GTP-dependent Regulation of Myometrial K_{Ca} Channels Incorporated into Lipid Bilayers

L. TORO, J. RAMOS-FRANCO, and E. STEFANI

From the Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT The regulation of calcium-activated K (K_{Ca}) channels by a G protein–mediated mechanism was studied. K_{Ca} channels were reconstituted in planar lipid bilayers by fusion of membrane vesicles from rat or pig myometrium. The regulatory process was studied by exploring the actions of GTP and GTPγS on single channel activity. K_{Ca} channels had a conductance of 260 ± 6 pS (n = 25, ± SE, 250/50 mM KCl gradient) and were voltage dependent. The open probability (P_o) vs. voltage relationships were well fit by a Boltzmann distribution. The slope factor (11 mV) was insensitive to internal Ca^{2+}. The half activation potential ($V_{1/2}$) was shifted −70 mV by raising internal Ca^{2+} from pCa 6.2 to pCa 4. Addition of GTP or GTPγS activated channel activity only in the presence of Mg^{2+}, a characteristic typical of G protein–mediated mechanisms. The P_o increased from 0.18 ± 0.08 to 0.49 ± 0.07 (n = 7, 0 mV, pCa 6 to 6.8). The channel was also activated (P_o increased from 0.03 to 0.37) in the presence of AMP-PNP, a nonphosphorylating ATP analogue, suggesting a direct G protein gating of K_{Ca} channels. Upon nucleotide activation, mean open time increased by a factor of 2.7 ± 0.7 and mean closed time decreased by 0.2 ± 0.07 of their initial values (n = 6). Norepinephrine (NE) or isoproterenol potentiated the GTP-mediated activation of K_{Ca} channels (P_o increased from 0.17 ± 0.06 to 0.35 ± 0.07, n = 10). These results suggest that myometrium possesses β-adrenergic receptors coupled to a GTP-dependent protein that can directly gate K_{Ca} channels. Furthermore, K_{Ca} channels, β-adrenergic receptors, and G proteins can be reconstituted in lipid bilayers as a stable, functionally coupled, molecular complex.

INTRODUCTION

Uterine smooth muscle relaxes in response to NE binding to its β-receptor on the cell surface (Bülbring et al., 1968; Diamond and Marshall, 1969; Johansson et al., 1980; Piercy, 1987). This interaction produces a rise in cAMP leading to uptake of cytosolic Ca^{2+} by intracellular stores, and to lower sensitivity of the contractile proteins to Ca^{2+} (for review, see Riemer and Roberts, 1986). In addition, β-receptor stimulation hyperpolarizes smooth muscle via an increase in K^+ permeability

Address reprint requests to Dr. Enrico Stefani, Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

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(Kroeger and Marshall, 1973; Yamaguchi et al., 1988). Accordingly, we have observed that NE enhances a K^+ current in patch clamped myometrial myocytes (Toro et al., 1987; Toro et al., 1990).

Stimulation of β-receptors in myometrium and other tissues activates a G protein and subsequently adenylate cyclase (Fortier et al., 1983; Birnbaumer et al., 1987; Levitzki, 1988). Thus, at least two mechanisms may be proposed to explain the increase in myometrial K^+ permeability after β-adrenergic stimulation: (*a*) cAMP-dependent channel phosphorylation (DePeyer et al., 1982), and (*b*) direct (in the absence of second messengers) G protein gating (Brown et al., 1989). Several types of K^+ channels are G protein gated (Birnbaumer et al., 1989). Examples are: the atrial K^+ channel (K^+[ACh]) coupled to the muscarinic receptor (mAChR) (Breitweiser and Szabo, 1985; Pfaffinger et al., 1985; Kurachi et al., 1986; Logothetis et al., 1987, 1988; Yatani et al., 1987; Cerbai et al., 1988; Kirsch et al., 1988); a 50-pS K^+ channel from clonal pituitary GH_3 cells (Codina et al., 1987), and four K^+ conductances from hippocampal pyramidal cells (VanDongen et al., 1988). Therefore, we decided to explore the possibility that myometrial K_{Ca} channels were activated by β-receptor activation coupled to G proteins.

G protein–dependent processes are mediated by a sequence of chemical reactions involving a G protein, a membrane effector (enzymes or ionic channels), and a catalyst (hormone or neurotransmitter). They can be recognized by the activation of membrane effector systems with: (*a*) GTP plus Mg^{2+} in the absence ("agonist-independent") or presence of the agonist ("agonist-dependent") (Birnbaumer et al., 1980; Iyengar and Birnbaumer, 1982; Birnbaumer et al., 1987; Okabe et al., 1990); (*b*) nonhydrolyzable analogues of GTP such as GTPγS, which is the most potent (Breitweiser and Szabo, 1988); or (*c*) by demonstrating that the agonist action is GTP and Mg^{2+} dependent (Pfaffinger et al., 1985; Brown and Birnbaumer, 1988). Thus, we used these criteria to define if K_{Ca} channels were G protein gated.

In this study we demonstrate that myometrial K_{Ca} channels are activated by intracellular GTP or GTPγS only in the presence of Mg^{2+}. Furthermore, extracellular NE or isoproterenol (β-agonist) potentiate the activity of K_{Ca} channels in the presence of intracellular GTP + Mg^{2+}. Thus, a direct activation of K_{Ca} channels by G proteins coupled to β-adrenergic receptors is proposed as one of the mechanisms involved in the hyperpolarization and relaxation of myometrium induced by β-adrenergic agents. Part of this work has been communicated in abstract form (Ramos-Franco et al., 1989).

MATERIALS AND METHODS

Isolation of Plasma Membrane Vesicles from Myometrium

Membrane vesicles were isolated from uterus of Wistar rats (150–200 g) or pigs (45–150 lb) using a modification of the procedure of Meissner (1984). Connective tissue and endometrium were removed from the uteri in Ringer-Krebs solution supplemented with protease inhibitors (in mM): 0.1 phenylmethylsulfonylfluoride, 1×10^{-3} pepstatin A, 1×10^{-3} *p*-aminobenzamidine, 1 μg/ml aprotinin, and 1 μg/ml leupeptin. The tissue was homogenized in isotonic sucrose solution (300 mM sucrose and 20 mM Tris-HEPES, pH 7.2) in the presence of the protease inhibitors. The homogenate was centrifuged for 30 min at 700 *g*. The supernatant was recentrifuged for 40 min at 14,000 *g* and 20 min at 9,500 *g*. We assumed that

the bulk of the plasma membrane was sedimented during the first 40 min and that contamination with mitochondria was diminished during the second 20 min. The pellet (heavy microsomes) was resuspended in a hypertonic salt solution (600 mM KCl and 5 mM Na-PIPES, pH 6.8) with a motor-driven Teflon pestle homogenizer. Samples were incubated on ice for 1 h and ultracentrifuged for 1 h at 42,000 *g* in a 70.1 Ti rotor. The pellets were resuspended in 400 mM KCl and 5 mM Na-PIPES, pH 6.8 (buffer 1)/10% sucrose (wt/wt). The heavy microsomes were placed on top of a discontinuous sucrose gradient/buffer 1 (wt/wt): 20:25:30:35:40%. The gradient was ultracentrifuged at 67,500 *g* in a SW28.1 swinging rotor for 18 h. At the end of the run the sucrose interfaces were collected, diluted with 5 mM Na-PIPES, pH 6.8, and centrifuged for 1 h at 42,000 *g* in a 60 Ti rotor. The pellets were resuspended in 300 mM sucrose, 100 mM KCl, and 5 mM Na-PIPES, pH 6.8, to a final concentration of 10–40 mg protein/ml, frozen in liquid N_2, and stored at −70°C until used. Protein was measured by the method of Lowry (1951) using BSA as a standard. Membranes obtained from the 20:25% and 25:30% sucrose interfaces were used in this study since they contained significant dihydropyridine [^{3}H]PN200-110 binding capacity (~ 0.3 and 0.5 pmol/mg protein, respectively). Five different preparations were used, three from rat and two from pig myometrium. K_{Ca} channel activation by GTP or GTPγS was similar regardless of the animal source. The results are expressed as mean values ± SE with the number of observations (*n*).

Incorporation of Channels into Lipid Bilayers

The incorporation of membrane vesicles into planar lipid bilayers was performed according to Miller and Racker (1976) and Latorre et al. (1982). For a detailed description of the procedure and amplifier, see Hamilton et al. (1989). Bilayers of the Mueller-Rudin type were made across 200-μm apertures in delrin cups (wall thickness of ~150 μm). The insertion of the cup divides a polyvinyl chloride block into two chambers (500 μl and 4 ml). The *cis* chamber (500 μl) was the voltage control side connected to the negative input of a voltage to current converter amplifier, while the *trans* side (4 ml) was referred to ground. For comparison with whole cell clamp recordings, the voltage values indicated in all figures are referenced to the potential on the myoplasmic side of the bilayer. Bilayers were cast from a phospholipid "painting" solution containing a 1:1 mixture (25 mg/ml) of phosphatidylethanolamine (PE) and phosphatidylserine (PS) (charged bilayers) or a 3:7 mixture (50 mg/ml) of palmitoyl-oleyl-phosphatidylcholine (POPC)/palmitoyl-oleyl-phosphatidyl-ethanolamine (POPE) (neutral bilayers) (Avanti Polar Lipids, Inc., Birmingham, AL) in *n*-decane (gold label grade; Aldrich Chemical Co., Milwaukee, WI). The GTP- or GTPγS-dependent activation was observed in both cases. To access the capacity (~200 pF) of the bilayer a ±50 mV per 70 ms ramp was used. To incorporate channels the membrane vesicles were added to the *cis* chamber (stirring constantly) to a final protein concentration of 200 μg/ml. Pulses of ±100 mV per 1 s were applied at 0.5 Hz until a channel was detected.

Voltage- and calcium-dependency were used to determine the sidedness of the channel. Channels were incorporated in a solution with symmetrical 100 μM free Ca^{2+} concentration and their orientation was initially determined by the P_o vs. voltage relationship. For example, if the P_o diminished with negative potentials, the internal side of the channel was facing the *cis* chamber. At 100 μM free Ca^{2+}, K_{Ca} channels have a P_o near 1.0 at potentials close to 0 mV; thus, to reduce the P_o value, the free Ca^{2+} concentration facing the intracellular side was lowered by adding K_2-EGTA. This maneuver confirmed the sidedness of the channel in the bilayer. Most of the channels (~80%) were incorporated with the Ca-sensitive site facing the *trans* chamber, indicating that myometrial membrane vesicles were primarily oriented outside-out (Hanke, 1986).

The majority of the experiments were performed in a 250/50 KCl gradient. The *cis*

chamber contained (in mM): 250 KCl, 10 K-MOPS, 0.5 K_2-EGTA, 0.6 $CaCl_2$, pH 7.4, pCa 4. The *trans* chamber contained (in mM): 50 KCl, 10 K-MOPS, 0.5 K_2-EGTA, 0.6 $CaCl_2$, pH 7.4, pCa 4. In some instances, to facilitate incorporation of channels, a larger osmotic gradient (450/50 KCl) was used (Hanke, 1986). After channel incorporation, the K^+ gradient was equilibrated to avoid further incorporation of channels. Adrenergic agents were added to the external side. Nucleotides and Mg^{2+} were applied to the myoplasmic side. Free calcium concentration was calculated according to Fabiato (1988).

Data Collection and Analysis

A custom-made voltage to current converter amplifier was used (Alvarez, 1986; Hamilton et al., 1989). pCLAMP software with a 12-bit A/D D/A converter was used (Axon Instruments, Burlingame, CA). Single-channel analysis was performed with custom-made programs or with programs kindly provided by Dr. O. Alvarez and Dr. R. Latorre from the Faculty of Science, University of Chile, Santiago, Chile. Data were filtered at 200–500 Hz with an 8-pole Bessel filter, digitized at 1–2 kHz, and collected on line with a personal computer for further analysis. Recordings were performed at constant potential or during 2-s pulses from 0 mV at regular intervals (0.1–0.05 Hz). Bilayers were generally stable (recordings up to 4 h) at steady potentials of ±40 mV. However, when larger potentials were applied (±100 mV) bilayers became unstable. Pulses were used to avoid membrane breakdown at the large potentials. Open and closed time distributions were obtained from experiments with only one active channel. P_o was calculated from total amplitude histograms by fitting a sum of Gaussian distributions using the nonlinear least-squares method (Levenberg-Marquardt fitting algorithm) or as the fraction of total time in each dwell class. The threshold for event detection was set at 50% of the average channel amplitude obtained from the amplitude histogram of all points. The resulting idealized records were corrected for dead time due to the sampling frequency and filter characteristics (Colquhoun and Sigworth, 1983). Logarithmically binned dwell time histograms were fitted by the maximum likelihood method to obtain the point estimate of the time constant(s) of the probability density function (PDF) (Colquhoun and Sigworth, 1983; Sigworth and Sine, 1987). The estimated values ± SD are given in Tables I and II.

RESULTS

Uterine smooth muscle, like other smooth muscles from blood vessels, trachea, or the gastrointestinal tract (Benham et al., 1985; 1986; Inoue et al., 1985; Cecchi et al., 1986; McCann and Welsh, 1986; Singer and Walsh, 1987; Bregestovski et al., 1988; Sadoshima et al., 1988*b*), possesses large conductance K^+ channels that are calcium- and voltage sensitive (Fig. 1) (Toro et al., 1988). Myometrial K_{Ca} channels in lipid bilayers (charged bilayers) have a conductance of 260 ± 6 pS ($n = 25$, 250/50 mM KCl gradient). Under these conditions their reversal potential measured from the current to voltage relationships is −35 mV, close to the theoretical E_K (−38 mV). This similitude indicates that K_{Ca} channels have a high selectivity towards K^+. Ca^{2+} sensitivity of K_{Ca} channels from skeletal muscle varies from channel to channel (Moczydlowski and Latorre, 1983; Oberhauser et al., 1988). We also observed variability in the Ca^{2+} concentration ($[Ca^{2+}]$) sensitivity of myometrial K_{Ca} channels. In most cases, K_{Ca} channels were active at myoplasmic $[Ca^{2+}]$ as low as pCa 6.8. For example, in eight experiments at myoplasmic pCa 6.5–6.8 the average P_o was 0.18 ± 0.06 ($V_H = 0$ mV). The K_{Ca} channel was the most frequent channel

recorded in our membrane preparations, which may indicate an important physiological relevance of this channel in situ.

General Properties of Myometrial K_{Ca} Channels in Bilayers

The voltage and Ca^{2+} dependence of myometrial K_{Ca} channels in bilayers is illustrated in Fig. 1. Channel recordings at different holding potentials and at two different internal pCa values are shown (*A* at pCa 4.0 and *B* at pCa 6.2). The curves

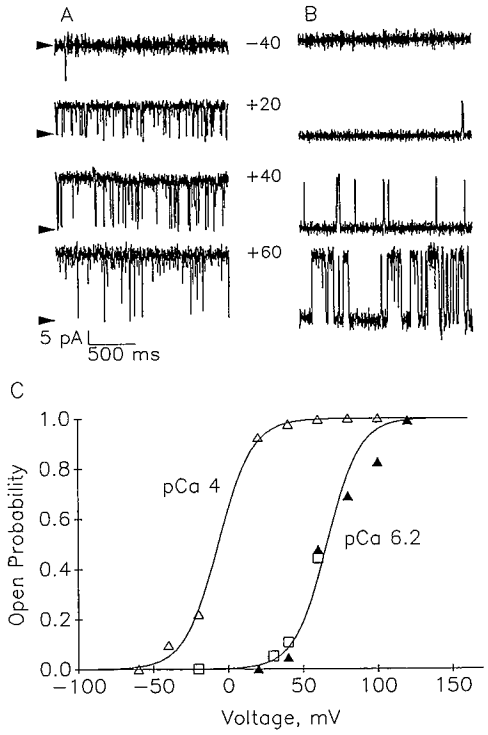

FIGURE 1. Calcium and voltage sensitivity of K_{Ca} channels from uterine smooth muscle. Channel activity was enhanced with depolarization and by increasing Ca^{2+} concentration. (*A*) Currents from porcine K_{Ca} channels (pCa 4) at different holding potentials (V_H) (numbers between *A* and *B*). In all figures, voltages are those sensed by the internal side of the channel. (*B*) Same channel after addition of EGTA to give a pCa of 6.2. Holding potentials are the same as in *A*. At 60 mV the P_o diminished from 0.99 to ~0.42 when pCa was changed from 4 to 6.2, respectively. (*C*) P_o vs. holding voltage curves at two pCa. Experimental data were fitted to a Boltzmann distribution (continuous lines): $P_o = 1/[1 + \exp[(V_{1/2} - V)/k]]$ where P_o = open probability; $V_{1/2}$ = half activation voltage; V = applied voltage, and k = slope factor. At pCa 4 (100 μM Ca^{2+}, *open triangles*), $V_{1/2}$ was −6.5 mV and k = 11 mV. At pCa 6.2, k was not significantly modified and $V_{1/2}$ was shifted to 66 mV (*open squares*, without Mg^{2+}; *filled triangles*, plus 1 mM Mg^{2+}). In all figures arrows mark the closed state of the channel. Neutral bilayer, symmetric 450 KCl.

in *C* are the corresponding activation curves (P_o vs. voltage relationship) for the same channel. The voltage dependency can be seen at both Ca^{2+} concentrations. When the internal side of the channel was depolarized from −40 to 60 mV the probability of opening increased, as would be expected for a K_{Ca} channel in the intact cell (arrows mark the closed state). For example, the P_o was 0 at −40 mV, and 0.42 at 60 mV (at pCa 6.2). The Ca^{2+} dependency is shown by the negative shift in the voltage axis of the activation curve when increasing the calcium concentration facing the

cytoplasmic side. Thus at pCa 4, channel openings could be detected at more negative potentials (P_o = 0.09 at −40 mV) than at pCa 6.2 (P_o = 0.05 at 30 mV).

The experimental values of P_o vs. voltage were fitted to a Boltzmann distribution (continuous lines in Fig. 1 *C*): $P_o = 1/[1 + \exp[(V_{1/2} - V)/k]]$, where P_o = open probability, $V_{1/2}$ = half activation voltage, V = applied voltage, and k = slope factor. At pCa 4 (*open triangles*), $V_{1/2}$ was −6.5 mV and k was 11 mV, corresponding to an effective valence of 2.3 ($k = RT/zF$, where z = effective valence, and R, T, and F have their usual meanings). At pCa 6.2 (*open squares*), the value of k was not significantly modified and $V_{1/2}$ was shifted to 66 mV, indicating that intracellular Ca^{2+} displaces the equilibrium between open and closed states without changing the voltage dependence of the gating process. Similar findings in the voltage dependency and in the $V_{1/2}$ shift by Ca^{2+} were reported for K_{Ca} channels of smooth and skeletal muscle in bilayers (Latorre et al., 1982; Cecchi et al., 1986; Oberhauser et al., 1988; Latorre et al., 1989). However, it seems that myometrial K_{Ca} channels could open at lower intracellular Ca^{2+} concentrations, bringing their Ca^{2+} sensitivity closer to K_{Ca} channels observed in inside-out patches from fetal human (Bregestovski et al., 1988) and rat aorta (Sadoshima et al., 1988*b*).

Mg^{2+} activates K_{Ca} channels from salivary acinar cells (Squire and Petersen, 1987) and from skeletal muscle membranes (Golowasch et al., 1986; Oberhauser et al., 1988). This property was investigated in K_{Ca} channels from myometrium. Addition of 1 mM $MgCl_2$ to the internal side of the channel did not modify either the value of k (Fig. 1 *C*, *solid triangles*) or the value of $V_{1/2}$, indicating that channel voltage dependency and Ca^{2+} sensitivity were not modified by this Mg^{2+} concentration (see also Fig. 2). Equivalent observations were obtained in another 39 bilayers at comparable pCa and at steady holding potentials between 0 and 40 mV. The average initial P_o was 0.20 ± 0.05 (n = 31) and remained almost the same after adding 1 mM Mg^{2+} ($\Delta P_o = -0.006 \pm 0.006$). In another seven bilayers 1 mM Mg^{2+} increased the P_o (0.16 ± 0.04 to 0.40 ± 0.07, n = 7). We also observed that Mg^{2+} slightly diminished the amplitude of K_{Ca} channels. The reduction (6 ± 1%, n = 27; Figs. 2, 3, and 8) of K_{Ca} channel conductance was consistent regardless of the presence of GTP.

In summary, myometrial K_{Ca} channels have two properties in common with other K_{Ca} channels in bilayers: (*a*) similar voltage dependency, and (*b*) equivalent voltage shift in the voltage–activation curve by cytoplasmic Ca^{2+}. Two particular aspects of myometrial K_{Ca} channels in bilayers are: (*a*) they may be active at lower intracellular Ca^{2+} concentrations, and (*b*) they are practically unaffected by 1 mM Mg^{2+}.

GTP and GTPγS Activate K_{Ca} Channels Only in the Presence of Mg^{2+}

G protein–dependent processes require Mg^{2+} and either GTP or a nonhydrolyzable analogue of GTP (Birnbaumer et al., 1987; Gilman, 1987; Brown and Birnbaumer, 1988). On this basis, experiments were designed to determine if the isolated membrane vesicles contained endogenous G proteins, which modulate K_{Ca} channel activity. This appeared to be the case since K_{Ca} channels were activated by GTP or GTPγS (10–100 μM) only if Mg^{2+} was present. The average P_o increase was from 0.18 ± 0.08 to 0.49 ± 0.07 (n = 7) at 0 mV and pCa from 6 to 6.8.

Fig. 2 illustrates one of these experiments in which GTPγS added after Mg^{2+}

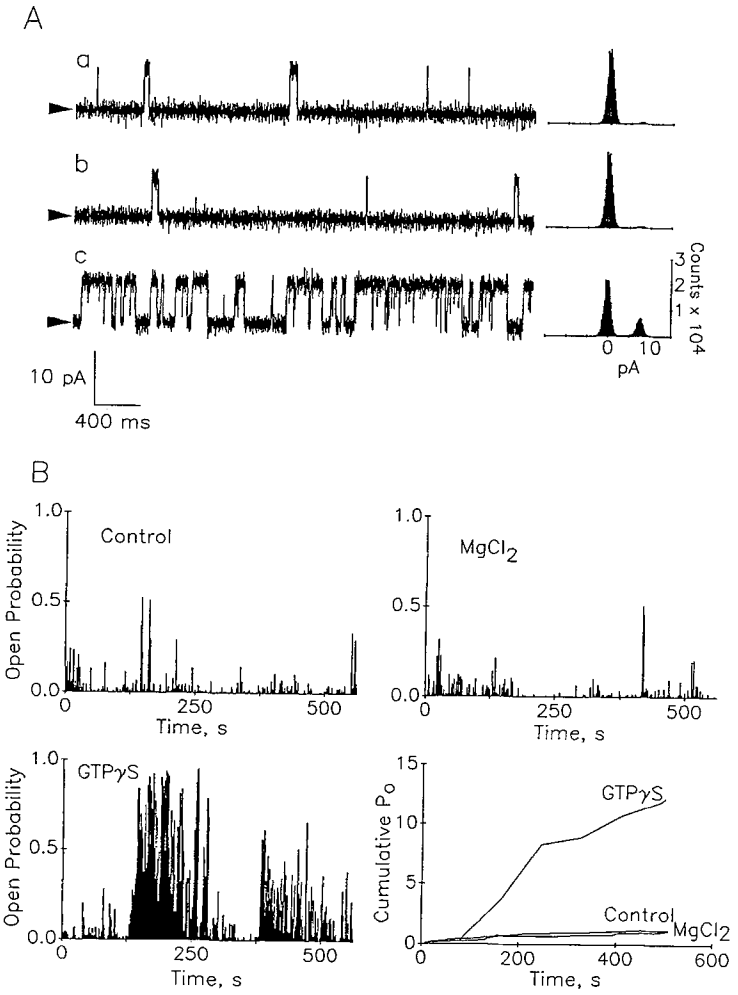

FIGURE 2. Activation of K_{Ca} channels mediated by a G protein process. (*A*) Records from porcine membrane vesicles and corresponding total amplitude histograms. Control records ($P_o = 0.01$) (*a*); same channel after sequential addition of 1 mM $MgCl_2$ ($P_o = 0.01$) (*b*), and 100 μM GTPγS (mean $P_o = 0.25$) (*c*). $V_H = +30$ mV, pCa 6.1, neutral bilayer and symmetrical 250 mM KCl. (*B*) Open probability vs. time graphs (*top*, and *bottom left*) and cumulative P_o vs. time graph (*bottom right*) of the experiment illustrated in *A*. Each value corresponds to the average P_o during 1,024 ms. Control (*top left*) shows spontaneous variations in P_o during time. This was not modified by the presence of 1 mM $MgCl_2$ (*top right*). A well-defined activation of the channel was depicted when GTPγS was added to the bath, while the variations in P_o persisted (*bottom left*).

enhanced the P_o of the channel. To discard the possibility that Mg^{2+} has a direct effect on channel activity, we added Mg^{2+} before GTPγS. Fig. 2 *A* shows selected single channel records (*a–c*, V_H = 30 mV, pCa 6.1, 250/250 KCl) and the corresponding total amplitude histograms for all the data. Under control conditions (trace *a*) the mean P_o was 0.01. Addition of 1 mM $MgCl_2$ (trace *b*) to the internal side did not alter channel activity (mean P_o = 0.01). Trace *c* shows the stimulatory effect of 100 μM GTPγS added to the internal side (mean P_o = 0.25).

Since some K_{Ca} channels presented spontaneous variations in their P_o (Cecchi et al., 1986; Oberhauser et al., 1988) we analyzed channel records continuously for at least 5–10 min in each condition. The stimulatory effect was always much more pronounced than the spontaneous shift of activity (Fig. 2 *B*). Graphs in Fig. 2 *B*

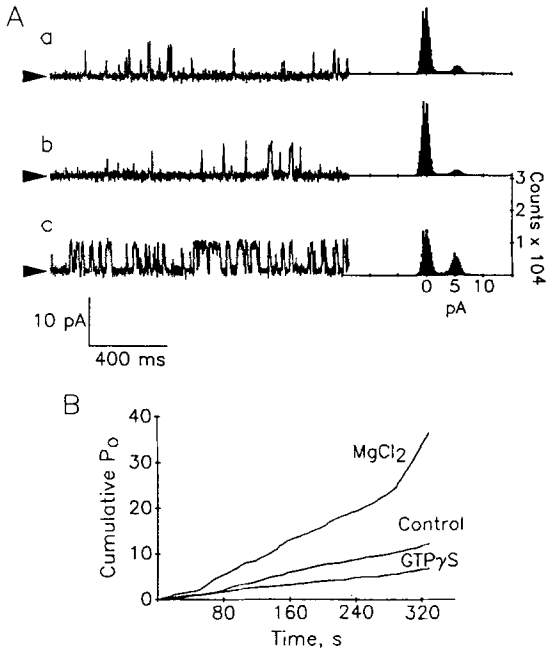

FIGURE 3. Activation of K_{Ca} channels by GTPγS only in the presence of Mg^{2+}. GTPγS by itself did not activate K_{Ca} channels. (*A*) Records from rat membranes and corresponding total amplitude histograms. Control channel recordings (*a*), after addition of 10 μM GTPγS (*b*), and 1 mM $MgCl_2$ (*c*). P_o (0.1) was the same in *a* and *b*, and increased to 0.32 only after addition of $MgCl_2$ in *c*. V_H = 0 mV, pCa 6.8, and charged bilayers. (*B*) Cumulative P_o vs. time curves. Each value corresponds to the average of 1,024 ms.

illustrate the time course of the experiment shown in Fig. 2 *A*. The P_o vs. time plots show the spontaneous variations in P_o, and illustrate that GTPγS had a definite activation effect (*bottom left*). The spontaneous variations in activity to very low levels even after activation with GTPγS and Mg^{2+} seem to be a common feature of several types of channels (for various examples see Birnbaumer et al., 1989). The molecular mechanism of this interesting behavior is a question that remains open. The increase in activity is better illustrated by graphing the cumulative P_o vs. time curves (Fig. 2 *B*, *bottom right*). These findings strongly suggest that K_{Ca} channels are G protein gated.

To test a possible direct activation of K_{Ca} channels by GTPγS, GTP, or contaminant Ca^{2+} in the nucleotide solutions, each nucleotide was added to the internal side before $MgCl_2$. Neither GTPγS (10 μM, n = 3; 100 μM, n = 4) nor GTP (100 μM,

$n = 2$) alone affected the P_o of the channel. This is exemplified for GTPγS in Fig. 3, where current traces and total amplitude histograms are shown for each case (Fig. 3 *A*, *a–c*), $V_H = 0$ mV at pCa 6.8. The top trace (*a*) represents the control experiment ($P_o = 0.1$). Addition of 10 μM GTPγS (*b*) did not increase the P_o of the channel (0.1). This lack of activation was always observed, and demonstrates that GTPγS or GTP by themselves or a hypothetical contaminant Ca^{2+} were not responsible for the activation of K_{Ca} channels. As expected for a G protein gating mechanism, subsequent addition of 1 mM $MgCl_2$ resulted in an increase of P_o (trace *c*, $P_o = 0.32$). The cumulative P_o vs. time graph (Fig. 3 *B*) was constructed from continuous records of channel activity acquired for 5 min in each condition. It is clear that the channel was activated only after Mg^{2+} was added to the bath containing GTPγS. These experiments eliminate the possibility that channel opening induced by GTPγS plus Mg^{2+} or GTP plus Mg^{2+} were due to a direct and independent effect of each individual agent on the channel.

These results suggest that K_{Ca} channels are directly regulated by G proteins. They also suggest that G proteins and K_{Ca} channels form stable complexes that do not diffuse away from each other during incorporation into the bilayer.

Kinetics of K_{Ca} Channels Activated by GTPγS and Mg^{2+}

Upon activation of K_{Ca} channels with GTPγS and Mg^{2+}, openings lasted longer (mean open time increased 2.7 ± 0.7 times its control value, $n = 6$) and closings were shorter (mean closed time was 0.2 ± 0.07 the initial value, $n = 6$).

Myometrial K_{Ca} channels had at least two open and three closed states. These were defined by the number of exponentials that could be fitted to the dwell time histograms (Fig. 4). The values for the fast time constants may be overestimated because of the time resolution of our system.

Open and closed time distributions of the experiment in Fig. 2 are shown in Fig. 4 (see values in Tables I and II, experiment 1). This channel had openings with corresponding fast (τ_{o1}, 1–9 ms) and medium (τ_{o2}, 10–100 ms) time constants, and closings with corresponding fast (τ_{c1}, 1–10 ms), medium (τ_{c2}, 11–100 ms), and long (τ_{c3}, >100 ms) time constants (Fig. 4, *A* and *B, top*). Mg^{2+} increased the proportion of the long closures (τ_{c3}) but did not produce an appreciable change in the rest of the kinetic parameters (Fig. 4, *A* and *B, middle*). Addition of GTPγS in the presence of Mg^{2+} augmented the values of τ_{o1} and τ_{o2} about three times, while their ratios were not greatly modified (Fig. 4 *A, bottom*). GTPγS plus Mg^{2+} had a marked effect on the closed state of the channel (Fig. 4 *B, bottom*). The relative occurrence and the value of τ_{c3} diminished. The contribution ratios of τ_{c1} and τ_{c2} were augmented (three and two times, respectively), while their values were not greatly modified.

The action of GTPγS on the different open and closed states had some variations (Tables I and II, $n = 5$). Some major conclusions can be drawn from the tables: (*a*) For the open state, addition of GTPγS does not greatly modify the value of τ_{o1}, while it increases τ_{o2} or makes more evident a third long component (Table I, experiments 3 and 5). (*b*) For the closed state, after GTPγS the value of τ_{c1} remained practically the same, but its ratio increased; the value of τ_{c3} was markedly decreased with a concomitant diminution in its contribution ratio.

In conclusion, K_{Ca} channels possess various open and closed states that can be differentially regulated. The mean durations of fast closings and openings are not affected by GTPγS. On the contrary, the mean durations of medium openings and long closings are the main targets of G protein activation.

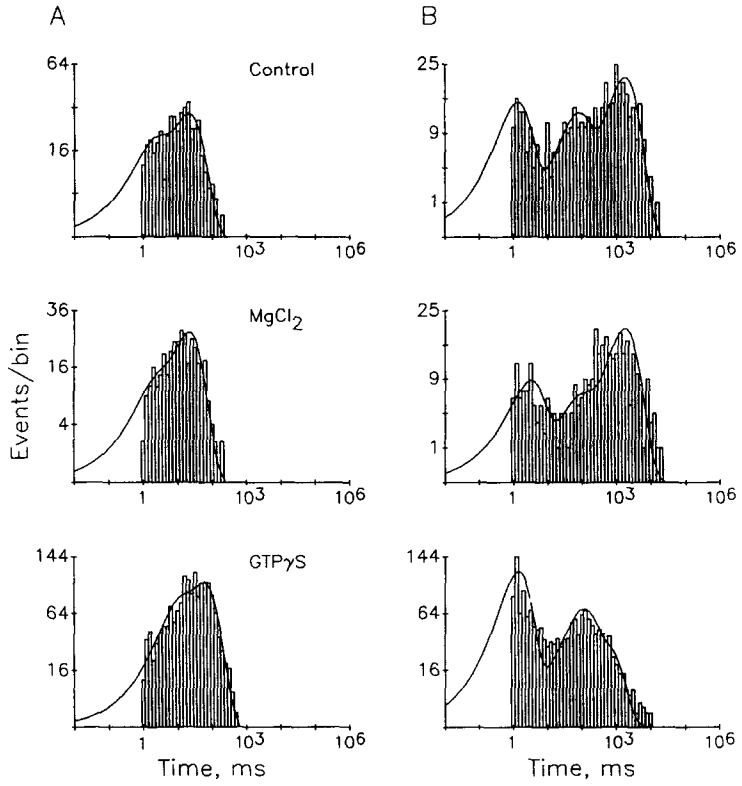

FIGURE 4. Kinetics of K_{Ca} channel after activation with GTPγS and Mg^{2+}. The major effect upon activation by GTPγS plus Mg^{2+} was on the closed state of the channel. Histograms correspond to the experiment in Fig. 2. Data were logarithmically binned and graphed using a square root ordinate. Number of transitions was: control = 600 (*top*); plus 1 mM Mg^{2+} (*middle*) = 518; and plus 100 μM GTPγS (*bottom*) = 2,875. Fitted histograms (*continuous lines*) give the time constants (corresponding peak values) and the relative contribution of each component. (*A*) Open time histograms fitted to two exponentials (open time constants, τ_{o1} and τ_{o2}). (*B*) Closed time histograms fitted to three exponentials (closed time constants, τ_{c1}, τ_{c2}, and τ_{c3}). Addition of 100 μM GTPγS in the presence of Mg^{2+} (*bottom*) diminished τ_{c3} from 2,465 to 870 ms, as well as its contribution ratio (from 0.62 to 0.1). Other values for the open and closed time constants, and contribution ratios in each condition are given in Tables I and II (experiment 1).

Adrenergic Stimulation Enhances the Activation of K_{Ca} Channels by GTP Plus Mg^{2+}

After demonstrating that K_{Ca} channels could be regulated by GTP or GTPγS only in the presence of Mg^{2+}, we decided to test if NE or a β-agonist could be the G protein

TABLE I

Open Time Constants after GTPγS + Mg Activation

Experiment No.	P_o	τ_{o1}	τ_{o2}	τ_{o3}	a_1	a_2	a_3
			ms				
1. Control	0.018	2.0 ± 0.24	23 ± 1	—	0.28	0.72	—
+$MgCl_2$	0.016	3.2 ± 1.27	26 ± 2	—	0.19	0.81	—
+GTPγS	0.250	8.7 ± 1.58	68 ± 3	—	0.30	0.70	—
2. Control	0.004	1.5 ± 0.73	15 ± 2	—	0.11	0.89	—
+$MgCl_2$	0.010	2.0 ± 0.94	25 ± 2	—	0.20	0.80	—
+GTPγS	0.050	2.0 ± 0.94	35 ± 2	—	0.07	0.93	—
3. Control	0.051	1.5 ± 0.24	10 ± 2	—	0.28	0.72	—
+$MgCl_2$	0.071	1.0 ± 0.57	11 ± 2	—	0.30	0.70	—
+GTPγS	0.920	1.5 ± 1.30	—	103 ± 49	0.05	—	0.95
4. Control	0.027	1.6 ± 1.90	25 ± 2	—	0.15	0.85	—
+$MgCl_2$	0.022	3.0 ± 0.41	30 ± 0.4	—	0.14	0.86	—
+GTPγS	0.115	2.0 ± 0.96	55 ± 2	—	0.13	0.87	—
5. Control	0.530	1.5 ± 0.25	23 ± 0.2	100 ± 3	0.18	0.44	0.38
+$MgCl_2$	0.380	—	35 ± 4	130 ± 14	—	0.63	0.37
+GTPγS	0.610	2.5 ± 0.70	30 ± 10	104 ± 3	0.10	0.02	0.88

Open time constants (τ_o ± SD) in five different experiments (1–5) are shown.
Experiment 1 is the same as in Figs. 2 and 4. The time constants were calculated as in Fig. 4. For explanation see text. P_o = open probability; a = fraction of the total events.

TABLE II

Closed Time Constants after GTPγS + Mg Activation

Experiment No.	P_o	τ_{c1}	τ_{c2}	τ_{c3}	a_1	a_2	a_3
			ms				
1. Control	0.018	1.0 ± 0.15	66 ± 12	1972 ± 135	0.34	0.20	0.46
+$MgCl_2$	0.016	2.3 ± 0.52	100 ± 28	2465 ± 212	0.22	0.16	0.62
+GTPγS	0.250	1.9 ± 0.34	99 ± 11	870 ± 78	0.63	0.27	0.10
2. Control	0.004	—	62 ± 56	4223 ± 565	—	0.08	0.91
+$MgCl_2$	0.010	2.0 ± 0.001	74 ± 2	3177 ± 1	0.05	0.16	0.79
+GTPγS	0.050	2.5 ± 0.60	317 ± 43	673 ± 2	0.10	0.25	0.65
3. Control	0.051	2.0 ± 1.79	23 ± 0.2	284 ± 2	0.05	0.16	0.79
+$MgCl_2$	0.071	1.5 ± 1.21	84 ± 1	402 ± 2	0.10	0.63	0.27
+GTPγS	0.920	1.0 ± 0.31	6 ± 7	20 ± 2	0.55	0.42	0.027
4. Control	0.027	1.0 ± 0.86	80 ± 2	1000 ± 1	0.12	0.13	0.75
+$MgCl_2$	0.022	2.0 ± 0.35	80 ± 2	1419 ± 0.4	0.10	0.08	0.82
+GTPγS	0.115	1.5 ± 0.27	50 ± 19	484 ± 46	0.18	0.10	0.72
5. Control	0.530	1.3 ± 0.24	10 ± 2	45 ± 1	0.08	0.08	0.84
+$MgCl_2$	0.380	1.5 ± 0.30	38 ± 2	200 ± 7	0.16	0.40	0.44
+GTPγS	0.610	1.8 ± 0.57	24 ± 3	113 ± 8	0.21	0.42	0.37

Closed time constants (τ_c ± SD) of channels 1–5 of Table I. Experiment 1 is the same as in Figs. 2 and 4. The time constants were calculated as in Fig. 4. For explanation, see text. P_o = open probability; a = fraction of the total events.

activators. Extracellular NE (1–20 μM) or the β-agonist isoproterenol (1–10 μM) potentiated the intracellular GTP + Mg^{2+} or GTPγS + Mg^{2+} effect on K_{Ca} channels. Experiments performed at various holding potentials (between −40 and 40 mV) and pCa (between 4 and 6.8) gave positive results (n = 10). For example, at 0 mV and pCa near 7 the P_o increased from 0.34 ± 0.04 to 0.57 ± 0.06 (n = 3). β-Adrenergic potentiation was not observed in the absence of intracellular GTP + Mg^{2+} or GTPγS + Mg^{2+}.

Fig. 5 *A* shows records (V_H = 0 mV, pCa 4.3) taken in the presence of 100 μM GTP and 1 mM $MgCl_2$ (*a*) and after addition of 1 μM isoproterenol (*b*) . The P_o of the

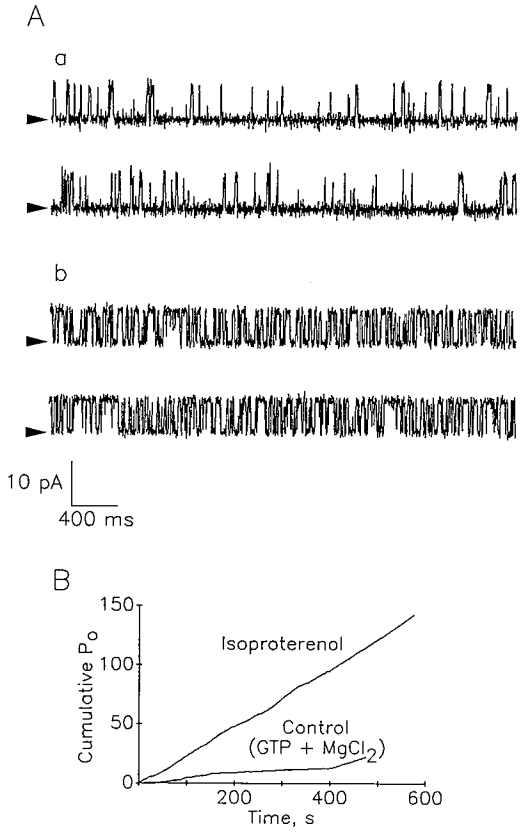

FIGURE 5. G protein activation of K_{Ca} channels is potentiated by a β-agonist. Records (*A*) and corresponding cumulative P_o vs. time curves (*B*). Each value in *B* corresponds to the average P_o during 512 ms. Control traces (*a*) show the activity of a channel (P_o = 0.1), in the presence of 100 μM GTP and 1 mM $MgCl_2$. Lower records (*b*) correspond to the activation due to addition of 1 μM isoproterenol (P_o = 0.5). Rat membranes in a neutral bilayer. V_H = 0 mV, pCa 4.3.

channel increased from 0.1 to 0.5 after isoproterenol. Data collected from 10 min of continuous recording in both conditions are shown in the cumulative P_o vs. time curves (Fig. 5 *B*). Isoproterenol definitely augmented the slope of the control curve. These results suggest that NE may activate K_{Ca} channels through the occupancy of β-receptors coupled to a G protein. Furthermore, in one experiment the effect of 5 μM isoproterenol was partially inhibited by the β-antagonist propanolol (100 μM).

Fig. 6 shows the open time (*A*) and closed time (*B*) histograms of another experiment (V_H = −40 mV, pCa 4), before (*top*) and after addition of isoproterenol (*bottom*). Isoproterenol increased the mean open time of the channel from 2.5 to 4.6 ms, and diminished the mean closed time from 285 to 50 ms.

Open time histograms (Fig. 6 *A*) could be fitted to two exponentials (τ_{o1} and τ_{o2}). Control openings (*top*) had a τ_{o1} of 1.6 ± 0.06 ms, which contributed 99% (a_1) of the total events, and a medium τ_{o2} of 20 ± 0.08 ms, which represented the remaining 1% (a_2) of the events. Isoproterenol (*bottom*) shifted the peak of the curve to a higher τ_{o1} of 4 ± 0.25 ms, while the second component remained unchanged (τ_{o2} = 20 ± 0.8 ms). The contributing ratios of each component were practically the same (a_1 = 0.94 and a_2 = 0.06).

Isoproterenol had a predominant effect on the closed state of the channel. Closed time histograms (Fig. 6 *B*) were fitted to three exponentials. Under control conditions (*top*), τ_{c1} was 3 ± 1.9 ms, τ_{c2} was 110 ± 16 ms, and τ_{c3} was 580 ± 22 ms. Their relative occurrences were 0.02 (a_1), 0.2 (a_2), and 0.78 (a_3). Isoproterenol (*bottom*)

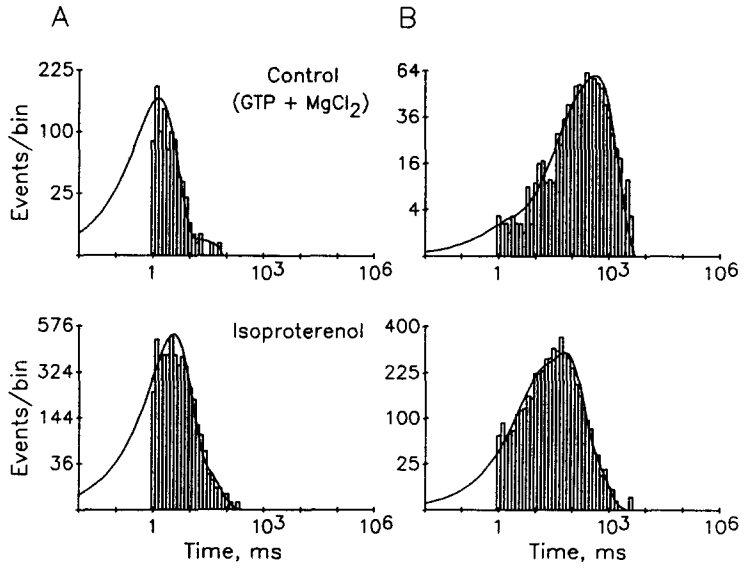

FIGURE 6. Kinetics of K_{Ca} channels after isoproterenol potentiation. Isoproterenol had a predominant effect on the closed state. Data were treated as in Fig. 4. Number of transitions was: control (100 μM GTP + 1 mM Mg^{2+}) = 1,566, and plus 10 μM isoproterenol = 7,469. Control (*upper panels*), plus isoproterenol (*lower panels*). (*A*) Open time histograms were fitted to two exponentials. (*B*) Closed time histograms were fitted to three exponentials. Values are given in the text. V_H = −40 mV, pCa 4.

changed the position of the peaks in the histogram, showing an isoproterenol-dependent change in the magnitude of the closed time constants (τ_{c1} = 10 ± 2 ms, τ_{c2} = 70 ± 1 ms, and τ_{c3} = 260 ± 23 ms). The relative contribution of each time constant also varied (a_1 = 0.26, a_2 = 0.64, and a_3 = 0.1).

GDPβS Inhibits Adrenergic Activation of K_{Ca} Channels

G proteins may be inhibited by the nonhydrolyzable analogue of GDP, guanosine 5′-[β-thio]diphosphate (GDPβS) (Eckstein et al., 1979). Therefore, to confirm the involvement of a G protein in the activation of K_{Ca} channels by NE we tested the

effect of GDPβS. Such an experiment is illustrated in Fig. 7. *A* shows selected traces of channel activity in the presence of 200 μM GTP plus 1 mM $MgCl_2$ (*a*); after addition of 10 μM NE (*b*); and after inhibition with 420 μM GDPβS (*c*) (V_H = 20 mV, pCa 4.4). *B* shows the time course of the experiment. The open probability vs. time

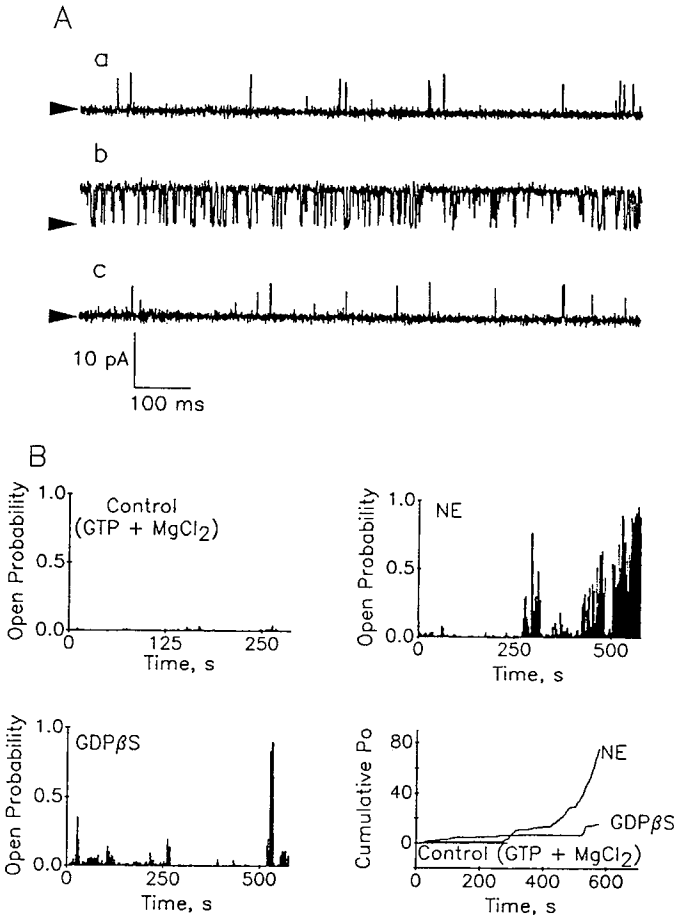

FIGURE 7. Inhibition of K_{Ca} channel activity with GDPβS after adrenergic stimulation. (*A*) Control traces (*a*) are in the presence of 200 μM GTP plus 1 mM $MgCl_2$ (P_o = 0.002). Addition of 10 μM NE (*b*) augmented channel activity to a P_o near 0.9, as shown in the corresponding P_o vs. time histogram (*B*, top right) (mean P_o = 0.13). Subsequent addition of 420 μM GDPβS (*c*) inhibited channel activity (P_o = 0.02). V_H = 20 mV, pCa 4.4, charged bilayers, symmetrical 450 KCl. (*B*) Open probability vs. time graphs (control, *top left;* plus NE, *top right;* plus GDPβS, *bottom left*) and cumulative P_o vs. time curves (*bottom right*). Each value corresponds to the average P_o during 512 ms.

curves show that NE enhanced the activity of the channel (compare *top left* and *right*) and that GDPβS diminished this activity to near control conditions (*bottom left*). The cumulative P_o vs. time curves in the three conditions are also shown (*bottom right*). This experiment demonstrates that GDPβS could revert the activation of the K_{Ca}

channel previously stimulated by NE, supporting the involvement of a G protein–mediated process.

AMP-PNP Does Not Inhibit the GTPγS + Mg^{2+} Effect

The activation of channels by G proteins may be direct or by triggering adenylate cyclase with a subsequent cAMP-mediated phosphorylation of the channel. In fact, after occupancy of β-receptors an increased production of cAMP in uterine and vascular smooth muscle is observed (Fortier et al., 1983; Hofmann, 1985). Moreover, cAMP increases the activity of K_{Ca} channels in aortic smooth muscle via phosphorylation (Sadoshima et al., 1988*a*). However, under our experimental conditions this mechanism was unlikely due to the absence of added ATP in our solutions. Nevertheless, to eliminate the possibility of phosphorylation by endogenous ATP in the membrane vesicles, the ATP analogue AMP-PNP was used to inhibit phosphorylation (Yount, 1975). Fig. 8 demonstrates that the activation of K_{Ca} channels by GTPγS plus $MgCl_2$ was unaffected by AMP-PNP. Records in Fig. 8 *A* are examples of the activity of a K_{Ca} channel (pCa 6.2, 0 mV) before (*a*), after addition of 100 μM AMP-PNP plus 10 μM GTPγS (*b*), and after activation of the putative G

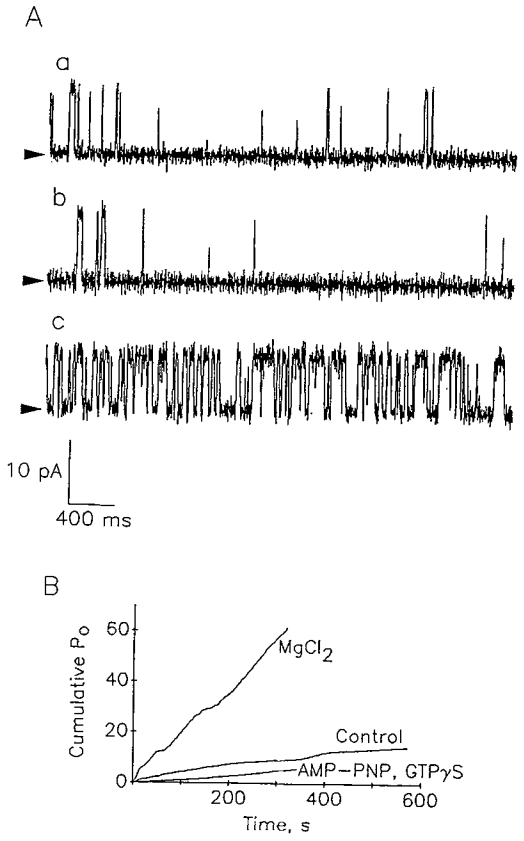

FIGURE 8. Activation of K_{Ca} channels by GTPγS and Mg^{2+} in the presence of AMP-PNP. (*A*) Top traces (*a*) correspond to the control experiment using rat membranes. Middle traces (*b*), same channel after the addition of 100 μM AMP-PNP and 10 μM GTPγS. Note that the P_o remained unchanged. Bottom traces (*c*) after addition of 1 mM $MgCl_2$ (12-fold increment in P_o). V_H = 0 mV, pCa 6.2, charged bilayers. (*B*) Cumulative P_o vs. time curve. Each value corresponds to the average P_o during 512 ms.

protein with Mg^{2+} (*c*). A 12-fold increment in P_o was observed. Fig. 8 *B* is the corresponding cumulative P_o vs. time graph of the experiment illustrated in Fig. 8 *A*. Channel activity was collected for 5–10 min. The slopes of the curves clearly show that GTPγS + Mg^{2+} activates the channel in the presence of AMP-PNP. Thus, the possibility that G proteins activate the channel through a phosphorylation from ATP is very unlikely.

DISCUSSION

K_{Ca} channels from myometrium can be adequately studied in lipid bilayers, since we have shown that reconstituted channels can retain important physiological and regulatory properties. In this work we have described the Ca^{2+} and voltage dependence of K_{Ca} channels and their regulation by G proteins coupled to β-adrenergic receptors. These findings in lipid bilayers are an indication that the membrane vesicles contain the molecules (receptors, channels, and G proteins) involved in this mechanism.

Calcium Dependency

K_{Ca} channels with different Ca^{2+} sensitivities and conductances have been demonstrated in many other tissues using the patch and bilayer techniques (for review see Latorre et al., 1989). The reported myometrial Ca^{2+}-activated K^+ channels can be categorized as being "maxi-K" or "large-conductance" K_{Ca} channels (100–300 pS) (Latorre et al., 1989). With respect to their Ca^{2+} sensitivity, K_{Ca} channels from myometrium are similar to stomach smooth muscle (Singer and Walsh, 1987), human astrocytoma cells (Pallotta et al., 1987), fetal human aorta (Bregestovski et al., 1988), rat aorta (Sadoshima et al., 1988*b*), and GH_3 anterior pituitary cells (Lang and Ritchie, 1987) (Fig. 1, *open squares*); they are more sensitive to Ca^{2+} than K_{Ca} channels from skeletal (Latorre et al., 1982) and intestinal smooth muscles (Cecchi et al., 1986), and less responsive to Ca^{2+} than those from mesenteric artery, jejunum (Benham et al., 1986), airway smooth muscles (McCann and Welsh, 1986), and adult human aorta (Bregestovski et al., 1988).

Magnesium Effects

The majority of myometrial K_{Ca} channels were unaffected by Mg^{2+} (~80%). The activation by Mg^{2+} we observed in a few channels may be due to a direct effect on the channel that increases its apparent affinity for Ca^{2+} (Golowasch et al., 1986; Oberhauser et al., 1988). In view of our findings on G protein regulation, it can also be thought that endogenous GTP in combination with the added Mg^{2+} was responsible for the activation. Intracellular addition of Mg^{2+} always slightly reduced the channel conductance by 6%, indicating a fast blockade mechanism as was reported for Cd^{2+} in K_{Ca} channels of skeletal muscle (Oberhauser et al., 1988).

Kinetics of K_{Ca} Channels

K_{Ca} channels from myometrium had several open and closed time constants, like K_{Ca} channels from other tissues (Magleby and Pallotta, 1983; Moczydlowski and Latorre, 1983; Benham et al., 1986; Smart, 1987; Bregestovski et al., 1988; Capiod and Ogden, 1989; Reinhart et al., 1989). The time constants (two for the open state and

three for the closed state) are qualitatively consistent with the model proposed by Magleby and Pallotta (1983) for skeletal muscle where two main open states and three closed states describe the K_{Ca} channel. The authors observed that increasing internal Ca^{2+} concentration ($[Ca^{2+}]_i$) gave rise to a third, longer, open distribution and proposed a third open state for the channel. This was also the case for K_{Ca} channels from myometrium, where a long open state was detected when the P_o was >0.38 (Table I).

G Protein Gating of K_{Ca} Channels

The GTP + Mg^{2+}– or GTPγS + Mg^{2+}–dependent stimulation of myometrial K_{Ca} channels suggest that an activated G protein increases channel activity (Gilman, 1987; Birnbaumer et al., 1989). The extracellular signal catalyzing the G protein activation is the occupancy of β-receptors by NE or isoproterenol. This mechanism was confirmed by the lack of activation of K_{Ca} channels with NE when GTP + Mg^{2+} or GTPγS + Mg^{2+} were absent in the cytoplasmic side. In accordance, the NE- and GTP-dependent stimulation of the channel could be reversed by GDPβS, an inhibitor of G protein activation (Eckstein et al., 1979). The nature of the G protein and subunit(s) involved is under study.

G proteins may also activate their targets (enzymes or channels) in the absence of the agonist when GTP and Mg^{2+} are present (Birnbaumer et al., 1980; Iyengar and Birnbaumer, 1982; Okabe et al., 1990). These findings can be interpreted as a displacement of the equilibrium towards the active state of the reaction, inactive G protein + GTP → activated G protein. This reaction is catalyzed by the occupancy of the receptors. In accordance we have shown that channel activity may be enhanced when GTP + Mg^{2+} are added to the cytoplasmic side of the channel in the absence of the agonist. This basal activity was further potentiated by the agonist.

K_{Ca} channels can also be activated by phosphorylation (DePeyer et al., 1982; Farley and Rudy, 1988; Sadoshima et al., 1988*a*) and by arachidonic acid (Ordway et al., 1989). The variety of regulatory agents that modulate K_{Ca} channel activity may explain the diversity in Ca^{2+} sensitivities of K_{Ca} channels found in different tissues. Our data are consistent with this hypothesis, since GTP-dependent activation made K_{Ca} channels behave as if they had a higher Ca^{2+} sensitivity. It is interesting that GTP-dependent proteins also increase the Ca^{2+} sensitivity of phospholipase C (Gold et al., 1987; Taylor and Exton, 1987; McDonough et al., 1988). Thus, changing Ca^{2+} sensitivity may be a common expression of G protein activation of different Ca^{2+}-dependent enzymes, including Ca^{2+}-activated K^+ channels.

In our experiments, other possible mechanisms that could explain the GTP-dependent activation of myometrial K_{Ca} channels are phosphorylation or activation by arachidonic acid. In these cases our membranes must contain adenylate cyclase, ATP, protein kinases, and/or phospholipases. Our findings with AMP-PNP exclude the possibility that contaminant ATP and protein kinases in our system were part of a phosphorylating mechanism that modulated channel activity. Furthermore, since the GTP-dependent activation of myometrial K_{Ca} channels was potentiated by a β-agonist and inhibited by a β-antagonist, the possibility that we were activating a phospholipase A_2 and producing arachidonic acid to activate K_{Ca} channels was rather low (for review see Birnbaumer et al., 1987). Therefore, we conclude that GTPγS

and Mg^{2+} triggered a G protein that directly gates K_{Ca} channels. In any event, our data do not exclude the possibility that myometrial K_{Ca} channels can be modulated in a parallel way by phosphorylation as in T tubule Ca^{2+} channels (Yatani et al., 1988) or other mechanisms that remain to be investigated. Consistent with the possibility of several parallel modulatory inputs is our recent observation of a direct activation of myometrial K_{Ca} channels by arachidonic acid (10 μM) in the absence of nucleotides (Ramos-Franco, J., L. Toro, and E. Stefani, unpublished observations).

In conclusion, our results indicate that one of the mechanisms by which β-adrenergic stimulation hyperpolarizes and relaxes uterine smooth muscle is via a direct G protein activation of K_{Ca} channels. Furthermore, it can be concluded that the β-adrenergic receptor, the G protein, and the K_{Ca} channel form a functional complex that is not dissociated or uncoupled during the fractionation procedure, and that this complex can be reconstituted in lipid bilayers. Similar conclusions were reported by Yatani et al. (1988) in reconstitution experiments with the Ca^{2+} channel from skeletal muscle T tubules.

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REFERENCES

Alvarez, O. 1986. How to set up a bilayer system. *In* Ion Channel Reconstitution. C. Miller, editor. Plenum Publishing Corp., New York. 115–130.

Benham, C. D., T. B. Bolton, R. J. Lang, and T. Takewaki. 1985. The mechanism of action of Ba^{2+} and TEA on single Ca^{2+}-activated K^+-channels in arterial and intestinal smooth muscle cell membranes. *Pflügers Archiv.* 403:120–127.

Benham, C. D., T. B. Bolton, R. J. Lang, and T. Takewaki. 1986. Calcium-activated potassium channels in single smooth muscle cells of rabbit jejunum and guinea-pig mesenteric artery. *Journal of Physiology.* 371:45–67.

Birnbaumer, L., J. Codina, R. Mattera, A. Yatani, N. Scherer, M. J. Toro, and A. M. Brown. 1987. Signal transduction by G proteins. *Kidney International.* 32:S14–S37.

Birnbaumer, L., T. L. Swartz, J. Abramowitz, P. W. Mintz, and R. Iyengar. 1980. Transient and steady state kinetics of the interaction of guanyl nucleotides with the adenylyl cyclase system from rat liver plasma membranes. *Journal of Biological Chemistry.* 255:3542–3551.

Birnbaumer, L., A. A. M. J. VanDongen, J. Codina, A. Yatani, R. Mattera, R. Graf, and A. M. Brown. 1989. Identification of G protein-gated and G protein-modulated ionic channels: molecular basis for G protein action. *Society of General Physiologists Series.* 44:17–54.

Bregestovski, P. D., O. Yu. Printseva, V. Serebryakov, J. Stinnakre, A. Turmin, and V. Zamoyski. 1988. Comparison of Ca^{2+}-dependent K^+ channels in the membrane of smooth muscle cells isolated from adult and foetal human aorta. *Pflügers Archiv.* 413:8–13.

Breitweiser, G. E., and G. Szabo. 1985. Uncoupling of cardiac muscarinic and beta-adrenergic receptors from ion channels by a guanine nucleotide analogue. *Nature* 317:538–540.

Breitweiser, G. E., and G. Szabo. 1988. Mechanism of muscarinic receptor-induced K^+ channel activation as revealed by hydrolysis-resistant GTP analogues. *Journal of General Physiology.* 91:469–493.

Brown, A. M., and L. Birnbaumer. 1988. Direct G protein gating of ion channels. *American Journal of Physiology.* 254:H401–H410.

Brown, A. M., A. Yatani, J. Codina, and L. Birnbaumer. 1989. G protein-gated channels: a third major category of ionic channels. *American Journal of Hypertension.* 56:421–437.

Bülbring, E., R. Casteels, and H. Kuriyama. 1968. Membrane potential and ion content in cat and guinea-pig myometrium and the response to adrenaline and noradrenaline. *British Journal of Pharmacology.* 34:388–407.

Capiod, T., and D. C. Ogden. 1989. The properties of calcium-activated potassium ion channels in guinea-pig isolated hepatocytes. *Journal of Physiology.* 409:285–295.

Cecchi, X., O. Alvarez, and D. Wolff. 1986. Characterization of a calcium-activated potassium channel from rabbit intestinal smooth muscle incorporated into planar bilayers. *Journal of Membrane Biology.* 91:11–18.

Cerbai, E., U. Klöckner, and G. Isenberg. 1988. The alpha subunit of the GTP binding protein activates muscarinic potassium channels of the atrium. *Science.* 240:1782–1783.

Codina, J., D. Grenet, A. Yatani, L. Birnbaumer, and A. M. Brown. 1987. Hormonal regulation of pituitary GH_3 cell K^+ channels by G_k is mediated by its α-subunit. *FEBS Letters.* 216:104–106.

Colquhoun, D., and F. Sigworth. 1983. Fitting and statistical analysis of single channel records. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York. 191–264.

DePeyer, J. E., A. B. Cachelin, I. B. Levitan, and H. Reuter. 1982. Ca^{++}-activated K^+ conductance in internally perfused snail neurons is enhanced by protein phosphorylation. *Proceedings of the National Academy of Sciences USA.* 79:4207–4211.

Diamond, J., and J. M. Marshall. 1969. A comparison of the effects of various smooth muscle relaxants on the electrical and mechanical activity of the rat uterus. *Journal of Pharmacology and Experimental Therapeutics.* 168:21–30.

Eckstein, F., D. Cassel, H. Levkovitz, M. Lowe, and Z. Selinger. 1979. Guanosine 5′-O′-(2-thiodiphosphate): an inhibitor of adenylate cyclase stimulation by guanine nucleotides and fluoride ions. *Journal of Biological Chemistry.* 254:9829–9834.

Fabiato, A. 1988. Computer programs for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods in Enzymology.* 157:378–417.

Farley, J., and B. Rudy. 1988. Multiple types of voltage-dependent Ca^{2+}-activated K^+ channels of large conductance in rat brain synaptosomal membranes. *Biophysical Journal.* 53:919–934.

Fortier, M., D. Chase, S. G. Korenman, and J. F. Krall. 1983. β-Adrenergic catecholamine-dependent properties of rat myometrium primary cultures. *American Journal of Physiology.* 245:C84–C90.

Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Annual Review of Biochemistry.* 56:615–649.

Gold, M. R., J. P. Jakway, and A. L. DeFranco. 1987. Involvement of a guanine nucleotide-binding component in membrane IgM-stimulated phosphoinositide breakdown. *The Journal of Immunology.* 139:3604–3613.

Golowasch, J., A. Kirkwood, and C. Miller. 1986. Allosteric effects of Mg^{2+} on the gating of Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Journal of Experimental Biology.* 124:5–13.

Hamilton, S. L., R. Mejia Alvarez, M. Fill, M. J. Hawkes, K. L. Brush, W. P. Schilling, and E. Stefani. 1989. [^{3}H]-PN200-110 and [^{3}H]-ryanodine binding and reconstitution of ion channel activity with skeletal muscle membranes. *Analytical Biochemistry.* 183:31–41.

Hanke, W. 1986. Incorporation of ion channels by fusion. *In* Ion Channel Reconstitution. C. Miller, editor, Plenum Publishing Corp., New York. 141–153.

Hofmann, F. 1985. The molecular basis of second messenger systems for regulation of smooth muscle contractility. *Journal of Hypertension.* 3:S3–S8.

Inoue, R., K. Kitamura, and H. Kuriyama. 1985. Two Ca-dependent K-channels classified by the application of tetraethylammonium distribute to smooth muscle membranes of the rabbit portal vein. *Pflügers Archiv.* 405:173–179.

Iyengar, R., and L. Birnbaumer. 1982. Hormone receptor modulates the regulatory component of adenylyl cyclase by reducing its requirement for Mg^{2+} and enhancing its extent of activation by guanine nucleotides. *Proceedings of the National Academy of Sciences USA.* 79:5179–5183.

Johansson, S. R. M., R. G. G. Andersson, and J. E. S. Wikberg. 1980. Comparison of β_1- and β_2-receptor stimulation in oestrogen or progesterone dominated rat uterus. *Acta Pharmacologica et Toxicologica.* 47:252–258.

Kirsch, G. E., A. Yatani, J. Codina, L. Birnbaumer, and A. M. Brown. 1988. α-Subunit of G_k activates atrial K^+ channels of chick, rat and guinea pig. *American Journal of Physiology.* 254:H1200–H1205.

Kroeger, E. A., and J. M. Marshall. 1973. Beta-adrenergic effects on rat myometrium: mechanisms of membrane hyperpolarization. *American Journal of Physiology.* 225:1339–1345.

Kurachi, Y., T. Nakajima, and T. Sugimoto. 1986. Acetylcholine activation of K^+ channels in cell-free membranes of atrial cells. *American Journal of Physiology.* 251:H681–H684.

Lang, D. G., and A. K. Ritchie. 1987. Large and small conductance calcium-activated potassium channels in the GH_3 anterior pituitary cell line. *Pflügers Archiv.* 410:614–622.

Latorre, R., A. Oberhauser, P. Labarca, and O. Alvarez. 1989. Varieties of calcium-activated potassium channels. *Annual Review of Physiology.* 51:385–399.

Latorre, R., C. Vergara, and C. Hidalgo. 1982. Reconstitution in planar lipid bilayers of a Ca^{2+}-dependent K^+ channel from transverse tubule membranes isolated from rabbit skeletal muscle. *Proceedings of the National Academy of Sciences USA.* 79:805–809.

Levitzki, A. 1988. From epinephrine to cyclic AMP. *Science.* 241:800–806.

Logothetis, D. E., D. Kim, J. K. Northup, E. J. Neer, and D. E. Clapham. 1988. Specificity of action of guanine nucleotide-binding regulatory protein subunits on the cardiac muscarinic K^+ channel. *Proceedings of the National Academy of Sciences USA.* 85:5814–5818.

Logothetis, D. E., Y. Kurachi, J. Galper, E. J. Neer, and D. E. Clapham. 1987. The beta-gamma subunits of GTP-binding proteins activate the muscarinic K^+ channel in heart. *Nature.* 325:321–326.

Lowry, O. H., N. J. Rosebrough, D. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *Journal of Biological Chemistry.* 193:265–275.

Magleby, K. L., and B. S. Pallotta. 1983. Calcium dependence of open and shut interval distributions from calcium-activated potassium channels in cultured rat muscle. *Journal of Physiology.* 344:584–604.

McCann, J. D., and M. J. Welsh. 1986. Calcium-activated potassium channels in canine airway smooth muscle. *Journal of Physiology.* 372:113–127.

McDonough, P. M., D. Goldstein, and J. H. Brown. 1988. Elevation of cytoplasmic calcium concentration stimulates hydrolysis of phophatidylinositol bisphosphate in chick heart cells: effect of sodium channel activators. *Molecular Pharmacology.* 33:310–315.

Meissner, G. 1984. Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+}-release in sarcoplasmic reticulum. *Journal of Biological Chemistry.* 259:2365–2374.

Miller, C., and E. Racker. 1976. Calcium-induced fusion of fragmented sarcoplasmic reticulum with artificial planar bilayers. *Journal of Membrane Biology.* 30:283–300.

Moczydlowski, E., and R. Latorre. 1983. Gating kinetics of Ca^{2+}-activated K^+ channels from rat muscle incorporated into planar lipid bilayers. *Journal of General Physiology.* 82:511–542.

Oberhauser, A., O. Alvarez, and R. Latorre. 1988. Activation by divalent cations of a Ca^{2+}-activated K^+ channel from skeletal muscle membrane. *Journal of General Physiology.* 92:67–86.

Okabe, K., A. Yatani, T. Evans, Y. K. Ho, J. Codina, L. Birnbaumer, and A. M. Brown. 1990. $\beta\gamma$ dimers of G proteins inhibit muscarinic K^+ channels. *Journal of Biological Chemistry.* In press.

Ordway, R. W., L. H. Clapp, A. M. Gurney, J. J. Singer, and J. V. Walsh, Jr. 1989. Fatty acids directly activate large conductance, calcium-activated K^+ channels in pulmonary artery smooth muscle cells from rabbit. *Journal of General Physiology.* 94:37*a*. (Abstr.)

Pallotta, B. S., J. R. Hepler, S. A. Oglesby, and T. K. Harden. 1987. A comparison of calcium-activated potassium channel currents in cell-attached and excised patches. *Journal of General Physiology.* 89:985–997.

Pfaffinger, P. J., J. M. Martin, D. D. Hunter, N. M. Nathanson, and B. Hille. 1985. GTP-binding proteins couple cardiac muscarinic receptors to a K^+ channel. *Nature.* 317:536–638.

Piercy, V. 1987. The β-adrenoreceptors mediating uterine relaxation throughout the oestrous cycle of the rat are predominately of the β_2-subtype. *Journal of Autonomic Pharmacology.* 8:11–18.

Ramos-Franco, J., L. Toro, and E. Stefani. 1989. GTP-g-S enhances the open probability of K_{Ca} channels from myometrium incorporated into bilayers. *Biophysical Journal.* 55:536*a*. (Abstr.)

Reinhart, P. H., S. Chung, and I. B. Levitan. 1989. A family of calcium-dependent potassium channels from rat brain. *Neuron.* 2:1031–1041.

Riemer, R. K., and J. M. Roberts. 1986. Endocrine modulation of myometrial response. *In* The Physiology and Biochemistry of the Uterus in Pregnancy and Labor. G. Huszar, editor. CRC Press, Inc., Boca Raton, FL. 53–71.

Sadoshima, J. I., N. Akaike, H. Kanaide, and M. Nakamura. 1988*a*. Cyclic AMP modulates Ca-activated K channel in cultured smooth muscle cells of rat aortas. *American Journal of Physiology.* 255:H754–H759.

Sadoshima, J.-I., N. Akaike, H. Tomoike, H. Kanaide, and M. Nakamura. 1988*b*. Ca-activated K channel in cultured smooth muscle cells of rat aortic media. *American Journal of Physiology.* 255:H410–H418.

Sigworth, J., and S. M. Sine. 1987. Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophysical Journal.* 52:1047–1054.

Singer, J. J., and J. V. Walsh, Jr. 1987. Characterization of calcium-activated potassium channels in single smooth muscle cells using the patch clamp technique. *Pflügers Archiv.* 408:98–111.

Smart, T. G. 1987. Single calcium-activated potassium channels recorded from cultured rat sympathetic neurones. *Journal of Physiology.* 389:337–360.

Squire, L. G., and O. H. Petersen. 1987. Modulation of Ca^{2+}- and voltage-activated K^+ channels by internal Mg^{2+} in salivary acinar cells. *Biochimica et Biophysica Acta.* 899:171–175.

Taylor, S. J., and J. H. Exton. 1987. Guanine-nucleotide and hormone regulation of polyphosphoinositide phospholipase C activity of rat liver plasma membranes. *Biochemical Journal.* 248:791–799.

Toro, L., E. Stefani, and S. Erulkar. 1987. Ca^{2+} and K^+ currents in uterine smooth muscle. Differential effect of norepinephrine on two K^+ currents. *Society for Neuroscience Abstracts.* 13:1441. (Abstr.)

Toro, L., E. Stefani, and S. Erulkar. 1990. Hormonal regulation of potassium currents in single myometrial cells. *Proceedings of the National Academy of Sciences USA.* 87:2892–2895.

Toro, L., H. Valdivia, R. Coronado, J. S. Smith, and E. Stefani. 1988. Single channel activity in membrane vesicles from myometrial cells incorporated into lipid bilayers. *Biophysical Journal.* 53:148*a*. (Abstr.)

VanDongen, A. M. J., J. Codina, J. Olate, R. Mattera, R. Joho, L. Birnbaumer, and A. M. Brown. 1988. Newly identified brain potassium channels gated by the guanine nucleotide binding protein G_o. *Science.* 242:1433–1437.

Yamaguchi, H., T. W. Honeyman, and F. S. Fay. 1988. β-Adrenergic actions on membrane electrical properties of dissociated smooth muscle cells. *American Journal of Physiology.* 254:C423–431.

Yatani, A., J. Codina, A. M. Brown, and L. Birnbaumer. 1987. Direct activation of mammalian atrial muscarinic potassium channels by GTP regulatory protein G_k. *Science.* 235:207–211.

Yatani, A., Y. Imoto, J. Codina, S. L. Hamilton, A. M. Brown, and L. Birnbaumer. 1988. The stimulatory G protein of adenylyl cyclase, G_s, also stimulates dihydropyridine-sensitive Ca^{2+} channels. *Journal of Biological Chemistry.* 263:9887–9895.

Yount, R. C. 1975. ATP analogs. *Advances in Enzymology and Related Areas of Molecular Biology.* 43:1–56.