done in these oocytes by taking this contamination into account.

In Fig. 5, we examined the voltage-dependent kinetics of ISP and nucleotide-induced inhibition of the inwardly rectifying K^+ current expressed in oocytes. The K^+ current under application of ISP (1 μM) was divided by that in the absence of ISP to obtain the blocking time-course (Fig. 5A). ISP hardly inhibited the current at the onset of the command steps both at −80 and at −60 mV. The inhibition became more pronounced during command steps in both cases, but more rapidly at −80 mV than at −60 mV. The blocking time-courses of the current by ISP both at −80 mV and at −60 mV could be well fitted with a single exponential. Similarly, those by dB-cAMP and dB-cGMP were also fitted with a single exponential at each command potential (not shown). Therefore, the ISP- and cyclic nucleotide-induced block of the inwardly rectifying K^+ current could be described as follows [1]:

$$O \underset{k_{-b}}{\overset{k_b[\text{blocker}]}{\rightleftharpoons}} B$$

$$k_b' = k_b[\text{blocker}]$$

$$(I_{Css} - I_{Bss})/I_{Css} = k_b'/(k_b' + k_{-b})$$

$$\tau = 1/(k_b' + k_{-b})$$

where O and B are open and blocked states of the channel; k_b, k_b' and k_{-b}, the blocking and unblocking rate constants; [blocker], concentration of ISP, dB-cAMP or dB-cGMP; I_{Css}

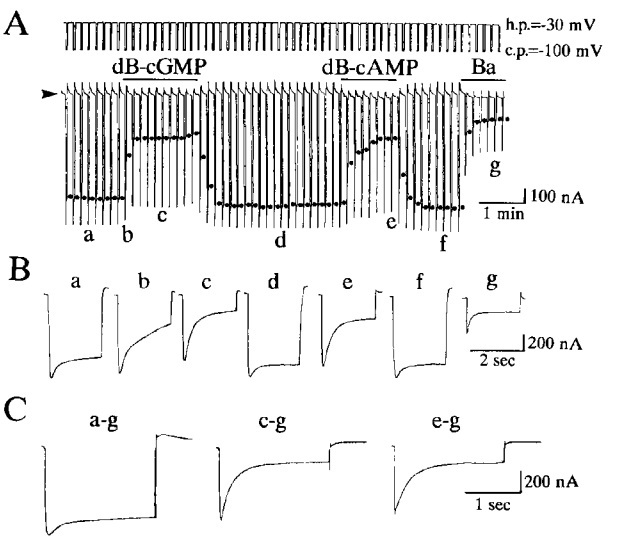

Fig. 3. The inhibitory effect of dibutylyl cGMP (dB-cGMP) on the inwardly rectifying K^+ current. A: Protocol for the drug application was indicated by the bars above the trace. B: Time-expanded current traces. C: Ba^{2+}-sensitive currents in control (a-g), in the presence of dB-cGMP (c-g) or dB-cAMP (e-g).

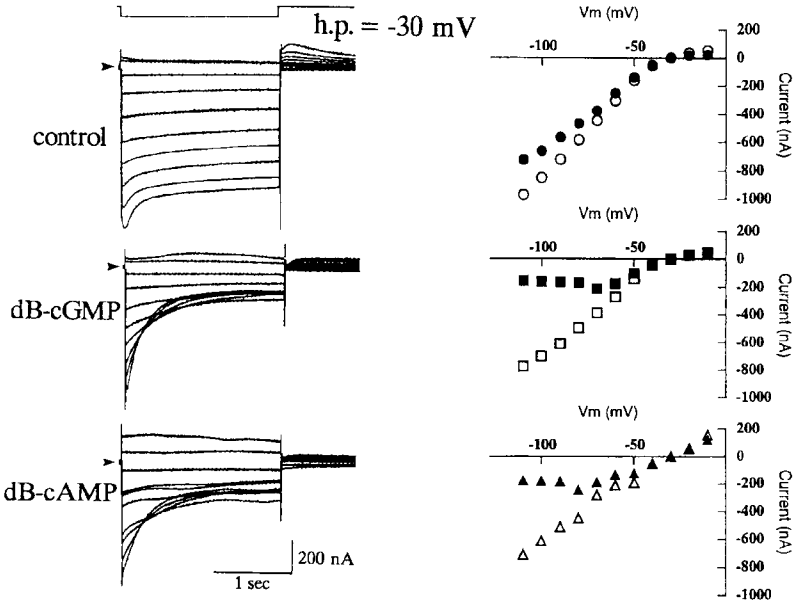

Fig. 4. Voltage-dependent effect of dB-cGMP and dB-cAMP on the Ba^{2+}-sensitive component of the oocyte membrane current. Command pulses were applied from −130 mV to 0 mV in 10 mV steps from a holding potential of −30 mV before and during application of cyclic nucleotides. Then Ba^{2+} (2 mM) was perfused and residual current was subtracted from the record at each voltage. In the left panel, the families of current traces at various potentials in the control, under application of dB-cGMP (500 μM) or dB-cAMP (500 μM) were shown. Right panel: Current-voltage relations of each condition are depicted. Open symbols are the currents at the onset of the pulses and the closed ones are those at the end of the pulses.

and I_{Bss}, the steady-state current amplitudes in control condition and in the presence of blockers; τ, the time constant for block development. In the analysis, k_b[blocker] was treated as a single parameter (k_b'), because the concentration of intracellular cAMP produced by ISP or those of dB-cAMP or dB-cGMP in the oocytes were unknown.

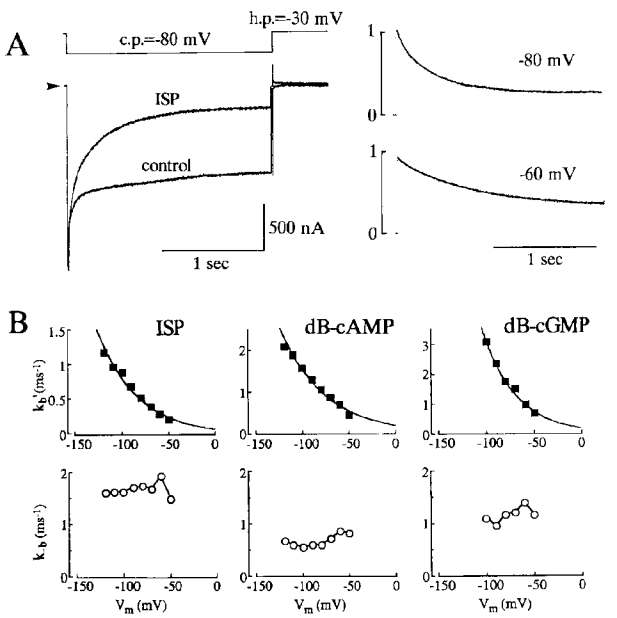

Fig. 5. A: The Ba^{2+}-sensitive component of inwardly rectifying K^+ currents at −80 mV in control and under application of ISP (1 μM) were shown overlapped in the left. In the right, the normalized currents under application of ISP during the voltage steps to −80 mV and −60 mV are shown. The normalized currents were obtained by dividing the current under application of ISP with the control one during command steps to each potential. B: Voltage-dependent kinetics of ISP-, dB-cAMP-, and dB-cGMP-induced inhibition of the expressed K^+ current. The fit curves are: $k_b = 0.062\exp(-0.024\ Vm)$ for ISP, $k_b = 0.154\exp(-0.021\ Vm)$ for dB-cAMP, and $k_b = 0.134\exp(-0.028\ Vm)$. k_{-b} was almost constant in all cases.

Fig. 5B shows calculated blocking and unblocking rate constants in the cases of ISP, dB-cAMP and dB-cGMP as a function of command potentials. In all cases, k_b' increased with hyperpolarization, while k_{-b} was practically voltage-independent.

$$k_b' = k_b'(0)\exp(z'FE/RT)$$

where $k_b'(0)$ is k_b' at 0 mV; z', effective valence of the blocking reaction; F, R, and T have their usual thermodynamic meanings. The z' values were 0.61, 0.53 and 0.71 for ISP, dB-cAMP and dB-cGMP, respectively. This similarity suggests that these agents utilizes a common voltage-dependent mechanism to block the K^+ current.

4. Discussion

In the present study, we have shown that isoproterenol, a β-adrenergic agonist, inhibited brain inwardly rectifying K^+ current heterologously expressed in *Xenopus* oocytes in a time- and voltage-dependent manner. The effect of ISP was likely mediated by an increase of intracellular cAMP. Unexpectedly, however, protein phosphorylation by A-kinase was not involved in this β-adrenergic modulation of the K^+ current.

In this study, we injected poly(A)$^+$ mRNA prepared from guinea pig whole brain into oocytes. Thus, the expressed inwardly rectifying K^+ current could be derived from either neurons or glial cells. Various inwardly rectifying K^+ channel clones have already been obtained and their expression in the brain has been confirmed [12–17], including glia-dominant K_{AB}-2 [18]. Most of these K^+ channel clones are equally well blocked by Ba^{2+} and have similar activation/inactivation

time-courses except G protein-gated GIRKs, it is difficult to decide which channel was mainly expressed in oocytes in this study. Therefore further study is needed to identify which clone(s) of the inwardly rectifying K^+ channel superfamily is inhibited by intracellular cyclic nucleotides.

Inwardly rectifying K^+ channels are expressed in a variety of cells including neuronal cells and play pivotal roles in formation of deep resting potential and in regulation of action potential duration [1]. Inhibition of K^+ currents results in an increase of neuronal cellular excitability by decreasing resting membrane potential, increasing the action potential duration and reducing afterhyperpolarization. It has been reported that substance P and thyrotropin-releasing hormone inhibit neuronal inwardly rectifying K^+ current [19–21]. This inhibition is not voltage-dependent, and involves PTX-insensitive G proteins and protein kinase C activation in their signalling.

In the present study, the inhibition of the inwardly rectifying K^+ current expressed in *Xenopus* oocytes by isoproterenol was mimicked by dB-cAMP. This finding meets the expectation that β-adrenergic effect is mediated by activation of adenylyl cyclase (AC) via stimulatory G protein (Gs). Surprisingly, β-adrenergic inhibition of the K^+ current seemed to be independent of A-kinase mediated phosphorylation: direct application of C subunit of A-kinase was without effects and A-kinase inhibitors, WIPTIDE and H8, did not prevent the inhibition by dB-cAMP. Furthermore, dB-cGMP blocked the K^+ current with similar voltage dependence to that of ISP- or dB-cAMP-induced block. Because the z′ values of blocking effects of ISP, cAMP and cGMP were similar, a common binding site for cAMP and cGMP may exist in the pore region of this inwardly rectifying K^+ channel.

A group of non-specific cation channels, present in retinal and olfactory receptor cells, are directly gated by cAMP and/or cGMP [22]. The hyperpolarization-activated channel (I_f) in cardiac pacemaker cells, which is also a non-specific cation channel, is reported to be activated directly by cAMP through modification of voltage dependence of activation [23]. Recently, it was reported that an inwardly rectifying K^+ current in astrocytes are directly inhibited by cAMP [24]. β-Adrenergic inhibition of the glial K^+ current was mimicked by forskolin, an activator of AC, and dB-cAMP, and not prevented by protein kinase inhibitors. These findings agree with ours. However, the inhibition by the agonists or cAMP was not voltage-dependent in the glial cells. It may present an interesting possibility that the voltage dependence is important for inhibition of inwardly rectifying K^+ channels in excitable neurons but not in non-excitable glial cells, because the resting membrane potential could be modified without severely changing action potential configurations when the effect is voltage-dependent. Because it is known that other transmitter receptors, i.e. dopamine D1 and D5, histamine H_2, serotonin, 5-HT_4 and adenosine A_2, can activate the Gs-AC cascade and raise the intracellular cAMP concentration [25], further study is needed to evaluate the physiological roles of this modulatory system on brain inwardly rectifying K^+ channels in regulation of brain function. The mechanism underlying the cyclic nucleotide inhibition of inwardly rectifying K^+ channels should be further elucidated at molecular level, because a number of cDNAs encoding classical inwardly rectifying K^+ channels have been isolated from brain [14–16,18].

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