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

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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 (Bühlbring 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.

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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 GH3 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 KCa 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 Mg2+ 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 Mg2+ dependent (Pfaffinger et al., 1985; Brown and Birnbaumer, 1988). Thus, we used these criteria to define if KCa channels were G protein gated.

In this study we demonstrate that myometrial KCa channels are activated by intracellular GTP or GTPγS only in the presence of Mg2+. Furthermore, extracellular NE or isoproterenol (β-agonist) potentiate the activity of KCa channels in the presence of intracellular GTP + Mg2+. Thus, a direct activation of KCa 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 β-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 N2, 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 [3H]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+ 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 Ca2+ concentration and their orientation was initially determined by the P0 vs. voltage relationship. For example, if the P0 diminished with negative potentials, the internal side of the channel was facing the cis chamber. At 100 μM free Ca2+, K+ channels have a P0 near 1.0 at potentials close to 0 mV; thus, to reduce the P0 value, the free Ca2+ concentration facing the intracellular side was lowered by adding K2-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 K2-EGTA, 0.6 CaCl2, pH 7.4, pCa 4. The trans chamber contained (in mM): 50 KCl, 10 K-MOPS, 0.5 K2-EGTA, 0.6 CaCl2, 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 Mg2+ 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 by 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 Walsh, 1986; Singer and Walsh, 1987; Bregestovski et al., 1988; Sadoshima et al., 1988b), possesses large conductance K+ channels that are calcium- and voltage sensitive (Fig. 1) (Toro et al., 1988). Myometrial KCa 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 similarity indicates that KCa channels have a high selectivity towards K+ . Ca2+ sensitivity of KCa channels from skeletal muscle varies from channel to channel (Moczydlowski and Latorre, 1983; Oberhauser et al., 1988). We also observed variability in the Ca2+ concentration ([Ca2+]i) sensitivity of myometrial KCa channels. In most cases, KCa channels were active at myoplasmic [Ca2+]i 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 KCa 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 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 internal side of the channel. The 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): \(P_o = 1/[1 + exp \left(\frac{\left(V_{1/2} - V\right)}{k}\right)]\) where \(P_o\) = open probability; \(V_{1/2}\) = half activation voltage; \(V\) = applied voltage, and \(k\) = slope factor. At pCa 4 (100 \(\mu\)M \(Ca^{2+}\), 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.
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 $P_o = \text{open probability}$, $V_{1/2} = \text{half activation voltage}$, $V = \text{applied voltage}$, and $k = \text{slope factor}$. 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 $z = \text{effective valence}$, 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$_c$ 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$_c$ channels could open at lower intracellular Ca$^{2+}$ concentrations, bringing their Ca$^{2+}$ sensitivity closer to K$_c$ channels observed in inside-out patches from fetal human (Bregestovski et al., 1988) and rat aorta (Sadoshima et al., 1988).

Mg$^{2+}$ activates K$_c$ 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$_c$ 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 $0.20 \pm 0.05$ ($n = 31$) 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 $P_o$ ($0.16 \pm 0.04$ to $0.40 \pm 0.07$, $n = 7$). We also observed that Mg$^{2+}$ slightly diminished the amplitude of K$_c$ channels. The reduction ($6 \pm 1\%$, $n = 27$; Figs. 2, 3, and 8) of K$_c$ channel conductance was consistent regardless of the presence of GTP.

In summary, myometrial K$_c$ channels have two properties in common with other K$_c$ 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$_c$ 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$_c$ 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$_c$ channel activity. This appeared to be the case since K$_c$ channels were activated by GTP or GTPγS (10–100 µM) only if Mg$^{2+}$ was present. The average $P_o$ increase was from $0.18 \pm 0.08$ to $0.49 \pm 0.07$ ($n = 7$) 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 $K_v$ 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 $\mu$M GTP$\gamma$S (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 GTP$\gamma$S 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 GTP$\gamma$S. 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 $\mu$M GTP$\gamma$S 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 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 GTP$\gamma$S had a definite activation effect (bottom left). The spontaneous variations in activity to very low levels even after activation with GTP$\gamma$S 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 GTP$\gamma$S, GTP, or contaminant Ca$^{2+}$ in the nucleotide solutions, each nucleotide was added to the internal side before MgCl$_2$. Neither GTP$\gamma$S (10 $\mu$M, $n = 3$; 100 $\mu$M, $n = 4$) nor GTP (100 $\mu$M,
$n = 2$ alone affected the $P_o$ of the channel. This is exemplified for GTP$_{yS}$ in Fig. 3, where current traces and total amplitude histograms are shown for each case (Fig. 3 A, a–c), $V_H = 0 \text{ mV at } pCa \text{ 6.8}$. The top trace (a) represents the control experiment ($P_o = 0.1$). Addition of 10 $\mu$M GTP$_{yS}$ (b) did not increase the $P_o$ of the channel (0.1). This lack of activation was always observed, and demonstrates that GTP$_{yS}$ 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 GTP$_{yS}$. These experiments eliminate the possibility that channel opening induced by GTP$_{yS}$ 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 GTP$_{yS}$ and Mg$^{2+}$**

Upon activation of $K_{Ca}$ channels with GTP$_{yS}$ and Mg$^{2+}$, openings lasted longer (mean open time increased $2.7 \pm 0.7$ times its control value, $n = 6$) and closings were shorter (mean closed time was $0.2 \pm 0.07$ 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 ($\tau_{o1}$, 1–9 ms) and medium ($\tau_{o2}$, 10–100 ms) time constants, and closings with corresponding fast ($\tau_{c1}$, 1–10 ms), medium ($\tau_{c2}$, 11–100 ms), and long ($\tau_{c3}$, > 100 ms) time constants (Fig. 4, A and B, top). Mg$^{2+}$ increased the proportion of the long closures ($\tau_{c3}$) but did not produce an appreciable change in the rest of the kinetic parameters (Fig. 4, A and B, middle). Addition of GTP$_{yS}$ in the presence of Mg$^{2+}$ augmented the values of $\tau_{o1}$ and $\tau_{o2}$ about three times, while their ratios were not greatly modified (Fig. 4 A, bottom). GTP$_{yS}$ 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 $\tau_{c3}$ diminished. The contribution ratios of $\tau_{c1}$ and $\tau_{c2}$ were augmented (three and two times, respectively), while their values were not greatly modified.

The action of GTP$_{yS}$ 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 GTP$_{yS}$ does not greatly modify the value of $\tau_{o1}$, while it increases $\tau_{o2}$ or makes more evident a third long component (Table I, experiments 3 and 5). (b) For the closed state, after GTP$_{yS}$ the value of $\tau_{c1}$ remained practically the same, but its ratio increased; the value of $\tau_{c3}$ 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, $\tau_{o1}$ and $\tau_{o2}$). (B) Closed time histograms fitted to three exponentials (closed time constants, $\tau_{c1}$, $\tau_{c2}$, and $\tau_{c3}$). Addition of 100 μM GTPγS in the presence of Mg$^{2+}$ (bottom) diminished $\tau_{c3}$ 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 GTP\textsubscript{yS} + Mg Activation

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>( P_o )</th>
<th>( \tau_{sl} )</th>
<th>( \tau_{sl} )</th>
<th>( \tau_{sl} )</th>
<th>( a_1 )</th>
<th>( a_2 )</th>
<th>( a_3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0.018</td>
<td>2.0 ± 0.24</td>
<td>23 ± 1</td>
<td>---</td>
<td>0.28</td>
<td>0.72</td>
<td>---</td>
</tr>
<tr>
<td>+ MgCl\textsubscript{2}</td>
<td>0.016</td>
<td>3.2 ± 1.27</td>
<td>26 ± 2</td>
<td>---</td>
<td>0.19</td>
<td>0.81</td>
<td>---</td>
</tr>
<tr>
<td>+ GTP\textsubscript{yS}</td>
<td>0.250</td>
<td>8.7 ± 1.58</td>
<td>68 ± 5</td>
<td>---</td>
<td>0.30</td>
<td>0.70</td>
<td>---</td>
</tr>
<tr>
<td>2. Control</td>
<td>0.004</td>
<td>1.5 ± 0.73</td>
<td>15 ± 2</td>
<td>---</td>
<td>0.11</td>
<td>0.89</td>
<td>---</td>
</tr>
<tr>
<td>+ MgCl\textsubscript{2}</td>
<td>0.010</td>
<td>2.0 ± 0.94</td>
<td>25 ± 2</td>
<td>---</td>
<td>0.20</td>
<td>0.80</td>
<td>---</td>
</tr>
<tr>
<td>+ GTP\textsubscript{yS}</td>
<td>0.050</td>
<td>2.0 ± 0.94</td>
<td>35 ± 2</td>
<td>---</td>
<td>0.07</td>
<td>0.93</td>
<td>---</td>
</tr>
<tr>
<td>3. Control</td>
<td>0.051</td>
<td>1.5 ± 0.24</td>
<td>10 ± 2</td>
<td>---</td>
<td>0.28</td>
<td>0.72</td>
<td>---</td>
</tr>
<tr>
<td>+ MgCl\textsubscript{2}</td>
<td>0.071</td>
<td>1.0 ± 0.57</td>
<td>11 ± 2</td>
<td>---</td>
<td>0.30</td>
<td>0.70</td>
<td>---</td>
</tr>
<tr>
<td>+ GTP\textsubscript{yS}</td>
<td>0.920</td>
<td>1.5 ± 1.30</td>
<td>---</td>
<td>103 ± 49</td>
<td>0.05</td>
<td>0.95</td>
<td>---</td>
</tr>
<tr>
<td>4. Control</td>
<td>0.027</td>
<td>1.6 ± 1.90</td>
<td>25 ± 2</td>
<td>---</td>
<td>0.15</td>
<td>0.85</td>
<td>---</td>
</tr>
<tr>
<td>+ MgCl\textsubscript{2}</td>
<td>0.022</td>
<td>3.0 ± 0.41</td>
<td>30 ± 0.4</td>
<td>---</td>
<td>0.14</td>
<td>0.86</td>
<td>---</td>
</tr>
<tr>
<td>+ GTP\textsubscript{yS}</td>
<td>0.115</td>
<td>2.0 ± 0.96</td>
<td>55 ± 2</td>
<td>---</td>
<td>0.13</td>
<td>0.87</td>
<td>---</td>
</tr>
<tr>
<td>5. Control</td>
<td>0.530</td>
<td>1.5 ± 0.25</td>
<td>23 ± 0.2</td>
<td>100 ± 3</td>
<td>0.18</td>
<td>0.44</td>
<td>0.38</td>
</tr>
<tr>
<td>+ MgCl\textsubscript{2}</td>
<td>0.380</td>
<td>---</td>
<td>35 ± 4</td>
<td>130 ± 14</td>
<td>0.63</td>
<td>0.37</td>
<td>---</td>
</tr>
<tr>
<td>+ GTP\textsubscript{yS}</td>
<td>0.610</td>
<td>2.5 ± 0.70</td>
<td>30 ± 10</td>
<td>104 ± 3</td>
<td>0.10</td>
<td>0.02</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Open time constants \((\tau_s ± SD)\) 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 GTP\textsubscript{yS} + Mg Activation

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

Closed time constants \((\tau_c ± SD)\) 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 + Mg²⁺ or GTPγS + Mg²⁺ effect on Kca 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 Po 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 + Mg²⁺ or GTPγS + Mg²⁺.

Fig. 5 A shows records (VH = 0 mV, pCa 4.3) taken in the presence of 100 μM GTP and 1 mM MgCl₂ (a) and after addition of 1 μM isoproterenol (b). The Po of the 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 Po vs. time curves (Fig. 5 B). Isoproterenol definitely augmented the slope of the control curve. These results suggest that NE may activate Kca 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 (VH = −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 ($\tau_{o1}$ and $\tau_{o2}$). Control openings (top) had a $\tau_{o1}$ of $1.6 \pm 0.06$ ms, which contributed 99% ($a_1$) of the total events, and a medium $\tau_{o2}$ of $20 \pm 0.08$ ms, which represented the remaining 1% ($a_2$) of the events. Isoproterenol (bottom) shifted the peak of the curve to a higher $\tau_{o1}$ of $4 \pm 0.25$ ms, while the second component remained unchanged ($\tau_{o2} = 20 \pm 0.8$ 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), $\tau_{c1}$ was $3 \pm 1.9$ ms, $\tau_{c2}$ was $110 \pm 16$ ms, and $\tau_{c3}$ was $580 \pm 22$ ms. Their relative occurrences were 0.02 ($a_1$), 0.2 ($a_2$), and 0.78 ($a_3$). Isoproterenol (bottom) changed the position of the peaks in the histogram, showing an isoproterenol-dependent change in the magnitude of the closed time constants ($\tau_{c1} = 10 \pm 2$ ms, $\tau_{c2} = 70 \pm 1$ ms, and $\tau_{c3} = 260 \pm 23$ ms). The relative contribution of each time constant also varied ($a_1 = 0.26$, $a_2 = 0.64$, and $a_3 = 0.1$).

**GDP\(\beta\)S Inhibits Adrenergic Activation of K\(_{Ca}\) Channels**

G proteins may be inhibited by the nonhydrolyzable analogue of GDP, guanosine 5'-[\(\beta\)-thio]diphosphate (GDP\(\beta\)S) (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 MgCl₂ (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

![Diagram A](image1)

**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 MgCl₂ (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 + Mg2+ 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 Kca channels in aortic smooth muscle via phosphorylation (Sadoshima et al., 1988a). 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 Kca channels by GTPγS plus MgCl2 was unaffected by AMP-PNP. Records in Fig. 8 A are examples of the activity of a Kca 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](https://example.com/fig8.png)
protein with Mg\(^{2+}\) (c). A 12-fold increment in \(P_o\) was observed. Fig. 8B is the corresponding cumulative \(P_o\) vs. time graph of the experiment illustrated in Fig. 8A. Channel activity was collected for 5–10 min. The slopes of the curves clearly show that GTP\(\gamma\)S + 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\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) channels and their regulation by G proteins coupled to \(\beta\)-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\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) channels (100–300 pS) (Latorre et al., 1989). With respect to their Ca\(^{2+}\) sensitivity, K\(_{\text{Ca}}\) 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), and GH\(_3\) anterior pituitary cells (Lang and Ritchie, 1987) (Fig. 1, open squares); they are more sensitive to Ca\(^{2+}\) than K\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) channels of skeletal muscle (Oberhauser et al., 1988).

**Kinetics of K\(_{\text{Ca}}\) Channels**

K\(_{\text{Ca}}\) channels from myometrium had several open and closed time constants, like K\(_{\text{Ca}}\) channels from other tissues (Magleby and Pallotta, 1983; Moczydowski 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\textsubscript{ca} channel. The authors observed that increasing internal Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) gave rise to a third, longer, open distribution and proposed a third open state for the channel. This was also the case for K\textsubscript{ca} channels from myometrium, where a long open state was detected when the \( P_o \) was >0.38 (Table 1).

**G Protein Gating of K\textsubscript{ca} Channels**

The GTP + Mg\textsuperscript{2+} or GTP\textgamma\textsubscript{S} + Mg\textsuperscript{2+}-dependent stimulation of myometrial K\textsubscript{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 \( \beta \)-receptors by NE or isoproterenol. This mechanism was confirmed by the lack of activation of K\textsubscript{ca} channels with NE when GTP + Mg\textsuperscript{2+} or GTP\textgamma\textsubscript{S} + Mg\textsuperscript{2+} were absent in the cytoplasmic side. In accordance the NE- and GTP-dependent stimulation of the channel could be reversed by GDP\textgamma\textsubscript{S}, 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\textsuperscript{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 \( \rightarrow \) 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\textsuperscript{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\textsubscript{ca} channels can also be activated by phosphorylation (DePeyer et al., 1982; Farley and Rudy, 1988; Sadoshima et al., 1988a) and by arachidonic acid (Ordway et al., 1989). The variety of regulatory agents that modulate K\textsubscript{ca} channel activity may explain the diversity in Ca\textsuperscript{2+} sensitivities of K\textsubscript{ca} channels found in different tissues. Our data are consistent with this hypothesis, since GTP-dependent activation made K\textsubscript{ca} channels behave as if they had a higher Ca\textsuperscript{2+} sensitivity. It is interesting that GTP-dependent proteins also increase the Ca\textsuperscript{2+} sensitivity of phospholipase C (Gold et al., 1987; Taylor and Exton, 1987; McDonough et al., 1988). Thus, changing Ca\textsuperscript{2+} sensitivity may be a common expression of G protein activation of different Ca\textsuperscript{2+}-dependent enzymes, including Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels.

In our experiments, other possible mechanisms that could explain the GTP-dependent activation of myometrial K\textsubscript{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\textsubscript{ca} channels was potentiated by a \( \beta \)-agonist and inhibited by a \( \beta \)-antagonist, the possibility that we were activating a phospholipase \( \alpha \) and producing arachidonic acid to activate K\textsubscript{ca} channels was rather low (for review see Birnbaumer et al., 1987). Therefore, we conclude that GTP\textgamma\textsubscript{S}
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 \(\mu\)M) 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 \(\beta\)-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 \(\beta\)-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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