Modulation of Calcium-activated Potassium Channels from Rat Brain by Protein Kinase A and Phosphatase 2A

Peter H. Reinhart,¹ Sungkwon Chung,¹ Bruce L. Martin,² David L. Brautigan,² and Irwin B. Levitan¹

¹Graduate Department of Biochemistry and Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02254 and ²Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

By incorporating plasma membrane vesicles into planar lipid bilayers, we previously characterized a family of four types of Ca^{2+} -activated K^+ channels from rat brain (Reinhart et al., 1989). Two of these are "large-conductance" or "maxi"- K^+ channels, which differ in their gating kinetics and toxin sensitivity and are henceforth referred to as "type 1" and "type 2" channels. Here we show that the gating of these two channel types can be modulated by phosphorylation and dephosphorylation. The effects of cAMP-dependent protein kinase catalytic subunit (PK-A) on type 1 maxi- K^+ channels are complex in that, while half of these channels are upregulated by the kinase, about one out of seven channels is downregulated. Thus, there may be several distinct channels within the type 1 category. Type 2 maxi- K^+ channels are consistently downregulated by PK-A. The effects of PK-A on both channel types are reversed by the catalytic subunit of protein phosphatase 2A (PP-2A), but not by protein phosphatase 1 (PP-1). Furthermore, some of the type 1 maxi- K^+ channels can be modulated by PP-2A, even without any prior PK-A treatment, indicating they are in a phosphorylated state when they are incorporated into the bilayer. The results demonstrate that (1) type 1 and type 2 maxi- K^+ channels are substrates for PK-A; (2) phosphorylation can shift the open probability of channels in either direction, by a mechanism involving multiple phosphorylation sites; (3) phosphorylation alters the Ca^{2+} /voltage sensitivity of these channels; and (4) dephosphorylation of type 1 and type 2 channels is catalyzed by specific phosphatases.

Ca^{2+} -activated K^+ channels can be divided into a number of subclasses based on properties such as single-channel conductance, voltage dependence, the sensitivity to pharmacological agents such as tetraethylammonium (TEA), apamin, and scorpion toxins, and inactivation kinetics (Hille, 1984; Petersen and Maruyama, 1984; Elkins et al., 1986; Moczydlowski et al., 1988; Rudy, 1988; Castle et al., 1989; Reinhart et al., 1989). Some cells possess more than one type of Ca^{2+} -activated K^+ channel, each type apparently playing a different role in the overall regulation of the cell's electrical activity (Romey and Lazdunski,

1984; Pennefather et al., 1985; Blatz and Magleby, 1986). These channels are intriguing because they provide a link between second-messenger systems, such as the inositol triphosphate (IP_3)-induced release of intracellular Ca^{2+} , and the membrane potential. Some members of this channel subgroup are also targets for other intracellular modulators such as protein kinase C (Baraban et al., 1985; Doerner et al., 1988; Kaczmarek, 1988; Shearman et al., 1989) and protein kinase A (PK-A; De Peyer et al., 1982; Ewald et al., 1985; Lechleiter et al., 1988; Kume et al., 1989; Sikdar et al., 1989).

Using reconstitution techniques, we have described two distinct types of maxi- K^+ channels in brain plasma membrane preparations (Reinhart et al., 1989). These two channel types share a number of properties such as the unitary conductance, voltage dependence, and TEA sensitivity. The main differences are that type 1 or "fast-gating" channels are blocked by charybdotoxin (CTX), while type 2 or "slow-gating" channels show different gating kinetics and are not blocked by nanomolar concentrations of CTX. The ability to incorporate these two channel types from brain plasma membrane vesicles into planar lipid bilayers opens the possibility to study the modulation of these channels in a defined environment, devoid of interactions with any but tightly associated intracellular components. In the present study, we show that protein phosphorylation by PK-A can modulate the gating kinetics of type 1 and type 2 maxi- K^+ channels. The direction of modulation is dependent on the individual channel type. These effects of PK-A can be reversed by protein phosphatase 2A (PP-2A), but not by protein phosphatase 1 (PP-1). The effect of such phosphorylation and dephosphorylation cycles may be to adjust precisely the Ca^{2+} /voltage sensitivity of target ion channels.

Some of these data have been previously presented in abstract form (Reinhart and Levitan, 1987).

Materials and Methods

Vesicle preparation. Rat brain plasma membrane vesicles were prepared as described previously (Reinhart et al., 1989). Briefly, brains were rapidly excised and immediately homogenized in an ice-cold isotonic sucrose buffer [0.25 M sucrose, 2.5 mM KCl, 0.1 mM EGTA, 0.1 mM dithiothreitol, and 20 mM HEPES (pH, 7.2)]. The homogenate was centrifuged at $800 \times g$ for 3 min, the pellet was washed once, and the supernatants were centrifuged at $30,000 \times g$ for 10 min. The pellet was resuspended in a hypotonic "lysis buffer" and allowed to stand on ice for 20 min. The membranes were then rehomogenized, pelleted, and layered under a discontinuous step gradient of Percoll (25%, 18%, 10%, and 0%, v/v). The gradients were centrifuged at $45,000 \times g$ for 2 min, and the membrane fractions banding at the 0–10% interface were collected. The Percoll was removed by centrifugation, and the membranes were frozen at -70°C .

Planar bilayers and membrane vesicle fusion. Single Ca^{2+} -activated

Received Oct. 9, 1990; revised Dec. 31, 1990; accepted Jan. 4, 1991.

We are grateful to Chris Miller for his interest and advice throughout the course of this work and to Terry Egan and Paul Katz for helpful comments on an earlier version of the manuscript. This work was supported by NIH Grants NS17910 to I.B.L., GM31768 to C. Miller, and DK31374 to D.L.B.

Correspondence should be addressed to Dr. Irwin B. Levitan, Graduate Department of Biochemistry and Center for Complex Systems, Brandeis University, 415 South Street, Waltham, MA 02254.

Copyright © 1991 Society for Neuroscience 0270-6474/91/111627-09\$03.00/0

K^+ channels were inserted into planar lipid bilayers (Mueller and Rudin, 1969) formed from a mixture of 1-palmitoyl, 2-oleoyl-phosphatidylethanolamine (PE) and 1-palmitoyl, 2-oleoyl-phosphatidylserine (PS; 3:1) in *n*-decane, as described previously (Reinhart et al., 1989). Membranes were added to the cytoplasmic solution consisting of 150 mM KCl, 1.05 mM CaCl_2 , 1 mM EGTA, 1 mM MgCl_2 , 7 mM KOH, and 10 mM HEPES (pH, 7.2). The extracellular solution consisted of 0.1 mM EGTA, 7 mM KOH, and 10 mM HEPES (pH, 7.2). For experiments involving the addition of PK-A, 500 μM Mg-ATP was added to this solution. For some experiments, the Ca^{2+} concentration was adjusted by adding more EGTA or more CaCl_2 . Ca^{2+} -activated K^+ channels inserted predominantly in one orientation, that is, with the Ca^{2+} -sensing side facing the cytoplasmic solution. After the incorporation of a channel, further fusion was suppressed by adding 50 mM KCl to the extracellular side, thereby reducing the transmembrane osmotic gradient. The electrical conductance of the bilayer was measured under voltage-clamp conditions at room temperature. Voltages are expressed in cellular convention, that is, the voltage of the cytoplasmic side with respect to the extracellular side. Solution changes were made by perfusing either the cytoplasmic or the extracellular chambers.

Electrical recordings and analysis. Single-channel currents were amplified using a 10-G Ω feedback resistor, filtered at 1–4 kHz using an 8-pole Bessel filter, and recorded on a β -videocassette recorder after 16-bit digitization at 44 kHz with a pulse code modulator (Sony PCM-701 ES). Data were analyzed off line using an MS-DOS-compatible computer (Everex 386, 20 MHz) equipped with a Tecmar Labmaster A/D board coupled to an Indec IBX Instrumentation Interface, and utilizing an Indec BASIC-Fastlab software library. Using operator-assisted computer programs, single-channel currents were sampled at either 4 or 8 kHz (unless otherwise indicated) and searched for transitions away from baseline. The threshold for detecting opening and closing transitions was set to 50% of the open-level current for each individual event. In this way, current levels corresponding to each closed/open transition were reduced to three values corresponding to the closed time, open time, and amplitude. Such reduced data lists were then further transformed to yield the open probability, mean amplitude, closed-time distributions, and open-time distributions. All experiments were repeated at least four times.

Kinase and phosphatase preparations. The catalytic subunit of the cAMP-dependent protein kinase was prepared from bovine heart essentially as described previously (Demaille et al., 1977; Peters et al., 1977). The final purification was achieved by HPLC using a Du Pont GF-250 gel filtration column. Catalytic subunits of protein phosphatases 1 and 2A were prepared by a modification of the procedure of Brautigan et al. (1985) as described by Pallis et al. (1990).

Materials. All lipids were from Avanti Polar Lipids (Birmingham, AL). Percoll was from Pharmacia (Boston, MA). Decane was from Aldrich (Milwaukee, WI).

Results

Rat brain Ca^{2+} -activated K^+ channels were incorporated into planar lipid bilayers (PE/PS, 3:1) and clamped at +10 mV using asymmetric ionic conditions (150 vs. 50 mM KCl, cytoplasmic vs. extracellular). Previous experiments have shown that under such conditions two different types of maxi- K^+ channels can be incorporated (Reinhart et al., 1989). The channels can be differentiated on the basis of a number of criteria. Type 1 channels are characterized by shorter mean open times and shorter mean closed times; that is, they appear to be fast-gating channels and are blocked by nanomolar concentrations of CTX. Type 2 channels are slow gating, less sensitive to Ca^{2+} by a factor of 2–5, and are not blocked by CTX. Hence, we can unequivocally assign brain maxi- K^+ channels as being either type 1 or type 2.

Shown in Figure 1 is a single-channel record of a type 1 maxi- K^+ channel (openings appear as upward deflections). Following addition of 400 nM PK-A, there was an upregulation of type 1 channels in 22 of 43 experiments, resulting in the channel being open most of the time (Fig. 1, center trace). Addition of 30 nM catalytic subunit of PP-2A resulted in the reversal of the PK-A-induced upregulation of this maxi- K^+ channel (Fig. 1, bottom

trace) in five of six experiments. Addition of comparable levels of PP-1 instead of PP-2A was without effect (four experiments; data not shown). We often observed that, after PK-A upregulation, the addition of PP-2A lowered the open probability of channels to below control levels (cf. Fig. 1, top and bottom traces). Presumably, the channel is incorporated in a partially phosphorylated form prior to PK-A addition, and these endogenous phosphates can be removed by the addition of PP-2A.

In 15 of 43 experiments with type 1 channels, PK-A had no significant effect on channel gating. To investigate whether this resulted from the incorporation of already highly phosphorylated type 1 channels, we examined the effects of adding PP-2A in the absence of any added PK-A. The data in Figure 2 show that adding PP-2A to type 1 channels prior to the addition of any PK-A caused a pronounced decrease in the open probability of the channel (four out of six experiments). To confirm that PP-2A addition after PK-A treatment (Fig. 1) does indeed reverse the actions of the kinase and is not removing endogenous phosphate groups present on the channel prior to PK-A administration, we first treated a type 1 channel with PP-2A and then perfused the cytoplasmic side of the channel extensively. After this treatment, we then added PK-A to activate the channel. Under these conditions, addition of PP-2A is still able to reverse the effect of PK-A (data not shown). In contrast to these results with type 1 channels, PP-2A added to type 2 channels prior to the addition of PK-A is without effect (four out of four experiments). Heat-inactivated PP-2A is without effect on either type 1 or type 2 channels. In all experiments, there was a time lag ranging between 30 sec and 3 min from the addition of either PK-A or PP-2A to the change in the activity of the channel, consistent with an enzymatic reaction taking place.

Initially we observed that PK-A significantly decreased the open probability of type 1 channels in one out of eight experiments. Increasing the number of experiments, we found this response in 6 of 43 experiments. Therefore, it seems that a subgroup of type 1 (CTX-sensitive) channels is downregulated by PK-A. This subgroup appears to represent a channel type that cannot be distinguished from the majority of type 1 channels by the criteria of gating kinetics and toxin sensitivity.

In contrast to the upregulation observed with most type 1 channels, the addition of PK-A to type 2 maxi- K^+ channels resulted in a decrease in the open probability of these channels in seven out of eight experiments (Fig. 3, center trace). Again, addition of PP-2A reversed these effects on type 2 channels (four out of four experiments; Fig. 3, bottom trace). Hence, the effects of PK-A and PP-2A on this channel type are opposite to that on type 1 maxi- K^+ channels. Note that the open probability of the channel after kinase and phosphatase treatment is about the same as the open probability of the untreated channel (Fig. 3, top and bottom traces).

The data in Figures 4 and 5 show the time dependence of the PK-A and PP-2A effects on single-channel open probabilities. For these experiments, single-channel fluctuations were measured in 60-sec sweeps, and the channel open probability for that sweep was calculated. The addition of PK-A to type 1 channels (Fig. 4A) results in a steep rise in the open probability of the channel, peaking within 5 min of administration. In these experiments, the open probability of the channel after PK-A administration is not constant, but oscillates between the control value and a much higher open-probability value (p_o). The open probability of type 1 channels is reduced within minutes of PP-2A addition. Note that the p_o after PP-2A addition is lower than

Type-1 Channel

Figure 1. Upregulation of type 1 Ca^{2+} -activated K^+ channels by cAMP-dependent protein kinase and reversal of this effect by PP-2A. Single-channel current records were obtained by incorporating plasma membrane vesicles from rat brain into PE/PS (3:1) planar bilayers. To facilitate detection and characterization of ion channels, asymmetric salt conditions were used (150 mM KCl on the cytoplasmic side, 50 mM KCl on the extracellular side). The free Ca^{2+} concentration was adjusted to approximately $0.5 \mu\text{M}$ using either CaCl_2 or EGTA, and the voltage across the bilayer was clamped at $+10 \text{ mV}$. For all traces, channel openings (O) are shown as upward deflections from the closed-level current (C). The *top trace* shows a 2.25-sec control sweep just prior to the addition of PK-A; the *center trace*, a 2.25-sec sweep 3 min after the addition of 400 nM PK-A; and the *bottom trace*, a 2.25-sec sweep obtained 3 min after the addition of 30 nM of PP-2A and while still in the presence of PK-A. Current fluctuations were filtered at 2 kHz and digitized at 4 kHz.

the control value (0.1 vs. 0.3; Fig. 4A; 0–20 min vs. 45–60 min). Furthermore, after PP-2A treatment, there was no more obvious oscillation in the open probability. The addition of the catalytic subunit of PP-1 instead of PP-2A never caused significant shifts in the open probability, whether added alone or after PK-A treatment. In the absence of PK-A or PP-2A, we never observed rapid, large changes in the open probability of single ion channels (Fig. 4B).

The effects of PK-A and PP-2A treatment on type 2 channels are shown in Figure 5A. After addition of PK-A, there is a steep

decrease in the single-channel open probability, reaching a new steady state within 5 min. The new open probability tends to be constant, without the large oscillations observed for type 1 channels. Addition of PP-2A produces a sharp increase in p_o , reversing the effects of PK-A by restoring the p_o to initial values over a 5-min period. Thus, the effects of PK-A and PP-2A appear to be mirror images on type 2 channels. Even though spontaneous open-probability shifts for type 2 channels are typically larger than those observed for type 1 channels, we never observed sudden large open-probability shifts in the absence of

Type-1 Channel

Figure 2. PP-2A alone can inactivate type 1 Ca^{2+} -activated K^+ channels. Single type 1 Ca^{2+} -activated K^+ channels were incorporated into lipid bilayers and clamped at $+10 \text{ mV}$ as described in Figure 1. The *top trace* shows a 2.25-sec sweep of a control channel, prior to any kinase or phosphatase treatment; the *bottom trace*, a 2.25-sec sweep 3 min after the addition of 30 nM PP-2A.

Type-2 Channel

Control

PK-A

PK-A + PP-2A

Figure 3. Downregulation of type 2 Ca²⁺-activated K⁺ channels by cAMP-dependent protein kinase and reversal of this effect by PP-2A. Single-channel currents were obtained as described in Figure 1. The *top trace* shows a 2.25-sec control sweep of a channel clamped at +10 mV; the *center trace*, a 2.25-sec sweep 3 min after the addition of 400 nM PK-A; and the *bottom trace*, a 2.25-sec sweep 3 min after the addition of 30 nM PP-2A and while still in the presence of PK-A.

added PK-A or PP-2A (Fig. 5B). We never observed any effects of heat-inactivated PK-A ($n = 3$) or of PK-A added in the absence of ATP ($n = 4$).

An analysis of the open-time and closed-time distributions (Table 1) revealed that both type 1 and type 2 channels can be described by three closed states and two open states. The fit for the open-time distribution for type 1 channels was only slightly improved by fitting to a sum of two rather than a single exponential term. Table 1 shows that the effect of PK-A on type 1 channel gating is to shorten closed times and increase open times, though the change in closed times is predominant. There appears to be little effect on the fastest "flickery" closed state, but this state is not clearly resolved in our experiments. It can also be seen that PP-2A modifies the same kinetic parameters

as PK-A but in the opposite direction. The effect of PK-A on type 2 channels is also to alter both the open-time and the closed-time distributions. For this channel type, the effect on open times may be larger than on type 1 channels. Thus, the effects of phosphorylation on channel gating are complex in that at least two open states and two closed states are modulated. Because these effects are very similar to those produced by altering Ca²⁺ concentrations, we examined the relationship between Ca²⁺ and phosphorylation. The open probability of a type 1 channel was examined as a function of the intracellular free Ca²⁺ concentration. The results are plotted in Figure 6 according to the Hill equation, showing a straight line (slope = 1.9). Following reaction with PK-A, there is a shift in the Ca²⁺ dependence, the channel now being two to three times more sensitive to Ca²⁺

Table 1. Kinetic analysis of PK-A and phosphatase effects on type 1 and type 2 Ca²⁺-activated K⁺ channels

	<i>n</i>	<i>p</i> _o ± SE	τ _{C1} ^a	τ _{C2}	τ _{C3}	τ _{O1}	τ _{O2}
Type 1 channels							
Control	6	0.44 ± 0.102	0.5 (50)	8.6 (46)	30.2 (4)	4.8 (70)	9.6 (30)
PK-A (400 nM)	6	0.81 ± 0.096	0.5 (71)	4.6 (26)	11.5 (3)	6.7 (68)	11.2 (32)
Control	5	0.55 ± 0.121	0.5 (51)	12.4 (40)	18.9 (9)	5.9 (62)	15.5 (38)
PP-2A (30 nM)	5	0.19 ± 0.086	0.5 (39)	22.6 (44)	41.5 (17)	4.1 (42)	9.2 (58)
Type 2 channels							
Control	4	0.47 ± 0.087	0.5 (71)	67 (16)	140 (13)	23.2 (48)	40.6 (30)
PK-A (400 nM)	4	0.07 ± 0.067	0.5 (40)	113 (30)	506 (30)	7.1 (75)	17.7 (25)

Digitized single-channel records were analyzed by the 50% threshold-crossing method, and the individual closed times and open times from at least 5000 transitions were placed into 1 of 255 bins. The threshold value was calculated for each individual closed-to-open transition. The data were then fit by exponential functions, allowing the extraction of the time constants and percentages.

^a Tau values are expressed in milliseconds, with the percentage shown in parentheses.

Figure 4. Time course of PK-A and PP-2A effects on the open probability of type 1 Ca^{2+} -activated K^+ channels. Single type 1 channels were incorporated into lipid bilayers as described in Figure 1. The open probability of these channels was adjusted to approximately 0.3, using either CaCl_2 or EGTA. Open probabilities were continuously calculated, and each point represents the value for a 60-sec sweep. *A*, The arrows mark the time at which either 400 nM PK-A or 30 nM PP-2A was added to the cytoplasmic side of the channel. *B*, The open probability of a channel was adjusted to approximately 0.5, using either EGTA or CaCl_2 , and continuously monitored for 60 min without addition of any enzymes. Under such conditions, we never observed large sudden shifts in the open probability of single ion channels ($n = 5$).

(curve shifted to the left), apparently without a significant change in the number of Ca^{2+} binding sites (slope not changed). We were unable to examine the effect of phosphorylation on the Ca^{2+} dependence of type 2 channels, due to the experimental difficulties of obtaining single channels with minimal spontaneous open-probability fluctuations over a 1–2-hr period. In contrast to experiments with PK-A and PP-2A for which only end-point measurements are required, Ca^{2+} titration experiments require exceedingly stable recordings, which we were unable to obtain for this channel type.

Figure 5. Time course of PK-A and PP-2A effects on the open probability of type 2 Ca^{2+} -activated K^+ channels. Single type 2 channels were incorporated into lipid bilayers as described in Figure 1. The open probability of these channels was adjusted to approximately 0.5 and measured as described in Figure 4. *A*, The arrows mark the time at which either 400 nM PK-A or 30 nM PP-2A was added to the cytoplasmic side of the channel. *B*, The open probability of a control channel was monitored for 60 min as described in Figure 4.

Discussion

Phosphorylation modulates maxi- K^+ channel gating

We have shown here that phosphorylation and dephosphorylation of maxi Ca^{2+} -activated K^+ channels from rat brain causes upregulation and downregulation under defined conditions. Both type 1 and type 2 channel types can be modulated by a protein phosphorylation and dephosphorylation cycle, catalyzed by PK-A and PP-2A. The most consistent effect of PK-A on type 1 channels is to increase the open probability of these channels. The extent of this effect is dependent on the open probability of the channel prior to treatment. The largest effects are observed when initial open probabilities are between 0.3 and 0.6. The effects of PK-A are minimized when the open probability is outside this range, as a result of changing either the holding

Figure 6. Phosphorylation increases the Ca^{2+} sensitivity of type 1 channels. Single type 1 Ca^{2+} -activated K^+ channels were incorporated into lipid bilayers as described in Figure 1. The Ca^{2+} concentration on the cytoplasmic side of the channel was increased in a step-wise manner, and the open probability was calculated (open circles). The cytoplasmic side of the channel was then extensively perfused, 300 nM PK-A was added, and the Ca^{2+} concentration again changed in a step-wise manner (solid circles). The Hill coefficient for individual channels ranged between 1.4 and 2.5 ($n = 5$).

voltage or the free Ca^{2+} concentration. Upregulation of Ca^{2+} -activated K^+ channels by PK-A has been described in a number of tissues (Ewald et al., 1985; Farley and Rudy, 1988; Lechleiter et al., 1988; Sadoshima et al., 1988; Kume et al., 1989), and this may represent a common type of response to elevation of cellular cAMP levels. However, there is evidence that Ca^{2+} -activated K^+ channels from pituitary gonadotrophs, like type 2 channels from brain, may also be downregulated by PK-A (Sikdar et al., 1989).

Protein phosphatases can reverse the effects of PK-A

An unexpected observation is the specificity of the purified catalytic subunit of PP-2A in reversing the effect of PK-A on both types of maxi- K^+ channels. Because PP-2A exactly reverses the effects of PK-A, the observed changes in gating kinetics must be due to protein phosphorylation. Significantly, PP-1 cannot mimic the effects of PP-2A, though these phosphatases have been shown to have broad and overlapping specificities on a number of other substrate proteins (Ingebritsen and Cohen, 1983; Ingebritsen et al., 1983; Cohen, 1989). However, recent *in vivo* studies have demonstrated a similar degree of specificity for PP-1 versus PP-2A, where only PP-1 dephosphorylates myosin light chains to produce rearrangement of the microfilament network in fibroblasts (Fernandez et al., 1990). This specificity of phosphatases for maxi- K^+ channels opens the possibility that PP-2A is another locus for the modulation of ion channel activity. Evidence that there is partial endogenous phosphorylation of Ca^{2+} and Na^+ channels (Curtis and Catterall, 1985; Hosey et al., 1986; Armstrong and Eckert, 1987; Rossie and Catterall, 1987; De Jongh et al., 1989; Emerick and Agnew, 1989) and Ca^{2+} -activated K^+ channels (present results) highlights the possible role of PP-2A in regulation. Regulation of phosphatase

activity, even without a change in kinase activity, may lead to an alteration in channel-gating kinetics. It is known that brain contains significant amounts of all four major classes of phosphatases (PP-1, PP-2A, PP-2B, and PP-2C) bound to membranes (see Cohen, 1989), and that brain may also contain two novel phosphatases (LP-1 and LP-2), which have been proposed to play a unique role in the regulation of brain functions (Yang et al., 1986, 1987).

Phosphorylation alters the Ca^{2+} /voltage dependence of maxi- K^+ channels

To gain further insight into how phosphorylation alters channel gating, we have analyzed the effects of PK-A on channel open-time and closed-time distributions (Table 1). Channel gating could be described in terms of three closed states and two open states. As has been reported previously (McManus and Magleby, 1985), many channel closings are very brief (0.5 msec or less) and are only poorly resolved in our experiments. The effect of PK-A on type 1 channels can be thought of as destabilizing at least one closed state and stabilizing at least one open state. The effects on the closed states are usually larger than the effects on the open states. This is similar to findings made in cultured smooth-muscle cells, where PK-A was also shown to change predominantly the closed state (Sadoshima et al., 1988). However, the latter experiments were carried out at very low open probabilities ($p_o = 0.015$ – 0.06), at which the channel open times were only approximately 1 msec, and small changes in the open-time distribution may not have been detected at a filter cutoff of 2 kHz.

Treating type 2 channels with PK-A has the opposite effect to that on type 1 channels: open states are destabilized, and closed states are stabilized. These alterations of both open-time and closed-time distributions (with little if any effect on the fast flickery closed state) are very similar to changes due to the addition of either Ca^{2+} or EGTA to channels (Magleby and Pallotta, 1983; Moczydlowski and Latorre, 1983; Vergara and Latorre, 1983; McManus and Magleby, 1985; Singer and Walsh, 1987). Hence, the kinetic "fingerprint" produced by phosphorylation is consistent with the possibility that phosphorylation alters the Ca^{2+} sensitivity of Ca^{2+} -activated K^+ channels. This possibility, first proposed by De Peyer et al. (1982), was examined more directly by comparing the effect of PK-A on type 1 channels at a range of free Ca^{2+} concentrations. Phosphorylation seems to increase the Ca^{2+} affinity of the channel by a factor of two to three; that is, less Ca^{2+} is required to open the phosphorylated channel to any given open probability. The number of Ca^{2+} ions involved in channel upregulation does not appear to change after PK-A treatment, as judged by the similarity in Hill coefficients. Thus, phosphorylation of this type of channel may not alter channel gating per se, but rather alter gating indirectly by stabilizing the binding of Ca^{2+} . Alternatively, our data could be interpreted to indicate that the voltage dependence of the channels is being modulated. The voltage dependence of these channels is not clearly understood, and it is possible that the Ca^{2+} -binding step itself may be voltage sensitive (Moczydlowski and Latorre, 1983; but see Methfessel and Boehm, 1982, for an alternative proposal). Hence, phosphorylating a "voltage-sensor" region of the channel could induce a significant alteration in the voltage and calcium dependence.

A correlation between phosphorylation and the shift in the Ca^{2+} sensitivity of maxi- K^+ channels may account for the large differences in the Ca^{2+} sensitivities of these channels observed

by numerous workers (Marty, 1983, 1989; Singer and Walsh, 1986; Wolff et al., 1986). Biochemical studies on the phosphorylation of Ca^{2+} and Na^{+} channels have revealed that PK-A often produces little or no incorporation of phosphate into channel subunits because the target residues are already phosphorylated when the channel is isolated (Rossie and Catterall, 1987; Emerick and Agnew, 1989).

Modulation may involve multiple phosphorylation sites

The time delay between the addition of PK-A and the onset of any change in gating kinetics is clearly longer than for Ca^{2+} or EGTA, indicating either that PK-A must overcome some diffusion barrier, that multiple sites must be phosphorylated before gating is affected, or that an endogenous phosphatase activity is competing with the PK-A activity. We believe that, because a shift in gating can take some minutes before a maximal effect is observed, and because the shift is gradual rather than occurring in a discrete jump, multiple sites are being phosphorylated in individual ion channels. For Na^{+} channels, Ca^{2+} channels, and ACh receptor/channels, it is known that multiple phosphorylation sites exist (Huganir et al., 1986; Rossie et al., 1987; Catterall et al., 1988; Hosey and Lazdunski, 1988; De Jongh et al., 1989; Rossie and Catterall, 1989).

To examine whether the channel itself is being phosphorylated, rather than some intermediate "regulator" protein which in turn modulates the channel, we extensively perfused the cytoplasmic side of the channel after vesicle fusion, thereby removing any unfused vesicles or soluble regulator proteins. These conditions did not significantly alter the actions of PK-A on either type 1 or type 2 channels. Because any proteins incorporated into the planar bilayer are essentially at infinite dilution, this indicates that phosphorylation of either the channel itself or a tightly associated protein is responsible for channel modulation.

After PK-A has induced a shift in gating kinetics, we often see large fluctuations in the open probability of individual ion channels. These fluctuations are on a time scale of many seconds to minutes and may indicate either that the effects of PK-A on channel gating are very complex, allowing the channel to move between a number of kinetic states, or that a phosphatase activity tightly associated with the channel is bringing the open probability back to control levels. This latter possibility suggests that the phosphorylated and dephosphorylated states of an ion channel are actively "cycled," so small changes in the activity of either the kinase or the phosphatase can result in rapid changes in the phosphorylation state, and hence the activity of the channel. Evidence that such mechanisms may occur *in vivo* has recently been presented by Klumpp et al. (1990). These workers made use of okadaic acid, a membrane-permeable polyether inhibitor of PP-1 and PP-2A, to show that PP-1 is responsible for dephosphorylation and closure of calcium channels in *Paramecium*. It has been proposed that such dephosphorylated channels represent a "closed-inactivated" state and must be rephosphorylated to become resensitized to depolarizing stimuli (Armstrong and Eckert, 1987; Klumpp et al., 1990). In another study on tracheal myocytes, okadaic acid was shown to activate Ca^{2+} -activated K^{+} channels in cell-attached membrane patches (Kume et al., 1989). These findings indicate that in some cell types an ion channel phosphorylation/dephosphorylation cycle is continuously active, and that channel gating can be altered by modulating either the kinase or the phosphatase portion of the cycle.

Families of modulatable maxi- K^{+} channels

The properties of large-conductance Ca^{2+} -activated K^{+} channels from a wide variety of tissues appear to be very similar (Latorre and Miller, 1983; Latorre, 1986; Singer and Walsh, 1986; Wolff et al., 1986; Marty, 1989). However, our previous characterization of four types of Ca^{2+} -activated K^{+} channels from rat brain revealed the existence of at least two types of maxi- K^{+} channels differing in their gating kinetics, Ca^{2+} sensitivity, and sensitivity to CTX (Reinhart et al., 1989). These differences between maxi- K^{+} channels are further highlighted in the present study showing that PK-A can upregulate one and downregulate the other maxi- K^{+} channel type from brain. Hence, there may be a family of modulatable maxi- K^{+} channels in brain, differing not only in toxin-binding and gating properties, but also in their response to phosphorylation. Even channels that appear kinetically and pharmacologically similar may be affected in very different ways by protein phosphorylation. Similarly, channels that have common toxin binding sites may be kinetically distinct (MacKinnon et al., 1988).

This concept of families of closely related potassium channels is highlighted by the large number of channel types isolated from rat brain cDNA libraries by cross-hybridization with a *Shaker* cDNA probe (Baumann et al., 1988; Iverson et al., 1988; Tempel et al., 1988; Stuehmer et al., 1989; Koren et al., 1990). It was surprising to find that even very minor structural variations have been found to underlie differences in gating properties and the sensitivity to some blocking agents (Iverson et al., 1988; Kamb et al., 1988; Timpe et al., 1988; Pongs, 1989). Similarly, the cloning of Na^{+} channels has revealed a family of different α -subunits (Noda et al., 1986). Subunits of neuronal origin were found to be unique in that they contain an insert embracing a cluster of PK-A phosphorylation sites (Rossie et al., 1987; Rossie and Catterall, 1989). The phosphorylation of these channels has been implicated in an altered binding to neurotoxins and may modulate the voltage dependence of inactivation (Rossie and Catterall, 1987). Furthermore, two different forms of L-type Ca^{2+} -channel α -subunits have recently been characterized (De Jongh et al., 1989), only one of which contains a C-terminal segment embracing multiple potential PK-A phosphorylation sites. Hence, while the ion-conduction and voltage-sensing components of many ion channels may be similar, there may be considerable variation in modulatory components of ion channels.

Finally, the results presented in this report highlight how diverse responses to a single second-messenger system can be generated. Different cell types, or regions within a cell, could respond to PK-A by either upregulating or downregulating maxi- K^{+} channels in response to a given stimulus. The magnitude and duration of the effect can be precisely modulated by setting the phosphorylation state of multiple sites in a channel not only by modulating the kinase activity, but also by modulating the phosphatase activity. If multiple phosphorylation sites modulate channel gating, then one can envision schemes in which not only the total number of phosphate groups, but also the order of phosphorylation and dephosphorylation that would generate different combinations of phosphate groups, may take on a special significance. The possibility of functional complexes involving ion channels, together with kinase and phosphatase activities, allows for an even greater diversity of responses to extracellular stimuli than that already afforded by the great diversity of different K^{+} channel types.

References

- Armstrong D, Eckert R (1987) Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc Natl Acad Sci USA* 84:2518–2522.
- Baraban JM, Snyder SH, Alger BE (1985) Protein kinase C regulates ionic conductance in hippocampal pyramidal neurons: electrophysiological effects of phorbol esters. *Proc Natl Acad Sci USA* 82:2538–2542.
- Baumann A, Grupe A, Achermann A, Pongs O (1988) Structure of the voltage dependent potassium channel is highly conserved from *Drosophila* to vertebrate central nervous system. *EMBO J* 7:2457–2463.
- Blatz AL, Magleby KL (1986) Single apamin-blocked Ca^{2+} -activated K^{+} channels of small conductance in cultured rat skeletal muscle. *Nature* 323:718–720.
- Brautigan DL, Shriner CL, Grippo PA (1985) Phosphorylase phosphatase catalytic subunit. Evidence that the $M_r = 33,000$ enzymatic fragment is derived from a native protein of $M_r = 70,000$. *J Biol Chem* 260:4295–4302.
- Castle NA, Haylett DG, Jenkinson DH (1989) Toxins in the characterization of potassium channels. *Trends Neurosci* 12:59–65.
- Catterall WA, Seagar MJ, Takahashi M (1988) Molecular properties of dihydropyridine-sensitive calcium channels in skeletal muscle. *J Biol Chem* 263:3535–3538.
- Cohen P (1989) The structure and regulation of protein phosphatases. *Annu Rev Biochem* 58:453–508.
- Curtis BM, Catterall WA (1985) Phosphorylation of the calcium antagonist receptor of the voltage-sensitive calcium channel by cAMP-dependent protein kinase. *Proc Natl Acad Sci USA* 82:2528–2532.
- De Jongh KS, Merrick DK, Catterall WA (1989) Subunits of purified calcium channels: a 212-kDa form of α_1 and partial amino acid sequence of a phosphorylation site of an independent β subunit. *Proc Natl Acad Sci USA* 86:8585–8589.
- Demaille J, Peters KA, Fisher EH (1977) Isolation and properties of rabbit skeletal muscle protein inhibitor of adenosine 3',5'-monophosphate dependent protein kinases. *Biochemistry* 16:3080–3086.
- De Peyer JE, Cachelin AB, Levitan IB, Reuter H (1982) Ca^{2+} -activated K^{+} conductance in internally perfused snail neurons is enhanced by protein phosphorylation. *Proc Natl Acad Sci USA* 79:4207–4211.
- Doerner D, Pitler TA, Alger BA (1988) Protein kinase C activators block specific calcium and potassium current components in isolated hippocampal neurons. *J Neurosci* 8:4069–4078.
- Elkins T, Ganetzki B, Wu C-F (1986) A *Drosophila* mutation that eliminates a calcium-dependent potassium current. *Proc Natl Acad Sci USA* 83:8415–8419.
- Emerick MC, Agnew WS (1989) Identification of phosphorylation sites for adenosine 3',5'-cyclic phosphate dependent protein kinase on the voltage-sensitive sodium channel from *Electrophorus electricus*. *Biochemistry* 28:8367–8380.
- Ewald D, Williams A, Levitan IB (1985) Modulation of single Ca^{2+} -dependent K^{+} channel activity by protein phosphorylation. *Nature* 315:503–506.
- Farley J, Rudy B (1988) Multiple types of voltage-dependent Ca^{2+} -activated K^{+} channels of large conductance in rat brain synaptosomal membranes. *Biophys J* 53:919–934.
- Fernandez A, Brautigan DL, Mumby M, Lamb NJC (1990) Protein phosphatase type-1, not type-2A, modulates actin microfilament integrity and myosin light chain phosphorylation in living nonmuscle cells. *J Cell Biol* 111:103–112.
- Hille B (1984) Ionic channels of excitable membranes. Sunderland, MA: Sinauer.
- Hosey MM, Borsotto M, Lazdunski M (1986) Phosphorylation, and dephosphorylation of dihydropyridine-sensitive voltage-dependent Ca^{2+} channel in skeletal muscle membranes by cAMP- and Ca^{2+} -dependent processes. *Proc Natl Acad Sci USA* 83:3733–3737.
- Hosey MM, Lazdunski M (1988) Calcium channels: molecular pharmacology, structure and regulation. *J Membr Biol* 104:81–105.
- Huganir RL, Delcour AH, Greengard P, Hess GP (1986) Regulation of desensitization of the nicotinic acetylcholine receptor by protein phosphorylation. *Nature* 321:774–776.
- Ingebritsen TS, Cohen P (1983) The protein phosphatases involved in cellular regulation. 1. Classification and substrate specificities. *Eur J Biochem* 132:255–261.
- Ingebritsen TS, Stewart AA, Cohen P (1983) The protein phosphatases involved in cellular regulation. 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. *Eur J Biochem* 132:297–307.
- Iverson LE, Tanouye MA, Lester HA, Davidson N, Rudy B (1988) Potassium channels from *Shaker* RNA expressed in *Xenopus* oocytes. *Proc Natl Acad Sci USA* 85:5723–5727.
- Kaczmarek LK (1988) The regulation of neuronal calcium and potassium channels by protein phosphorylation. *Adv Sec Mess Phosph Res* 22:113–139.
- Kamb A, Tseng-Crank J, Tanouye MA (1988) Multiple products of the *Drosophila Shaker* gene may contribute to potassium channel diversity. *Neuron* 1:421–430.
- Klumpp S, Cohen P, Schultz JE (1990) Okadaic acid, an inhibitor of protein phosphatase 1 in *Paramecium*, causes sustained Ca^{2+} -dependent backward swimming in response to depolarizing stimuli. *EMBO J* 9:685–689.
- Koren G, Liman ER, Logothetis DE, Nadal-Ginard B, Hess P (1990) Gating mechanism of a cloned potassium channel expressed in frog oocytes and mammalian cells. *Neuron* 2:39–51.
- Kume H, Tokuno H, Tomita T (1989) Regulation of Ca^{2+} -dependent K^{+} -channels in tracheal myocytes by phosphorylation. *Nature* 341:152–154.
- Latorre R (1986) The large calcium-activated potassium channel. In: *Ion channel reconstitution* (Miller C, ed), pp 431–467. New York: Plenum.
- Latorre R, Miller C (1983) Conduction and selectivity in potassium channels. *J Membr Biol* 71:11–30.
- Lechleiter JD, Dartt DA, Brehm P (1988) Vasoactive intestinal peptide activates Ca^{2+} -dependent K^{+} channels through a cAMP pathway in mouse lacrimal cells. *Neuron* 1:227–235.
- MacKinnon R, Reinhart PH, White MM (1988) Charybdotoxin block of *Shaker* K^{+} channels expressed in *Xenopus* oocytes suggests that functionally different K^{+} channels share common structural features. *Neuron* 1:997–1001.
- Magleby KL, Pallotta BS (1983) Calcium dependence of open and shut interval distributions from calcium-activated potassium channels in cultured rat muscle. *J Physiol (Lond)* 344:585–604.
- Marty A (1983) Ca^{2+} -dependent K^{+} -channels with large unitary conductance. *Trends Neurosci* 6:262–265.
- Marty A (1989) The physiological role of calcium-dependent channels. *Trends Neurosci* 12:420–424.
- McManus OB, Magleby KL (1985) The large-conductance calcium-activated potassium channel in cultured rat muscle has at least three open states of similar conductance and six shut states. *Biophys J* 47:137a.
- Methfessel C, Boheim G (1982) The gating of single calcium-dependent potassium channels is described by an activation/blockade mechanism. *Biophys Struct Mech* 9:35–60.
- Moczydlowski E, Latorre R (1983) Gating kinetics of Ca^{2+} -activated K^{+} channels from rat muscle incorporated into planar lipid bilayers. *J Gen Physiol* 82:511–542.
- Moczydlowski E, Lucchesi K, Ravindran A (1988) An emerging pharmacology of peptide toxins targeted against potassium channels. *J Membr Biol* 105:91–111.
- Mueller P, Rudin DO (1969) Translocators in bimolecular lipid membranes: their role in dissipative and conservative energy transduction. *Curr Top Bioenerg* 3:157–249.
- Noda M, Ikeda T, Kayano T, Suzuki H, Takeshima T, Kurasaki M, Takahashi H, Numa S (1986) Existence of distinct sodium channel messenger RNAs in rat brain. *Nature* 320:188–192.
- Pallis DC, Shahrik LK, Martin BL, Jaspers S, Miller TB, Brautigan DL, Roberts TM (1990) Polyoma small and middle T antigens and SV-40 small T antigen form stable complexes with protein phosphatase 2A. *Cell* 60:167–176.
- Pennafather P, Lancaster B, Adams PR, Nicoll RA (1985) Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells. *Proc Natl Acad Sci USA* 82:3040–3044.
- Peters K, Demaille J, Fisher E (1977) Adenosine 3':5'-monophosphate dependent protein kinase from bovine heart. Characterization of the catalytic subunit. *Biochemistry* 16:5691–5697.
- Petersen OH, Maruyama Y (1984) Calcium-activated potassium channels and their role in secretion. *Nature* 307:693–696.
- Pongs O (1989) Molecular basis of potassium channel diversity. *Pfluegers Arch* 414:S71–S75.

- Reinhart PH, Levitan IB (1987) Modulation of charybdotoxin-sensitive calcium-dependent potassium channels by phosphorylation. *Soc Neurosci Abstr* 13:1439.
- Reinhart PH, Chung S, Levitan IB (1989) A family of calcium-dependent potassium channels from rat brain. *Neuron* 2:1031-1041.
- Romey G, Lazdunski M (1984) The coexistence in rat muscle cells of two distinct classes of Ca^{2+} -dependent K^{+} -channels with different pharmacological properties and different physiological functions. *Biochem Biophys Res Commun* 118:669-674.
- Rossie S, Catterall WA (1987) Cyclic AMP-dependent phosphorylation of voltage-sensitive sodium channels in primary cultures of rat brain neurons. *J Biol Chem* 262:12735-12744.
- Rossie S, Gordon D, Catterall WA (1987) Identification of an intracellular domain of the sodium channel having multiple cAMP-dependent phosphorylation sites. *J Biol Chem* 262:17530-17535.
- Rossie S, Catterall WA (1989) Phosphorylation of the α subunit of rat brain sodium channels by cAMP-dependent protein kinase at a new site containing Ser⁶⁸⁶ and Ser⁶⁸⁷. *J Biol Chem* 264:14220-14224.
- Rudy B (1988) Diversity and ubiquity of K channels. *Neuroscience* 25:729-749.
- Sadoshima J, Akaike N, Kanaide H, Nakamura M (1988) Cyclic AMP modulates Ca-activated K channel in cultured smooth muscle cells of rat aortas. *Am J Physiol* 255:H754-H759.
- Shearman MS, Sekiguchi K, Nishizuka Y (1989) Modulation of ion channel activity: a key function of the protein kinase C enzyme family. *Pharmacol Rev* 41:211-237.
- Sikdar SK, McIntosh RP, Mason WT (1989) Differential modulation of Ca^{2+} -activated K^{+} -channels in ovine pituitary gonadotrophs by GnRH, Ca^{2+} and cyclic AMP. *Brain Res* 496:113-123.
- Singer JJ, Walsh JV (1986) Large-conductance Ca^{2+} -activated K^{+} -channels in freshly dissociated smooth muscle cells. *Membr Biochem* 6:83-110.
- Singer JJ, Walsh JV (1987) Characterization of calcium-activated potassium channels in single smooth muscle cells using the patch-clamp technique. *Pfluegers Arch* 408:98-111.
- Stuehmer W, Ruppersberg JP, Schroeter KH, Sakmann B, Stocker M, Giese KP, Perschke A, Baumann A, Pongs O (1989) Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *EMBO J* 8:3235-3244.
- Tempel BL, Yan YN, Jan LY (1988) Cloning of a probable potassium channel gene from mouse brain. *Nature* 332:837-839.
- Timpe LC, Schwarz TL, Tempel BL, Papazian DM, Jan YN, Jan LY (1988) Expression of functional potassium channels from *Shaker* cDNA in *Xenopus* oocytes. *Nature* 331:143-145.
- Vergara C, Latorre R (1983) Kinetics of Ca^{2+} -activated K^{+} channels from rabbit muscle incorporated into planar bilayers. *J Gen Physiol* 82:543-568.
- Wolff D, Vergara C, Cecchi X, Latorre R (1986) Characterization of large-unitary-conductance calcium-activated potassium channels in planar lipid bilayers. In: *Ionic channels in cells and model systems* (Latorre R, ed), pp 307-334. New York: Plenum.
- Yang S-D, Yu J-S, Fong Y-L (1986) Purification and characterization of two inactive/latent protein phosphatases from pig brain. *J Biol Chem* 261:5590-5596.
- Yang S-D, Liu J-S, Fong Y-L, Yu J-S, Tzen T-C (1987) Endogenous basic protein phosphatases in the brain myelin. *J Neurochem* 48:160-166.