

# Phosphatase Is Responsible for Run Down, and Probably G Protein-mediated Inhibition of Inwardly Rectifying $K^+$ Currents in Guinea Pig Chromaffin Cells

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**ABSTRACT** The mechanism of G protein-mediated inhibition of an inwardly rectifying  $K^+$  current ( $I_{IR}$ ) in adrenal chromaffin cells was investigated using the whole-cell version of the patch clamp technique. In case of recording with use of ATP-containing patch solution, the  $I_{IR}$  was well maintained; otherwise, it ran down within 15 min. This run down was not prevented by replacement with adenylyl-imidodiphosphate, a nonhydrolysable analogue of ATP, but was markedly reduced by the addition to the ATP-free solution of 1  $\mu$ M calyculin A, a specific inhibitor of serine/threonine phosphatase 1 (PP1) and 2A (PP2A). The addition of alkaline phosphatase to the ATP-containing solution facilitated run down of the current, and application of 100  $\mu$ M H-7, a general kinase inhibitor, reversibly suppressed  $I_{IR}$ . These results taken together suggest that inwardly rectifying  $K^+$  channels are under the influence of kinase and phosphatase without external signals.

Infusion of nonhydrolysable analogues of GTP, guanosine-5'-O-(3-thiophosphate) (GTP $\gamma$ S) or guanylyl-imidodiphosphate, through the pipette produced little inward current at  $-55$  mV, but completely inhibited  $I_{IR}$  within  $\sim 5$  or 6 min in all cells tested in the presence of 12  $\mu$ M  $Mg^{2+}$  inside the cell. In contrast, infusion of aluminum fluoride (AlF) complex, another GTP binding (G) protein activator, consistently produced large inward currents, but did not alter  $I_{IR}$  noticeably for 15 min in 17% of the cells tested. In the other cells, the inhibition of  $I_{IR}$  developed slowly after long latent periods. This inhibitory potency of AlF was not enhanced by an increase in  $Mg^{2+}$  concentrations. Subtraction of the current-voltage relationship before from that noted during the generation of inward current by AlF complex revealed that the inward current diminished progressively with hyperpolarizations, as is the case with a nonselective cation current ( $I_{NS}$ ) induced by a muscarinic agonist. Thus, AlF complex seems to be potent with the generation of  $I_{NS}$ , but not with  $I_{IR}$  inhibition. The addition of 3  $\mu$ M calyculin A significantly retarded the  $I_{IR}$  inhibition by GTP $\gamma$ S, whereas that of 1  $\mu$ M okadaic acid, another inhibitor of PP1 and PP2A, markedly prevented the decline of  $I_{IR}$  by AlF complex. Our observations suggest that the low potency of AlF complex in inhibiting  $I_{IR}$  may be due to

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interference with phosphatase activity and that the activation of G protein suppresses  $I_{IR}$ , probably by enhancing the apparent activity of phosphatase, which may explain run down of the current.

#### INTRODUCTION

Activity of inwardly rectifying (IR)  $K^+$  channels contributes to the resting membrane potential in a variety of cells. In some cells, IR  $K^+$  channels are targets for neurotransmitters and hormones (reviewed by Inoue and Yoshii, 1992). Substance P was first found to inhibit IR  $K^+$  channels through a pertussis toxin (PTX)-insensitive guanosine-5'-triphosphate (GTP) binding protein (G protein) in cultured rat brain neurons (Nakajima, Nakajima, and Inoue, 1988). Subsequently, similar inhibitions were noted for thyrotropin releasing hormone in rat GH<sub>3</sub> pituitary cells (Bauer, Meyerhof, and Schwarz, 1990; Barros, Delgado, del Camino, and de la Peña, 1992),  $\alpha 1$  agonists in rabbit ventricular cells (Fedida, Braun, and Giles, 1991), angiotensin II in mouse renal juxtaglomerular cells (Kurtz and Penner, 1989). Furthermore, the intracellular application of nonhydrolysable analogues of GTP resulted in the inhibition of inwardly rectifying  $K^+$  currents ( $I_{IR}$ ) in a rat leukemia cell line (McCloskey and Cahalan, 1990). Although attention has been directed to mechanisms related to  $I_{IR}$  inhibition, much remains to be clarified. All agents so far shown to inhibit  $I_{IR}$  are capable of facilitating phosphatidylinositol (PI) hydrolysis in a PTX-insensitive manner (Cockcroft and Thomas, 1992). Accumulating evidence, however, negates involvement of either of the second messengers, diacylglycerol or inositol 1,4,5-trisphosphate, resulting from PI hydrolysis in the inhibition (Kurtz and Penner, 1989; McCloskey and Cahalan, 1990; Barros et al., 1992).

Function of IR  $K^+$  channels in guinea pig ventricular myocytes (Takano, Qin, and Noma, 1990), and probably in leukemia cells (McCloskey and Cahalan, 1990), was maintained by phosphorylation of the channel or its closely associated protein. The suppression of phosphorylation process, such as removal of ATP, resulted in run down of  $I_{IR}$ , probably due to spontaneous activity of phosphatase. However, little attention has been directed to the relation between dephosphorylation-dependent washout of and G protein-mediated inhibition of IR  $K^+$  channels. The intracellular application of guanosine-5'-O-(3-thiophosphate), a nonhydrolysable analogue of GTP, to the chromaffin cell induced the inhibition of IR  $K^+$  channels in a PTX-insensitive manner (Inoue and Imanaga, 1993b). The primary objective of the present experiments was to address the issue of whether IR  $K^+$  channels in the chromaffin cell wash out in a dephosphorylation-dependent manner. We also investigated possible involvement of phosphatase in the G protein activator-induced inhibition. We obtained evidence that serine/threonine protein phosphatase may mediate the washout and inhibition of IR  $K^+$  channels.

#### METHODS

##### *Preparation*

Female guinea pigs (250–350 g) were killed by a blow to the neck, and the adrenal glands removed and immediately put into ice-cooled  $Ca^{2+}$ -free balanced salt solution. Adrenal

medullae were dissected from the cortex under a dissecting microscope, then were cut into three to six pieces and incubated for 30 min with 0.25% collagenase dissolved in  $\text{Ca}^{2+}$ -free solution. During the initial 10 min of this enzyme treatment, the preparation was gently agitated by bubbling with 99.9%  $\text{O}_2$  to facilitate digestion. After the incubation, the tissues were washed three or four times in  $\text{Ca}^{2+}$ -free solution and gently dissociated with a fire-polished Pasteur pipette. The isolated cells were kept in  $\text{Ca}^{2+}$ -free solution at room temperature (23–25°C) from 3–10 h.

#### *Current Recording*

The whole-cell current was recorded using a conventional patch clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Dispersed chromaffin cells were left for a few minutes to facilitate attachment to bottom of the bath before being constantly perfused with a standard external solution at a rate of  $\sim 1$  ml/min. After recorded with an EPC-7 amplifier (List, Germany), currents were passed through a high-cut filter of 5 or 3 Hz and then fed into a pen recorder. The resistance of a patch pipette was 2.0–2.5  $\text{M}\Omega$ , when filled with internal solution. The series resistance in whole-cell recording was about two or three times larger than the pipette resistance. To investigate the current-voltage ( $I$ - $V$ ) relationship, a 50-ms pulse was applied every 5 s in 10-mV steps from a holding potential of  $-55$  mV. At the same time, currents were stored on a disc of a computer at a sampling interval of 0.1 ms after filtering at 3 kHz. When the amplitude of  $I_{\text{IR}}$  was monitored during a recording, a 50-ms pulse was applied from  $-55$  to  $-155$  mV every 10 or 20 s and the current amplitude at the end of pulse was measured. For extracellular application, the drug was added to the perfusate and the solution exchange was completed within 5 s.

#### *Solutions*

The standard external solution contained (in millimolar): 137 NaCl, 5.4 KCl, 1.8  $\text{CaCl}_2$ , 0.5  $\text{MgCl}_2$ , 0.53  $\text{NaH}_2\text{PO}_4$ , 5 D-glucose, 5 HEPES, and 4 NaOH. The pH was adjusted to 7.4. In  $\text{Ca}^{2+}$ -free solution,  $\text{Ca}^{2+}$  was removed from the standard external solution. The standard K internal (pipette) solution contained (in millimolar): 110 K aspartate, 20 KCl, 1  $\text{MgCl}_2$ , 5 EGTA, 5 HEPES, 5  $\text{Na}_2\text{ATP}$ , and 19.4 KOH. When ATP was removed from the pipette solution, it was replaced with 10 mM NaCl. In the standard aluminum fluoride (AlF) solution, 10 mM KF were substituted for 10 mM KCl, 0.1 mM  $\text{AlCl}_3$  was added, and the other constituents were the same as in the standard K solution. The concentration of free  $\text{Mg}^{2+}$  ( $[\text{Mg}^{2+}]$ ) in the standard internal solutions was 12  $\mu\text{M}$  (Inoue and Imanaga, 1993a). When  $[\text{Mg}^{2+}]$  was increased, an appropriate amount of  $\text{MgCl}_2$  was added. The pH in all the internal solutions was 7.2. The liquid junction potential between the external and internal solutions was  $\sim -12$  mV and was corrected for membrane potential measurements. All the experiments were done at 23–25°C. The results are expressed as means  $\pm$  SD, unless otherwise noted and  $t$  test was used to determine the statistical significance.

#### *Chemicals*

Sources of chemicals are as follows: guanosine-5'-O-(3-thiophosphate) tetralithium salt (GTP $\gamma$ S), guanylyl-imidodiphosphate (GMP-PNP), and guanosine-5'-O-(2-thiophosphate) tetralithium salt (GDP $\beta$ S), alkaline phosphatase, and adenylyl-imidodiphosphate tetralithium salt (AMP-PNP) (Boehringer Mannheim, Germany); adenosine-5'-triphosphate disodium salt (ATP) (Sigma Chemical Co., St. Louis, MO); okadaic acid and calyculin A (Wako Pure Chemical Industries, Japan); collagenase (Yakult, Japan); 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide hydrochloride (HA1004) (Seikagaku Co., Japan). Okadaic acid and calyculin A were dissolved in dimethyl sulfoxide (DMSO) in such a way that the final concentration of the solvent was 0.1% or less.

## RESULTS

*Run Down of  $I_{IR}$  Due to Dephosphorylation*

We reported that IR  $K^+$  channels mediate an inward-going rectification in the chromaffin cell (Inoue and Imanaga, 1993b). This channel washed out rapidly when recorded with an ATP-free solution inside the patch electrode. Fig. 1 illustrates currents in response to 50-ms pulses and the  $I$ - $V$  relationships in the absence of ATP. Just after intracellular access, larger inward currents were generated with increasing hyperpolarizations from a holding potential of  $-55$  mV, and with the pulses to  $-135$  mV or more negative, currents were rapidly inactivated to a plateau level. These properties of evoked currents were reflected in the  $I$ - $V$  relationship measured at the end of pulse (plateau  $I$ - $V$  relationship), which exhibited an inwardly going rectifica-

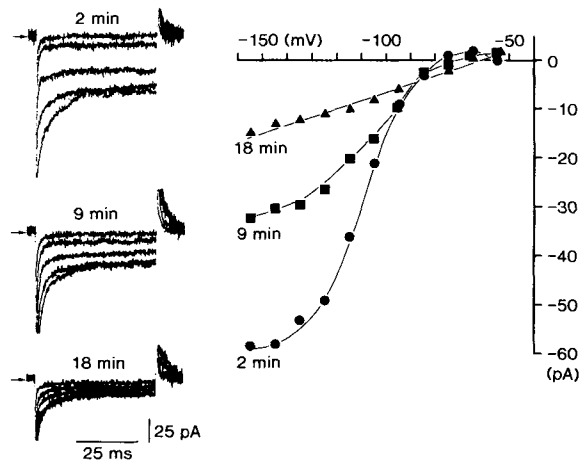


FIGURE 1. Run down of inwardly rectifying  $K^+$  current ( $I_{IR}$ ). Internal solution contained no ATP. (Left) Families of current traces in response to pulses applied from  $-55$  to  $-75$ ,  $-95$ ,  $-115$ ,  $-135$ , and  $-155$  mV. (Arrows) Zero current level. (Right)  $I$ - $V$  relationships during recording. 50-ms pulse was applied from  $-55$  mV in 10-mV steps every 5 s. Amplitudes of current at the end of pulse (plateau current) were measured and plotted against membrane potentials. Time after break in is indicated. Each family of current traces corresponds to the  $I$ - $V$  curve at the same time.

tion and a decrease in slope below  $-125$  mV. This rectification diminished markedly at 9 min of recording, and then disappeared at 18 min, resulting in a linear  $I$ - $V$  curve. The second and third  $I$ - $V$  relationships crossed the first one at about the same membrane potential of  $-85$  mV, a value which is identical to the equilibrium potential of  $K^+$  ( $-85$  mV) estimated from a Nernst equation. On average, the reversal potential for run down currents was  $-82.2 \pm 4.1$  mV ( $n = 5$ ). This result, together with an inwardly going property, implies that IR  $K^+$  channels were washed out. Fig. 2A summarizes time courses of washout of IR  $K^+$  channels in the presence (●) and absence (▲) of 2 mM ATP. The amplitude of plateau current evoked by 50-ms pulse from  $-55$  to  $-155$  mV was measured and expressed relative to that elicited 1 min after break in. This value started to decline soon after intracellular access in the absence of ATP, whereas in its presence, such a decrease was not observed during 20

min of recording. This prevention of washout could not be mimicked by substitution of 2 mM AMP-PNP, a nonhydrolysable analogue of ATP (■).

The foregoing results suggest that the IR  $K^+$  channel or its closely associated protein is under the influence of kinase and phosphatase and thus, the lack of ATP

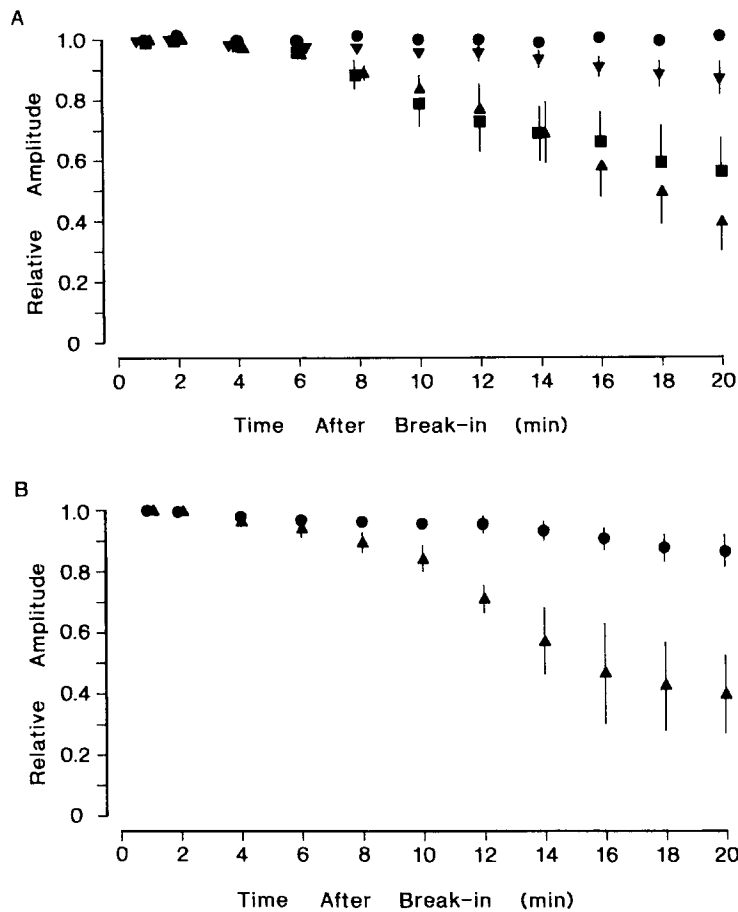


FIGURE 2. Phosphorylation-dependent run down of  $I_{IR}$ . 50 ms pulse was applied from  $-55$  to  $-155$  mV every 10 or 20 s, and the amplitude of plateau current was measured, expressed relative to that seen 1 min after break in, and plotted against time of recording. (A) Substitution of AMP-PNP for ATP did not prevent run down of  $I_{IR}$ . Internal solution contained 2 mM ATP (●,  $n = 4$ ), no ATP (▲,  $n = 5$ ), 2 mM AMP-PNP (■,  $n = 6$ ). (B) Calyculin A reduced run down of  $I_{IR}$ . Calyculin A at 1  $\mu$ M (●,  $n = 6$ ) or 0.5  $\mu$ M (▲,  $n = 3$ ) was added to ATP-free K solution. Data represent means  $\pm$  SEM.

inside the cell shifts the balance between phosphorylation and dephosphorylation over the latter. If the hypothesis is correct, then block of the dephosphorylation process would retard the run down of  $I_{IR}$  under ATP-free conditions. This possibility was examined by adding calyculin A, a specific inhibitor of serine/threonine protein

phosphatase 1 (PP1) and 2A (PP2A) (Ishihara, Martin, Brautigam, Karaki, Ozaki, Kato, Fusetani, Watabe, Hashimoto, Uemura, and Hartshorne, 1989), to the ATP-free solution. Fig. 2 *B* demonstrates that this is the case. The decline of relative plateau current at  $-155$  mV was significantly diminished in the presence of  $1 \mu\text{M}$  calyculin A ( $\bullet$ ), but not  $0.5 \mu\text{M}$  ( $\blacktriangle$ ). On the contrary, the enhancement of phosphatase activity by adding alkaline phosphatase, a nonspecific phosphatase (Kim, 1991; McComb, Bowers, and Posen, 1979), induced a run down of current even with  $5$  mM ATP inside the pipette (Fig. 3). In two of the three cells examined with  $40$  U/ml of alkaline phosphatase, the amplitude of plateau elicited by pulse to  $-155$  mV began to decrease  $\sim 5$  min after intracellular access, whereas in one cell, the current was not altered noticeably during  $20$  min of recording. Fig. 3 summarizes the relative amplitude of plateau currents in these three cells ( $\blacktriangle$ ) and that in six control cells ( $\bullet$ ), none of which showed a progressive decline. It is evident that application of alkaline phosphatase shifted the equilibrium between phosphorylation and dephosphorylation toward the latter.

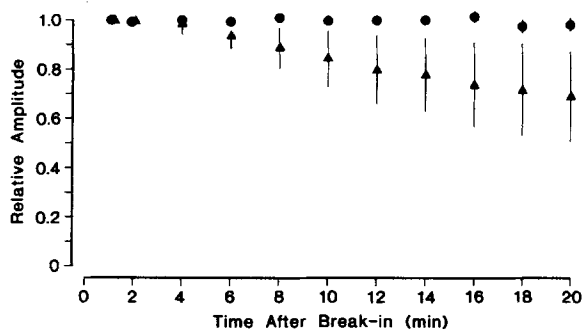


FIGURE 3. Facilitation of  $I_{IR}$  run down by alkaline phosphatase. Alkaline phosphatase ( $40$  U/ml) was added to  $5$  mM ATP containing K solution. Relative amplitudes of plateau current at  $-155$  mV in the presence of ( $\blacktriangle$ ,  $n = 3$ ) and absence ( $\bullet$ ,  $n = 6$ ) of phosphatase were plotted against time of recording. Relative amplitude of plateau current was measured in the same way as in Fig. 2. Data represent means  $\pm$  SEM.

Our thesis was further supported by the reversible suppression of  $I_{IR}$  by H-7, a general kinase inhibitor (Hidaka, Inagaki, Kawamoto, and Sasaki, 1984) (Fig. 4). When  $100 \mu\text{M}$  H-7 was included in the perfusate, the amplitude of plateau by pulse to  $-155$  mV gradually decreased over  $140$  s ( $104 \pm 34$  s,  $n = 5$ ) and reached  $81\%$  of the control (*A*). After the termination of exposure, the current reverted to the control level in a time course similar to that seen with wash in. As shown by the current traces (*B*) and I-V curves (*C*), the inhibition by H-7 did not alter noticeably the properties of voltage and time dependence of inactivation of  $I_{IR}$ . This result is consistent with the properties of  $I_{IR}$  running down partially under ATP-free conditions (Fig. 1). On average, the plateau amplitude at  $-155$  mV in the presence of  $50 \mu\text{M}$ ,  $100 \mu\text{M}$ ,  $150 \mu\text{M}$ , and  $300 \mu\text{M}$  H-7 was  $97.5 \pm 2.1$  ( $n = 2$ ),  $84.9 \pm 6.4$  ( $n = 5$ ),  $69.7 \pm 3.8$  ( $n = 3$ ), and  $52.5 \pm 0.7\%$  ( $n = 2$ ) of that in its absence. In contrast,  $100 \mu\text{M}$  HA 1004, which is the same isoquinolinesulfonamide as H-7 but has different potencies against protein kinases (Hidaka et al., 1984), had no effect on the plateau current. In the

three cells exposed to H-7 and HA1004, the plateau current measuring 140 s after application was  $83.9 \pm 6.9\%$  and  $98.4 \pm 1.0\%$  of control, respectively.

#### *Effects of G Protein Activators on IR K<sup>+</sup> Channel*

The results with phosphatase and kinase inhibitors are all consistent with the notion that IR K<sup>+</sup> channels are under the influence of kinase and phosphatase, even without external signals. A G-protein activator such as GTP $\gamma$ S might enhance apparent phosphatase activity, thereby producing an inhibition of  $I_{IR}$ . To explore this possibil-

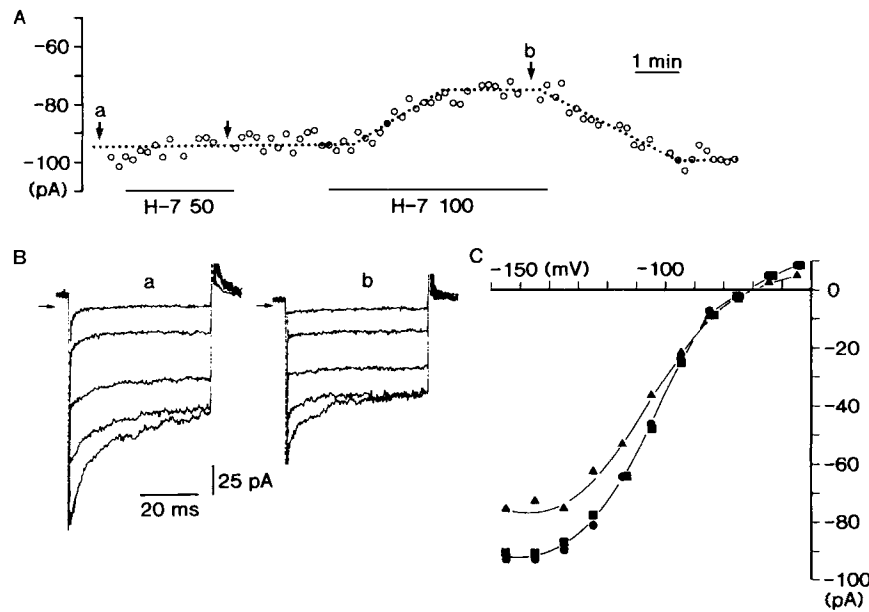


FIGURE 4. Reversible suppression of  $I_{IR}$  by H-7. (A) Time course of inhibition of  $I_{IR}$  by H-7. 50-ms pulse was applied from  $-55$  to  $-155$  mV every 10 s, and the amplitude of plateau current was measured and plotted against time. 50 or 100  $\mu$ M H-7 was added to the perfusate during the period indicated by the bar. Arrows indicate the time of investigation of  $I$ - $V$  relationships. (B) Families of current traces induced by pulses to  $-75$ ,  $-95$ ,  $-115$ ,  $-135$ , and  $-155$  mV. (a and b) correspond to a and b in A. (C) Plateau  $I$ - $V$  relationship in the absence ( $\bullet$ ) and presence of 50 ( $\blacksquare$ ) or 100  $\mu$ M ( $\blacktriangle$ ) H-7.  $\bullet$ ,  $\blacksquare$ , and  $\blacktriangle$  correspond to the first, second, and third examinations of  $I$ - $V$  relationship.

ity, we compared inhibitory potencies of GTP analogues and AIF complex on the K<sup>+</sup> channel. This complex acts as a G protein activator, similarly to GTP analogues (Sternweis and Gilman, 1982; Bigay, Deterre, Pfister, and Chabre, 1987; Antony and Chabre, 1992) and F<sup>-</sup> is an inhibitor of phosphatases (Shenolikar and Nairn, 1991). Fig. 5 A illustrates current records with 0.2 mM GMP-PNP-containing standard K solution. The current level at  $-55$  mV ( $\circ$ ) was not altered markedly during the recording and was  $+0.5 \pm 1.3$  pA ( $n = 3$ ) relative to the zero level 4 min after intracellular access. On the other hand, the amplitude of plateau current induced by

-100 mV pulse began to decrease 120 s ( $121 \pm 57$  s,  $n = 3$ ) after break in, then the inhibition developed with half time of decay ( $T_{1/2}$ ) of 60 s ( $42 \pm 16$  s,  $n = 3$ ). The plateau  $I$ - $V$  relationship ( $\blacktriangle$  in C) measured after completion of the inhibition was linear and crossed that ( $\bullet$ ) before the beginning of suppression at -85 mV, thereby indicating that the suppressed current is  $I_{IR}$ . In all three cells examined,  $I_{IR}$  was completely suppressed. Similarly, the internal application of 0.1 mM GTP $\gamma$ S produced a complete inhibition of  $I_{IR}$  within 5 min of recording in all 10 cells tested,

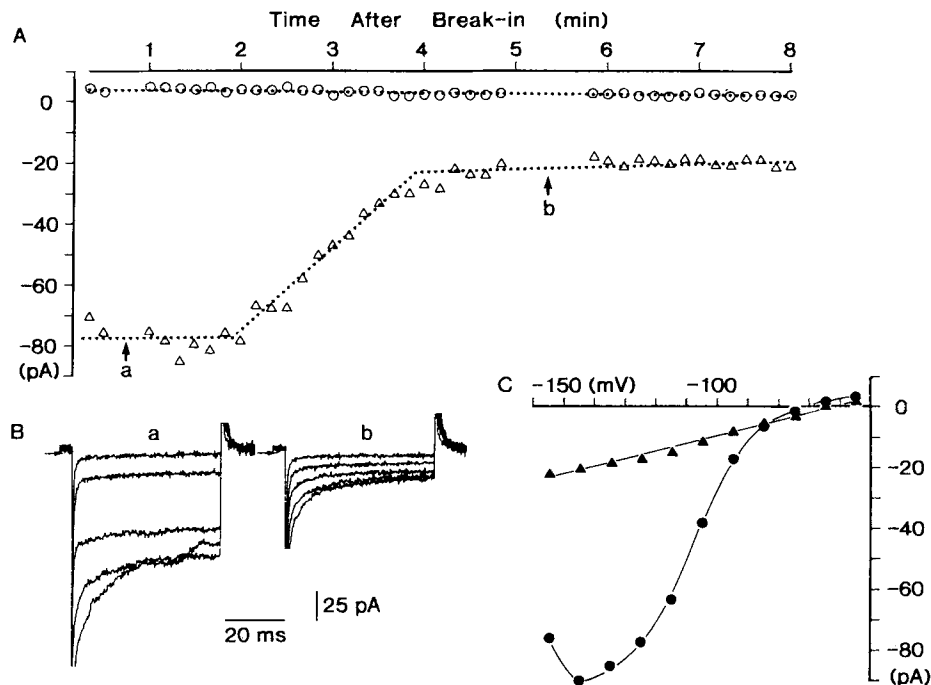


FIGURE 5. Effects of guanylyl-imidodiphosphate (GMP-PNP) on current level at -55 mV and plateau current at -155 mV. 0.2 mM GMP-PNP was added to standard K solution. (A) amplitude of current at -55 mV (○) and of plateau current elicited by 50-ms pulse to -155 mV (△) was plotted against time of recording. (Arrows) Time of examination of  $I$ - $V$  relationships. (B) Families of current traces in response to pulses to -75, -95, -115, -135, and -155 mV. (a and b) Correspond to first and second examinations in A. (C) Plateau  $I$ - $V$  relationships examined before and after inhibition of plateau current. (● and ▲) First and second examination in A.

while it altered little the current level at -55 mV (see Fig. 8). The latency and  $T_{1/2}$  for the GTP $\gamma$ S inhibition were  $103 \pm 26$  s and  $40 \pm 15$  s ( $n = 10$ ), respectively.

In contrast to GTP analogues, AIF complex was weak in inhibiting  $I_{IR}$ , but did induce a large inward current at -55 mV. This current reached a maximum  $187 \pm 69$  s ( $n = 10$ ) after break in and the maximum amplitude was  $-22.4 \pm 10.5$  pA with reference to the zero current level (Fig. 6A). This evoked current was then inactivated by  $84.5 \pm 20.6\%$  with a  $T_{1/2}$  of  $180 \pm 38$  s. During gradual development



of the inward current, amplitudes of plateau in response to  $-100$  mV pulse were not noticeably altered, but began to decrease 5 min 30 s after break in (Fig. 6A). This decrease in current response developed with a  $T_{1/2}$  of 167 s and the time course coincided with that of the decrease of inward current. In other cells, however, suppression of the plateau current either did not occur or began to develop after the inward current disappeared, thereby suggesting no causal relation between the decrease of inward current and the inhibition of  $I_{IR}$ . To determine the kind of channel responsible for the inward current,  $I$ - $V$  relationships were studied with 50-ms pulses just after break in, at a maximum of the inward current, and after the

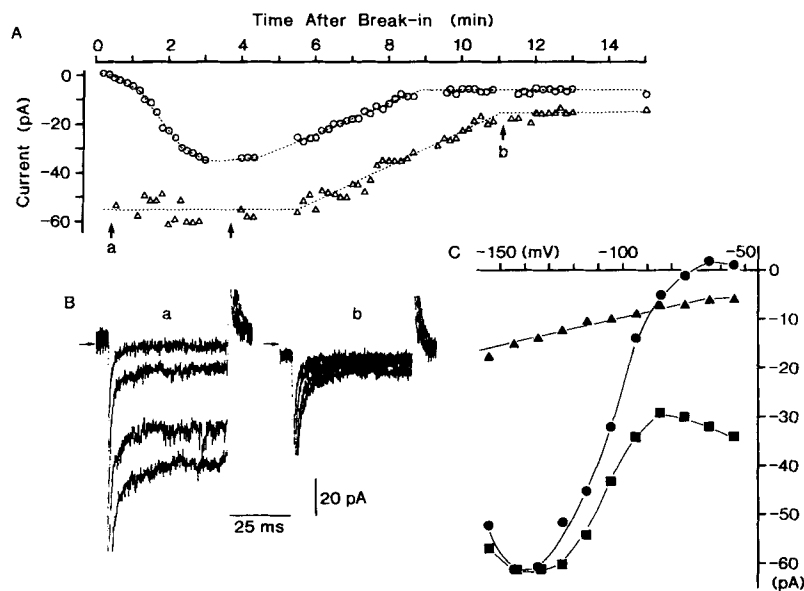


FIGURE 6. Effects of AIF complex on current level at  $-55$  mV and plateau current at  $-155$  mV. Standard AIF solution. (A) Amplitudes of current at  $-55$  mV ( $\circ$ ) and of plateau current elicited by 50-ms pulse to  $-155$  mV ( $\Delta$ ) were plotted against time of recording. Arrows indicate the time of investigation of  $I$ - $V$  relationships. (B) Families of current traces in response to pulses to  $-85$ ,  $-105$ ,  $-125$ , and  $-145$  mV. (Left and right) First and third examination in A. (C) Plateau  $I$ - $V$  relationships examined just after break in, at maximum development of and after disappearance of inward current.  $\bullet$ ,  $\blacksquare$ , and  $\blacktriangle$  correspond to first, second, and third examinations in A.

completion of  $I_{IR}$  inhibition (Fig. 6C). The first  $I$ - $V$  relationship ( $\bullet$ ) exhibited characteristics of  $I_{IR}$ : an inward-going rectification and upward curvature below  $-135$  mV due to  $\text{Na}^+$  block (Inoue and Imanaga, 1993b). The second  $I$ - $V$  relationship ( $\blacksquare$ ) coincided with the first one at  $-135$  mV and more negative membrane potentials. Subtraction of the first from the second  $I$ - $V$  curve revealed that the AIF complex-induced inward current diminished progressively with increasing hyperpolarizations and no inward current was elicited below  $-125$  mV. The last  $I$ - $V$  relationship ( $\blacktriangle$ ), which was measured after the completion of inhibition, was completely linear and crossed the first one at  $-88$  mV, which is close to the equilibrium potential of  $\text{K}^+$ . In

9 of 12 cells,  $I_{IR}$  was completely inhibited during 15 min of recording, whereas in two cells, the inhibition was not noticeable. In the remaining one cell, the inhibition was not completed during the recording. Fig. 7 summarizes the decline of relative amplitude of the plateau evoked by  $-100$  mV pulse in the presence of AIF complex (■,  $n = 12$ ), GTP $\gamma$ S (●,  $n = 10$ ) and GMP-PNP (▲,  $n = 3$ ). The AIF-induced inhibition occurred with a markedly long latency and a slow rate, compared with the GTP $\gamma$ S and GMP-PNP inhibition. These characteristics of  $I_{IR}$  inhibition by AIF complex might be due to low  $[Mg^{2+}]$  present in the standard AIF solution, although the GTP $\gamma$ S inhibition depended little on  $[Mg^{2+}]$ , at least in the limited range between  $12 \mu M$  and  $1$  mM (Inoue and Imanaga, 1993b). To examine this possibility,  $[Mg^{2+}]$  was increased to  $\sim 1$  mM by adding  $5$  mM  $MgCl_2$  to the standard AIF solution. In six of seven cells tested, plateau currents in response to  $-100$  mV pulse unexpectedly began to diminish  $\sim 1$  min after break in, then the inhibition developed slowly. At 15 min of recording, the relative plateau current ( $0.55 \pm 0.10$ ,  $n = 7$  in Fig. 7) in the presence of high  $Mg^{2+}$  did not differ from that ( $0.50 \pm 0.30$ ,  $n = 12$ ) in the presence of low  $Mg^{2+}$ .

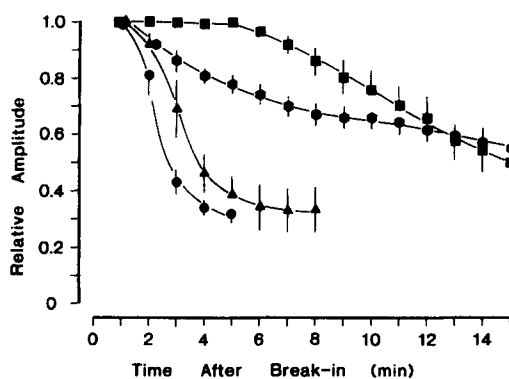


FIGURE 7. Summary of time course of  $I_{IR}$  inhibition induced by guanosine-5'-O-(3-thiophosphate) (GTP $\gamma$ S), GMP-PNP, and AIF complex. Relative amplitude of plateau current at  $-155$  mV was plotted against time of recording, as in Fig. 2. Whole-cell current was recorded with  $0.1$  mM GTP $\gamma$ S (●,  $n = 10$ ),  $0.2$  mM GMP-PNP (▲,  $n = 3$ )-containing standard K solution, standard AIF solution (■,  $n = 12$ ), or high  $Mg^{2+}$ -containing AIF solution (●,  $n = 7$ ). Data represent means  $\pm$  SEM.

Atypical inhibition by AIF complex might leave doubt concerning the involvement of G protein. Hence, we investigated the effects of  $0.1$  mM GDP $\beta$ S on AIF inhibition. Consistent with the GTP $\gamma$ S-induced inhibition (Inoue and Imanaga, 1993b), AIF complex failed to inhibit  $I_{IR}$  in the presence of GDP $\beta$ S in all four cells tested. The amplitude of plateau current elicited at  $-155$  mV 15 min after break in was  $103.3 \pm 13.6\%$  ( $n = 4$ ) of that measured just after it.

#### Effects of Phosphatase Inhibitors

The different potency between GTP analogues and AIF complex in inhibiting  $I_{IR}$  raises the possibility that phosphatase mediates inhibition of IR  $K^+$  channels. Thus, we examined effects of calyculin A on GTP $\gamma$ S-induced inhibition of  $I_{IR}$ . Fig. 8A illustrates the current level at  $-55$  mV (○) and amplitude of plateau current by  $-100$  mV pulse (Δ) in the presence of  $0.1$  mM GTP $\gamma$ S and  $3 \mu M$  calyculin A. The plateau current began to decrease  $140$  s ( $138 \pm 38$  s,  $n = 7$ ) after break in. This latency did

not differ from that in the presence of 0.1 mM GTP $\gamma$ S and 0.1% DMSO ( $158 \pm 34$  s,  $n = 6$ ). However, the inhibition developed slowly with  $T_{1/2}$  of 90 s. Fig. 8 *B* shows average time courses of decline of the plateau current in the cells perfused with GTP $\gamma$ S and calyculin A ( $\circ$ ) and with GTP $\gamma$ S and DMSO ( $\nabla$ ). The time of onset of the

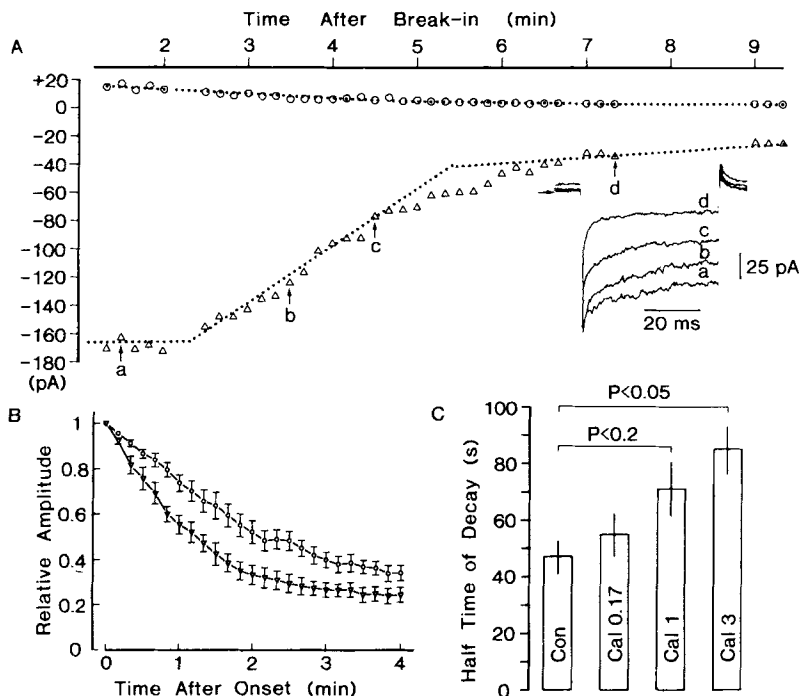


FIGURE 8. Slowing of GTP $\gamma$ S-induced inhibition of  $I_{IR}$  by calyculin A. 3  $\mu$ M calyculin A was added to 0.1 mM GTP $\gamma$ S-containing standard K solution. (A) Amplitude of current at  $-55$  mV ( $\circ$ ) and of plateau current elicited by 50-ms pulse to  $-155$  mV ( $\Delta$ ) was plotted against time. (Inset) Family of current traces in response to  $-100$  mV pulse. (a-d) Correspond to a-d in A. (Arrow) Indicates zero current level. (B) Time courses of decline of plateau currents in the presence of 3  $\mu$ M calyculin A ( $\circ$ ) and 0.1% dimethyl sulfoxide (DMSO) alone ( $\nabla$ ) in 0.1 mM GTP $\gamma$ S solution. Amplitudes normalized to that before onset of decline are plotted against time after the onset. Data for calyculin A and DMSO are means  $\pm$  SEM of seven and six observations, respectively. (C) Summary of half time of decay for GTP $\gamma$ S-induced inhibition of  $I_{IR}$  in the presence of 0.1% DMSO alone (Con,  $n = 6$ ) or of 0.17  $\mu$ M (Cal 0.17,  $n = 3$ ), 1  $\mu$ M (Cal 1,  $n = 4$ ), or 3  $\mu$ M (Cal 3,  $n = 7$ ) calyculin A. Half time of decay represents time required for half inhibition of plateau current. Indicated concentrations of calyculin A or 0.1% DMSO alone was added to 0.1 mM GTP $\gamma$ S solution. Data are means  $\pm$  SEM. Statistical significance is given.

decline in each cell was aligned to be 0 min. Addition of the inhibitor apparently retarded the decline of plateau current. Fig. 8 *C* summarizes  $T_{1/2}$  of  $I_{IR}$  inhibition in the presence of various concentrations of calyculin A or of the solvent. The inhibitor at 0.17  $\mu$ M tended to retard the decline and 3  $\mu$ M significantly slowed it.

As calyculin A did not prevent  $I_{IR}$  inhibition by GTP $\gamma$ S, we investigated the effects of 1  $\mu$ M okadaic acid, another specific inhibitor of PP1 and PP2A (Bialojan and Takai, 1988), on the AIF complex-induced inhibition of  $I_{IR}$ . In two of the five cells tested, no noticeable inhibition of  $I_{IR}$  occurred during 15 min of recording, whereas in one cell,  $I_{IR}$  was completely suppressed, resulting in a linear  $I$ - $V$  relationship. In the remaining two cells, the inhibition ceased in the middle of the process. Fig. 9 illustrates this partial suppression. The amplitude of plateau current at  $-155$  mV

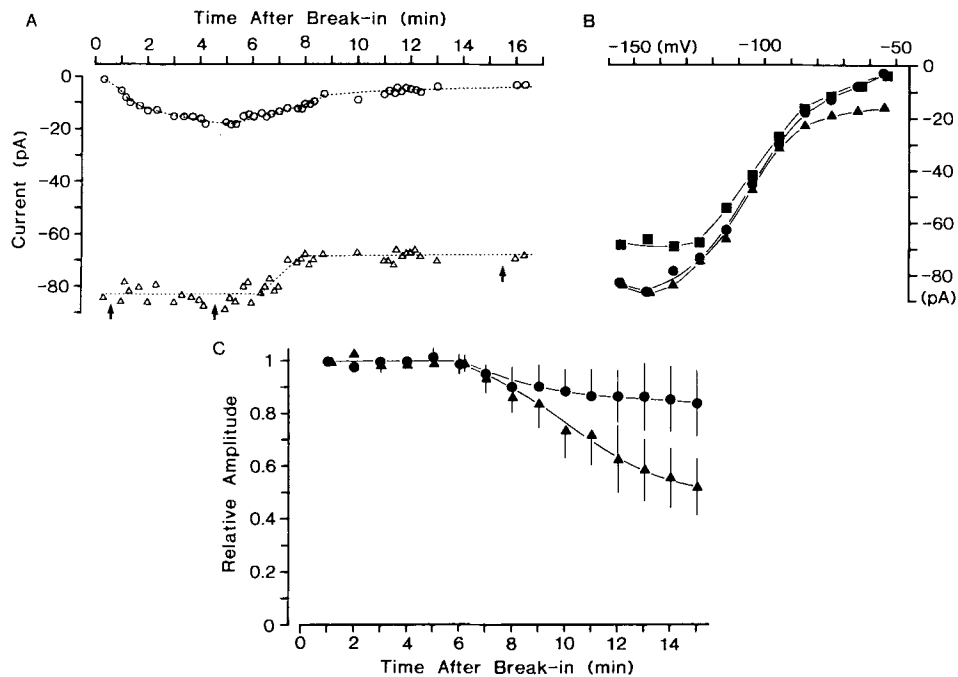


FIGURE 9. Prevention of AIF complex-induced inhibition of  $I_{IR}$  by okadaic acid. 1  $\mu$ M okadaic acid was added to standard AIF solution. (A) Amplitudes of current at  $-55$  mV ( $\circ$ ) and of plateau current elicited by 50-ms pulse to  $-155$  mV ( $\Delta$ ) were plotted against time of recording. (B) Plateau  $I$ - $V$  relationship just after break in, at maximum development of, and after disappearance of inward current.  $\bullet$ ,  $\blacktriangle$ , and  $\blacksquare$  correspond to first, second, and third examinations of A. (C) Relative amplitudes of plateau current at  $-155$  mV were plotted against the time of recording.  $\bullet$  and  $\blacktriangle$  represent currents recorded in the presence of okadaic acid ( $n = 5$ ) and of 0.1% DMSO alone ( $n = 6$ ), respectively. Data represent means  $\pm$  SEM.

began to decrease 6 min 30 s after break in, but the inhibition continued only for 90 s, after which the current was stabilized at a lower level. The  $I$ - $V$  relationship studied after stabilization shows that the majority of IR  $K^+$  channels were functioning. The slope conductance between  $-95$  and  $-115$  mV decreased only by 24% during the recording. For the control experiment, six cells were perfused with the standard AIF solution containing 0.1% DMSO. In three of these cells, the  $I_{IR}$  was completely suppressed within 15 min, whereas in one cell, there was no inhibition. In the

remaining two cells, the inhibition was not completed within the period of recording. Fig. 9 C summarizes the relative amplitudes of plateau current evoked by  $-100$  mV pulse in the presence of  $1 \mu\text{M}$  okadaic acid and of  $0.1\%$  DMSO during the recording. In the presence of the inhibitor, the amplitude decreased to  $0.84 \pm 0.28$  of the initial value 15 min after break in. This value differed from  $0.56 \pm 0.31$  seen in the presence of DMSO (statistically with  $P < 0.1$ ) and from  $0.50 \pm 0.30$  in the absence of any additive (with  $P < 0.05$ ) (Fig. 6). Thus, okadaic acid significantly prevents the ALF complex-induced inhibition of  $I_{\text{IR}}$ .

## DISCUSSION

### *Washout of the IR $K^+$ Channel Due to Dephosphorylation*

The IR  $K^+$  channel in the chromaffin cells was washed out rapidly in case of recordings with an ATP-free solution in the patch electrode. This property agreed with that of IR  $K^+$  channels in ventricular myocytes (Takano et al., 1990) and in leukemia cells (McCloskey and Cahalan, 1990). In the former, the washout was suggested to be due to dephosphorylation of the channel or its closely associated protein, because exposure of the cytosolic side of membrane to ATP, but not to AMP-PNP, restored the channel activity. Similarly, the run down of  $I_{\text{IR}}$  in the present experiment could not be prevented by substitution of AMP-PNP for ATP, and was facilitated by addition of alkaline phosphatase to the ATP-containing solution. Thus, the run down of  $I_{\text{IR}}$  in the chromaffin cell would also be due to dephosphorylation of the channel or its closely related protein. Furthermore, the addition of calyculin A to the ATP-free solution diminished the decline of current. This prevention of  $I_{\text{IR}}$  run down suggests the possible involvement of PP1 or PP2A in dephosphorylation of the  $K^+$  channel or its regulator, although the concentration used here was about three orders of magnitude higher than the concentration producing 50% inhibition ( $\text{IC}_{50}$ ) for purified PP1 or PP2A (Ishihara et al., 1989). This difference of concentration may not be crucial because calyculin A was applied through the pipette and the equilibrium between the inhibitor and phosphatase involved may not have been established. Furthermore,  $\text{IC}_{50}$  determined on purified enzymes may not apply to findings in cells (cf. Cohen, Klumpp, and Schelling, 1989). In fact, a similar concentration of calyculin A was required to enhance the hyperpolarization-induced inward current or  $i_f$  in canine Purkinje fibers and myocytes (Yu, Chang, and Cohen, 1993) and to potentiate an increase in  $\text{Ca}^{2+}$ -dependent  $K^+$  current in smooth muscle cells of the canine colon induced by the application of protein kinase A (Carl, Kenyon, Uemura, Fusetani, and Sanders, 1991). In addition,  $1 \mu\text{M}$  calyculin A had no effect on several alkaline and acid phosphatases (Ishihara et al., 1989).

The bath application of H-7 at  $100 \mu\text{M}$  or over reversibly suppressed  $I_{\text{IR}}$  in a dose-dependent manner. This weak potency might be accounted for not by the inhibition of kinase, but by a direct suppression of the channel. The inhibition constants ( $K_i$ ) of H-7 against cyclic nucleotide-dependent kinases and protein kinase C (PKC) were reported to be  $5.8\text{--}3.0 \mu\text{M}$  and  $6.0 \mu\text{M}$ , respectively (Hidaka et al., 1984), and the  $\text{IC}_{50}$  against calmodulin-dependent kinase II was  $20\text{--}32 \mu\text{M}$  (Malenka, Kauer, Perkel, Mauk, Kelly, Nicoll, and Waxham, 1989). However, the inhibitory potency of H-7 is expected to be markedly reduced under the present

experimental condition where the standard K solution contained 5 mM ATP, because it is a competitive inhibitor of these kinases with respect to ATP (Hidaka et al., 1984). In the canine Purkinje fiber, similar concentrations of H-7 were needed to reverse enhancement of  $i_f$  induced by isoprenaline or cyclic adenosine monophosphate (cAMP)-related agents (Chang, Cohen, DiFrancesco, Rosen, and Tromba, 1991). In addition, HA 1004, which is the same isoquinolinesulfonamide derivative as H-7 but has different potencies against kinases, had no effect on  $I_{IR}$  whereas H-7 inhibited it by 16% in the same cells. Thus, the inhibition by H-7 may be attributed to suppression of the basal activity of kinase responsible for the maintenance of  $I_{IR}$ . The finding that H-7 is more potent than HA 1004 might suggest the participation of  $Ca^{2+}$ -dependent PKC (i.e., classical PKC: Nishizuka, 1992) in this phosphorylation, since  $K_i$  of the former was 6.7 times smaller than that of the latter. However, this is unlikely because  $[Ca^{2+}]$  present in the standard K solution was strongly buffered to be  $10^{-9}$  M or less. Thus, a possible kinase would be  $Ca^{2+}$ -independent PKCs (i.e., new and atypical PKCs). Alternatively, a yet to be identified kinase might mediate phosphorylation of the channel or its regulator to maintain channel activity. This notion is consistent with the possible presence of a specific  $K^+$  channel kinase in rat brain (Rehm, Pelzer, Cochet, Chambaz, Tempel, Trautwein, Pelzer, and Lazdunski, 1989). The purified protein from rat brain was found to display a  $K^+$  channel activity and kinase activity. This kinase activity was not affected by a cAMP-dependent kinase inhibitor, a  $Ca^{2+}$ /calmodulin mixture, or phorbol ester. The presence of a similar kinase was also suggested with respect to the  $Ca^{2+}$ -dependent  $K^+$  channel in rat brain because the activity of the channel incorporated into planar lipid bilayers was enhanced by the addition of ATP without exogenous protein kinase (Chung, Reinhart, Martin, Brautigan, and Levitan, 1991). The results taken together suggest that the IR  $K^+$  channel is constantly under the influence of kinase and phosphatase without external signals, as was noted for nonselective cation (NS) channels (Inoue and Imanaga, 1993a). The kind of kinase and phosphatase involved differs between IR  $K^+$  and NS channels. The putative phosphorylation of the latter was sensitive to HA1004 (Inoue, unpublished observation) and the dephosphorylation depended on  $[Mg^{2+}]$ .

*Different Potencies of GTP Analogues and AIF Complex in  $I_{IR}$  Inhibition and  $I_{NS}$  Generation*

Internal application of GTP $\gamma$ S or GMP-PNP rapidly suppressed  $I_{IR}$  in all the cells tested, but induced little inward current. On the other hand, AIF complex was weak in inhibiting  $I_{IR}$  but produced a large inward current. This inward current is due to activation of NS channels since it exhibited a voltage dependence (Fig. 6) similar to that induced by GTP $\gamma$ S in the presence of high  $Mg^{2+}$  and by a muscarinic agonist (Inoue and Kuriyama, 1991) and its reversal potential was  $\sim +10$  mV (Inoue, unpublished observation). The differences between GTP analogues and AIF complex might be accounted for by a difference of  $Mg^{2+}$  dependence because the activation of G proteins is known to depend on  $[Mg^{2+}]$  (Birnbaumer, Abramowitz, and Brown, 1990; Yatani and Brown, 1991). However, the weak action of AIF complex on IR  $K^+$  channels cannot be attributed to high dependence on  $[Mg^{2+}]$  because an increase of  $[Mg^{2+}]$  in the AIF solution markedly shortened the latency for  $I_{IR}$  inhibition, but the

suppression developed slowly and the extent of inhibition at 15 min of recording did not differ from that under low  $Mg^{2+}$  conditions. Alternatively, AIF complex might have a low affinity for the G protein involved in the  $I_{IR}$  inhibition, a possibility which seems unlikely. The inhibition by AIF complex is mediated by G protein since the addition of GDP $\beta$ S to the AIF solution completely prevented the inhibition during 15 min of recording. This G protein responsible for  $I_{IR}$  inhibition was consistently found to be PTX-insensitive in a variety of cells (Inoue and Yoshii, 1992). This PTX-insensitive G protein is probably a trimeric G protein because the AIF complex failed to activate monomeric G proteins with a molecular mass in the range of 20–25 kD (Kahn, 1991). Thus, candidate G proteins would be a stimulatory G protein (Gs) coupled to adenylate cyclase and a G protein (Gq) linked to phospholipase C. These G proteins were activated similarly by nonhydrolysable GTP analogues and AIF complex, when cAMP generation (Howlett, Sternweis, Macik, Van Arsdale, and Gilman, 1979) or production of inositol phosphates (Guillon, Mouillac, and Balestre, 1986) was measured. Furthermore, when activation of Gs was measured as an increase in tryptophan fluorescence, the time course of activation by AIF complex was more rapid than that caused by GTP $\gamma$ S (Higashijima, Ferguson, Sternweis, Ross, Smigel, and Gilman, 1987).

A plausible explanation for the poor potency of AIF complex is that this complex may interfere with an inhibitory mechanism downstream to G protein. The fact that  $F^-$  is an inhibitor of serine/threonine phosphatases is compatible with this notion. Serine/threonine phosphatases are classified into four groups, depending on susceptibility to inhibitors and the requirement of divalent cations for activity (Cohen, 1989). Activity of PP2B and PP2C is entirely dependent on  $Ca^{2+}$  and  $Mg^{2+}$ , respectively; thus, the possible contribution of either would be, if any, little under the present experimental conditions. In fact, the GTP $\gamma$ S-induced inhibition of  $I_{IR}$  was not affected by  $Ca^{2+}$  or  $Mg^{2+}$  present in the pipette solution. Thus, if phosphatase mediates the  $I_{IR}$  inhibition, PP1 or PP2A would be responsible. The activity of PP1 or PP2A decreased to half the maximum in the presence of 3 to 15 mM  $F^-$  (Shacter-Noiman and Chock, 1983; Khandelwal and Enno, 1985; Mieskes and Söling, 1987). Thus, PP1 or PP2A activities were expected to diminish at least in part in the presence of 10 mM  $F^-$ , even if PP in vivo might have a lower sensitivity to  $F^-$  (Mieskes and Söling, 1987). This diminished phosphatase activity may account for the failure of AIF complex to produce  $I_{IR}$  inhibition in 17% cells tested or the slow rate of inhibition in the remaining cells.

#### *Involvement of Phosphatase in $I_{IR}$ Inhibition*

The addition of calyculin A to the GTP $\gamma$ S solution significantly retarded  $I_{IR}$  inhibition and that of okadaic acid to the AIF solution almost abolished the inhibition. Thus, the decline of  $I_{IR}$  in the presence of AIF complex and okadaic acid markedly contrasted with that of  $I_{IR}$  in the presence of GTP $\gamma$ S alone. This behavior under the former condition may be due to the combination of  $F^-$  and okadaic acid as an inhibitor of PP1 and PP2A. Although the concentrations of both specific inhibitors were far higher than  $IC_{50}$ s for PP1 and PP2A, this apparent poor potency may relate, at least in part, to the present experimental condition, as discussed previously. In addition, the failure of calyculin A to prevent the GTP $\gamma$ S inhibition might be accounted for by

a putative enhancement of apparent phosphatase activity by GTP $\gamma$ S, whereas its prevention of  $I_{IR}$  run down was probably due to a low level of phosphatase activity involved. These results taken together with the weak potency of AIF complex suggest that phosphatase, probably PP1 or PP2A, mediates the inhibition of IR K<sup>+</sup> channels induced by G protein activators. This phosphatase might be responsible for washout of IR K<sup>+</sup> channels observed under ATP-free conditions. At present, it remains an open question whether G protein stimulates phosphatase itself or enhances the affinity of target protein for the enzyme through a conformational change. If the former is the case, an indirect activation through diffusible messengers would be feasible as serine/threonine protein phosphatases are present in the cytosolic fraction (Shenolikar and Nairn, 1991). The activity of glycogen-associated PP1 in skeletal muscle is known to be regulated in the opposite manner through phosphorylation of different sites by adrenaline and insulin (Hubbard and Cohen, 1993).

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