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# A G Protein-Activated $K^+$ Current in Bovine Adrenal Chromaffin Cells: Possible Regulatory Role in Exocytosis

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**SUMMARY**

Guanine nucleotide binding proteins (G proteins) act as signal transducers between membrane receptors and ion channels. In the present study, the whole-cell arrangement of the patch clamp technique was used to examine the effect of G proteins on $K^+$ channels in cultured bovine adrenal chromaffin cells. Internal dialysis of chromaffin cells with guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) or external application of $AlF^-_4$, to stimulate G proteins, resulted in a voltage-dependent increase in the amplitude of the outward $K^+$ currents. The half-maximal voltage required for activation of the currents was shifted by −16 mV in the presence of GTPγS. The augmentation in the $K^+$ currents was accompanied by the appearance of a fast component of current activation measured at potentials positive to 0 mV. The GTPγS-sensitive current could not be detected when internal $K^+$ was replaced with $Cs^+$ and was reversibly inhibited by tetraethylammonium ($IC_{50}$, 2 mM). In contrast, the scorpion venom charybdotoxin (50 nM) and the bee venom apamin (250 nM) only slightly reduced the $K^+$ currents during stimulation by GTPγS and did not alter the activation kinetics. In addition, the GTPγS-sensitive $K^+$ current could be activated in the absence of internal $Ca^{2+}$ and when the inward $Ca^{2+}$ current was inhibited with $CdCl_2$. Treatment of the chromaffin cells with fluoride decreased nicotine-evoked secretion of catecholamines in a concentration-dependent manner. Thus, bovine chromaffin cells contain a G protein-stimulated $K^+$ channel that may play a regulatory role in secretagogue-mediated exocytosis.

Chromaffin cells in the adrenal medulla secrete catecholamines in response to stimulation by the splanchnic nerve. Studies of the ionic requirements for catecholamine release have demonstrated that $Ca^{2+}$ entry plays a central role in stimulus-secretion coupling in these cells (1) and that ion channels directly participate in this process (2–4). Several types of ion channels including $Na^+$ (5), $Ca^{2+}$ (5–7), $Cl^-$ (8, 9), and $K^+$ channels have been characterized in cultured bovine chromaffin cells. Based on single channel conductance measurements, Marty and Neher (10) identified three different $K^+$ channel subtypes in these cells. This included a large-conductance $Ca^{2+}$-activated channel (the BK channel) and two smaller conductance channels that were not dependent on $Ca^{2+}$ for activity (10). Whole-cell macroscopic recordings have also revealed several different outward $K^+$ currents that can be distinguished according to their $Ca^{2+}$ dependence, drug-sensitivity, and kinetic properties (10, 11).

G proteins can regulate $K^+$ channels through both indirect and direct mechanisms (12). By stimulating the enzyme adenylate cyclase and increasing intracellular levels of cAMP, the $G_s$ indirectly modulates the activity of potassium channels found in various cell types (13–15). Another G protein, designated $G_K$, directly couples the cardiac muscarinic receptor to a $K^+$ channel in atrial myocytes (16, 17). In a number of neuronal preparations, G proteins couple serotonin and adrenergic receptors to inward-rectifying $K^+$ channels (18, 19). In addition, direct G protein activation of an outward-rectifying $K^+$ channel in a rat histamine-secreting basophilic leukemia cell line (RBL-2H3) has been reported (20).

Pretreatment of chromaffin cells with pertussis toxin, which uncouples the $G_i$ from receptor stimulation, enhances catecholamine secretion in response to secretagogues including acetylcholine, nicotine, histamine, vasoactive intestinal peptide, and elevated external $K^+$ (21–23). These findings suggest that exocytosis in chromaffin cells is under tonic inhibition mediated by pertussis toxin-sensitive G proteins. Thus, we wondered whether G proteins regulate catecholamine secretion in the chromaffin cells through effects on ion channels. In the present study, the effects of G protein stimulation were determined on $K^+$ channels in bovine chromaffin cells. Intracellular application of GTPγS or external addition of fluoride increased the amplitude and altered the activation kinetics of the outward $K^+$ currents. The G protein-stimulated $K^+$ current was blocked by TEA, but could be measured in the presence of charybdo-

**ABBREVIATIONS:** G protein, guanine nucleotide binding protein; $G_s$, G stimulatory protein; $G_i$, inhibitory G protein; TEA, tetraethylammonium; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); DMEM, Dulbecco's modified Eagle's medium; I/V, current versus voltage; DIDS, diidothiocyanate-stilbene-2,2′-disulfonic acid; ChTX, charybdotoxin; AP, apamin; PKA, protein kinase A; PKC, protein kinase C; 8-CPT, 8-chlorphylthio.

toxin and during dialysis of the cells with $Ca^{2+}$-free internal solution. The ability of fluoride to reduce nicotine-evoked catecholamine secretion implicates this $K^+$ channel in the regulation of exocytosis.

## Materials and Methods

**Preparation of chromaffin cells.** Primary cultures of bovine adrenal chromaffin cells were prepared by collagenase digestion and centrifugation on Renografin gradients as described previously (24). In some experiments, 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 20,000 × *g* for 20 min at 20° in a final concentration of 42.5% Percoll. Cells recovered from the upper half of the centrifuge tube contained approximately 50% norepinephrine versus 20 to 30% in unfractionated cells. For patch clamp recording, cells were plated and maintained in serum-containing medium on glass coverslips in 35 × 10-mm polystyrene dishes (Corning) at a density of $1 \times 10^6$ cells/2.5 ml. Coverslips were then transferred to a recording chamber containing a physiological external solution (see below). For secretion experiments, cells were purified and plated at a density of 500,000 cells/$cm^2$ in 24-well tissue culture plates (Corning) as described (23). Cultures were maintained in a humidified atmosphere of 5% $CO_2$ at 37° and used between 1 and 7 days after plating.

**Recording procedure and measurement $K^+$ currents.** The patch clamp method of Hamill *et al.* (25) was used to record whole-cell chromaffin $K^+$ currents using a Warner PC-501 amplifier (Warner Instrument Corp., Hamden, CT). Microelectrodes were made from Gold Seal Accu-fill 90 Micropets (Clay Adams Inc.) and had resistances of 2 to 4 MΩ when filled with the potassium glutamate internal solution (see below). 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 arose between the pipette solution and bath solution. Liquid junction potential values were usually between −5 and +5 mV. Uncompensated series resistance in this study was typically ≤1.5 MΩ and was stable over the time course (10–15 min) of most experiments. Thus, even for the large $K^+$ currents (4–5 nA) recorded in some experiments, voltage errors caused by series resistance were not greater than 7 mV.

Chromaffin cell membrane currents were recorded during 40-msec voltage steps to −60 through +70 mV applied from a holding potential of −80 mV. Data was acquired and analyzed using a Softek-386 computer in conjunction with an A/D, D/A converter (Scientific Associates, Rochester, NY). Data were sampled at 10 kHz, filtered at 2 kHz with a low pass Bessel filter, and stored on the computer's hard drive. Linear leak and capacity transients were removed from test currents using records obtained during four to six hyperpolarizing pulses to −100 mV. These records were averaged and subtracted from the test currents. Use of this protocol was justified because voltage- and/or time-dependent conductances were not present at this potential.

**Recording solutions and dialysis of GTPγS.** The standard internal solution, which was nominally $Ca^{2+}$-free, consisted of (mM): potassium glutamate, 145; NaCl, 8; $MgCl_2$, 2; ATP ($K^+$ or $Na^+$ salt), 0.5; and HEPES, 10; pH adjusted to 7.2 with KOH (total [$K^+$], 155 mM). In some experiments the internal solution was supplemented with 11 mM EGTA and 1 mM $CaCl_2$ [calculated free $Ca^{2+}$, 10 nM according to Fabiato (26)]. Similar effects of G protein stimulation on the $K^+$ currents were observed with the two solutions. The standard external solution consisted of (mM): NaCl, 150; KCl, 5; $CaCl_2$, 1; glucose, 7.8; and HEPES, 5; pH adjusted to 7.4 with NaOH. In some experiments, NaCl and KCl were replaced with Na-aspartate and K-aspartate, respectively. Tetrodotoxin (5 μM) was added to the external solution to eliminate the inward $Na^+$ currents present in the chromaffin cells. In several experiments, 100 μM $CdCl_2$ was added to the external solution to inhibit $Ca^{2+}$ entry *via* the voltage-dependent $Ca^{2+}$ channels present in these cells (5–7). This was not a general procedure, because $Cd^{2+}$ was found to be toxic to the cells. The external and internal solutions were maintained at room temperature (22–25°).

For experiments using internal cellular dialysis of GTPγS to stimulate G proteins, a concentration of 100 to 200 μM GTPγS was included in the internal pipette solution. After disruption of the cell membrane, chemicals move from the pipette into the cell by diffusion. Based on theoretical studies (27), a nucleotide such as GTPγS (molecular mass, 560 Da) should reach equilibrium with a time constant of 30 to 40 sec given the size of the cells and electrodes used in this study. Doroshenko *et al.* (8) have identified recently a novel chloride conductance in bovine chromaffin cells that is activated by intracellular GTPγS. Measurement of this time-independent background current provided a valuable monitor for determining the onset of G protein stimulation by GTPγS. In general, activation of membrane currents during dialysis with GTPγS occurred within 30 to 120 sec after membrane breakthrough with the patch pipette. The ability of GTPγS to activate the $Cl^-$ current and modulate the $K^+$ current was observed in approximately one-third of the chromaffin cells studied. This low success rate may have resulted from GTPγS-sensitive and -insensitive populations of chromaffin cells present in the study. In preliminary experiments, it was found that the frequency of activating the $Cl^-$ and $K^+$ currents was higher in cell preparations enriched in norepinephrine-secreting cells.

$AlF^-_4$ was introduced in these experiments by dilution of a 1 M stock of NaF, which was stored in a glass container. Under these conditions, $Al^{3+}$ is present in small amounts in the NaF solutions (28). Yatani and Brown (29) have shown that a 10 to 30 mM concentration of fluoride is effective in activating G proteins in cardiac atrial cells.

Activation kinetics of the $K^+$ currents were fit with either the single exponential function, $I_K = I_{Kmax} \cdot [1 - \exp(-t/\tau)]$ or the biexponential function, $I_K = I_{K1} \cdot [1 - \exp(-t/\tau_1)] + I_{K2} \cdot [1 - \exp(-t/\tau_2)]$. The curve-fitting procedure used a nonlinear least squares algorithm. Goodness of fit was judged by comparing standard deviations and by inspecting the fitted $K^+$ current records. Activation curves were fit using the Boltzmann equation, $g_K = g_{Kmax}/(1 + \exp[-(V_m - V_{1/2})/k])$, where $g_{Kmax}$ is the maximal conductance, $V_m$ is the membrane potential and $V_{1/2}$ and $k$ are constants that determine the potential at which the activation curve is half-saturated and that affect the slope of the curve, respectively.

**Catecholamine secretion.** Culture medium was removed from cell cultures in the 24-well culture plate and replaced with secretion medium consisting of (mM): NaCl, 150; $CaCl_2$, 2; KCl, 5; glucose, 7.8; HEPES, 5; pH 7.4 adjusted to 7.4 with NaOH. The secretion medium also contained NaF ± the secretagogue nicotine as indicated in Fig. 6. At the end of the incubation, an aliquot of the secretion medium was removed and acidified with 1 M acetic acid. After aspiration of the remaining solution, cells were lysed with 0.2 M acetic acid. Catecholamines secreted into the medium and those remaining in the cells were determined by reverse-phase HPLC with electrochemical detection (24). Each experiment represents the averages of triplicate determinants. Results are expressed as the percentage of total cell catecholamine released. The catecholamine content of the cultures averaged 140 nmol/$10^6$ cells.

**Materials.** DMEM, ascorbic acid, tetrodotoxin, GTPγS, NaF, nicotine, apamin, and TEA were purchased from Sigma Chemical Co. (St. Louis, MO). Charybdotoxin was obtained from Peptides International (Louisville, KY).

**Data presentation.** Data are expressed as means ± standard error (number of observations). Where indicated, statistical significance was evaluated using ANOVA with post-hoc Bonferroni *t* tests.

## Results

**Regulation of chromaffin $K^+$ currents by GTPγS.** The upper left panel of Fig. 1 shows an example of outward voltage-dependent $K^+$ currents obtained during 40-msec voltage steps applied from a holding potential of −80 mV to potentials ranging from −40 to +60 mV. As described previously in bovine

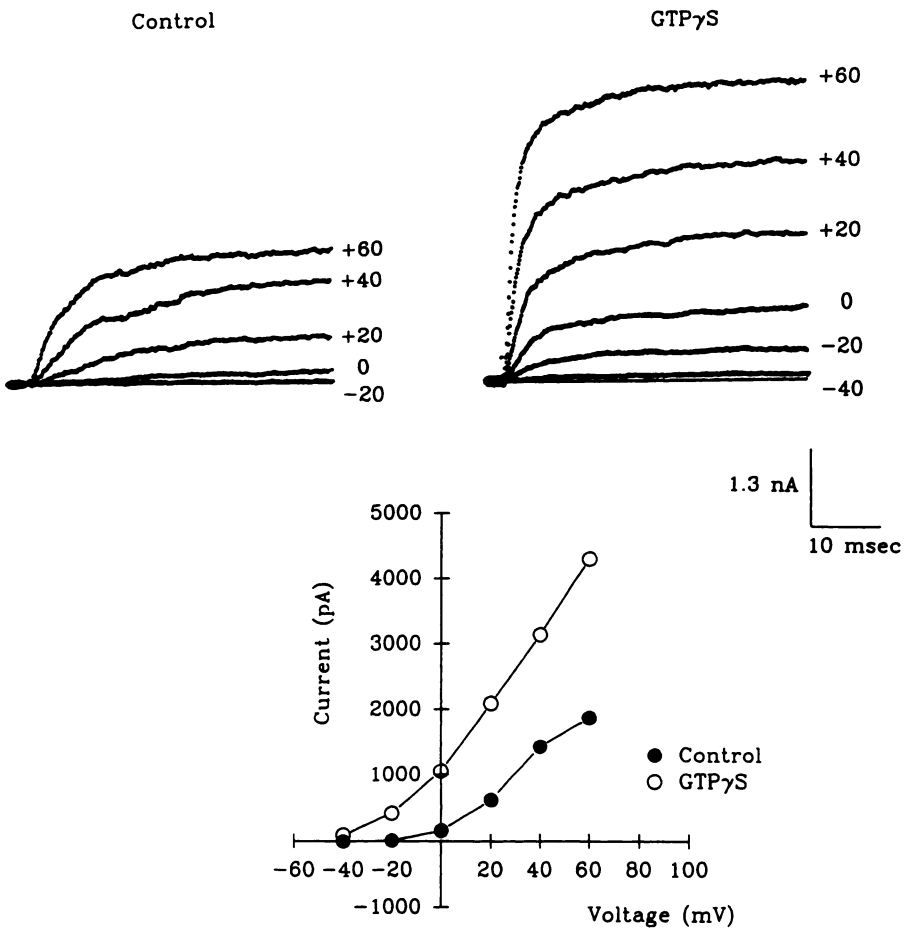


**Fig. 1.** Modulation of whole-cell $K^+$ currents by GTPγS in cultured bovine adrenal chromaffin cells. *Top (left)*: control currents recorded during 40-msec voltage steps, given in 20-mV increments, applied from a holding potential of −80 mV to potentials ranging from −20 to +60 mV. *Top (right)*: currents activated in the same cell during dialysis of 200 μM GTPγS during voltage steps to −40 through +60 mV. *Bottom*: I/V relationship for $K^+$ currents shown in *top panels*. Cell CHR109.

chromaffin cells (10, 11), these currents are activated when the membrane potential is depolarized positive to −40 mV. With increased depolarization, these currents respond with an increase in the rate of activation as well as an increase in current amplitude (10, 11). The right panel of Fig. 1 displays $K^+$ currents recorded in the same cell after dialysis with 200 μM GTPγS. In the presence of GTPγS the currents activated more rapidly, and there was an increase in the current amplitude. Moreover, dialysis with GTPγS caused the $K^+$ currents to become activated at more negative potentials as shown in the current versus voltage (I/V) relationship (*bottom panel* of Fig. 1). This had the overall effect of shifting the I/V relationship to the left.

In addition to altering the properties of the outward $K^+$ currents, dialysis of the chromaffin cells with GTPγS resulted in the appearance of a time-independent background current (results not shown). Doroshenko *et al.* (8) have reported that GTPγS activates a time-independent $Cl^-$ current in these cells. Because activation of the $Cl^-$ channels by GTPγS would result in an outward current, using the recording protocol of the present experiments ($[Cl]_o$, 157 mM; $[Cl]_i$, 12 mM; $E_{Cl}$, −63 mV), it was important to determine the contribution of the $Cl^-$ current to the GTPγS-mediated effects. Fig. 2 shows the results of an experiment in which NaCl and KCl in the external solution were replaced with Na-Aspartate and K-Aspartate ($[Cl]_o$, 2 mM; $[Cl]_i$, 12 mM; $E_{Cl}$ = +45 mV) and outward currents measured during voltage steps to −60 mV through +40 mV. Dialysis with GTPγS caused changes in the outward currents (e.g., increased amplitude, shifted I/V relationship, and speeded activation kinetics) (see Fig. 2) identical to that observed in the normal NaCl, KCl external solution. In addition, exposure of the cells to DIDS (10 μM), which inhibits the chromaffin $Cl^-$ current (8), caused no change in the time-dependent outward currents (results not shown). Thus, although both the $Cl^-$ channels (8) and the $K^+$ channels are targets of GTPγS action, the changes observed in the time-dependent outward currents result primarily from effects on $K^+$ currents.

As would be expected from the negative shift in the I/V relationship displayed in Fig. 1, dialysis with GTPγS resulted in a voltage-dependent increase in the $K^+$ currents. The increase in current amplitude caused by GTPγS was significantly greater at the more negative potentials of −40 mV, −20 mV and 0 mV (increases of 105 ± 3, 96 ± 1, and 81 ± 3%, respectively) when compared to the more positive potentials of +20 mV, +40 mV, and +60 mV (increases of 61 ± 4, 52 ± 5, and 49 ± 5%, respectively) ($n$ = 10 cells, $p < 0.05$). In order to further quantify this effect, the normalized conductance for the currents is plotted as a function of the test voltage in Fig. 3. The *continuous lines* represent the best fits of the data points to the Boltzmann equation. The half-maximal voltage ($V_{1/2}$) required for activation was +17 mV under control conditions, and decreased to +1 mV in the presence of GTPγS. The slope of the fitted curve decreased by 30% with GTPγS.

**Activation kinetics of chromaffin $K^+$ currents.** In addition to increasing the peak $K^+$ current amplitude, dialysis of the chromaffin cells with GTPγS also caused the currents to activate more rapidly (see Fig. 1, *right*). In order to quantify this effect, $K^+$ current records were fit using either the equation,

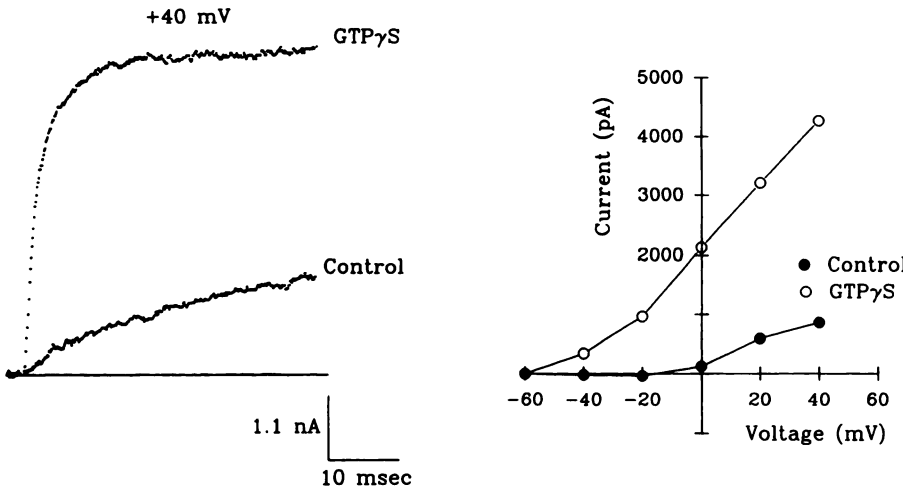


Fig. 2. Modulation of outward currents by GTPγS in low chloride-containing external solution. *Left panel*: control and GTPγS-activated $K^+$ currents recorded during voltage steps to +40 mV in a chromaffin cell bathed in a Na-Aspartate, K-Aspartate external solution. Under the conditions of this experiment ($[Cl]_o$, 2 mM; $[Cl]_i$, 12 mM, $E_{Cl}$, +45 mV), $Cl^-$ currents should be negligible at +40 mV. *Right panel*: I/V relationship for control and GTPγS-regulated $K^+$ currents. Cell CHR126.

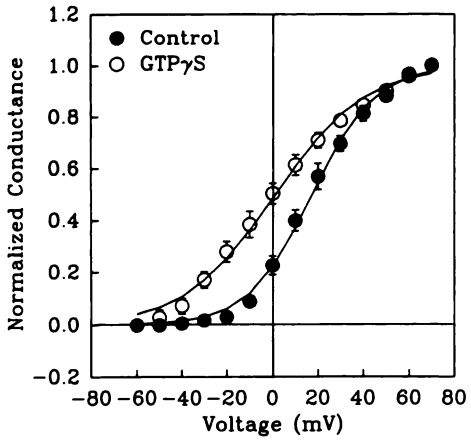


Fig. 3. Voltage dependence of $K^+$ current increase during dialysis with GTPγS. Activation curve for currents obtained in five chromaffin cells during voltage steps applied in 10-mV increments to −60 through +70 mV. Conductance was determined by dividing the peak current amplitude at each potential by the driving force for $K^+$ ($V_m - E_K$). The *continuous line* represents the best fit of the Boltzmann equation, $g_K = g_{Kmax}/(1 + \exp[-(V_m - V_{1/2})/k])$, where $V_{1/2}$ is the half-maximal voltage required for activation and $k$ gives the steepness of the voltage dependence, to the data points. The fitted parameters were $V_{1/2}$ = +17 mV and $k$ = 14 for control, and $V_{1/2}$ = +1 mV and $k$ = 20 for GTPγS curves.

$I_K = I_{Kmax} \cdot [1 - \exp(-t/\tau)]$, or the equation, $I_K = I_{K1} \cdot [1 - \exp(-t/\tau_1)] + I_{K2} \cdot [1 - \exp(-t/\tau_2)]$. Fig. 4 displays $K^+$ currents recorded at +20 mV and +60 mV both before and after stimulation by GTPγS. Superimposed on the current *traces* are fits determined using the exponential equations. Control currents were well fit using a single exponential, with a time constant ($\tau$) or 14.1 msec at +20 mV and 12.5 msec at +60 mV. It should be noted that variances in the activation kinetics of the control currents were occasionally observed (see Fig. 8). After dialysis with GTPγS, a biexponential was required to account for both a fast component present only in the GTPγS-modulated currents ($\tau_1$ = 1.4 msec at +20 mV; $\tau_1$ = 1.1 msec at +60 mV) and a slow component similar to that seen in the control records ($\tau_2$ = 12.9 msec at +20 mV; $\tau_2$ = 11.8 msec at +60 mV). Time constant data obtained during voltage steps to +20, +40, and +60 mV from 10 chromaffin cells is summarized in Table 1. At more negative potentials (−20, −10, and 0 mV), $K^+$ currents recorded both under control conditions and in the presence of GTPγS were fit using the single exponential equation. In this case, dialysis of GTPγS caused over a 4-fold decrease in $\tau$ (control $\tau$ = 19.8 ± 0.3 msec; GTPγS $\tau$ = 4.7 ± 0.9 msec; $n$ = 8).

**Properties of GTPγS-sensitive $K^+$ current.** As shown in Figs. 1–4, dialysis of the chromaffin cells with GTPγS caused a voltage-dependent increase in the $K^+$ currents that was accompanied by the appearance of a rapidly activating component in the current records. For convenience, this rapidly activating current observed in the presence of GTPγS will be referred to as the GTPγS-sensitive $K^+$ current. The GTPγS-sensitive $K^+$ current was not detected when potassium glutamate in the internal solution was replaced with $CsCl_2$ ($n$ = 3, results not shown). However, the time-independent $Cl^-$ current (8) (see above) could be activated under these conditions, indicating that GTPγS was diffusing into the cells in these experiments. The GTPγS-sensitive $K^+$ current could be observed when 100 μM $CdCl_2$ was added to the external solution to inhibit $Ca^{2+}$ entry through the voltage-dependent $Ca^{2+}$ channels present in the cells (5, 6).

In Figs. 5 and 6 the results of experiments obtained using the $K^+$ channel blockers TEA, ChTX, and AP are shown. Although it was difficult to quantify accurately the effects of these agents on the GTPγS-sensitive $K^+$ current, because of the presence of multiple $K^+$ channels in the chromaffin cells active at the recording potentials (10, 11), these experiments did supply information concerning the pharmacological sensitivity of this channel. As shown on the *left* of Fig. 5, a 5 mM concentration of TEA caused a large reduction in the GTPγS-sensitive current. In this experiment, TEA reversibly inhibited the current by 85% at +60 mV and by 90% at −40 mV. The $IC_{50}$ for this inhibition was 2 mM (see Fig. 5, *right*). In contrast to the results with TEA, ChTX, a blocker of large conductance $Ca^{2+}$-activated $K^+$ channels (BK channels) (30), caused only a slight decline in the $K^+$ currents recorded during GTPγS dialysis (Fig. 6, *left*). In three cells examined, 50 nM ChTx reduced the GTPγS-sensitive current by 11 ± 1%. Moreover, ChTX did not alter the kinetic properties of the GTPγS-sensitive current (Fig. 6, *left*, Table 1). AP, a blocker of small conductance $Ca^{2+}$-activated $K^+$ channels (31), was also relatively ineffective in reducing the GTPγS-sensitive $K^+$ current when used at a concentration of 250 nM (Fig. 6, *right*).

**Pharmacological regulation of chromaffin $K^+$ currents.** The results displayed in Figs. 1–6 suggest that stimula-

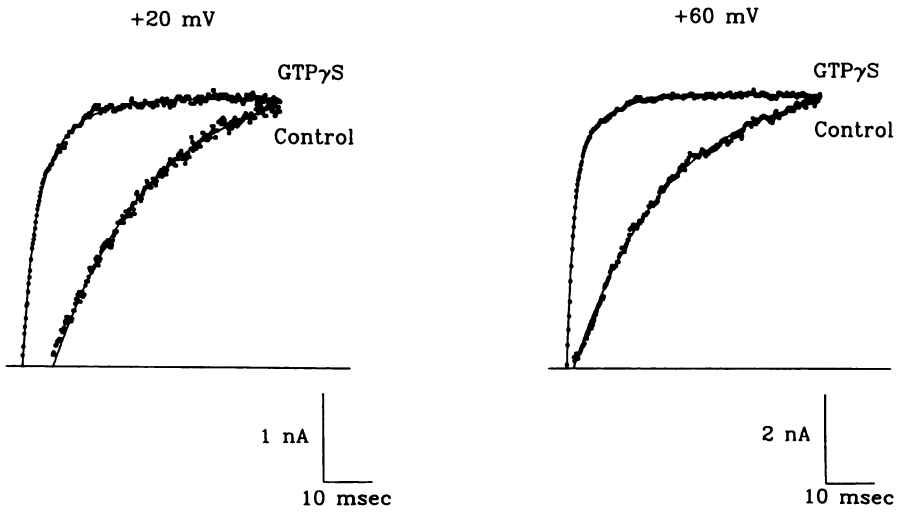


**Fig. 4.** Activation kinetics of control and GTPγS-activated $K^+$ current. Records were obtained at +20 mV (*left*) and +60 mV (*right*). Control records were scaled to the maximum current with GTPγS. Control $K^+$ currents were well fit using the single exponential equation, $I_K = I_{Kmax} \cdot [1 - \exp(-t/\tau)]$ with $\tau$ = 14.1 msec at +20 mV and 12.5 msec at +60 mV. GTPγS-activated currents were fit using the biexponential equation, $I_K = I_{K1} \cdot [1 - \exp(-t/\tau_1)] + I_{K2} \cdot [1 - \exp(-t/\tau_2)]$ with $\tau_1$ = 1.4 msec and $\tau_2$ = 12.9 msec at +20 mV and $\tau_1$ = 1.1 msec and $\tau_2$ = 11.8 msec at +60 mV. The gap seen at the beginning of the control record measured at +20 mV was caused by a delay in current activation. See Table 1 for summary of data. Cell CHR170.

**TABLE 1**

**Kinetics of $K^+$ current activation**

Time constants ($\tau$, in ms) were obtained as described in the text and displayed in Fig. 4. Control values are listed under $\tau_2$ for comparison with GTPγS time constants.

| Conditions | Voltage | | | | | |
|---|---|---|---|---|---|---|
| | +20 mV | | +40 mV | | +60 mV | |
| | $\tau_1$ | $\tau_2$ | $\tau_1$ | $\tau_2$ | $\tau_1$ | $\tau_2$ |
| Control $n = 10$ | | 15.1 ± 0.4 | | 13.0 ± 0.7 | | 11.6 ± 0.7 |
| GTPγS $n = 10$ | 1.9 ± 0.2 | 12.5 ± 1.0 | 1.8 ± 0.1 | 11.9 ± 0.8 | 1.3 ± 0.1 | 9.4 ± 0.7 |
| GTPγS + ChTX $n = 3$ | 1.9 ± 0.4 | 11.3 ± 1.3 | 1.3 ± 0.2 | 8.8 ± 1.8 | 1.2 ± 0.2 | 6.7 ± 0.3 |

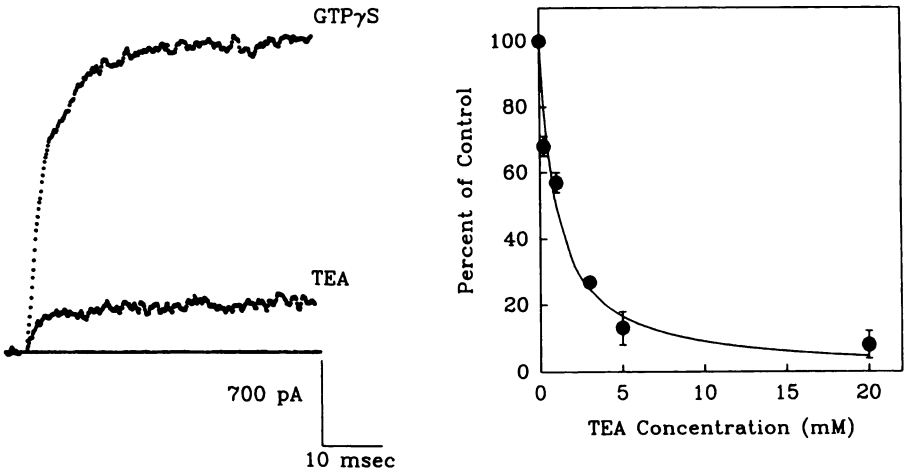


**Fig. 5.** Inhibition of GTPγS-sensitive $K^+$ current by TEA. *Left panel*: $K^+$ currents recorded at +60 mV during dialysis of a chromaffin cell with GTPγS in the presence and absence of 5 mM TEA. In this experiment TEA reversibly inhibited the current by 85%. Cell CHR178. *Right panel*: concentration versus response curve for inhibition of the GTPγS-sensitive $K^+$ current by TEA determined at +60 mV. Each *point* represents the mean of two to four experiments. When appropriate, standard error bars have been included. In some experiments two concentrations of TEA were tested on a single cell. The *smooth curve* is given by $1/(1 + [TEA]/IC_{50})$ with an $IC_{50}$ of 2 mM providing the best least squares fit to inhibition.

tion of chromaffin G protein(s) by GTPγS alters the properties of the outward $K^+$ currents. To determine whether receptor stimulation causes similar changes, the effects of several agents known to regulate adrenal medullary function were examined on the $K^+$ currents. Dopamine (10–30 μM, $n$ = 8), γ-aminobutyric acid (10 μM, $n$ = 4), neuropeptide Y (10 μM, $n$ = 4), carbachol (100 μM, $n$ = 5), and substance P (10 μM, $n$ = 4) produced no change in the outward $K^+$ currents (results not shown). Interestingly, adenosine (10 μM) increased the amplitude of the currents (measured at +60 mV) by 36 ± 15% ($n$ = 3). However, in contrast to the results with GTPγS, adenosine caused no change in the activation kinetics or voltage dependence of the $K^+$ currents, suggesting that adenosine is acting through a different mechanism.

G proteins are involved in stimulating cAMP-dependent protein kinase (PKA) and increase the activity of PKC by augmenting the breakdown of phosphatidylinositol-4,5-bisphosphate. For this reason the possible involvement of PKA and PKC in regulating the $K^+$ currents was determined. Fig. 7 (*left*) shows inward $Ca^{2+}$ currents measured in the chromaffin cells with a Cs-containing internal solution. Addition of 200 μM of the membrane soluble cAMP analog 8-CPT cAMP resulted in a large increase in the $Ca^{2+}$ currents, indicating that 8-CPT cAMP is effective in stimulating PKA in these cells. In

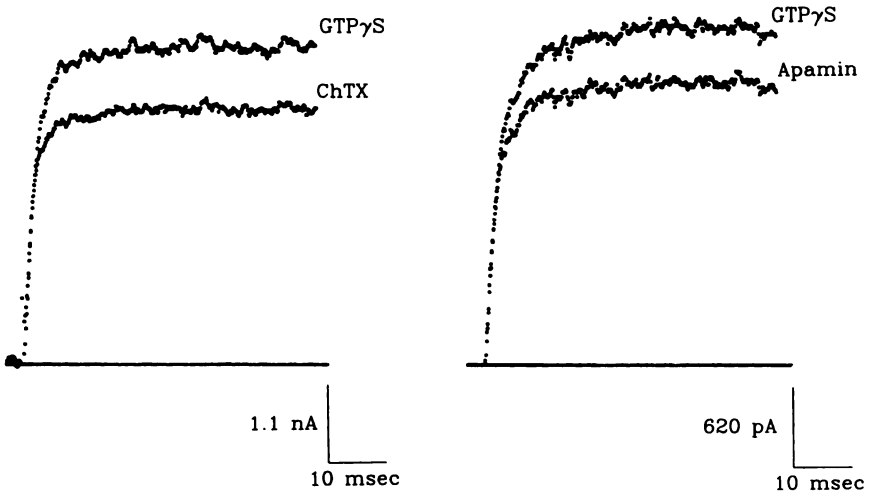


**Fig. 6.** Effects of charybdotoxin and apamin on GTPγS-sensitive $K^+$ current. Currents were recorded at +60 mV during dialysis of GTPγS either in the presence or absence of 50 nM ChTX (*left*) or 250 nM AP (*right*). Addition of charybdotoxin resulted in a 20% decrease in the current, whereas AP produced a 16% decrease in these experiments. Both ChTX and AP also reduced control $K^+$ currents recorded in the absence of GTPγS (results not shown), suggesting that venom-sensitive channels are present under basal and stimulated conditions. Cells CHR141 and CHR203.

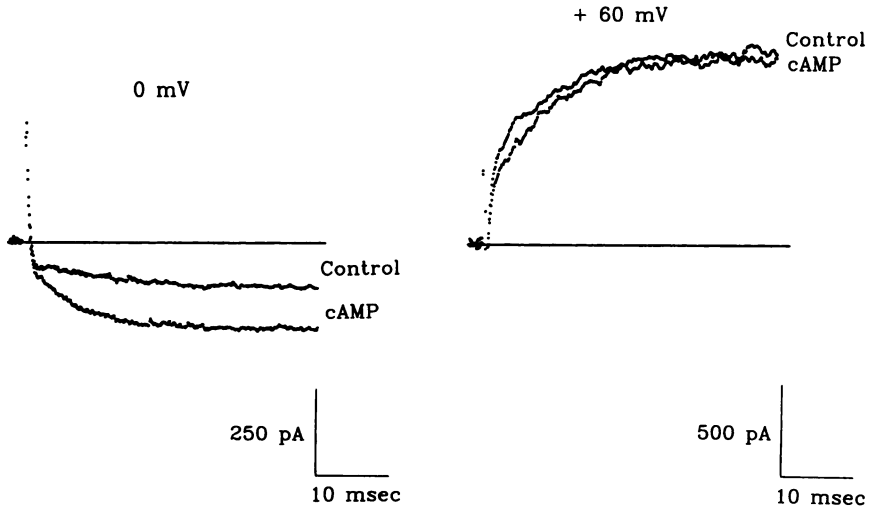


**Fig. 7.** Lack of regulation of chromaffin $K^+$ currents by cAMP. *Left*: inward $Ca^{2+}$ currents recorded during voltage steps to 0 mV before (control) and after (cAMP) the addition of the membrane-soluble cAMP analog 8-chlorphenylthio cAMP. In this experiment internal $K^+$ was replaced with $Cs^+$. Addition of 8-CPT cAMP resulted in a 100% increase in the $Ca^{2+}$ current. *Right*: outward $K^+$ currents from another cell recorded at +60 mV in the presence and absence of cAMP. $K^+$ currents were recorded with the normal internal solution (see Materials and Methods). Cells BCA1 and CHR265.

contrast, application of 8-CPT cAMP caused no change in the outward $K^+$ currents (% change = 3 ± 8, $n = 5$) when measured with the normal internal solution (Fig. 7, *right*). Exposure of the cells to 12,13 phorbol dibutyrate, to stimulate PKC, also had no effect on the $K^+$ currents.

**Effect of fluoride on chromaffin $K^+$ currents and catecholamine secretion.** $AlF^-_4$ permanently activates G proteins by binding with GDP on the α subunit of the protein and mimicking the action of the terminal phosphate group of GTP (29). To determine the effect of $AlF^-_4$ on the $K^+$ currents, chromaffin cells were exposed to various concentrations of NaF. As was the case with GTPγS dialysis, addition of 20 mM fluoride to the external solution caused both an increase in the $K^+$ current amplitude and a speeding in the activation kinetics (Fig. 8, *left*). Similar results were obtained in five other cells with either 10 or 20 mM fluoride. These changes in the $K^+$ currents were not caused by nonspecific effects brought about by increasing external $Na^+$, because substitution of NaF with NaCl had no effect on the size or the kinetics of the currents (results not shown).

The effect of fluoride was also examined on secretion in the chromaffin cells. Fig. 8 (*right*) shows the effect of 2, 10, and 20 mM NaF on nicotine-evoked catecholamine secretion. At a concentration of 2 mM, NaF had no effect on nicotine-evoked secretion. In addition, at this and higher concentrations (10 and 20 mM), NaF produced no change in basal catecholamine secretion (*open columns*). However, addition of 10 or 20 mM NaF to the secretion medium inhibited catecholamine release stimulated by nicotine to 50 and 11%, respectively, of that obtained in the control cells ($p < 0.05$, $n = 5$) (*hatched columns*).

## Discussion

**Effects of G protein stimulation on bovine chromaffin cells.** In the present study, stimulation of cellular G proteins through either internal dialysis with GTPγS or external addition of $AlF^-_4$ resulted in a voltage-dependent increase in the size of the outward $K^+$ currents recorded in bovine chromaffin cells. This augmentation in current amplitude was accompanied by the appearance of a fast component of current activation, which was not observed under basal recording conditions. The ability of fluoride to reduce nicotine-evoked catecholamine secretion suggests that this $K^+$ channel can regulate secretion by increasing the rate of membrane repolarization and, thus, decreasing the amount of $Ca^{2+}$ that can enter the cells through the voltage-dependent $Ca^{2+}$ channels.

An opioid/G protein-activated delayed rectifier $K^+$ current has been identified recently in isolated hippocampal neurons

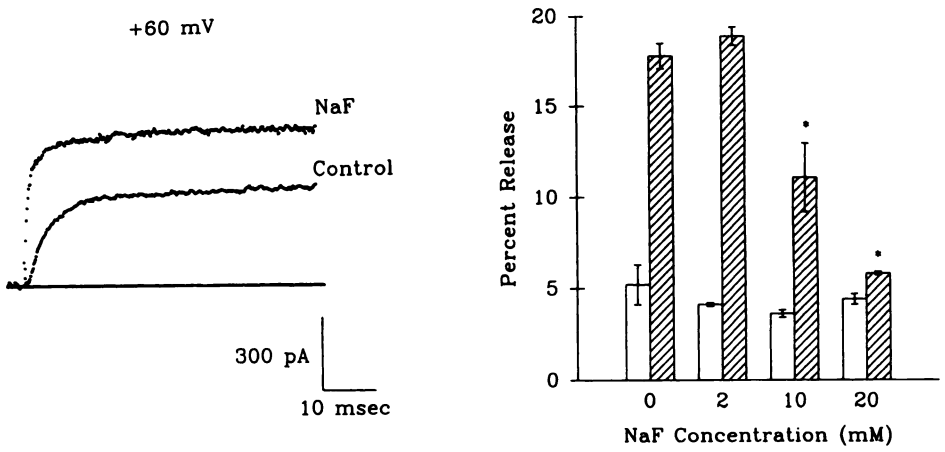


**Fig. 8.** Effects of fluoride on $K^+$ currents and nicotine-evoked exocytosis. *Left*: $K^+$ currents recorded at +60 mV either before or after addition of 20 mM NaF. At this concentration NaF caused both a voltage-dependent increase in the $K^+$ current amplitude and a speeding in the activation kinetics. Cell CHR53. *Right*: Effect of fluoride on nicotine-evoked catecholamine secretion. *Open columns* represent addition of the indicated concentrations of NaF to the secretion medium in the absence of nicotine. *Hatched columns* represent the addition of 5 μM nicotine and NaF to the medium. The ability of nicotine to enhance secretion was significantly reduced by 10 and 20 mM concentrations of fluoride ($p < 0.05$, $n = 5$). This effect was observed in two other experiments. Experiment Nic-NaF-#6.

(32). This current can be regulated by cAMP-dependent protein kinase (PKA) phosphorylation (32). In contrast, the chromaffin GTPγS-sensitive $K^+$ current was not activated during stimulation of PKA (see Fig. 7) or PKC. Thus, GTPγS does not appear to regulate the chromaffin $K^+$ currents by stimulating adenylate cyclase or phospholipase C. The involvement of other second messengers in G protein action, including metabolites of arachidonic acid and phosphatidylinositol, will require further study.

**Characterization of the G protein-sensitive $K^+$ channel.** There are two possible schemes that can be proposed to account for the effects of GTPγS and fluoride on the outward $K^+$ currents described in this study. According to the first scheme, GTPγS and fluoride, by stimulating G proteins in the chromaffin cells, modify the properties of a $K^+$ channel that is present under control conditions and opens during membrane depolarization. Alternatively, stimulation of G proteins might activate a novel $K^+$ channel that is not normally active under the conditions of these experiments. This channel would open rapidly during depolarization and could be activated at relatively negative membrane potentials (−40 to −30 mV) compared with the basal $K^+$ channels. The possibility that G proteins regulate a $K^+$ channel previously identified in the chromaffin cells is discussed below.

By far the best characterized $K^+$ channel present in the bovine chromaffin cells is the $Ca^{2+}$-activated $K^+$ channel (BK channel). This channel has a conductance that ranges, depending on the recording conditions, from 96 to 310 pS, is relatively non-voltage dependent and requires internal $Ca^{2+}$ for activity (10, 33, 34). The BK channel can be activated under whole-cell conditions after the movement of $Ca^{2+}$ into the chromaffin cell through the membrane $Ca^+$ channels (10, 11). This activation is inhibited in the presence of external $Cd^{2+}$ (10, 11). The BK channel is blocked by external TEA both in single channel ($IC_{50}$, 0.2 mM) (34) and whole-cell (1 mM causes 90% block) (10) recordings. In addition, BK channels in various cell types are potently inhibited by charybdotoxin when applied at nanomolar concentrations ($IC_{50}$, 10 nM) (30). Based on these observations, it is unlikely that the GTPγS-sensitive $K^+$ current represents the activity of BK channels in these cells. The GTPγS-sensitive current could be activated during dialysis with a $Ca^{2+}$-free internal solution and was present when the external solution contained $CdCl_2$. Augmentation of the $K^+$ currents by GTPγS was greater at potentials close to the threshold for current activation (−40–0 mV) than at more positive potentials (+20–+60 mV). Although the GTPγS-sensitive current could be inhibited by TEA, higher concentrations of this compound were required for this block ($IC_{50}$, 2 mM) when compared to the BK channel. Finally, the GTPγS-sensitive current was only slightly inhibited by 50 nM charybdotoxin (11% reduction).

In addition to the BK channel, two smaller conductance $Ca^{2+}$-independent $K^+$ channels have been identified in bovine chromaffin cells (10). One of these channels (termed the SK channel) has a conductance of 8 pS, is not strongly voltage-dependent, and activates slowly during depolarization. In contrast, the other channel (termed the FK channel) has a conductance of 18 pS, possesses a steep voltage dependence that saturates between +20 and +40 mV and displays fast activation kinetics. Furthermore, the ensemble averaged current for the FK channel activates with a time to half-maximum current of 2 to 3 msec at +20 mV, which is comparable to the time course of activation of the GTPγS-sensitive current ($\tau_1$ = 2 msec at +20 mV). Thus, the FK channel could represent a possible candidate for the GTPγS-sensitive current. Although the pharmacology of the FK channel is not known, TEA (1 mM) reduces both the $Ca^{2+}$-sensitive and insensitive components of the whole-cell $K^+$ currents in chromaffin cells (10).

**Role of G proteins in chromaffin cell exocytosis.** The role of G proteins in hormone secretion from bovine adrenal medullary chromaffin cells is unclear. In certain studies analogs of GTP, including GTPγS and GMPPNP, cause $Ca^{2+}$-independent secretion and enhance the $Ca^{2+}$ sensitivity of secretion in chromaffin cells permeabilized by digitonin or bacterial toxins (35, 36). Activation of G proteins by fluoride stimulates secretion in ouabain-treated cells (37). In contrast, other studies demonstrate an inhibitory effect of GTPγS on $Ca^{2+}$-evoked secretion from permeabilized chromaffin cells (38, 39). These conflicting effects on secretion are not surprising considering that all of the G proteins in the chromaffin cell are activated by the treatments used.

Evidence for involvement of specific G proteins in secretion comes primarily from studies with pertussis toxin. Pretreatment of chromaffin cells with pertussis toxin leads to ADP ribosylation of several 39- to 41-kDa proteins and to enhancement of secretion evoked by various agents (21, 23, 40). The effects of pertussis toxin on secretion in permeabilized cells require cytoplasmic G protein α-subunits (40) and are mim-

icked by antibodies to the $\alpha$-subunit of Go (38). These findings suggest that Go$\alpha$ mediates a tonic inhibition of the secretory process. Such a role might be expected for the G protein-regulated $K^+$ channel described here. This $K^+$ channel may normally participate in the termination of secretion and attenuate secretion during prolonged stimulation *in vitro*. Inhibition of the channel by ADP ribosylation of its associated G protein would increase the secretory response. Conversely, activation of this $K^+$ channel through its associated G protein should inhibit evoked secretion, as observed in the fluoride-dependent inhibition of nicotine-evoked secretion (see Fig. 8). Hence, the G protein-regulated $K^+$ channel described here may play a regulatory role in secretagogue-mediated exocytosis.

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**References**

1. Douglas, W. W. Secretomotor control of adrenal medullary secretion: synaptic, membrane, and ionic requirements in stimulus secretion coupling, in *Handbook of Physiology* (H. Blaschko, ed.). American Physiological Society, Bethesda, MD, 367–388 (1975).
2. Biales, B., M. Dichter, and A. Tischler. Electrical excitability of cultured adrenal chromaffin cells. *J. Physiol. (Lond.)* **262**:743–753 (1976).
3. Brandt, B. L., S. Hagiwara, Y. Kidokoro, and S. Miyazaki. Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol. (Lond.)* **263**:417–439 (1976).
4. Kidokoro, Y. and A. K. Ritchie. Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *J. Physiol. (Lond.)* **307**:199–216 (1980).
5. Fenwick, E. M., A. Marty, and E. Neher. Sodium and calcium channels in bovine chromaffin cells. *J. Physiol. (Lond.)* **331**:599–635 (1982).
6. Hoshi, T., J. Rothlein, and S. J. Smith. Facilitation of $Ca^{2+}$ channel currents in bovine adrenal chromaffin cells. *Proc. Natl. Acad. Sci. USA* **81**:5871–5875 (1984).
7. Artalejo, C. R., M. K. Dahmer, R. L. Perlman, and A. P. Fox. Two types of $Ca^{2+}$ currents are found in bovine chromaffin cells: facilitation is due to the recruitment of one type. *J. Physiol. (Lond.)* **432**:681–707 (1991).
8. Doroshenko, P., R. Penner, and E. Neher. Novel chloride conductance in the membrane of bovine chromaffin cells activated by intracellular GTP$\gamma$S. *J. Physiol. (Lond.)* **436**:711–724 (1991).
9. Doroshenko, P. and E. Neher. Volume-sensitive chloride conductance in bovine chromaffin cell membrane. *J. Physiol. (Lond.)* **449**:197–218 (1992).
10. Marty, A. and E. Neher. Potassium channels in cultured bovine adrenal chromaffin cells. *J. Physiol. (Lond.)* **367**:117–141 (1985).
11. Sala, S. and B. Soria. Inactivation of delayed potassium current in cultured bovine chromaffin cells. *Eur. J. Neurosci.* **3**:462–472 (1991).
12. Brown, A. M. and L. Birnbaumer. Direct G protein gating of ion channels. *Am. J. Physiol.* **254**:H401–H410 (1988).
13. Siegelbaum, S. A., J. S. Camardo, and E. R. Kandel. Serotonin and cyclic AMP close single $K^+$ channels in *Aplysia* sensory neurones. *Nature (Lond.)* **299**:413–417 (1982).
14. Benson, J. A. and I. B. Levitan. Serotonin increases an anomalously rectifying K current in Aplysia neuron R15. *Proc. Natl. Acad. Sci. USA* **80**:3522–3525 (1983).
15. Walsh, K. B. and R. S. Kass. Regulation of a heart potassium channel by protein kinase A and C. *Science* **242**:67–69 (1988).
16. Yatani, A., J. Codina, A. M. Brown, and L. Birnbaumer. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein $G_K$. *Science* **235**:207–211 (1987).
17. Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. The $\beta(\gamma)$ subunits of GTP-binding proteins activate the muscarinic $K^+$ channel in heart. *Nature (Lond.)* **325**:321–326 (1987).
18. Andrade, R., R. C. Malenka, and R. A. Nicoll. A G protein couples serotonin and $GABA_B$ receptors to the same channels in hippocampus. *Science* **234**:1261–1265 (1986).
19. Tatsumi, H., M. Costa, M. Schimerlik, and R. A. North. Potassium conductance increased by noradrenaline, opioids, somatostatin and G-proteins: whole-cell recording from guinea pig submucous neurons. *J. Neurosci.* **10**:1675–1682 (1990).
20. McCloskey, M. A. and M. D. Cahalan. G protein control of potassium channel activity in a mast cell line. *J. Gen. Physiol.* **95**:205–227 (1990).
21. Tanaka, T., H. Yokohama, M. Negishi, H. Hayashi, S. Ito, and O. Hayaishi. Pertussis toxin facilitates secretagogue-induced catecholamine release from cultured bovine adrenal chromaffin cells. *Biochem. Biophys. Res. Commun.* **144**:907–914 (1987).
22. Brocklehurst, K. W. and H. B. Pollard. Pertussis toxin stimulates delayed-onset, $Ca^{2+}$-dependent catecholamine release and the ADP-ribosylation of a 40-kDa protein in bovine adrenal chromaffin cells. *FEBS Lett.* **234**:439–445 (1988).
23. Wilson, S. P. Vasoactive intestinal peptide is a secretagogue in bovine chromaffin cells pretreated with pertussis toxin. *Neuropept.* **23**:187–192 (1992).
24. Wilson, S. P. Purification of adrenal chromaffin cells on Renografin gradients. *J. Neurosci. Methods* **19**:163–171 (1987).
25. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and J. Sigworth. Improved patch-clamp techniques for high resolution current recordings from cells and cell-free membrane patches. *Pflugers Arch.* **391**:85–100 (1981).
25. Fabiato, A. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.* **157**:378–416 (1988).
27. Pusch, M. and E. Neher. Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflugers Arch.* **411**:204–211 (1988).
28. Sternweis, P. C. and A. G. Gilman. Aluminum: a requirement for activation of the regulatory component of adenylate cyclase by fluoride. *Proc. Natl. Acad. Sci. USA* **79**:4888–4891 (1982).
29. Yatani, A. and A. M. Brown. Mechanism of fluoride activation of G protein-gated muscarinic atrial $K^+$ channels. *J. Biol. Chem.* **266**:22872–22877 (1991).
30. Miller, C., E. Moczydlowski, R. Latorre, and M. Phillips. Charybdotoxin, a protein inhibitor of single Ca-activated K channels from mammalian skeletal muscle. *Nature (Lond.)* **313**:316–318 (1985).
31. Blatz, A. L. and K. L. Magleby. Single apamin-blocked Ca-activated $K^+$ channels of small conductance in cultured rat skeletal muscle. *Nature (Lond.)* **323**:718–720 (1986).
32. Wimpey, T. L. and C. Chavkin. 8-Bromo-cAMP blocks opioid activation of a voltage-gated potassium current in isolated hippocampal neurons. *Neurosci. Lett.* **137**:137–140 (1992).
33. Marty, A. Ca-dependent K channels with large unitary conductance in chromaffin cells. *Nature (Lond.)* **291**:497–500 (1981).
34. Yellen, G. Ionic permeation and blockade in $Ca^{2+}$-activated $K^+$ channels of bovine chromaffin cells. *J. Gen. Physiol.* **84**:157–186 (1984).
35. Bader, M.-F., J.-M. Sontag, D. Thierse, and D. Aunis. A reassessment of guanine nucleotide effects on catecholamine secretion from permeabilized adrenal chromaffin cells. *J. Biol. Chem.* **264**:16426–16434 (1989).
36. Bittner, M. A., R. W. Holz, and R. R. Neubig. Guanine nucleotide effects on catecholamine secretion from digitonin-permeabilized adrenal chromaffin cells. *J. Biol. Chem.* **261**:10182–10188 (1986).
37. Ito, S., M. Negishi, N. Mochizuki-Oda, H. Yokohama, and O. Hayaishi. Sodium fluoride mimics the effect of prostaglandin $E_2$ on catecholamine release from bovine adrenal chromaffin cells. *J. Neurochem.* **56**:44–51 (1991).
38. Ohara-Imaizumi, M., K. Kameyama, N. Kawae, K. Takeda, S. Muramatsu, and K. Kamakura. Regulatory role of the GTP-binding protein, $G_o$, in the mechanism of exocytosis in adrenal chromaffin cells. *J. Neurochem.* **58**:2275–2284 (1992).
39. Knight, D. E. and P. F. Baker. Guanine nucleotides and $Ca^{2+}$-dependent exocytosis: studies on two adrenal cell preparations. *FEBS Lett.* **189**:345–349 (1985).
40. Sontag, J.-M., D. Thierse, B. Rouot, D. Aunis, and M.-F. Bader. A pertussis-toxin-sensitive protein controls exocytosis in chromaffin cells at a step distal to the generation of second messengers. *Biochem. J.* **274**:339–347 (1991).

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