The human large conductance, calcium-activated potassium ( maxi-K) channel (α and β subunits) and β2-adrenergic receptor genes were coexpressed in Xenopus oocytes in order to study the mechanism of β-adrenergic modulation of channel function. Isoproterenol and forskolin increased maxi-K potassium channel currents in voltage-clamped oocytes expressing the receptor and both channel subunits by 33 ± 5% and 35 ± 8%, respectively, without affecting current activation or inactivation. The percentage of stimulation by isoproterenol and forskolin was not different in oocytes coexpressing the α and β subunits versus those expressing only the α subunit, suggesting that the α subunit is the target for regulation. The stimulatory effect of isoproterenol was almost completely blocked by intracellular injection of the cyclic AMP-dependent protein kinase (cAMP-PK) regulatory subunit, whereas injection of a cyclic GMP-dependent protein kinase inhibitory peptide had little effect, indicating that cellular coupling of β2-adrenergic receptors to maxi-K channels involves endogenous cAMP-PK. Mutation of one of several potential consensus cAMP-PK phosphorylation sites (serine 869) on the α subunit almost completely inhibited β-adrenergic receptor/channel stimulatory coupling, whereas forskolin still stimulated currents moderately (16 ± 4%). These data demonstrate that physiological coupling between β2-receptors and maxi-K channels occurs by the cAMP-PK mediated phosphorylation of serine 869 on the α subunit on the channel.

Hormones and neurotransmitters alter cellular excitability in part through the modulation of plasmalemmal ion channel function. Two prominent mechanisms by which hormone/neurotransmitter receptor occupation results in the modulation of membrane ion channels are the phosphorylation of one or more residues of the target channel protein(s) (for review see Ref. 1), and the binding of a heterotrimeric G protein subunit to a modulatory channel domain (for review see Ref. 2). Stimulation of β2-receptors relaxes smooth muscle by modulating the activity of several protein targets (3). One prominent target of β2-adrenergic signaling in smooth muscle is the large conductance, calcium-activated potassium (maxi-K) channel, the activity of which is markedly increased following receptor binding (4–7). Modulation of maxi-K channel activity appears to be a functionally important component of β-adrenergic relaxation of smooth muscle, since charybdotoxin and iberiotoxin, selective peptide inhibitors of maxi-K channels, markedly inhibit the relaxant ability of isoproterenol and other β-adrenergic agents (8–10). The molecular mechanism by which channel modulation occurs is unclear, however, since studies have indicated that single maxi-K channels are regulated by phosphorylation and by phosphorylation-independent G protein interactions (5, 6, 11–14). Further, with respect to channel phosphorylation, maxi-K channel stimulation has been attributed to channel phosphorylation by cAMP-dependent protein kinase (5, 6, 11, 15, 16), by cGMP-dependent protein kinase (17–21), and by channel dephosphorylation (19, 22, 23).

Maxi-K channels are composed of at least two dissimilar subunits: the α subunit, which forms the channel pore (24–27), and the β subunit (28–30), which modifies the voltage and calcium sensitivity of the pore-forming subunit (31). Both the human α (hSlo) and β (hKv1,4,β) subunit genes encode several consensus cAMP-PK, although the site(s) associated with physiological regulation have not been determined. We examined β-adrenergic receptor/maxi K coupling by coexpression of hSlo and hKv1,4,β and the human β2-adrenergic receptor gene (β2AR) in Xenopus laevis oocytes using the two-electrode voltage clamp technique. We demonstrate that isoproterenol and forskolin increase maxi-K currents in voltage-clamped oocytes, that this action is mediated by PKA phosphorylation of the α subunit, and that mutation of Ser-869 almost completely eliminates β2 receptor/channel coupling.

**EXPERIMENTAL PROCEDURES**

Expression of Maxi-K and β2 Receptor cRNAs in Xenopus Oocytes—hSlo was obtained from Drs. Ligio Toro, Enrico Stefani, and Martin Walner (Department of Anesthesiology, UCLA, Los Angeles, CA) and from Dr. Lawrence Salkoff (Department of Genetics, Washington University, St. Louis, MO), and the hβ2AR cDNA clone was obtained from Dr. Jeffery Benovic (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). We cloned hKv1,4,β (32) by reverse transcription and polymerase chain reaction. Double-stranded cDNA was synthesized from human tracheal RNA (CLONTECH) using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim); the cDNA was amplified with hKv1,4,β-specific sense (5′-ATGTTGAACCTGTT-GATGCC-3′) and antisense (5′-TACCTTCTGCGCCGAGGTGAGGGA-5′) primers, and the polymerase chain reaction fragment cloned in TA 2.1 cloning vector (Invitrogen) and sequenced.

Mutagenesis of the hSlo cDNA was carried out using the pAlter mutation kit (Promega). The full-length cDNA was subcloned in pAlter-1 vector at SmaI site after blunt ending with Klenow enzyme. The plasmid DNA was denatured with 2 M NaOH, 2 mM EDTA solution. The mutation primer, spanning from nucleotide 2596 to 2617 (5′-CGTG-GATGCC-3′), was used to amplify the cDNA with hKv1,4,β-specific sense (5′-CTGTTGAACCTGTT-GATGCC-3′) and antisense (5′-TACCTTCTGCGCCGAGGTGAGGGA-5′) primers, and the polymerase chain reaction fragment cloned in TA 2.1 cloning vector (Invitrogen) and sequenced.

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CAACCATCATCACGGGGA-3'), was annealed to the denatured DNA to mutate amino acid 869 from Ser to Ala. The annealed DNA was used to carry out site-specific mutagenesis using the manufacturer's protocol; mutated cDNA (hSloΔS869A) was confirmed by sequencing (33).

cRNA was prepared from the human maxi-K cDNAs (hSlo, hKv1.5β, hSloΔS869A) and hβ2AR cDNA using the Message Machine kit (Ambion). The plasmid DNAs were linearized with appropriate restriction enzymes and the cRNAs were synthesized using T7 (hSlo, hKv1.5β) and Sp6 (hβ2AR receptor) RNA polymerase enzymes (34). The integrity of the cRNAs was tested on ethidium bromide-stained agarose gels, and cRNA concentrations were estimated by spectrophotometric measurements.

Oocytes from X. laevis were prepared for injection as described previously (35). Channel and receptor cRNAs were mixed at final concentrations of 0.6–0.9 μg/μl and 0.35–0.45 μg/μl, respectively; the final concentration of hKv1.5β cRNA was adjusted to achieve a 1:2 molar ratio to hSlo, so that all expressed channel α subunits would likely interact with β subunits (32). Approximately 50 nl of these solutions were injected per oocyte, and oocytes were then incubated for 3–6 days in ND96 (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 5.0 mM HEPES, pH 7.5) with sodium pyruvate (5.0 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 18 °C.

Electrophysiology—Two-electrode voltage clamp measurements were made at room temperature in ND96, using pipettes of 0.3–1.0 megohms resistance filled with 3M KCl. Currents were amplified (OC-725C, Warner Instrument, Hamden, CT), filtered at 200 Hz (4-pole Bessel filter, model 902, Frequency Devices), digitized at 1 kHz (Digidata, Axon Instruments), monitored (pClamp, Axon Instruments), and simultaneously stored on disk. Maxi-K currents were monitored by holding the membrane potential constant at −60 mV and imposing a 500-ms test pulse to 60 mV, every 20 s. Following voltage clamp initiation, currents were monitored for a 5-min equilibration period; oocytes in which currents spontaneously varied in amplitude over this period were discarded. All currents are shown after leak subtraction. Results are expressed as means ± S.E. Statistical significance was determined using Student’s t test for paired observations.

Chemicals—Isoproterenol and forskolin were obtained from Sigma. Iberiotoxin was purchased from Peptide Institute (Osaka, Japan), and the PKA regulatory subunit was obtained from Promega (Madison, WI).

RESULTS

Reconstitution of β2-Adrenergic Stimulation of Maxi-K Channels—Xenopus oocytes expressing the human β2-adrenergic receptor (β2AR) and either the pore-forming (α) subunit, or the pore-forming and regulatory (α + β) subunits of the human maxi-K channel were examined in two-electrode voltage-clamp experiments. Functional expression of channel proteins was determined by activation of iberiotoxin sensitive outward currents. As reported previously (30, 31), average currents recorded from oocytes injected with both channel subunit cRNAs were larger at all voltages than those recorded from oocytes injected with hSlo alone (3265.3 ± 256.9 nA (n = 25) versus 2671.9 ± 365.5 nA (n = 14) at 60 mV). As shown in Fig. 1A, application of the β-adrenergic receptor agonist isoprotenerol (ISO) to oocytes injected with hSlo, hKv1.5β, and hβ2AR cRNAs resulted in a rapid stimulation of maxi-K current monitored with a clamp step to 60 mV. The current was maximally stimulated 78 ± 11 s after ISO addition (n = 17; current examined at 20-s intervals), and fell to prestimulation levels upon removal of ISO. Iberiotoxin, a highly specific peptidyl inhibitor of maxi-K channels (36), blocked almost all of the voltage-dependent current, and isoproterenol failed to activate a current in the presence of the maxi-K antagonist (Fig. 1A). As shown in Table I, the mean current stimulation by 10 μM ISO was 33.3 ± 5.1% (60 mV), whereas ISO had no effect on oocytes injected with cRNA encoding only the channel subunits (without hβ2AR), or on oocytes injected with only hβ2AR (without maxi-K channel genes). Qualitatively similar results were observed in oocytes injected with only hSlo and hβ2AR cRNAs (Fig. 1B), i.e. ISO stimulated a subunit maxi-K currents to a degree similar to that for αβ currents (25.8 ± 5.3 versus 33.3 ± 5.1% (Table I)). In both cases, ISO stimulation was not associated with an alteration in current activation or decay kinetics; rather, stimulated α/β or α maxi-K currents were scaled increases of control currents. Current stimulation was observed over the entire voltage range examined, resulting in a marked shift in the conductance/voltage relationship toward more negative potentials (Fig. 1C).

Mechanism of β2AR/Maxi-K Channel Coupling—To determine whether β2AR/maxi-K channel coupling occurs via stimulation of adenyl cyclase and activation of cAMP-PK, we first examined the stimulation of maxi-K currents by forskolin in oocytes expressing αβ or α channel subunits. As shown in Fig. 2A, forskolin (10 μM) stimulated maxi-K currents to a degree
### Table I

| ISO [10 μM] | Forskolin [10 μM] |
|-------------|-------------------|
| β<sub>2</sub>α | 33.3 ± 5.1 %*** n = 12 | 34.9 ± 7.8 %* n = 4 |
| β<sub>2</sub>α | 25.8 ± 5.2 %** n = 8 | 33.6 ± 7.8 %*** n = 6 |
| β<sub>2</sub>α | -1.0 ± 1.5 % n = 5 | 27.3 ± 5.4 %*** n = 4 |
| β<sub>2</sub>α | 0 % n = 4 | 0 % n = 6 |
| β<sub>2</sub>α/S869A | 5.5 ± 1.3 %** n = 9 | 16.0 ± 3.9 %** n = 9 |

* p < 0.05; ** p < 0.01; *** p < 0.001

that was similar to that observed with ISO (34.9 ± 7.8% stimulation at 60 mV), although the time to peak was substantially slower than observed following exposure to ISO (466 ± 35 s; n = 13). As with ISO, forskolin application did not result in an alteration of current kinetics, but in a scaled increase in the current at all voltages (Fig. 2B), and stimulated both α/β and α maxi-K currents to a similar degree (Table I). Taken together with results obtained with ISO, these data indicate that the regulatory target associated with β<sub>2</sub>AR receptor stimulation is the channel α subunit.

It has been suggested that β<sub>2</sub>AR-mediated stimulation of maxi-K currents is associated with stimulation of cAMP-dependent protein kinase (cGMP-PK) rather than cAMP-PK (23), and that stimulation may occur via a direct interaction between G protein subunits and the channels (12, 13). We directly examined the role of cAMP-PK in β<sub>2</sub>AR maxi-K coupling by intracellular injection cAMP-PK and cGMP-PK regulatory peptides. As shown in Fig. 3, ISO stimulation was quite similar before and after sham injection of oocytes, whereas injection of the PKA regulatory subunit markedly reduced ISO stimulation. In three of four experiments, injection of the regulatory subunit inhibited the previous ISO stimulation by 57% (28.2 ± 9.6 versus 12.2 ± 2.8% stimulation before and after injection of peptide), clearly indicating a role for cAMP-PK in receptor/channel coupling. Conversely, ISO stimulated maxi-K currents in oocytes injected with cGMP-PK inhibitory peptide (final concentration 80 μM) by 23.9 ± 6.2% (n = 6, data not shown). This level of stimulation was less than observed with ISO alone; however, the degree of current stimulation was consistent with the K<sub>i</sub> of the peptide for cAMP-PK (predicted to inhibit the kinase by about 13%). To determine whether the mode of receptor/channel coupling was sensitive to the amount of expressed receptor, we performed experiments in the amount of injected β<sub>2</sub> receptor cRNA was reduced 10-fold (2.5 ng); however, no current stimulation was observed under these conditions (-1.6 ± 0.4%, n = 6; data not shown).

The mammalian homologues of dSlo do not share the optimal cAMP-PK phosphorylation site of dSlo (24, 37), but share a conserved, less cAMP-PK-specific phosphorylation site at approximately the same position (e.g. 866–869, GenBank accession no. U11058), as well as several other potential phosphorylation sites. To determine the relevant phosphorylation site associated with physiological β<sub>2</sub>AR stimulation, we used site-directed mutagenesis to replace serine 869 with alanine (hSlo<hbox>(Δ869A)), and coexpressed the mutant channel subunit with the β subunit and the β<sub>2</sub>AR. As shown in Fig. 4, hSlo<sub>Δ869A</sub>/β currents were almost completely insensitive to modulation by ISO; ISO increased the current amplitude by 5.5 ± 1.3% (n = 9), whereas exposure to forskolin still produced a significant increase in current magnitude (16.0 ± 3.9%; n = 9), although substantially less than in the wild type channels (Table I). The amplitude and kinetics of hSlo<sub>Δ869A</sub>/β currents were not different from hSlo<sub>(wt)</sub>/β currents (data not shown). These data indicate that cAMP-dependent phosphorylation occurs at Ser-869.

**DISCUSSION**

The present study demonstrates the reconstitution of β<sub>2</sub> adrenergic receptor stimulatory coupling to maxi-K channels by heterologous expression of receptor and channel RNAs in X. laevis oocytes. Isoproterenol and forskolin increased maxi-K currents in oocytes expressing either the pore-forming α subunit alone, or co-expressing the α and regulatory β subunits (Figs. 1 and 2, Table I). The stimulated currents were not endogenous oocyte currents since there was no augmentation of outward current in oocytes expressing β<sub>2</sub> receptors but not maxi-K channels, and since forskolin, but not ISO, stimulated...
β-Adrenergic Modulation of Maxi-K Channels

Considerable controversy exists with respect to the mechanism of stimulatory coupling to maxi-K channels. Stimulatory mechanisms that have been proposed include cAMP-dependent phosphorylation (5, 6, 11, 15, 16), cGMP-dependent phosphorylation (17, 18, 20, 21, 40), cGMP-dependent dephosphorylation (19, 22, 23), by G protein subunits (12, 13), and direct stimulation by NO (41). We used the heterologous expression system and site-directed mutagenesis to identify the mechanism of β adrenergic stimulatory coupling to maxi-K channels and to identify the relevant channel modulatory site. Our data clearly implicate cAMP-PK in β2 receptor/channel stimulatory coupling since injection of the regulatory subunit of cAMP-PK disrupts β2 receptor/maxi-K channel stimulatory coupling (Fig. 3). In addition to disrupting stimulatory coupling, injection of oocytes with the cAMP-PK regulatory subunit also consistently reduced the amplitude of the basal current before application of isoproterenol (Fig. 3B), suggesting that phosphorylation by cAMP-PK regulates maxi-K channel activity under resting conditions. Conversely, a semi-selective (6-fold) cGMP-PK inhibitory peptide only slightly reduced ISO current stimulation, consistent with the predicted inhibition of cAMP-PK. Further evidence suggesting functional modulation of maxi-K channels by cAMP-PK was the finding that heterologously expressed maxi-K channels are stimulated by forskolin, and that this stimulation occurs with or without expression of the β2 receptor (Fig. 2, Table I).

dSlo, the Drosophila maxi-K channel gene, contains a single optimal consensus sequence for PKA phosphorylation in the C-terminal region of the protein (RRGS at 959–962; Ref. 24), and mutations at this site were later found to prevent channel activation by exogenous PKA in inside-out patches (42). The mammalian homologues of dSlo contain a less cAMP-PK-specific phosphorylation site at approximately the same position (RQPS at 866–869, e.g. GenBank accession no. U11058), which is conserved in mammalian genes, and which occupies a similar position upstream of a highly conserved region, aspartate-rich region that is a likely calcium-binding site (43). However, as many as 10 other potential cAMP-PK consensus phosphorylation sites exist throughout the coding region of the α subunit (37). To ascertain the physiologically relevant phosphorylation site, we mutated serine 869 to alanine, and coexpressed the mutant α subunit with the wild type β subunit and the β2 receptor. This single mutation markedly reduced, but did not abolish, β2 receptor stimulation of the current (from 33.3 ± 5.1% to 5.5 ± 1.3%, Table I), strongly implicating serine 869 in receptor/effecter coupling. The low degree of current stimulation consistently observed in the mutant may relate to a slight role for additional cAMP phosphorylation sites, coupling through other kinases, or direct G protein interactions. Moreover, forskolin stimulation was only decreased by about half in mutant channels, suggesting that additional sites of channel modulation exist. It is possible that the dominant mechanism of stimulatory coupling observed in our experiments relates to factors unique to the Xenopus oocyte, such as cell size or endogenous G proteins, and that other regulatory mechanisms may be more prominent in mammalian cells. However, our results are consistent with the major mechanism of β2 receptor/

currents in oocytes expressing maxi-K channels, but not β2 receptors (Table I). Moreover, virtually all of the outward current was inhibited by iberiotoxin (Figs. 1 and 2), a selective peptidyl inhibitor of maxi-K channels (36). Since heterologous expression avoids complications associated with expression of multiple receptor or channel subtypes in target tissues, the present results clearly establish stimulatory coupling between human β2 receptors and maxi-K channels. Further, since stimulation was achieved with expression of only the β2 receptor and the α subunit and the degree of stimulation was similar with or without the regulatory β subunit (Table I), these studies localize the modulatory site to the channel α subunit. It should be noted that the degree of stimulation observed (30–35%) is somewhat less than reported in mammalian cells. In smooth muscle cells, current stimulation varies from approximately 50% to 100%, and is somewhat dependent on the step voltage and [Ca2+]i (7, 38, 39).

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![Fig. 3. Inhibition of cAMP-PK markedly decreases β2AR/maxi-K stimulatory coupling. A, in control stimulatory experiments, ISO (10 μM) application repeatedly augmented maxi-K currents. B, following initial ISO stimulation, oocyte was injected with 3.125 units of cAMP-PK regulatory subunit. Subsequent applications of ISO were markedly less effective in stimulation maxi-K currents. All currents were obtained by step depolarizations from −60 to +60 mV at 20-s intervals.](image)

![Fig. 4. Mutation of serine 869 abolishes β2AR/maxi-K stimulatory coupling. ISO application to an oocyte injected with wild type β2AR and maxi-K β subunit genes, and a mutant maxi-K α subunit gene (ΔS869A). Whereas ISO (10 μM) failed to stimulate the current, forskolin (10 μM) produced a modest increase in current magnitude with kinetics similar to that observed in wild type experiments. The experimental protocol was the same as described for Fig. 1.](image)
maxi-K channel stimulatory coupling occurring by cAMP-PK phosphorylation of serine 869.

Finally, the stimulatory effect of both the β2 receptor agonist ISO and forskolin was an increase in current amplitude at all voltages, without a change in current kinetics, similar to the modulatory action of β2 receptor stimulation on cardiac sodium channel α subunits heterologously expressed in *Xenopus* oocytes (44). In that study, mutation of all consensus cAMP-PK phosphorylation sites on the sodium channel failed to remove channel stimulation, leading to the suggestion that modulation might occur by phosphorylation of an unrelated protein resulting in a redistribution of channels to the plasma membrane (44). ISO stimulation of sodium currents was quite slow (approximately 10 min to maximum stimulation), which could be consistent with a redistribution of membranes within the oocyte. In the present study, however, stimulatory coupling occurred quite rapidly (approximately 1 min to maximum), which would be consistent with a direct effect on channel gating.

In summary, we have used a heterologous expression system to demonstrated β2-adrenergic receptor/maxi-K channel stimulatory coupling. Coupling involves a cAMP-PK-dependent phosphorylation of the channel α subunit; mutation of one consensus cAMP-PK phosphorylation site (serine 869) almost eliminates β2-adrenergic modulation of the maxi-K current. These findings do not rule out other modulatory mechanisms, but do confine the physiologically relevant molecular mechanisms associated with β2 receptor/maxi-K channel coupling.

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