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Stimulatory Regulation of the Large-Conductance, Calcium-Activated Potassium Channel by G Proteins in Bovine Adrenal Chromaffin Cells

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SUMMARY

G proteins regulate the electrical activity of various cells through their actions on membrane ion channels. In the present study, the effect of G proteins was examined on unitary, large conductance (BK), Ca^{2+}-activated K^+ channels measured in excised, inside-out patches of membrane obtained from cultured bovine adrenal chromaffin cells. Cytoplasmic application of either guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) or AlF^-_4 to stimulate G proteins resulted in a >4-fold increase in the open probability of the BK channel measured at +40 mV in the presence of a 1 μM concentration of Ca^{2+}. A similar stimulatory regulation was observed after the addition of an activated, mixed $G_i/G_{o\alpha}$ preparation. The increase in the open probability during G protein stimulation was associated with a large reduction in the duration of a long closed state of the channel and could be observed in the presence of a protein kinase inhibitor. The half-maximal voltage required for steady state activation of the BK channel decreased from +63 mV to +48 mV in the presence of GTPγS. In addition, the half-maximal Ca^{2+} concentration required for channel opening was reduced from 11.7 μM in control measurements to 1.3 μM during regulation by GTPγS. Thus, G proteins increase the open probability of the chromaffin BK Ca^{2+}-activated K^+ channel by shifting the voltage dependence of channel gating to more negative potentials and by enhancing the affinity of the channel for Ca^{2+}. Stimulatory regulation may provide a compensatory mechanism for decreasing the action potential duration during secretagogue-mediated exocytosis.

BK, Ca^{2+}-activated K^+ channels are a widely distributed group of integral membrane proteins found in muscle (1, 2), nerve (3, 4), and a variety of other tissues (5). Unitary BK channel measurements were first obtained by Marty (6) in excised membrane patches obtained from cultured bovine adrenal chromaffin cells. The adrenal chromaffin BK channel has a single-channel conductance that ranges, depending on the recording conditions, from 96 to 310 pS and requires micromolar concentrations of internal Ca^{2+} for activity (6–9). The chromaffin cell channel is blocked by a number of agents, including tetraethylammonium, quinidine, and Ba^{2+} (7–9). Of particular importance, the BK channel can be activated under whole-cell recording conditions after the movement of Ca^{2+} into the chromaffin cell through the plasma membrane Ca^+ channels (7), indicating a physiological mechanism for opening of this channel. Although previous studies have shown that Ca^{2+}-activated K^+ channels found in other tissues can be regulated by protein kinases (10–12) and G proteins (13, 14), there is limited information available concerning the regulation of the chromaffin BK channel.

In a previous study, we reported that dialysis of bovine adrenal chromaffin cells with GTPγS results in the activation of a voltage-dependent, outward-directed, whole-cell K^+ current (15). The GTPγS-sensitive K^+ current displays rapid activation kinetics (15) that distinguish this current from the basal K^+ current normally measured in the chromaffin cells (7, 16). The goal of the present investigation was to study the regulation of chromaffin K^+ currents on the single-channel level to determine the site of G protein action. It is reported that G proteins increase the P_o of the chromaffin BK Ca^{2+}-activated K^+ channel. This regulation results from the ability of the G proteins to shift the voltage dependence of the channel P_o and to increase the sensitivity of the BK channel to Ca^{2+}. These findings have been previously reported in preliminary form (17).

Materials and Methods

Preparation of chromaffin cells. Primary cultures of bovine adrenal chromaffin cells were prepared by collagenase digestion and

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ABBREVIATIONS: BK, large conductance; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PTX, pertussis toxin; DTT, dithiothreitol; PKI_{6-22}, protein kinase inhibitor; PKA, cAMP-dependent protein kinase; P_o, channel open-state probability; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

centrifugation on Renografin gradients as described previously (18). Cells were subjected to a Percoll density gradient to enrich the cell preparation for norepinephrine-secreting cells. Briefly, chromaffin cells obtained from Renografin gradients were washed to remove Renografin and centrifuged at 30,000 × g_{max} for 30 min at 20° in a final concentration of 42.5% Percoll. Cells recovered from the top half of the centrifuge tube contained ~50% of their total catecholamines as norepinephrine versus 20–30% in unfractionated cells. For patch-clamp recording, cells were plated onto glass coverslips coated with 1 $\mu g/cm^2$ laminin (generously supplied by Dr. Thomas Borg, University of South Carolina School of Medicine, Columbia, SC) in 35 × 10-mm polystyrene dishes (Corning). Cells were plated at a density of 1 × 10^6 cells in 2.5 ml of 95% Dulbecco's modified Eagle's medium/F-12 plus 5% bovine calf serum and maintained in this medium. Coverslips were then transferred to a recording chamber containing the normal bath solution (see below). Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37° and used at 1–5 days after plating.

Recording procedure and measurement of single-channel K^+ currents. Single-channel K^+ currents were recorded at room temperature (22–24°) by using the inside-out configuration of the patch-clamp technique (19). A reference electrode made from a Ag/AgCl pellet was connected to the bath using an agar salt bridge saturated with external solution. Data were adjusted for liquid junction potentials that are produced when there is an interface between dissimilar salt solutions. In this study, the membrane potential (V_m) was corrected for junction potentials that arose (i) between the pipette solution and the bath solution (V_{LJ}) and (ii) between the bath solution and the reference electrode ($V_{2,1}$) with the use of the equation $V_m = V - V_{LJ} - V_{2,1}$, as described previously (20). In this equation, V was the voltage reading on the patch-clamp amplifier, V_{LJ} was equal to the offset potential created during the zeroing of the pipette solution in the internal (bath solution), and $V_{2,1}$ was determined for the bath solution using a 3 M KCl filled patch pipette. Values of V_{LJ} were usually between +3 and +5 mV.

Steady state single-channel records were measured using a List L/M EPC-7 amplifier and stored on video tape using an analogue data recorder (Instrutech). Data were later digitized at 8 kHz and filtered at 2–4 kHz with an eight-pole Bessel filter (Frequency Devices). The microelectrodes (Accu-fill 90 Micropets, Clay Adams) used for single-channel recordings were treated with Sylgard (Dow Corning) to reduce background noise and had resistances of 3–5 MΩ when filled with external solution. The standard pipette solution (external solution) contained 5 mM KCl, 150 mM NaCl, 1 mM $CaCl_2$, 7.8 mM glucose, and 5 mM HEPES, pH adjusted to 7.4 with NaOH. The normal bath solution (internal solution) consisted of 50 mM KCl, 60 mM potassium aspartate, 2 mM $MgCl_2$, 5 mM EGTA, and 10 mM HEPES, pH adjusted to 7.3 with KOH (total $[K^+]$ = 140 mM). We varied the free internal Ca^{2+} concentration from 1 nM to 100 μM by adjusting the concentration of $CaCl_2$ (21).

Data acquisition and analysis were performed using pClamp (Axon Instruments) and SigmaPlot (Jandel) software installed on 486 personal computers. Channel openings were determined by setting a threshold detector at the 50% level of the open channel amplitude. To eliminate artifacts due to rapid channel transitions, openings and closings of <0.2 msec were excluded from analysis. P_o was determined from the following equation:

$$P_o = \sum_{i=1}^{n} \frac{t_i(i)}{Tn}$$

where t_i is the open time of each open current level (i.e., with i = 1, 2, ... number of open channels), n is the maximal number of channels active in the patch at the period of measurement. For the experiments reported in this article, n was normally the same under control and G protein conditions. P_o was also determined by comparing the areas obtained from fits of all-point amplitude histograms to gaussian curves (Fig. 1). P_o values obtained with the two methods were comparable. Control P_o was monitored over a period of 200–300 sec before G protein stimulation to ensure stationary activity. In addition, mock chemical additions were done before the addition of the test agents to rule out nonspecific changes in P_o. Single-channel

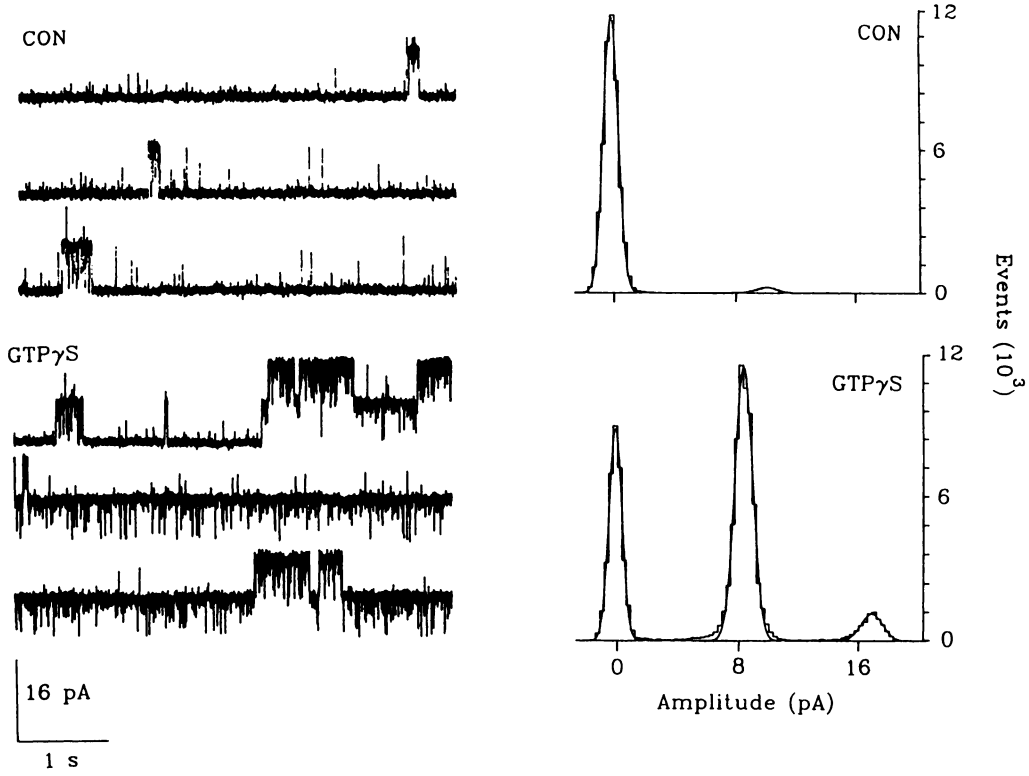

Fig. 1. Stimulatory regulation of the BK, Ca^{2+}-activated K^+ channel by GTPγS. *Left*, BK single-channel currents recorded at +40 mV with 1 μM internal Ca^{2+} before (*top*) and after (*bottom*) the addition of 100 μM GTPγS. *Right*, All-point amplitude histograms obtained for the channels displayed on left. Superimposed on the histograms are gaussian fits to the data. P_o was determined as described in the text. In this experiment, nP_o increased by 14-fold after the application of GTPγS (Patch A43F).

results are presented as the number of channels present in the patch (n) multiplied by P_o (nP_o).

Open and closed times for the channel were determined in patches containing single BK channels using the method of Sigworth and Sine (22) in that duration histograms are created by plotting the square root of the number of events versus logarithmically binned durations (Fig. 2). The advantage of this procedure is that it allows analysis of dwell times that range over many orders of magnitude. Duration histograms were fit with a probability density function with the following form:

$$f(x) = \sum_{j=1}^{m} a_j * \exp[\ln t - \ln\tau_j - \exp(\ln t - \ln\tau_j)]$$

where t is the binned time durations, τ is the jth time constant, and a is the fraction of total events of the jth state. The number of exponential components in the fit was initially chosen by inspection of the histogram and modified based on the outcome of an F test [$F = (RSS_s - RSS_c)/(RSS_c/df)$], where RSS_s and RSS_c are the respective residual sum of squares of the more simple and more complex (with one more parameter) models with degrees of freedom). Based on this analysis, it was determined that the open-time histogram was best described using a two-exponential model, whereas the closed-time histogram was best fit using a three-exponential model (see Fig. 2). Kinetic analysis was limited to patches containing one BK channel.

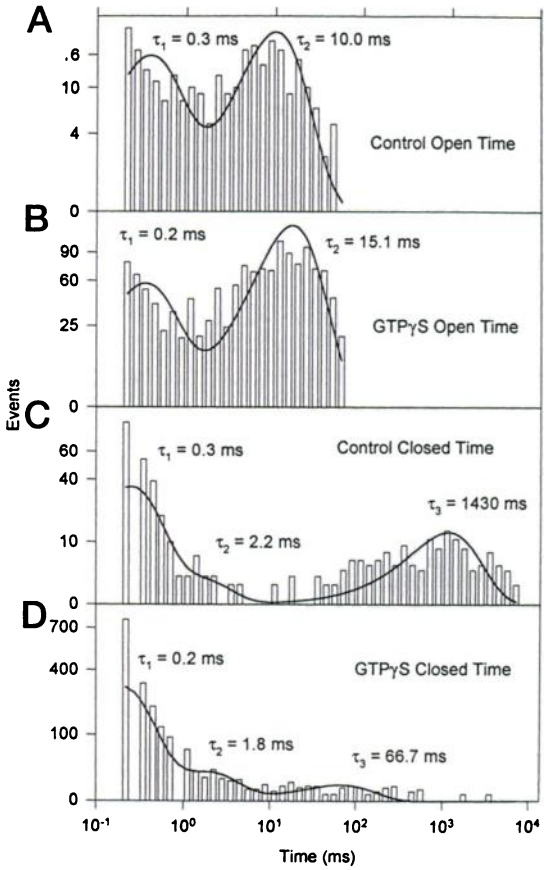

Fig. 2. Effect of G proteins on the open and closed times of the BK, Ca^{2+}-activated K^+ channel. *Top,* open-time distributions measured in the presence and absence of GTPγS. Open-time distributions were best described by two exponentials. Fitted time constants were τ_1 = 0.3 msec and τ_2 = 10.0 msec under control conditions and τ_1 = 0.2 msec and τ_2 = 15.1 msec with GTPγS. *Bottom,* closed-time distributions were best described by three exponentials. Fitted time constants were τ_1 = 0.3 msec, τ_2 = 2.2 msec, and τ_3 = 1427 msec under control conditions and τ_1 = 0.2 msec, τ_2 = 1.8 msec, and τ_3 = 66.7 msec with GTPγS. Open- and closed-time distributions were fit as described in the text (Patch G40F).

Stimulation of G proteins and preparation of $G_i/G_{o\alpha}$. G proteins present in the membrane patches were stimulated by the cytoplasmic application of either 100 μM GTPγS or AlF_4^- (2–20 mM NaF plus 100 μM $AlCl_3$). In some experiments, a preparation containing a mixture of the α subunits of the G_i and G_o proteins ($G_i/G_{o\alpha}$) was added to determine the direct effect of the G proteins on the K^+ channels. The $G_i/G_{o\alpha}$ preparation was a generous gift of Dr. John Hildebrandt (Medical University of South Carolina) and was purified from bovine brain as described previously (23). The specific activity of [^{35}S]GTPγS binding to the preparation was 8.5 pmol/μg. $G_i/G_{o\alpha}$ (33 μM) was preincubated with 33 μM GTPγS in 50 mM HEPES, 8 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol, pH 8.0, for 30 min at 25°. $G_i/G_{o\alpha}$ subunits were diluted in the same buffer to 300 nM and used at a final concentration of 1 nM in the internal solution.

PTX (10 μg/ml) was activated by incubation in a modified internal solution (containing 5 mM DTT and 3 mM ATP) for 30 min at 37°. The PTX solution was diluted 10-fold into normal internal solution containing 1 mM NAD and 200 μM GTP. The final concentrations of PTX, DTT, and ATP were 1 μg/ml, 0.5 mM, and 0.3 mM, respectively. Application of an internal solution containing DTT, ATP, and NAD, without PTX, had no effect on the P_o of the BK channel.

Materials. Dulbecco's modified Eagle's medium/F-12, ascorbic acid, GTPγS, GTP, PTX, NAD, PKI_{6-22}, NaF, and $AlCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine calf serum was obtained from Hyclone Laboratories (Logan, UT).

Results

Regulation of chromaffin BK Ca^{++}-activated K^+ channels by G proteins. Fig. 1 shows an example of unitary, BK, Ca^{2+}-activated K^+ channel currents measured from an inside-out patch of membrane obtained from an adrenal chromaffin cell. These channels displayed properties similar to those previously reported for the chromaffin BK channel, including a large conductance (mean slope conductance = 114 pS from 30 patches); block by tetraethylammonium ions (results not shown); and a strong dependence on internal Ca^{2+} for channel opening (see Fig. 7). As shown in Fig. 1, cytoplasmic application of 100 μM GTPγS, in the absence of ATP, resulted in a large increase in the P_o of the BK channel when measured at +40 mV with a 1 μM concentration of internal Ca^{2+}. In 11 patches, nP_o increased from 0.074 ± 0.016 under control conditions to 0.326 ± 0.078 after the addition of GTPγS to the patch. A similar increase in the P_o was obtained after the addition of AlF_4^- (10 mM NaF plus 100 μM $AlCl_3$) (five patches) (Fig. 3, *left*). AlF^-_4 permanently activates G proteins by binding with GDP to the α subunit of the protein and mimicking the action of the terminal phosphate group of GTP (24). The stimulatory effect of GTPγS and AlF^-_4 on the BK channels occurred within 5–10 min after addition to the internal solution and was irreversible during washout with control internal solution (data not shown).

$G_{\alpha i}$ and $G_{\alpha o}$ have been identified as G protein α subtypes in bovine adrenal chromaffin cells (25). To determine whether GTPγS and AlF^-_4 increased the P_o of the BK channel by stimulating these G proteins, we determined the effect of a purified preparation of $G_i/G_{o\alpha}$. As was the case with GTPγS and AlF^-_4, the addition of $G_i/G_{o\alpha}$ to the internal membrane resulted in a large and irreversible increase in the P_o of the BK channel (Fig. 3, *right*). In three patches examined, the addition of G_i/G_o caused an average 4.0-fold increase in nP_o.

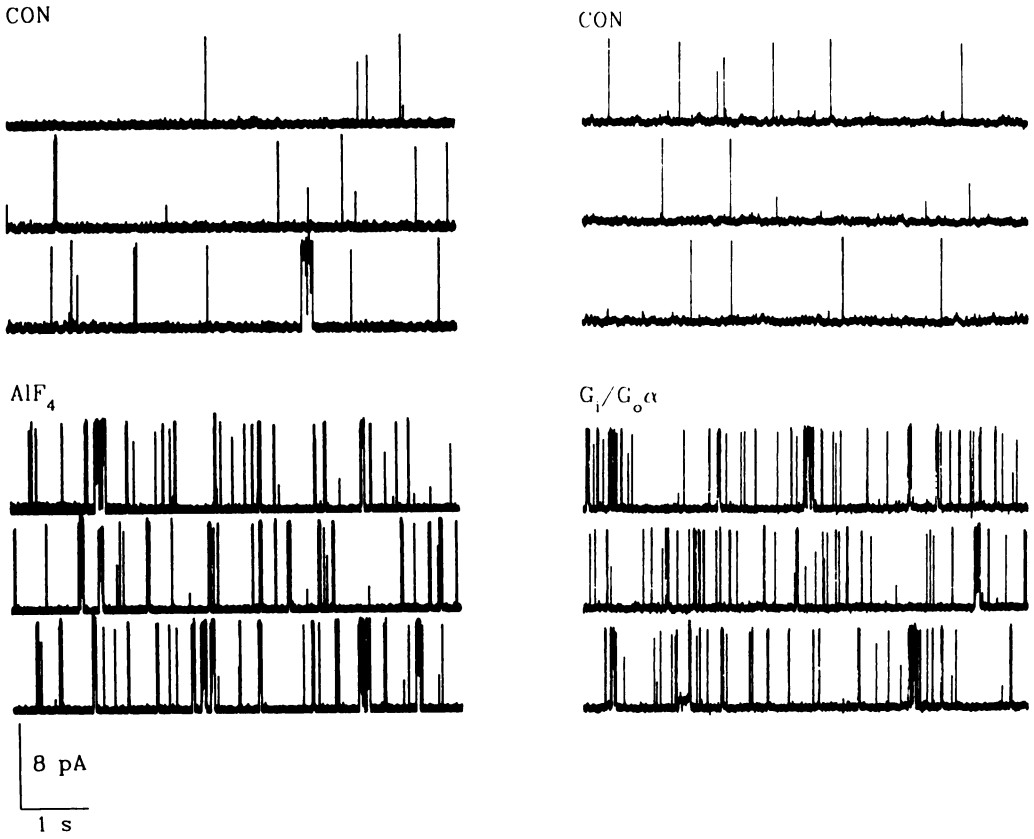

Fig. 3. Modulation of the BK, Ca^{2+}-activated K^+ channel activity by AlF_4^- and $G_i/G_{o\alpha}$. *Left*, BK single-channel currents recorded at +40 mV with 1 μM internal Ca^{2+} before (*top*) and after (*bottom*) the addition of AlF_4^- (10 mM NaF plus 100 μM $AlCl_3$). In this experiment, nP_o increased by 9-fold after the application of AlF_4^-. (Patch A41F.) *Right*, BK single-channel currents recorded at +40 mV with 1 μM internal Ca^{2+} before (*top*) and after (*bottom*) the addition of a $G_i/G_{o\alpha}$ preparation. In the presence of $G_i/G_{o\alpha}$, the channel nP_o increased by 18-fold (Patch G44F).

Large conductance, Ca^{2+}-activated K^+ channels are up-regulated by PKA in various tissues (10–12). To provide further evidence for a direct action of G proteins on the BK channels, excised patches of membrane were exposed to an internal solution containing 100 nM of the inhibitory peptide PKI_{6-22}. This peptide functions as a strong inhibitor of PKA (26). In the presence of PKI_{6-22}, application of the 100 μM GTPγS produced a 4.5-fold increase in nP_o (results not shown), suggesting that stimulation of PKA was not involved in this regulatory action.

PTX ADP-ribosylates the chromaffin G proteins $G_{i\alpha}$ and $G_{o\alpha}$ (25). Therefore, if up-regulation of the BK channels is mediated through one or both of these proteins, this modulation should be diminished in the presence of PTX. In Fig. 4, the effect of PTX was examined on a BK channel after stimulation with 200 μM GTP. We choose to examine the regulation by GTP, rather than GTPγS because previous work has shown that PTX-induced inhibition of G protein-coupled channels can be reversed in the presence of GTPγS (27). For the records shown in Fig. 4, the addition of GTP caused nP_o to increase from 0.06 to 0.28. Exposure of the patch of membrane to activated PTX (1 μg/ml) (see Materials and Methods) with 1 mM NAD reduced nP_o for the GTP-regulated channels to 0.07 but did not completely eliminate channel openings. In the absence of NAD, nP_o was not decreased by PTX. Furthermore, in separate experiments, PTX (plus NAD) had no inhibitory action on BK channels measured in the absence of GTP.

Kinetic effects of G proteins on the BK channel. Open- and closed-duration histograms for a single BK channel are shown in Fig. 2, and the kinetic results obtained from four membrane patches are summarized in Table 1. The histogram of the open time displayed a double exponential shape indicating two distinct open states for the chromaffin BK channel (Fig. 2, *top*, and Table 1). The addition of GTPγS caused no significant change in either the short or the medium open-time constants (Fig. 2, *top*, and Table 1). However, GTPγS significantly increased the area a under the fitted curve for the medium open-time constant while decreasing a for the short-time constant. This may indicate that G protein stimulation converts short open events to medium openings.

Fig. 2 show the histograms for the closed time. This distribution was fit with three time constants that defined a short, medium, and long closed state for the channel. The long time constant arose from the relatively long-duration closures that could be observed between bursts of channel openings under basal conditions (see Figs. 1, 3, and 4). Stimulation with GTPγS caused a 5.5-fold reduction in the time constant of this long closed state but produced no significant change in either the short or medium time constants (Fig. 2 and Table 1). In addition, GTPγS significantly decreased the a value for the long closings.

G proteins shift the voltage dependence of BK channel gating. The P_o of Ca^{2+}-activated K^+ channels is strongly influenced by both the voltage and the internal Ca^{2+} concentration (2, 5). The stimulatory action of GTPγS, AlF^-_4 and G_i/G_o on the chromaffin BK, Ca^{2+}-activated K^+ channel shown in Figs. 1 and 3 was measured at a fixed membrane potential (+40 mV) and a fixed internal Ca^{2+} concentration (1 μM). Thus, it seemed reasonable that G proteins might increase the P_o of the channel through one of at least two possible mechanisms. First, the G proteins might shift the voltage dependence of BK channel opening to more negative potentials so that the P_o would be increased at +40 mV.

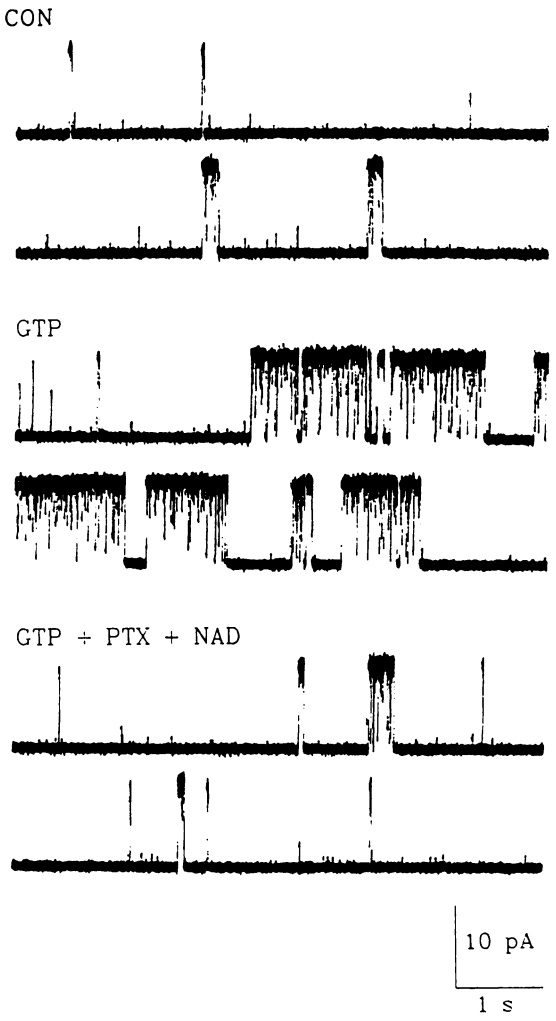

Fig. 4. PTX inhibits GTP regulation of the BK, Ca^{2+}-activated K^+ channel. A and B, BK channel currents recorded under control conditions at +40 mV in 1 μM internal Ca^{2+}. B and C, current records obtained after the addition of 200 μM GTP, which increased nP_o by 5-fold. C and D, BK channel currents measured in the presence of GTP, activated PTX (1 μg/ml), and 1 mM NAD. Although PTX inhibited channel opening, nP_o was not reduced below the control value (Patch AF0).

Alternatively, the G proteins might increase the Ca^{2+} sensitivity of the BK channel.

Fig. 5 plots the relationship of voltage to P_o for the BK channel measured in the presence and absence of GTPγS with 1 μM internal Ca^{2+}. Both the control and G protein-stimulated channels displayed strong voltage dependence when measured between 0 and +100 mV. To quantify this

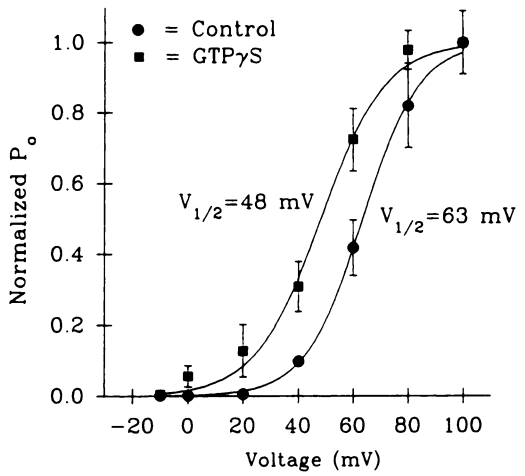

Fig. 5. G proteins shift the voltage dependence of BK, Ca^{2+}-activated K^+ channel gating. The normalized channel P_o is plotted versus the recording potential in the presence and absence of GTPγS. P_o was normalized to the value obtained at +100 mV. Each point represents the mean P_o ± standard error obtained from 3–30 patches. *Continuous lines*, best fits of the Boltzmann equation: $P_o = 1/(1 + \exp[-(V_m - V_{½})/k])$, where $V_{½}$ is the half-maximal voltage required for activation. The fitted parameters were $V_{½}$ = +63 mV for control and +48 mV for the GTPγS curves, respectively.

voltage dependence, the data points were fit using the Boltzmann equation, $P_o = 1/(1 + \exp[-(V_m - V_{½})/k])$, where V_m is the membrane potential and $V_{½}$ and k are constants that determine the potential at that the activation curve is half-saturated and that affect the slope of the curve, respectively. As shown in Fig. 5, GTPγS caused a shift in the voltage dependence of channel gating with $V_{½}$ values of +63 mV and +48 mV measured in control and GTPγS internal solutions, respectively.

G proteins increase the sensitivity of the BK channel to internal Ca^{++}. Under control conditions, increasing the internal Ca^{2+} concentration from 100 nM to 1 μM causes nP_o to increase from 0.003 ± 0.001 to 0.067 ± 0.021 (six patches). Fig. 6 (*top two traces*) shows control BK channel currents recorded in the presence of 100 nM internal Ca^{2+}. As expected from the experiments described above, the addition of 100 μM GTPγS to the patch of membrane resulted in a 10-fold increase in the P_o of the channel (*middle traces*). However, once stimulated by GTPγS, increasing the internal Ca^{2+} concentration from 100 nM to 1 μM produced only a marginal further increase (40%) in the activity of the channel (*bottom traces*). Furthermore, addition of the $G_i/G_{o\alpha}$ subunits to the 100 nM Ca^{2+} internal solution caused an 8.2-fold increase in

TABLE 1

Single-channel kinetics

Time constants (τ in msec) and fraction of events (a in %) were determined as described in Materials and Methods. Each value represents the mean ± standard error obtained from four different patches under control conditions and after the addition of either GTPγS (two patches), AlF^-_4 (one patch), or $G_i/G_{o\alpha}$ (one patch). In each individual patch examined, the fitted τ value for the long closed state was significantly decreased ($p < 0.005$) during G protein stimulation. In two experiments, the medium open τ value also was significantly changed but in different directions.

Condition	Openings		Closings		
	Short	Medium	Short	Medium	Long
Control τ	0.8 ± 0.5	25.5 ± 6.2	0.25 ± 0.03	1.3 ± 0.3	1219 ± 116[a]
Control a	68.8 ± 4.5[a]	31.8 ± 4.4[a]	57.5 ± 2.8[a]	13.0 ± 2.0	29.0 ± 4.1[a]
G protein τ	0.8 ± 0.5	20.1 ± 5.1	0.23 ± 0.03	1.6 ± 0.2	220.3 ± 80.0[a]
G protein a	48.3 ± 0.6[a]	51.8 ± 0.6[a]	68.8 ± 1.4[a]	18.3 ± 0.3	13.0 ± 1.4[a]

[a] Indicates a significant difference between control and G protein values with $p < 0.05$.

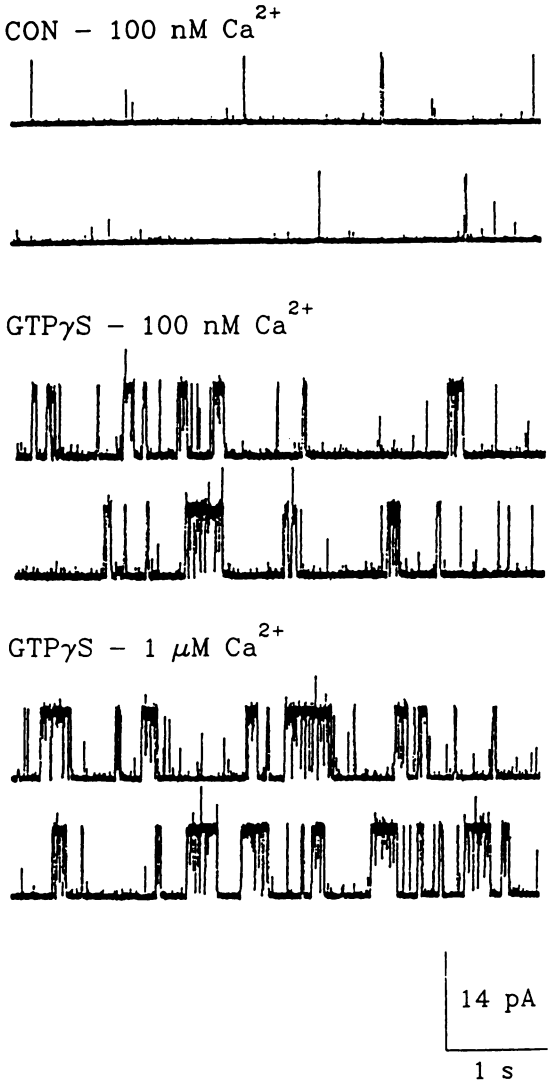

Fig. 6. Effect of increasing internal Ca^{2+} on the BK, Ca^{2+}-activated K^+ channels in the presence of GTPγS. *Top traces*, BK channel currents recorded under control conditions at +40 mV in 100 nM internal Ca^{2+}. *Middle traces*, current records obtained after the addition of 100 μM GTPγS, which increased nP_o by 10-fold. *Bottom traces*, BK channel currents measured in the presence of GTPγS after increasing the internal Ca^{2+} concentration to 1 μM. Increasing the internal Ca^{2+} concentration in the presence of GTPγS increased nP_o by only 1.4-fold (Patch V47F).

nP_o (two patches), which was more than that observed in 1 μM Ca^{2+} internal solution (see above). These results suggest that during G protein stimulation, the BK channels require less internal Ca^{2+} to open.

The concentration-versus-normalized P_o curve for opening of the BK channels by Ca^{2+} is plotted in Fig. 7. Each point represents the mean ± standard error for experiments obtained from a total of 32 membrane patches with internal Ca^{2+} concentrations ranging from 1 nM to 100 μM. The continuous lines represent the best fits of the data points to the Michaelis-Menton equation ($P_o$max * $[Ca^{2+}]^n/K_d + [Ca^{2+}]^n$) to records obtained in the presence or absence of GTPγS. The half-maximal Ca^{2+} concentration (K_d) required for activity was 11.7 μM under control conditions and decreased to 1.3 μM in the presence of GTPγS. The Hill coefficient, which represents the number of binding sites for Ca^{2+} on the channel,

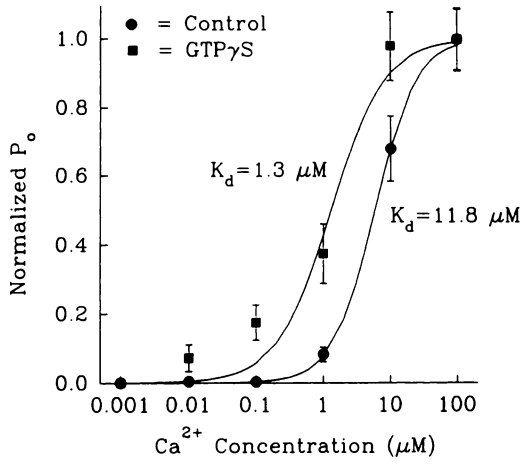

Fig. 7. G proteins increase the sensitivity of the BK, Ca^{2+}-activated K^+ channel for internal Ca^{2+}. The normalized channel P_o is plotted versus the Ca^{2+} concentration in the internal solution. P_o was normalized to the value obtained at 100 μM Ca^{2+}. Each point represents the mean P_o ± standard error obtained from 3–32 patches at +40 mV. The continuous lines are given by the Michaelis-Menton equation ($P_o$max * $[Ca^{2+}]^n/K_d + [Ca^{2+}]^n$). K_d values of 11.7 μM and 1.3 μM provided the best least-squares fit to the data under control conditions (●) and in the presence of GTPγS (■).

was 1.3 and 1.1 for the control and GTPγS curves, respectively. In addition, the maximal activity of the BK channel, measured with 100 μM Ca^{2+}, was not significantly different between the control patches and the GTPγS-stimulated patches (Fig. 7). Thus, the leftward shift of the curve in the presence of GTPγS indicates that GTPγS increases BK channel sensitivity to Ca^{2+}.

Discussion

Stimulatory regulation of the calcium-activated BK channel by G proteins. The major finding of this study was that G proteins regulate the opening of the adrenal chromaffin BK, Ca^{2+}-activated K^+ channel. The addition of the G protein activators AlF_4^-, GTPγS, and GTP, or a heterogeneous mixture of bovine brain $G_i/G_{o\alpha}$ subunits, caused an average 4–5-fold increase in the P_o of the BK channel. Stimulatory regulation by GTP could be inhibited through the application of activated PTX, suggesting a colocalization of the G protein with the BK channel. Similar kinetic changes and an increased Ca^{2+} sensitivity were observed in the channel after treatment with both GTPγS and the $G_i/G_{o\alpha}$ subunits. Thus, either G_i or G_o, which are found in adrenal chromaffin cells (27), may directly interact with the channel or a regulatory component associated with the channel to increase the P_o.

Mechanism of G protein/BK channel interaction. Several previous studies have examined the role of G proteins and protein kinases in the regulation of BK channels. Application of either GTPγS or $G_{s\alpha}$ produces an increased P_o of the BK channel measured in airway (14) and coronary artery smooth muscle (13, 28) cells. $G_{s\alpha}$ regulates the smooth muscle BK channel in the presence of PKI, suggesting that the effects of G proteins are not mediated via PKA stimulation. Thus, this G protein modulation can be distinguished from the up-regulation of the BK channel observed in various tissues after the addition of the catalytic subunit of PKA (10, 11, 28) and during stimulation of an endogenous membrane-

associated kinase with ATP (29, 30). Our findings that regulation of the chromaffin BK channel by GTPγS occurs in an ATP-free internal solution and in the presence of PKI_{6-22} supports the hypothesis that the increased P_o does not result from PKA-induced phosphorylation. However, in the absence of a reconstituted system, it is not possible to completely rule out the participation of an endogenous protein kinase in this regulation.

Stimulation of the chromaffin BK channel by G proteins caused a large decrease in the time constant for a long closed state of the channel and shifted the voltage dependence of the BK channel P_o to more negative potentials. Scornik *et al.* (28) reported that $G_{s\alpha}$ reduces a long closed state of the coronary artery BK channel while having no significant effect on the open time of the channel. Similarly, up-regulation of a neuronal BK channel by PKA is associated with decreases in the closed times of the channel (11). Consistent with the results of the present study, G protein stimulation of the smooth muscle channel causes a negative shift in the voltage dependence for channel opening (14, 28). Moczydlowski and Latorre (2) reported that the voltage dependence of activation of the skeletal muscle BK channel is shifted to more negative potentials by increasing the internal Ca^{2+} concentration. Based on a kinetic analysis, these authors hypothesized that the inherent voltage dependence of the BK channel results from the binding of Ca^{2+} to a site that senses the membrane voltage (2). Therefore, according to this model, the increased affinity of the chromaffin channel for Ca^{2+} should be reflected by a shift in the voltage dependence to more negative membrane potentials.

The *Drosophila slowpoke* (*slo*) (31, 32) and mouse *mslo* (33) genes encode for BK Ca^{2+}-activated K^+ channels found in *Drosophila* muscle and mouse brain and skeletal muscle. The deduced amino acid sequences for these channels contain consensus sites for protein kinase phosphorylation and a region proposed to function as a Ca^{2+} binding loop (31, 32). A putative nucleotide binding site has also been identified in the *slo* channel (31). Nucleotide binding sequences are found in the atrial muscarinic potassium channel (34) and the enzyme adenylate cyclase (35), two proteins that are stimulated by G proteins. Whether this sequence in the BK channel serves as a site of G protein regulation or as a site of direct nucleotide (GTP or ATP) binding (as with the ATP-sensitive channel) has not been addressed. Studies of the *slo* channel expressed in *Xenopus* oocytes will provide an important test for determining direct G protein interaction with the channel.

Although various kinetic schemes for the adrenal chromaffin cell BK channel can be proposed, the following model provides a good approximation to the results of this study and is supported by previous findings (11, 36).

$$\text{Long Closed} \overset{+Ca^{2+}}{\rightleftarrows} \text{Medium Closed} * Ca^{2+} \rightleftarrows \text{Short Open} * Ca^{2+}$$
$$\uparrow\downarrow \qquad\qquad \uparrow\downarrow$$
$$\text{Medium Open} * Ca^{2+} \rightleftarrows \text{Short Closed} * Ca^{2+}$$

Because channel openings can occur from the medium closed and short closed states of the channel (see Fig. 1), these conformations must represent Ca^{2+}-bound states of the protein. The observation that GTPγS and AlF^-_4 act primarily to decrease the long closed state of the BK channel suggests that G proteins affect the transition between the long and medium closed states and thus drive the gating reaction to the right. This may result from the ability of G proteins to increase the binding constant for Ca^{2+} at this site. By stabilizing the open state of the channel, G proteins would shift the activation curve to more negative potentials. It is not likely that G protein stimulation regulates the binding of Ca^{2+} to the channel during the transitions from the short and medium closed states to the open states, since we found no evidence for changes in the time constant of these states.

Pharmacological relevance of BK channel stimulatory regulation. We previously reported that dialysis of bovine adrenal chromaffin cells with GTPγS or external application of AlF^-_4 (20 mM NaF) results in the appearance of a rapidly activating, whole-cell K^+ current (15). The finding that NaF could inhibit nicotine-evoked catecholamine secretion in the chromaffin cells suggested that activation of this K^+ current could play a regulatory role in secretagogue-mediated exocytosis (15). Various neuromodulators, including the opioid peptides Leu-enkephalin and Met-enkephalin (37), dopamine (38), and adenosine (15), augment the chromaffin whole-cell Ca^{2+}-activated K^+ current. In addition, adenosine has been shown to inhibit exocytosis (39). Because chromaffin cells secrete enkephalins, as well as adenosine and dopamine (40), the present results suggest that these hormones may feedback to control exocytosis through an autoregulatory process. Future experiments examining the effect of GTPγS on catecholamine secretion in single chromaffin cells will provide valuable information concerning the regulatory role of the BK channels during excitation/secretion coupling.

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