Conditional and Unconditional Inhibition of Calcium-activated Potassium Channels by Reversible Protein Phosphorylation*

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Large conductance, calcium-activated potassium channels (BK$_{Ca}$, or maxi-K) are important determinants of membrane excitability in many cell types. We used patch clamp techniques to study the biochemical regulation of native BK$_{Ca}$ channel proteins by endogenous Ser/Thr-directed protein kinases and phosphatases in cell-free membrane patches from rat pituitary tumor cells (GH$_4$C$_1$). When protein kinase activity was blocked by removing ATP, endogenous protein phosphatases slowly increased BK$_{Ca}$ channel activity approximately 3-fold. Dephosphorylated channels could be activated fully by physiological increases in cytoplasmic calcium or membrane depolarization. In contrast, endogenous protein kinases inhibited BK$_{Ca}$ channel activity at two functionally distinct sites. A closely associated, cAMP-dependent protein kinase rapidly reduced channel activity in a conditional manner that could be overcome completely by increasing cytoplasmic free calcium 3-fold or 20 mV further depolarization. Phosphorylation at a pharmacologically distinct site inhibited channel activity unconditionally by reducing availability to approximately half that of maximum at all physiological calcium and voltages. Conditional versus unconditional inhibition of BK$_{Ca}$ channel activity through different protein kinases provides cells with a powerful computational mechanism for regulating membrane excitability.

Electrophysiological measurements indicate that the activity of BK$_{Ca}$ channels in native cells may be up- or down-regulated by reversible protein phosphorylation. For example, protein kinase-induced phosphorylation enhances calcium-activated potassium currents in smooth muscle (5, 6), whereas in photoreceptors (7), hippocampal neurones (8–11), and neuroendocrine cells (12), the currents are inhibited by kinase activity. BK$_{Ca}$ channels exist as multimeric protein complexes composed of two integral membrane subunits, the pore-forming α subunit and the regulatory β subunit (1). All α subunits are apparently coded by the same gene, the slo gene, but alternative RNA splicing during development produces functionally distinct channel proteins in different cell types (13–15). Hence, opposite modulatory effects of protein phosphorylation are likely to reflect differential modulation of BK$_{Ca}$ channel subtypes derived from alternatively spliced mRNA transcripts or could arise as a result of variations in the α and β subunit composition of the channel complexes (1, 16) or more indirect signaling cascades. We have used the patch-clamp technique to investigate the modulation of BK$_{Ca}$ channel behavior by reversible protein phosphorylation at the molecular level by recording channel opening behavior in cell-free patches of native membrane. Thus, we report studies of BK$_{Ca}$ channel modulation under controlled conditions in the absence of alternative splicing or diffusible regulatory proteins, where the open probability ($P_o$) of the channels was determined under a variety of experimental conditions to promote or inhibit protein phosphorylation.

In mammalian endocrine and nervous systems, most examples of BK$_{Ca}$ channel regulation by hormones and neurotransmitters involve channel inhibition by protein phosphorylation (1, 9–11). Similarly, the BK$_{Ca}$ channels in rat pituitary tumor cells (GH$_4$C$_1$ cells) are also inhibited by phosphorylation. In these cells, maximal stimulation of either protein kinase A (PKA) or C (PKC) completely suppresses BK$_{Ca}$ channel activity in the physiological voltage range (17, 18); conversely, hormones that inhibit secretion from GH$_4$C$_1$ cells have been shown to stimulate BK$_{Ca}$ channels through protein dephosphorylation (12, 19). Thus, the BK$_{Ca}$ channels in GH$_4$C$_1$ cells are regulated in the same way as many other physiologically relevant examples of BK$_{Ca}$ channels in the brain. For this reason, we have chosen to study the effects of reversible protein phosphorylation on the calcium and voltage dependence of single native BK$_{Ca}$ channels from GH$_4$C$_1$ cells. Previous studies at the single-channel level have focused on BK$_{Ca}$ channels that are stimulated by protein phosphorylation (5, 20–23). In this paper, we report evidence of BK$_{Ca}$ channel modulation using a cell-free system that qualitatively and quantitatively reproduces the effects of hormones on intact cells.

This is the first study of native BK$_{Ca}$ channels in native membranes that systematically varies all three of the molecular mechanisms known to regulate these channels: voltage, intracellular free calcium concentration, and protein phosphorylation. BK$_{Ca}$ channel modulation has not previously been
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examined at sufficiently high open probabilities to address the molecular mechanism of modulation across the complete range of channel activity. To measure the effects of reversible protein phosphorylation on BK<sub>Ca</sub> channel activity reproducibly over the entire physiological range, 0.01 ≤ P<sub>o</sub> ≤ 0.9, we have found it essential to use 1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)<sup>1</sup> and its dibromo analogue to buffer calcium on the cytoplasmic side of the membrane patches. EGTA, which saturates at submicromolar concentrations of calcium (24–26), is an ineffective buffer of physiological calcium transients. In contrast, BAPTA and dibromo-BAPTA bind calcium rapidly and independently of pH and physiological magnesium concentrations with an affinity that allows effective buffering of free calcium at concentrations up to and above 10 μM (27). With this experimental precaution, our measurements have revealed a novel property of BK<sub>Ca</sub> channel regulation by protein phosphorylation. Our findings have important implications for the control of BK<sub>Ca</sub> channel proteins and demonstrate that reversible protein phosphorylation cascades play an integral role in controlling cellular excitability through their actions on BK<sub>Ca</sub> channels.

**EXPERIMENTAL PROCEDURES**

Cell Culture—BK<sub>Ca</sub> channel activity was studied in GH<sub>3</sub> C cells, an immortal cell line derived from rat anterior pituitary tumor cells (28). Cells were maintained at 37 °C in sterile Ham's F-10 culture medium supplemented with fetal bovine serum (2.5%, vol), equine serum (12.5%, vol), and antibiotics (penicillin and streptomycin). Cells were plated onto plain or collagen-coated glass coverslips 1–6 days before patch clamp recording, and the culture medium was replaced every 3–4 days. Cells from culture passage 2–28 were used in these studies.

Electrophysiological Recording—The activity of single BK<sub>Ca</sub> channels was investigated using conventional patch clamp recording techniques (29). Patch pipettes were fabricated from borosilicate glass capillary tubing (7052; Garner Glass Co, Claremont, CA), the pipette shank was coated with Sylgard (Dow Corning, Midland, MI), and the tips were fire-polished just before use. These pipettes had resistances in the range of 4–8 megohms when filled with electrolyte (see below for composition). Single-channel activity was recorded at room temperature from cell-attached patches of membrane on intact cells and from cell-free, inside-out patches using an Axopatch 1C amplifier and TL-1 interface (Axon Instruments, Burlingame, CA). Data were recorded and analyzed using pClamp software, version 6.0.1 (Axon Instruments).

Voltage-dependent channel activity was measured over the range −40 to 100 mV, with the applied voltage stepped at random. BK<sub>Ca</sub> channels were identified by their conductance, ~120 picosiemens in a physiological [K+] gradient. The stochastic behavior of ion channels in these patches was interpreted using standard analytical procedures. Channel open probability (P<sub>o</sub>) was determined from continuous records of channel activity over 10, 20, or 30 s at a given potential; P<sub>o</sub> may show some variability when measured over shorter periods (30). Excised membrane patches commonly contained more than one active BK<sub>Ca</sub> channel. The number of active channels could be counted with confidence (N ≤ 3) when all the channels were stimulated to open simultaneously, giving high open probabilities (P<sub>o</sub> near 1) during a 10-s period. In these patches, P<sub>o</sub> was calculated according to the relationship

\[
P_o = \sum_i (i \times t_i) (N \times t_i)
\]

(Eq. 1)

where \(i = 1 \) to \(N\), \(N\) is the maximum number of channels open simultaneously at the most depolarized membrane potentials (positive to +60 mV; \(N \leq 3\), \(t_i\) is the total time of the recording and \(t_i\) is the cumulative time during which exactly \(i\) channels are open. When channel behavior in multichannel patches was not monitored across the complete voltage range, activity is expressed as \(N^i P_o\). Data are expressed as the mean value ± S.D. from multiple independent measurements (\(n \geq 3\). Statistical analyses were made by Student's t test (2-tailed) for paired or unpaired data as appropriate; the null hypothesis was rejected when \(p < 0.05\). Boltzmann curves were generated by best fit to complete data

**RESULTS**

When GH cells are voltage-clamped through perforated patches in a physiological salt solution, BK<sub>Ca</sub> channels dominate the steady-state membrane conductance at depolarized voltages (12, 32, 33). In contrast, individual channels in cell-attached patches on resting cells often showed surprisingly low activity: \(P_o < 0.1\) even at +40 mV. However, when membrane patches were excised into an ATP-free bathing solution (Fig. 1), the mean open probability (P<sub>o</sub>) increased slowly but significantly over 10–15 min, reaching a new steady-state level that was 258 ± 167% greater than the activity measured immediately following patch excision (\(n = 11\); \(p < 0.01\). In many patches, an increase in the number of BK<sub>Ca</sub> channels open simultaneously (N) was also observed. The rate of increase in channel activity was not affected by varying calcium concentration the range 0.1 to 1.0 μM or by varying the membrane potential of the patch from 0 to +40 mV (not shown).

**Dethosphorylated BK<sub>Ca</sub> Channels Respond to Voltage and Calcium in the Physiological Ranges—**Once BK<sub>Ca</sub> channel activity had stabilized in MgATP-free solution, it remained stable for the duration of the recording (up to 90 min, Fig. 2A). Nevertheless, the mean open probability of the channels in the patch could be increased independently by further depolarization (V) or by increasing the free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) at the intracellular face of the membrane, up to a maximum \(P_o = 0.86 ± 0.11\) (\(n = 10\) complete P<sub>o</sub>V curves at 5 different [Ca<sup>2+</sup>]<sub>i</sub> in 8 patches) (Fig. 2B). These effects were reproducible and fully reversible and showed no sign of inactivation during prolonged stimulation. At calcium concentrations between 0.1 and 10.0 μM, an e-fold increase in P<sub>o</sub> over the linear range from 0.1 to 0.8 was produced by increasing the voltage 14.6 ± 6.6 mV (\(n = 8\) independent curves, in 6 patches). On average, increasing free calcium 10-fold at the cytoplasmic surface reduced the voltage required to produce half-maximal activity (V<sub>1/2</sub>) by 55mV without changing the intrinsic voltage dependence of activity (Fig. 2C). Thus, when protein kinases are inhibited, native BK<sub>Ca</sub> channels are extremely sensitive to changes in voltage and calcium in the physiological ranges.

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1 The abbreviations used are: BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; PKA and PKC, protein kinases A and C, respectively; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A.
dissociation constant ($K_D$) for Ca$^{2+}$ binding was 1.8 $\mu$M (calculated from the linear fit to the data in Fig. 2C (34)). Hence, although depolarization greater than any action potential ($-155$ mV) would be required for half-maximal channel activity at the resting calcium level in the cell ($-0.1$ mM), half-maximal channel activity would be sustained below the resting membrane potential of the cell ($-255$ mV) as long as free Ca$^{2+}$ levels underneath the plasma membrane exceeded 10.0 $\mu$M.

Protein Dephosphorylation Increases BK$_{Ca}$ Channel Activity—Previous studies show that protein dephosphorylation stimulates BK$_{Ca}$ channel activity in GH$_4$C$_1$ cells (12, 19); this behavior is similar to that of BK channels in other neuronal cell types (3, 9–11). The increase in BK$_{Ca}$ channels activity we observed following patch excision into ATP-free solutions is consistent with this published data. Two independent pharmacological manipulations of the patches provide additional evidence that endogenous protein phosphatases are responsible for the increase in BK$_{Ca}$ channel activity. First, inhibition of serine/threonine-directed protein phosphatases with the structurally unrelated microbial toxins 1 $\mu$M microcystin ($n = 3$) or 1 nM okadaic acid ($n = 5$) to selectively inhibit the PP2A family of phosphatases (35) completely prevented the increase in channel activity when they were added before patch excision (Fig. 1B). In contrast, if the inhibitors were not added until after channel activity had increased in ATP-free solution, they had no effect on channel activity (not shown), which rules out direct effects of the toxins on channel gating. Second, subsequent addition of MgATP at the intracellular face of the membrane rapidly reduced activity back to the low level recorded immediately following patch excision (Fig. 3A; $n = 6$). This effect of MgATP was not readily reversible, but neither free Mg$^{2+}$ ions alone (as 1 mM MgCl$_2$; $n = 4$) nor 1 mM MgADP ($n = 4$) nor 0.1 mM GTP ($n = 2$) could mimic this effect of MgATP. These findings demonstrate a specific requirement for MgATP, which indicates a role for protein phosphorylation in the suppression of BK$_{Ca}$ channel opening. In support of this conclusion, the effect of MgATP was prevented when PKA was inhibited by preaddition of 10 $\mu$M Rp-cAMPS with 1 $\mu$M wiptide (PKI$_{5-22}$ (Peninsula Labs)).

Protein Phosphorylation Reduces the Sensitivity of BK$_{Ca}$ Channel Inhibition by Protein Phosphorylation

Fig. 1. BK$_{Ca}$ channel activity runs up in ATP-free solution. $A$, continuous records of unitary BK$_{Ca}$ currents recorded at 0 mV in the same excised, inside-out patch of membrane from a GH$_4$C$_1$ cell immediately after excision into ATP-free solution with 1 $\mu$M Ca and 22 min later. $B$, time course of channel activity ($N^oP_o$ calculated from continuous 20-s records) following patch excision into ATP-free solution (filled circles). Run up was prevented in cells that had been treated with 1 nM okadaic acid for 10 min before patch excision (open circles).

Fig. 2. Dephosphorylated BK$_{Ca}$ channels respond to physiological calcium and voltage. $A$, time course of BK$_{Ca}$ channel activity ($N^oP_o$ calculated from continuous 20-s records at discrete intervals throughout the experiment) during changes in the membrane voltage ($V_m$) and cytoplasmic calcium ([Ca$^{2+}$]) in the prolonged absence of exogenous ATP. Following run-up, activation by physiological [Ca$^{2+}$] and voltage was fully reversible. $B$, voltage dependence of BK$_{Ca}$ channel activity measured in the same patch in 0.1 mM (filled circles) and 1 mM (filled squares) free calcium following development of stable channel activity in the absence of exogenous ATP. The activity of dephosphorylated channels increases steeply with depolarization (e-fold/14.6 mV). $C$, plot of the voltage required for half-maximal channel activity ($V_{1/2}$) as a function of [Ca$^{2+}$] on the former cytoplasmic side of the membrane in the prolonged absence of exogenous ATP. Increasing [Ca$^{2+}$] 10-fold shifts the voltage dependence of channel activity by 55 mV in the depolarizing direction (data fit with a straight line ($r = 0.85$), which was then used to determine $K_D$ and $\delta$ (34)).
Channels to Calcium—The activity of BK<sub>Ca</sub> channels in dephosphorylated membrane patches was reduced dramatically by subsequent protein phosphorylation (Fig. 3). The addition of 1 mM MgATP (but not ADP or GTP; see above) to the solution at the intracellular face of the excised patch caused a shift of \(19 \pm 6\) mV (\(n = 5\) patches; \(p < 0.05\)) toward more depolarizing potentials in the voltage dependence of BK<sub>Ca</sub> channel activity without changing the slope of the relationship (Fig. 3C). We interpret the reduction in activity as a decrease in channel sensitivity to calcium because the same effect was observed at two calcium concentrations (1 and 5 \(\mu M\) Ca) in the same patch before (filled circles) and after (filled squares) the addition of 1 mM MgATP at the intracellular face of the membrane. Note that the intrinsic voltage dependence of channel activity, indicated by the slope of the relationship, does not change.

MgATP in excised patches; channels could be stimulated maximally by raising calcium or by depolarizing further (Fig. 3). In three patches, \(P_{o_{\text{max}}}\) was 0.76 ± 0.07 following run up in the absence of ATP and 0.71 ± 0.10 following the addition of MgATP (no significant difference; \(p > 0.1\)).

The inhibitory effect of MgATP on channel activity was not potentiated by subsequent addition of low concentrations (<10 nM) of okadaic acid or microcystin (not shown), indicating that the basal activity of PKA was substantially higher than that of the phosphatase. This conclusion is consistent with the low activity of BK<sub>Ca</sub> channels in cell-attached patches observed before excision and is supported by the observation that incubating cells in 1 nM okadaic acid and 1 mM cpt-cAMP for 10 min before patch excision also had no significant effect on channel activity in cell-attached patches (\(n = 4\)). However, the open probability of the channels after patch excision from cells treated with okadaic acid could not be increased beyond \(P_{o} = 0.47 \pm 0.19\) (\(n = 10\) complete \(P_{o}\)V curves over a range of [Ca\(^{2+}\)] in 6 patches) at any calcium concentration (<50 \(\mu M\)) or voltage (<100 mV) we examined (Fig. 4). In all but one of the patches treated with okadaic acid before excision, maximal opening
probability was <0.5. In contrast, in every single one of the dephosphorylated patches and those patches that were rephosphorylated after excision, maximal channel open probability could be driven to $P_\text{o} > 0.7$. Hence, we interpret this suppressed channel activity as an effect on all of the channels rather than on a subset of the channel population. Our data indicate that in intact cells there is an additional kinase regulating the activity of BK$_{ca}$ channels to produce a significantly different physiological effect on cell excitability. PKA-mediated phosphorylation reduces the sensitivity of BK$_{ca}$ channels to increases in voltage and calcium, but phosphorylation by the second kinase reduces the maximum attainable channel activity by half. In other words, the inhibition by PKA is conditional, but the inhibition by the second kinase is unconditional.

**DISCUSSION**

The data reported here establish clearly that native calcium-activated potassium channels with the largest conductance (BK$_{ca}$ or maxi-K) are inhibited by endogenous protein kinases and stimulated by endogenous protein phosphatases that remain closely associated with the channels in cell-free patches from an immortalized endocrine rat pituitary cell line (GH$_4$C$_1$). Our results are entirely consistent with the physiological stimulation of excitability and secretion in the anterior pituitary by hypothalamic neuropeptides, which increase protein kinase activity through $G_\alpha$- and $G_\beta\gamma$-coupled receptors (36, 37). Such kinase-mediated inhibition demonstrates that BK$_{ca}$ channels in GH$_4$C$_1$ cells behave in the same way as the calcium-activated potassium currents in hippocampal neurons of the mammalian central nervous system (3, 8–11). In contrast, most previous single-channel studies of BK$_{ca}$ channel modulation have been conducted on channels that are stimulated by protein kinases and inhibited by protein phosphatases, using either native channels in nonneuronal tissues (5, 21, 22) or recombinant channels that are expressed heterologously in Xenopus oocytes, where the channels are much less sensitive to calcium and phosphorylation (38–40). Nevertheless, single-channel studies in neurons have also shown stimulation of BK$_{ca}$ channel activity by protein kinases (20, 23, 40). Similar diversity in the response to protein kinases and phosphatases has been reported for BK$_{ca}$ channels in cells from the vascular system (42–44). As yet there is only one $slo$ gene encoding the pore-forming subunit of the BK$_{ca}$ channel in any single species; hence, such diametrically opposed differences in modulation by phosphorylation must reflect alternative splicing of the gene or interaction with additional regulatory subunits or more indirectly signaling cascades through which the protein kinases produce their effects.

We have used a number of criteria to identify the specific biochemical mechanism of the observed “run-up” of BK$_{ca}$ channel activity in excised membrane patches, although we have not characterized as clearly all of the enzymes or their substrates that are responsible for modulating the availability of BK$_{ca}$ channels for opening. This is the first study to investigate the effect of protein phosphorylation on BK$_{ca}$ channel activity at maximal open probabilities. As a result, we have discovered that pharmacologically distinct protein kinases produce functionally distinct effects on channel behavior. One kinase, probably PKA, reduces stimulation of BK$_{ca}$ channel activity by calcium and voltage without reducing the availability of the channels. For an enzyme, this would correspond to a reduction in $K_m$ without a change in $V_{\text{max}}$. A second, unidentified kinase reduces the availability of the channels at all calcium concentrations and voltages, corresponding to a decrease in $V_{\text{max}}$. The exact mechanisms by which the functional consequences of protein phosphorylation are achieved remain unclear. Both $\alpha$ and $\beta$ subunits of the channel complex contain potential sites for phosphorylation by protein kinase A and protein kinase C (16). The C-terminal “tail” of the $\alpha$ subunit has also been demonstrated to confer the calcium-sensing properties of BK$_{ca}$ channels (38); thus, the addition or removal of charged phosphate groups could alter the calcium sensitivity of channel activity by influencing the “Ca$^{2+}$ sensor” in the protein. Alternatively, co-expression of the $\beta$ subunit has been shown to enhance the calcium sensitivity of BK$_{ca}$ channels (13, 39, 45) so reversible phosphorylation could modulate channel behavior by promoting or inhibiting interactions between the $\alpha$ and $\beta$ subunits in the heteromultimeric channel complex.

**Fig. 5. Diagramatic summary of the experimental results.** Boltzmann curves generated from the experimental data (all values given in text). Solid lines represent the $P_\text{o}/V$ relationships when ATP was omitted; broken lines represent the relationships with ATP present. $A$, proteins dephosphorylated. The activity of dephosphorylated BK$_{ca}$ channel proteins is sensitive to [Ca$^{2+}$] and voltage in the physiological ranges (method: patches excised in the absence of exogenous nucleotides). $B$, phosphorylation at PKA/PP2A site only. Rephosphorylation of the excised patch reduces the [Ca$^{2+}$] sensitivity of channel activity conditionally, without altering $P_{\text{o,max}}$ (Method: ATP added following stable run up of channel activity). $C$, phosphorylation at both PKA/PP2A and PKC/PP1 sites. Maximally phosphorylated channel proteins have reduced [Ca$^{2+}$] sensitivity, and $P_{\text{o,max}}$ is reduced unconditionally to ~50% (Method: intact cells bathed in okadaic acid and patches excised into MgATP and cAMP).
Coupled receptors would inhibit BK Ca channel activity and activity of calcium- and voltage-activated potassium channels. Effects of endogenous protein kinases and phosphatases on the BKCa channels in other systems (23, 46). PP2A (31), and PP1 selectively reverses the effects of PKC on phosphatase less sensitive to okadaic acid than PP2A. Protein phosphatase 1 (PP1) is much less sensitive to okadaic acid than PP2A. Protein phosphatase 1 (PP1) is much less sensitive to okadaic acid than PP2A (31), and PP1 selectively reverses the effects of PKC on BKCa channels in other systems (23, 46).

In summary, we have identified two functionally distinct effects of endogenous protein kinases and phosphatases on the activity of calcium- and voltage-activated potassium channels. Phosphorylation at a putative PKA/PP2A site reduces the calcium sensitivity of BKCa channels without altering the voltage dependence of activation or suppressing maximum channel availability. In contrast, phosphorylation at a putative PKC/PP1 site reduces the availability of the channels at all calcium concentrations and voltages in the physiological range. Such conditional and unconditional regulation by two pharmacologically distinct signaling pathways provides the nervous and neuroendocrine systems with a powerful computational process at the cellular level. Activation of Gq-coupled receptors would inhibit BKCa channel activity and, hence, stimulate cell excitability in a conditional manner, an effect that could be reversed when the intracellular calcium concentration increased or the membrane depolarized further. In contrast, activation of Gq-coupled receptors would inhibit BKCa channel activity and stimulate excitability uncontrollably until the effects of PKC were reversed by protein phosphatase activity.

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