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Stimulatory and inhibitory regulation of calcium-activated potassium channels by guanine nucleotide-binding proteins

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ABSTRACT **The regulation of membrane ion channels by guanine nucleotide-binding proteins (G proteins) has been described in numerous tissues. This regulation has been shown to involve the membrane-delimited stimulatory action of G proteins on ion channels. We now show that single calcium-activated potassium channels (K_{Ca} channels) in airway smooth muscle cells are both stimulated and inhibited by G proteins in membrane patches. We demonstrate that the β-adrenergic agonist isoproterenol stimulates channel activity via the α subunit of the stimulatory G protein of adenylyl cyclase, G_s, and that channel opening is inhibited by the action of the muscarinic agonist methacholine, acting via a pertussis toxin-sensitive G protein. Isoproterenol stimulated and methacholine inhibited channel activity in the same outside-out patches when GTP was present at the cytosolic surface of the patch. In inside-out patches, addition of GTP and guanosine 5′-[γ-thio]triphosphate (GTP[γS]) augmented channel activity when isoproterenol was included in the patch pipette, and inhibited channel activity when methacholine was included in the pipette. Consistent with these results, in the presence of GTP[γS], the α subunit of G_s (α_s·GTP[γS] complex) opened K_{Ca} channels in a dose-dependent manner, whereas in the presence of guanosine 5′-[β-thio]diphosphate, α_s had no effect. By contrast, application of activated α_i or α_o proteins did not inhibit channel activity in inside-out patches, indicating that channel inhibition is more complex than a simple α subunit/channel interaction, similar to the complex inhibitory regulation of adenylyl cyclase. These results suggest that hormonal regulation of K_{Ca} channels shares substantial features with the regulation of adenylyl cyclase and demonstrate that a single ion channel may serve as the regulatory target for the membrane-delimited action of stimulatory and inhibitory G proteins. Moreover, they demonstrate a potentially important functional pathway by which β-adrenergic and other G_s-linked receptors stimulate relaxation of smooth muscle, independent of cAMP-dependent protein phosphorylation.**

Large-conductance, calcium-activated potassium channels (K_{Ca} channels) are ubiquitous membrane ion channels in smooth muscle, brain, and other tissues. One mechanism of K_{Ca} channel regulation involves the indirect activation of the channel by adenylyl cyclase-linked receptor stimulation, leading to phosphorylation of the channel by cAMP-dependent protein kinase (1–4). In smooth muscle, activation of adenylyl cyclase-linked receptors results in a marked augmentation of potassium channel activity (5–7), which is a principal mechanism of relaxation of vascular and nonvascular smooth muscle (8–11). For example, β_2-adrenergic receptor agonists are widely used clinically to relax airway smooth muscle, and it is known that receptor stimulation results in an increase in K_{Ca} channel activity in airway smooth muscle cells (5) and that these channels play an important functional role in β-adrenergic bronchodilation (12, 13). Conversely, muscarinic agonists (and other contractile agents) inhibit potassium conductances in smooth muscle (14–18), and this functional antagonism between adrenergic and muscarinic hormone action converges on a single potassium current, the M current, in nonmammalian smooth muscle cells (8). Although K_{Ca} channel activation by cAMP-dependent protein kinase has been documented in smooth muscle, membrane-delimited mechanisms of channel regulation analogous to those described in other cells (19–23) have not been explored. We report that β-adrenergic stimulation activates single K_{Ca} channels independently of channel phosphorylation, via the membrane-delimited action of the α subunit of the stimulatory guanine nucleotide-binding protein, G_s. Moreover, we demonstrate functionally antagonistic, hormone-linked stimulatory and inhibitory regulation of K_{Ca} channels at the single-channel level, indicating that the channel is regulated by stimulatory and inhibitory G proteins in a manner similar to the regulation of adenylyl cyclase (24–27).

MATERIALS AND METHODS

Cell Dissociation and Electrophysiology. Unitary currents from membrane patches were recorded by standard methods (28) from porcine, canine, and ferret tracheal myocytes that had been disaggregated as described (29). Cells from all species behaved identically; all experiments were performed at least once in patches from each species. Currents were filtered at 1 kHz and digitized at 5 kHz [−3 decibels (dB)] for open-state probability analysis. Displayed records were filtered at 250 or 500 Hz and digitized at 0.5 or 1 kHz for data compression. The cytosolic solution used was 126 mM KCl/5 mM NaCl/1 mM $MgCl_2$/2.5 mM EGTA/10 mM Hepes adjusted to pH 7.2 (KOH); 1 mM $CaCl_2$ was added to achieve a free Ca^{2+} concentration of ≈0.1 μM (30). Some experiments were performed with 5 mM $MgCl_2$; no difference in response was observed. The extracellular solution was 125 mM NaCl/5 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/10 mM Hepes adjusted to pH 7.4 (NaOH). Single-channel analysis was performed using software routines provided by M. Nelson (University of Vermont). The voltage dependence of open-state probability was analyzed by fitting the data to the Boltzmann equation: $nP_o = 1/\{1 + \exp[(V - V_m)/k]\}$. In each experimental condition evaluating the effect of added agonists or guanine nucleotides, nP_o was determined over a 3- to 5-min period before and immediately following the addition and ΔnP_o (nP_o experimental/nP_o control) was determined; analyzed periods were identical in any given experiment. Statistical comparisons were made by one-way analysis of variance; data are expressed as mean ± SE.

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Abbreviations: K_{Ca} channel, calcium-stimulated potassium channel; ATP[β,γNH], adenosine 5′-[β,γ-imido]triphosphate; GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP[βS], guanosine 5′-[β-thio]diphosphate.

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Guanine Nucleotides. Recombinant α_s (α_s-short, the 45-kDa form) was expressed in and purified from *Escherichia coli* (31). α_s (30 μM) was preincubated with 30 μM guanine 5′-[γ-thio]triphosphate (GTP[γS]) or 5′-[β-thio]diphosphate (GDP[βS]) in 50 mM Hepes/8 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol at pH 8 for 30 min at 23°C. α_s subunits were diluted in the same buffer to 100 times the final concentration and frozen in aliquots. For pertussis toxin (P-6659; Sigma) treatment, dissociated cells were incubated with toxin (0.1 μg/ml) for 4 hr at 37°C. This treatment completely blocks muscarinic channel inhibition in outside-out patches (18). cAMP-dependent protein kinase was inhibited by addition of A-kinase inhibitor (P-5636; Sigma) at 5 μg/ml, sufficient to inhibit all kinase activity from ≈5 mg of tissue (32). The inhibitor was added to the bath after inside-out patch formation, and the patch was incubated for 30 min.

RESULTS

Stimulation and Inhibition by Agonists in Outside-Out Patches. We have previously demonstrated G protein-dependent inhibition of single K_{Ca} channels in inside-out and outside-out patches (18). To examine analogous stimulatory coupling mechanisms between hormone receptors and K_{Ca} channels, channel activity was examined in outside-out patches under conditions of physiologic calcium concentration and GTP (100 μM) at the cytosolic patch surface, before and after exposure to the β-adrenergic agonist isoproterenol. Fig. 1*A* shows that bath addition of 1 μM isoproterenol to outside-out patches stimulated channel activity; following drug washout, channel activity was inhibited by charybdotoxin (100 nM), a specific inhibitor of the large-conductance K_{Ca} channel in this tissue (33). In seven similar experiments, isoproterenol stimulated channel activity 5.1 ± 0.5-fold and produced an apparent left shift in the voltage/nP_o relationship (Fig. 1*B*), although this effect could not be discriminated from a shift in the maximal open-state probability of the channel, since the patches contained too many channels to permit accurate estimation of the maximum nP_o. Although ATP was not added to the patch pipette, we sought to eliminate the possibility of channel phosphorylation through a localized activation of cAMP-dependent protein kinase supported by endogenous ATP, by performing experiments in the presence of the nonmetabolizable ATP analogue adenosine 5′-[β,γ-imido]diphosphate (ATP[β,γNH]) to inhibit phosphorylation. Channel stimulation was observed in the presence of ATP[β,γNH] (1 mM) in the patch pipette; in two such experiments, mean stimulation was 4.5-fold.

Functionally antagonistic channel regulation was directly demonstrated by sequential addition of stimulatory and inhibitory hormones to the same outside-out patches. Fig. 2*A* shows that addition of isoproterenol (1 μM) stimulated channel activity, and, following drug washout, the muscarinic agonist methacholine (10 μM) decreased channel activity. Receptor-linked inhibitory and stimulatory modulation of channel activity was not sequence-dependent; results from an experiment in which channel activity was inhibited by methacholine and then stimulated by isoproterenol are shown in Fig. 2*B*. Fig. 2*C* summarizes outside-out experiments that demonstrated functionally antagonistic hormone action on channel activity. Inhibition of channel activity by methacholine (10 or 50 μM) was similar to that reported (18); channel activity in five patches was inhibited by methacholine to 0.26 ± 0.05 of control values, and this inhibition was G protein-dependent and pertussis toxin-sensitive.

Stimulation and Inhibition by Guanine Nucleotides in Inside-Out Patches. We next sought to demonstrate membrane-delimited G-protein actions on K_{Ca} channels by the addition of exogenous guanine nucleotides to inside-out patches. Consistent with outside-out experiments, addition of guanine

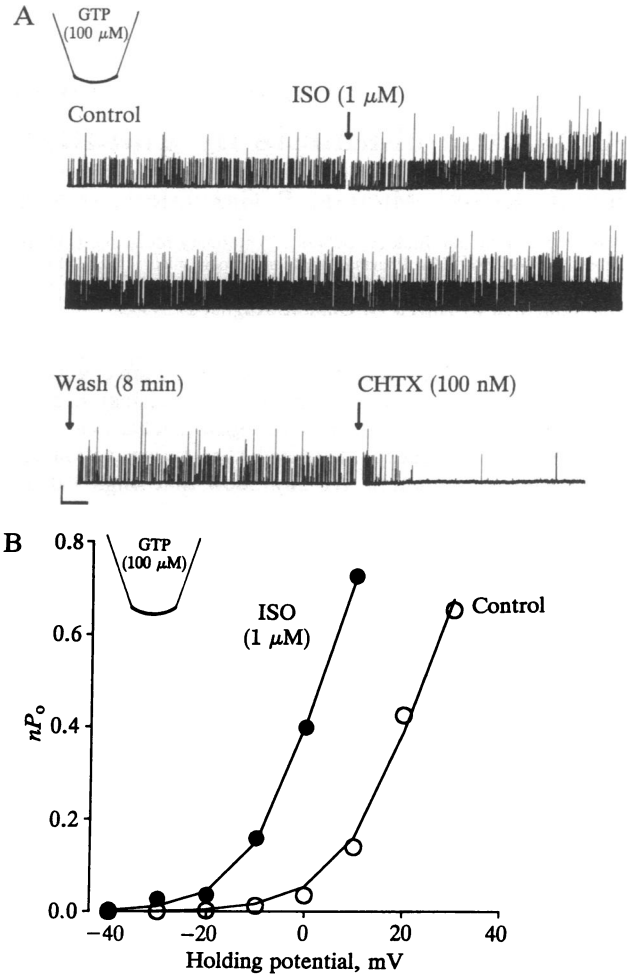

FIG. 1. Isoproterenol stimulates K_{Ca} channel activity in outside-out patches. (*A*) Addition of isoproterenol (ISO) to an outside-out patch opens K_{Ca} channels. Channel activation was reversible following bath wash; charybdotoxin (CHTX), a specific inhibitor of the large-conductance K_{Ca} channel, blocked channel activity. Trace shows continuous recording with addition artifacts blanked, except for an 8-min wash as indicated. Calibration: 10 s, 3 pA. Canine cell 0816C; holding potential, 0 mV. (*B*) Open-state probability versus holding potential for patch shown in *A*. Stimulated channel activity appears at all voltages; nP_o was determined over 30 s at each potential. Boltzmann fits to the data (lines) indicate the apparent shift in the voltage/open-state probability relationship.

nucleotides stimulated channel activity when isoproterenol was present at the extracellular membrane surface. Fig. 3*A* shows that addition of GTP[γS] (100 μM) to an inside-out patch (1 μM isoproterenol in the pipette) resulted in a rapid stimulation of channel activity; the kinetics of channel activation were quite rapid (mean time to maximum was 2.5 min). Fig. 3*B* summarizes a series of experiments in which guanine nucleotides were added to inside-out patches in the presence or absence of agonists. Addition of GTP[γS] to inside-out patches in the absence of any receptor agonist resulted in a slight increase in channel activity (1.23-fold increase, 10 patches), consistent with previous reports (34). Since K_{Ca} channels are inhibited by muscarinic receptor stimulation via a pertussis-sensitive G protein (18), we reasoned that the effect of GTP[γS] alone was consistent with the activation of both stimulatory and inhibitory G-protein regulators of K_{Ca} channels, resulting in an aggregate channel stimulation. Exposure of the external patch membrane to the β-agonist resulted in marked augmentation of guanine nucleotide-stimulated channel activity (GTP, 3.92 ± 0.91-fold stimulation, 4 patches; GTP[γS], 9.55 ± 2.25-fold, 5 patches). We

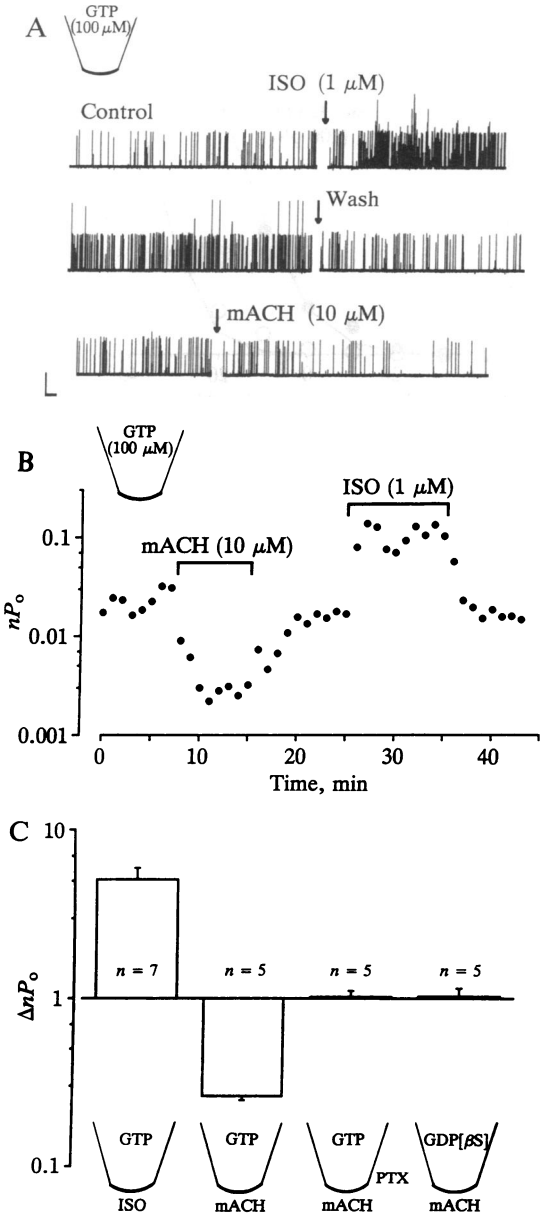

FIG. 2. Stimulation and inhibition of K_{Ca} channel activity in the same outside-out patch. (*A*) Exposure of an outside-out patch to isoproterenol (ISO) stimulates channel open-state probability, whereas, following wash, methacholine (mACH) inhibits activity. Trace shows continuous recording of agonist addition with addition artifacts blanked (4 min of wash recording was deleted at beginning of last trace). In this experiment, control, isoproterenol, control, and methacholine nP_o values were 0.011, 0.062, 0.0097, and 0.0006, respectively. Calibration: 10 s, 3 pA: Porcine cell 0304C; holding potential, 0 mV. (*B*) Open-state probability versus time for an experiment similar to *A*, with agonist added in reverse order, indicating that modulation of channel activity does not depend on sequence of hormone action. Conditions as in *A*. Ferret cell 0228B. (*C*) Summary of outside-out experiments demonstrating functional antagonism of agonists, and abolition of the inhibitory coupling by pretreatment with pertussis toxin (PTX) or addition of GDP[βS] to the pipette solution. Outside-out patches were exposed to a single agonist, or both agonists with washout between exposure, in which case ΔnP_o was calculated relative to the control state before addition of each agonist.

interpret these results to indicate that the inclusion of the β-adrenergic agonist promotes GDP release from G_s proteins bound to β-adrenergic receptors, similar to results obtained using reconstituted β-adrenergic receptors and G_s (35, 36),

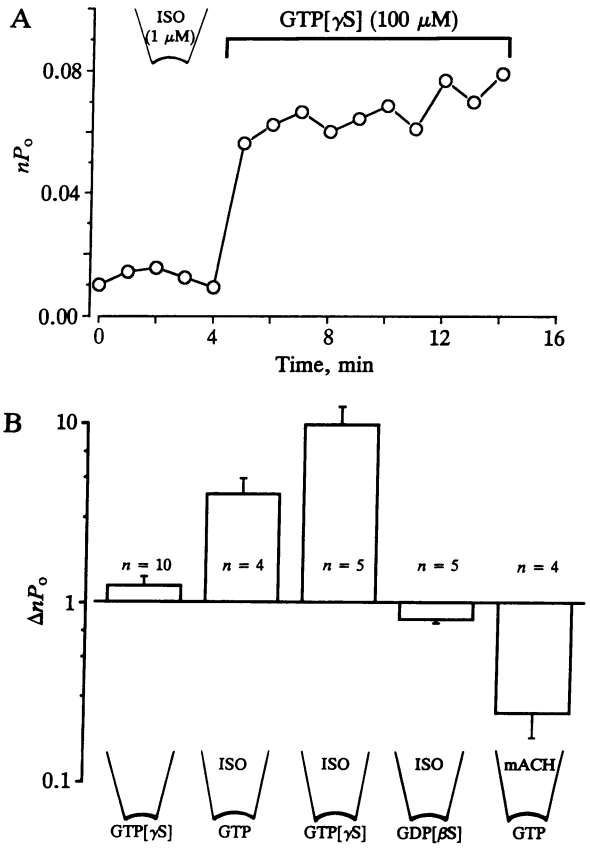

FIG. 3. Guanine nucleotides stimulate channel activity in inside-out patches exposed to isoproterenol. (*A*) Addition of GTP[γS] (100 μM) to an inside-out patch containing isoproterenol results in a rapid increase in channel open-state probability. Holding potential, 0 mV; cytosolic solution contained 1 mM $MgCl_2$. Patch 0917A. (*B*) Ratio of channel activity before and after guanine nucleotide addition to inside-out patches. In the absence of receptor agonist, GTP[γS] stimulated channel activity slightly. In the presence of isoproterenol (ISO, 1 μM), stimulation by GTP[γS] was greatly enhanced. Following preexposure of patches to methacholine (mACH, 50 μM), addition of GTP resulted in the opposite effect on channel activity compared to when the β-receptor agonist was present (compare bars 2 and 5), demonstrating the inhibitory coupling between muscarinic receptor and K_{Ca} previously reported (18). These results suggest that the effect of GTP[γS] on K_{Ca} in untreated patches is the aggregate effect of activation of stimulatory and inhibitory G proteins. GTP[γS] and GTP were added at 100 μM in all conditions; GDP[βS] was 1 mM.

and analogous to the effect of guanine nucleotide-releasing proteins in other systems (37). The rapid activation kinetics observed in the presence of the agonist (Fig. 3*A*) are also consistent with this notion. Moreover, in further support of the interpretation that the receptor agonist promotes GDP release from G proteins bound only to that receptor, preexposure of patches to the muscarinic agonist methacholine (50 μM), which is known to be coupled to G_i activity in this tissue (38), resulted in an opposite effect on channel activity upon addition of GTP (Fig. 3*B*; compare bars 2 and 5).

Stimulation of Channel Activity by Recombinant α_s. To unequivocally demonstrate the role of G proteins in the membrane-delimited stimulation of K_{Ca} channel activity, recombinant α_s proteins were applied to inside-out patches. Fig. 4*A* demonstrates an experiment in which α_s·GTP[γS] augmented channel activity in a dose-dependent fashion. Mean stimulation was 5.30 ± 0.79-fold (11 patches) at 100 pM and 15.6 ± 4.24-fold (7 patches) at 1 nM and caused an apparent leftward shift in the voltage dependence of channel open-state probability (Fig. 4*B*), similar to the effect of addition of isoproterenol to outside-out patches (Fig. 1*B*) or

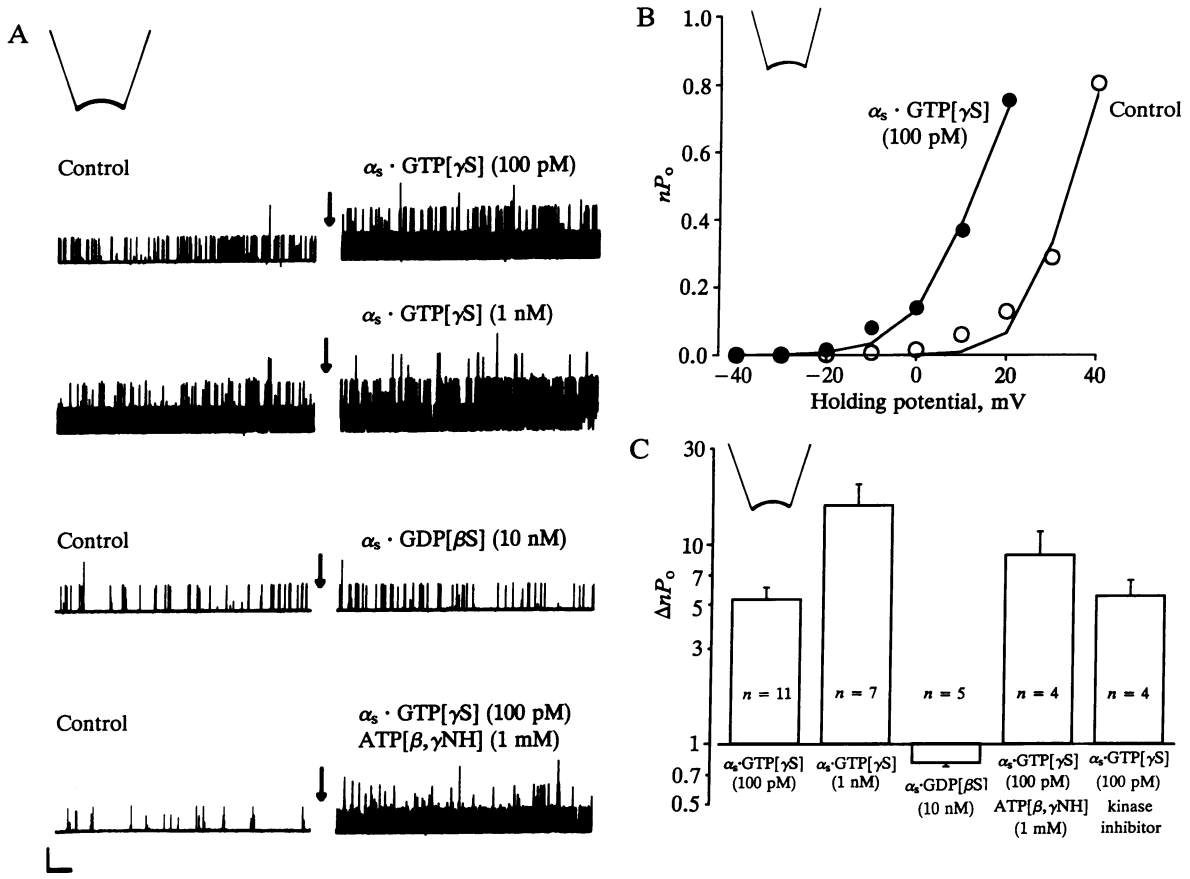

FIG. 4. α_s activates K_{Ca} channels in a phosphorylation-independent manner. (*A*) (*Top*) Addition of α_s·GTP[γS] to an inside-out patch results in a rapid, dose-dependent increase in channel activity. After addition of 1 nM α_s·GTP[γS], no closed state is observed for long periods. Patch 0627. (*Middle*) A different patch exposed to the same α_s (10 nM) that had been reacted with GDP[βS] indicates the GTP-dependent activity of the subunit. Patch 0709. (*Bottom*) Stimulation of K_{Ca} activity with 100 pM α_s·GTP[γS] occurs in the presence of 1 mM ATP[β,γNH] used to compete with endogenous ATP. Patch 0829. All traces are continuous recordings except for blanking of addition artifact. Calibration: 10 s, 5 pA. Porcine cells; holding potential, 0 mV: (*B*) Open-state probability versus voltage before and after exposure of an inside-out patch to 100 pM α_s·GTP[γS]; note shift in voltage dependence. Smooth lines are Boltzmann fits to the data. (*C*) Summary of experiments using α subunits. α_s·GTP[γS] increased nP_o in a dose-dependent manner. Channel stimulation of 100 pM α_s·GTP[γS] was not significantly altered by preexposure of the patch to ATP[β,γNH] (1 mM; $P > 0.5$) or incubation with A-kinase inhibitor (5 μg/ml for 30 min; $P > 0.5$).

of guanine nucleotides to inside-out patches. The stimulatory effect could not be explained by effects of unreacted GTP[γS] in picomolar concentration, since in inside-out patches 100 μM GTP[γS] had only modest stimulatory effects (Fig. 3*B*) and since stimulation was also observed after preincubation of the patch with 1 mM GDP (mean stimulation in 4 patches was 5.7 ± 0.62-fold at 100 pM α_s·GTP[γS]). Experiments demonstrating the effect of recombinant α_s proteins are summarized in Fig. 4*C*. Addition of α_s·GDP[βS] had no stimulatory effect, and boiling α_s·GTP[γS] eliminated stimulatory activity (5 patches, data not shown). Channel activation was equivalent in the presence of ATP[β,γNH] (1 mM) or an inhibitor of cAMP-dependent protein kinase (Fig. 4*C*). To eliminate the possibility that α_s effects on K_{Ca} were mediated by a localized rise in $[Ca^{2+}]$ through a stimulation of calcium channels in the patch, we examined the effect of α_s under conditions in which $[Ca^{2+}]_{pipette}$ was ≈100 times lower than $[Ca^{2+}]_{bath}$ (2.5 mM EGTA, 0 Ca^{2+} in pipette). In five such experiments, α_s·GTP[γS] stimulation was equivalent (14.6 ± 2.32-fold stimulation at 1 nM α_s). By contrast, addition of purified erythrocyte α_i, or α_o proteins that activate cardiac potassium channels at picomolar concentrations (20, 39), had no effect on channel activity (Table 1). It is unlikely that the lack of effect of pertussis toxin-sensitive α subunits was due to tissue specificity of the subunits, since a heterogeneous mixture of brain α_i/α_o proteins also was without effect (Table 1).

DISCUSSION

β-Adrenergic agonists are of clinical importance as a treatment for bronchial asthma and other disorders. The actions of these agents are generally believed to be associated with the G protein-dependent stimulation of adenylyl cyclase and the phosphorylation of target proteins by cAMP-dependent protein kinases. Our results show that β-adrenergic agonists can stimulate K_{Ca} channels by a membrane-delimited action of α_s that does not require cAMP-dependent phosphorylation. Channels were activated by physiological concentrations of isoproterenol in outside-out patches (Figs. 1 and 2) and by guanine nucleotides (Fig. 3) and GTP[γS]-activated α_s (Fig. 4) in inside-out patches. The membrane-delimited ef-

Table 1. Inhibition of K_{Ca} is not mediated by α_i or α_o proteins

Protein (100 pM)	*n*	ΔnP_o
α_{i-2}	4	1.31 ± 0.16
α_{i-3}	8	1.32 ± 0.15
α_{o-1}	9	0.95 ± 0.12
α_i/α_o	4	0.95 ± 0.18

Channel activity was not altered following exposure of inside-out patches to GTP[γS]-activated α_i or α_o proteins. Conditions were identical to those in experiments using α_s. Purified erythrocyte α_{i-2}, α_{i-3}, and α_{o-1} proteins (40) kindly provided by J. Codina and L. Birnbaumer. α_i/α_o was a highly purified mixture of α subunits from brain (41) and kindly provided by P. Casey.

fects of G_s demonstrated here are likely to be physiologically relevant for several reasons. (*i*) Relatively low concentrations of isoproterenol stimulated channel activity in outside-out patches independently of channel phosphorylation under physiological conditions of calcium at the cytosolic membrane surface, indicating that receptor–channel coupling is likely to occur in the intact cell. (*ii*) Channel stimulation was readily demonstrated in inside-out patches by using exogenous guanine nucleotides to activate endogenous G proteins in the patch. (*iii*) Charybdotoxin, a specific blocker of K_{Ca} channels in this tissue, antagonized the action of β-agonists, indicating that these channels comprise an important functional role in β-adrenergic relaxation of airway smooth muscle (12, 13). Thus the membrane-delimited G-protein regulation of these channels is likely to play an important role in the action of G_s-linked receptor function in smooth muscle. Our data do not exclude additional channel regulatory mechanisms, similar to the recently reported stimulatory effects of $\beta\gamma$ subunits on specific adenylyl cyclase isoforms (25).

K_{Ca} channels are also activated by cAMP-dependent protein kinase phosphorylation in airway smooth muscle (5). Thus, our results establish a second example of an ion channel stimulated by dual pathways—i.e., by membrane-delimited G-protein actions and by G protein-dependent phosphorylation—analogous to the regulatory control proposed for cardiac calcium channels (22, 42, 43). Whereas the vulnerability of calcium channels to patch excision makes the study of this regulation difficult, K_{Ca} channels should provide a more stable and convenient experimental preparation for the examination of dual stimulatory pathways of channel regulation. Regulation is readily observed off-cell, and channel activity is stable for many hours.

Finally, we have demonstrated the convergence of excitatory and inhibitory G-protein effects on the same ion channel (i.e., K_{Ca} channels) at the single-channel level. Since patches always contain multiple channels, our data do not definitely demonstrate that the same channel protein is subject to both stimulatory and inhibitory G protein-dependent modulation; it is possible that different subtypes of K_{Ca} channels with the same biophysical and pharmacological properties, but different regulatory sites, exist. G_s-linked receptor agonists stimulated, and G_i-linked receptor agonists inhibited, channels in the same outside-out patches (Fig. 2 *A* and *B*). By exploiting the guanine-nucleotide releasing activity of agonist–receptor coupling, we were able to demonstrate that addition of guanine nucleotides produces inhibition or stimulation of channel activity in inside-out patches, depending on the agonist that has been preexposed to the extracellular membrane surface. In the absence of agonist, addition of GTP[γS] stimulated channel activity slightly, consistent with previous reports of Mg^{2+}-dependent GTP[γS] stimulation of K_{Ca} channels reincorporated in planar lipid bilayers (34). Similar studies demonstrating GTP-dependent stimulation and inhibition of adenylyl cyclase have been reported (44). Whereas inhibition of channel activity by a pertussis toxin-sensitive G protein could be demonstrated in outside-out and inside-out patches, neither α_i nor α_o proteins inhibited channel activity in inside-out patches. Unlike α_s, purified or recombinant α_i/α_o proteins also fail to inhibit adenylyl cyclase, and their lack of effect on K_{Ca} activity may suggest an indirect inhibition of the channel, similar to that proposed for the inhibition of adenylyl cyclase by G_i (24–27). Thus the regulatory actions of inhibitory and stimulatory G proteins on K_{Ca} channels are reminiscent of their regulation of adenylyl cyclase and may indicate a degree of structural homology (45) and coordinated regulation between these membrane proteins.

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