Reconstitution of the ATP-sensitive Potassium Channel of Skeletal Muscle

Activation by a G Protein–dependent Process

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ABSTRACT Potassium channels inhibited by adenosine-5′-trisphosphate, K(ATP), found in the transverse tubular membrane of rabbit skeletal muscle were studied using the planar bilayer recording technique. In addition to the single-channel properties of K(ATP) we report its regulation of Mg^{2+} and by the guanosine-5′-trisphosphate analogue, GTP-y(gamma)-S. The K(ATP) channel (*a*) has a conductance of 67 pS in 250 mM internal, 50 mM external KCl, and rectifies weakly at holding potentials more positive than 50 mV, (*b*) is not activated by internal Ca^{2+} or membrane depolarization, (*c*) has a permeability ratio $P_K/P_{Na} > 50$, and (*d*) is inhibited by millimolar internal ATP. Activity of K(ATP), measured as open channel probability as a function of time, was unstable at all holding potentials and decreases continuously within a few minutes after a recording is initiated. After a decrease in activity, GTP-y-S (100 μM) added to the internal side reactivated K(ATP) channels but only transiently. In the presence of internal 1 mM Mg^{2+}, GTP-y-S produced a sustained reactivation lasting 20–45 min. Incubation of purified *t*-tubule vesicles with AlF_4 increased the activity of K(ATP) channels, mimicking the effect of GTP-y-S. The effect of AlF_4 and the requirement of GTP-y-S plus Mg^{2+} for sustained channel activation suggests that a nucleotide-binding G protein regulates ATP-sensitive K channels in the *t*-tubule membrane of rabbit skeletal muscle.

INTRODUCTION

Potassium channels with partial or mild inward rectification have been described in numerous cell types including skeletal muscle (Spruce et al., 1985, 1987), heart cells (Trube and Hescheler, 1984), Hela cells (Sauvé et al., 1983), pancreatic-beta cells (Findlay et al., 1985), proximal kidney cells (Parent et al., 1988), and endothelial cells (Sauvé et al., 1988). The detectable levels of outward single-channel current measured in all these cases and the lack of a region of negative slope conductance in the current-voltage relationship, separates this K channel type from the strong

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inward rectifiers described in heart and skeletal muscle (Sackmann and Trube, 1984; Payet et al., 1985; Standen et al., 1984). In physiological solutions, mild inward rectifiers have single-channel conductances which in the outward direction are 10–20 pS and in the inward direction are 70–80 pS (for review, see Ashcroft, 1988). Some of these channels appear to share common ligand-gating properties. Cellular ATP is a potent blocker in skeletal (Spruce et al., 1985, 1987) and cardiac muscle (Noma, 1983; Kakei and Noma, 1984; Trube and Hescheler, 1984), and pancreatic-beta cells (Cooks and Hales, 1984; Findlay et al., 1985; Rorsman and Trube, 1985). Also, in two cases it was shown that rectification of the outward current depends on internal Mg^{2+} (Horie et al., 1987; Findlay, 1987). Thus the mechanism by which outward current is partially restricted in this type of K channel could involve blockade of the pore by cellular divalent ions. Clearly its ubiquity, distinct sensitivity to key cellular ligands, and possible linkage to the sulfonylurea receptor (Sturgess et al., 1985; Fosset et al., 1988) suggest that ATP-sensitive K channels, or K(ATP), belong to a separate class of proteins.

Two reports indicate that K(ATP) channels can be activated by guanine nucleotides in the insulin-secreting cells RIN5mF (Dunne and Petersen, 1986; Findlay, 1987). GTP (>10 μM) and GDP (>100 μM) evoke a dose-dependent channel activation that is reversible upon washout of the nucleotide (Dunne and Petersen, 1986). It was also demonstrated that millimolar internal Mg^{2+} was necessary to trigger channel activation (Findlay, 1987). The requirement for GTP and Mg^{2+} is significant because it raises the possibility of a direct gating of the K(ATP) channel by a guanine nucleotide–binding protein or G protein, as in the case of the muscarinic K channel of cardiac atrial cells (Breitweiser and Szabo, 1985, 1988; Pfaffinger et al., 1985; Kurachi et al., 1986*a* and *b*; 1987 Yatani et al., 1987*a*; Codina et al., 1987*a*; Logothetis et al., 1987, 1988; Cerbai et al., 1988) and of K channels in GH_3 pituitary cells (Codina et al., 1987*b*). In the absence of receptor occupancy by an appropriate agonist, G proteins can be activated by nonhydrolyzable analogues of GTP such as GTP-γ-S, Gpp[NH]p, Gpp[CH_2]p, or by the combined presence of F^- and Al^{3+} (Gilman, 1987; Brown and Birnbaumer, 1988). Breitweiser and Szabo (1988) have shown that in the absence of muscarinic agonist, GTP-γ-S is the most effective activator of the atrial K channel. Our finding of ATP-sensitive K channels in purified transverse tubules of rabbit skeletal muscle (Parent and Coronado, 1988) led us to undertake a reconstitution study of its gating mechanism including a possible participation of guanine nucleotide–binding proteins. In the present paper we describe the blocking effect of K(ATP) channels by ATP and Mg^{2+} and the activation by GTP-γ-S in the presence of Mg^{2+}. We suggest that this nucleotide-dependent activity is the result of a direct stimulation of a GTP-binding protein associated with the K(ATP) channel. Part of this work has been published in an abstract form (Parent and Coronado, 1988; 1989).

MATERIALS AND METHODS

Purification of Transverse Tubules of Rabbit Skeletal Muscle

Transverse tubules were purified by modification of the microsome fractionation procedure of Meissner (1984) that yields light and heavy membranes from *t*-tubules and junctional SR,

respectively. 150–200 g of rabbit back and hind leg muscle are homogenized in buffer A (0.3 M sucrose; 20 mM Hepes-Tris, pH 7.2) and centrifuged for 30 min at 2,600 *g* (4,000 rpm) in a GSA-Sorvall rotor (DuPont Co., Newton, CT). The 2,600 *g* supernatant is reserved and certifuged again for 30 min at 10,000 *g* (8,000 rpm) in a GSA-Sorvall rotor. After this second centrifugation, pellets are resuspended and briefly homogenized in 0.6 M KCl, 5 mM NaPipes, pH 6.8, with two strokes of a motor-driven Teflon/glass homogenizer. This material is incubated on ice for 1 h. Salt-treated microsomes are sedimented at 90,000 *g* (32,000 rpm) in a model 35 rotor (Beckman Instruments, Inc., Fullerton, CA) for 1 h. Supernatants are discarded and pellets are resuspended in 0.4 M KCl, 5 mM NaPipes, pH 6.8 (buffer C) plus 10% sucrose (wt/wt). This material is layered onto discontinuous sucrose gradients (8 ml of 20%, 8 ml of 25%, 6 ml of 30%, 4 ml of 40% wt/wt sucrose) prepared in the same buffer. Centrifugation is carried out overnight for 18 h at 26,000 rpm in a Beckman SW 27 rotor. Membranes sedimenting in the 10–20% sucrose interface were identified as *t*-tubules by the specific binding activity of the dihydropyridine [^{3}H]PN200-110 (Galizzi et al., 1986). The 10–20% sucrose fraction showed the highest PN200-110 binding capacity of ~40 pmol/mg protein (Valdivia and Coronado, 1989). Membranes were diluted with ice cold bi-distilled water, pelleted at 90,000 *g* (32,000 rpm), and resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM NaPipes, pH 6.8, to give a final concentration of ~ 5 mg protein/ml. Storage was done at −80°C.

Bilayer Formation and Incorporation of Channels

Lipid bilayers were formed by the painting method of Mueller-Rudin on Delrin cups containing apertures of 300 μm in diameter. Each chamber, designated *cis* (current amplifier side) and *trans* (ground side), have a volume of 3 ml. Bilayer-forming solution was composed of a 1:1 weight ratio mixture of brain phosphatidylethanolamine and brain phosphatidylserin (Avanti Polar Lipids, Birmingham, AL) dissolved in decane (Aldrich Chemical Co., Milwaukee, WI) at a final concentration of 20 mg/ml. All experiments were performed at room temperature. To incorporate channels, 20–30 μg of *t*-tubule protein was added under stirring into the 3-ml *cis* chamber. The *cis* chamber contained 250 mM KCl and the *trans* chamber contained 50 mM KCl (see Solutions). Channel activity appeared within 5–15 min after protein addition. The rate of channel incorporation could be increased by repetitively breaking and repainting the bilayer in the presence of *t*-tubule vesicles in the *cis* chamber. Presumably, this procedure allows vesicles initially fused into the thick lipid annulus (White, 1986) to be dragged into the bilayer region where channels can be actually recorded. K(ATP) channels were recorded in six of nine different preparations. In preparations where we failed to record K(ATP) channels we also failed to record K(Ca) and Bay K8644-dependent Ca channels. In each of the active preparations, K(ATP) channels were recorded in ~85% of vesicle-bilayer fusion attempts. K(ATP) channels were recorded in 95 independent experiments, and the time digitally stored and analyzed was 60 h.

Single-Channel Recording and Analysis

Recordings were performed using a model L/M EPC-7 patch-clamp amplifier (List Electronic Medical Systems, Greenvale, NY). *Cis* solution (voltage command side) was connected via an Ag/AgCl electrode and an agar/KCl bridge to the headstage input of the amplifier. *Trans* solution was held at ground potential using the same electrode arrangement. Records were low-pass filtered at a corner frequency of 100 Hz using an eight-pole Bessel filter (Frequency Devices, Springerfield, MA); digitized at 1 point/ms using a 12-bit resolution A/D converter (Keithley Instruments, Inc., Cleveland, OH); and fed into a PC/AT computer for analysis (IBM Instruments Inc., Danbury, CT). Holding potential corresponds to the potential

applied directly into the *cis* chamber after correction for the electrode offset potential. Mean channel amplitude and duration of single events were measured after separation of open and closed current levels using a dual threshold detector program (Coronado and Affolter, 1986*a*). A third threshold detector was used to reject simultaneous openings. Histograms of durations were constructed by plotting the fraction of events of duration t or longer than time t (cumulative distribution). Histograms were fitted using a least-square regression routine. To compute the activity of K(ATP) channels as a function of time, records were divided into segments of 75 s. In each segment we calculated the fraction of time that one or more channels were simultaneously open. This was done by constructing an amplitude histogram of all the sampled current in the segment (75,000 points per segment). Samples corresponding to baseline current were fit to a Gaussian curve and were subtracted from the histogram of total samples. The percent of remaining samples, that is, the fraction of samples that do not fall under the baseline peak, was plotted as "channel activity" in the corresponding figures. Thus plotted "channel activity" does not specify the number of channels recorded at any given time. The number of channels per record could not be determined in most cases because of the fast rundown and the low open probability of K(ATP) in the absence of GTP-y-S.

Solutions

Incorporation of channels was performed using a 250 mM KCl solution bathing the *cis* side (voltage command side) and a 50 mM KCl solution bathing the *trans* side (ground side) of the bilayer. Unless stated otherwise, *cis* and *trans* solutions contained 1 mM K_2-EGTA, 25 mM Hepes, 10 mM KOH, pH 7.4 (<10 nM free divalent). No EGTA was added to adjust Ca^{2+} concentration above 10 μM. The total K^+ concentration, including the contribution from the Hepes-KOH buffer, was 260 mM (*cis*) and 60 mM (*trans*). Free concentration of Mg^{2+} and Ca^{2+} was calculated as described by Fabiato and Fabiato (1979). Divalents were added as chloride salts. Permeability ratios were calculated using the Goldman-Hodgkin-Katz equation without correction for ionic activities. All salts were analytical grade (Alfa Products, Danvers, MA). Glyburide was a gift of Dr. Lydia Navarro-Bryan at Baylor College of Medicine. Nucleotides (ATP, GTP-y-S, GTP, and ATP-y-S) were purchased from Sigma Chemical Co. (St. Louis, MO) as Na^+ or Li^+ salts and were shipped on dry ice. Stock solutions of nucleotides were buffered to pH 7.3 with either Hepes-KOH or MOPS-KOH and stored in liquid N_2 (−80°C) until use.

RESULTS

Separation of K(ATP) from K(Ca) Channels

Recordings in planar bilayers have shown that transverse tubules of rat or rabbit skeletal muscle contain at least four channel types: a large conductance calcium-activated potassium channel, K(Ca), (Latorre et al., 1982), chloride channels (Coronado and Affolter, 1986*b*; Valdivia and Coronado, 1989), dihydropyridine-sensitive calcium channels (Affolter and Coronado, 1985; Ma and Coronado, 1988), and tetrodotoxin-sensitive sodium channels (Moczydlowski et al., 1984). In the case of Ca and K(Ca) channels the reported sidedness of insertion is *cis*-intracellular (Latorre et al., 1982; Coronado and Affolter, 1986*b*) but mainly *trans*-intracellular for Na channels (Moczydlowski et al., 1984). Fig. 1 shows overlapped openings of two types of K channels routinely recorded in our preparation of t-tubules at vari-

ous *cis*-free Ca^{2+} concentrations. In this experiment and those that follow, the KCl concentration was 250 mM in the *cis* solution and 50 mM in the *trans* solution. A large conductance channel, described in Fig. 2 as K(Ca), could be recorded at positive potentials, provided that the Ca^{2+} concentration was >10 μM. At 0.1 μM Ca^{2+} and lower, a channel of much lower conductance was the most frequently observed. We refer to the latter as the K(ATP) channel due to the inhibitory effect of this nucleotide (see Fig. 10). Therefore, lowering of *cis* Ca^{2+} was sufficient to separate K(Ca) from K(ATP). Fig. 2 confirms that the large conductance channel is the maxi K(Ca) channel described in other preparations of *t*-tubules and is inserted in our system with the myoplasmic face in the *cis* chamber. A current-voltage curve is shown in Fig. 1 *A*. The slope conductance was 327 pS (SD = 10 pS, n = 8) and the reversal potential averaged −36 mV (E_{Cl} = +37 Mv, E_K = −37 mV). Both parame-

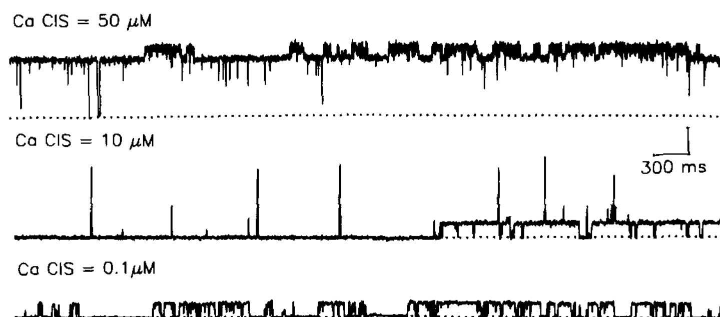

FIGURE 1. Simultaneous recordings of K(Ca) and K(ATP) channels in rabbit *t*-tubules incorporated into planar bilayers. Single-channel activity was measured in *cis* 250 mM, *trans* 50 mM KCl. Both solutions were buffered to pH 7.4 with 25 mM Hepes, 10 mM KOH. *Cis* free Ca^{2+} concentration is indicated in each record. Free Ca^{2+} was adjusted using 1 mM K_2-EGTA and 1 mM $CaCl_2$ (10 μM free Ca^{2+}) or 1 mM K_2-EGTA plus 0.87 mM $CaCl_2$ (0.1 μM free Ca^{2+}). No EGTA was used to adjust the 50 μM Ca^{2+} solution. Opening probability for the large conductance K(Ca) channel was 0.96 at 50 μM Ca, 0.01 at 10 μM Ca, and not measurable in 0.1 μM Ca. Holding potentials are +18 mV (*top*), 0 mV (*center*) 0 mV (*bottom*). Calibration bars are 8 pA, 300 ms (*top*) and 4 pA, 300 ms (*center and bottom*). Dotted line indicates baseline.

ters were independent of Ca^{2+} in the range of 5–60 μM. Fig. 2 *B* and records in Fig. 2, *C* and *D*, show that K(Ca) is gated into the open state by *cis* positive potentials and by *cis* micromolar Ca^{2+}. This interrelated dependence of *cis* Ca^{2+} and voltage is similar to that described in detail by Moczydlowski and Latorre (1983) in other preparations of *t*-tubules. In all membrane preparations tested (n = 9), *trans* Ca^{2+} had no effect on open probability. Thus the polarity of insertion was consistently *cis*-intracellular. The fact that K(Ca) and K(ATP) channels were incorporated simultaneously into the bilayer most of the time suggested that both channels were present in the same vesicle. We therefore assumed that the internal side of both channels faced into the *cis* solution. This sidedness was further supported by experiments in which K(ATP) was blocked by *trans*-added glyburide (not shown), a sulfonylurea that blocks K(ATP) channels extracellularly (Sturgess et al., 1985).

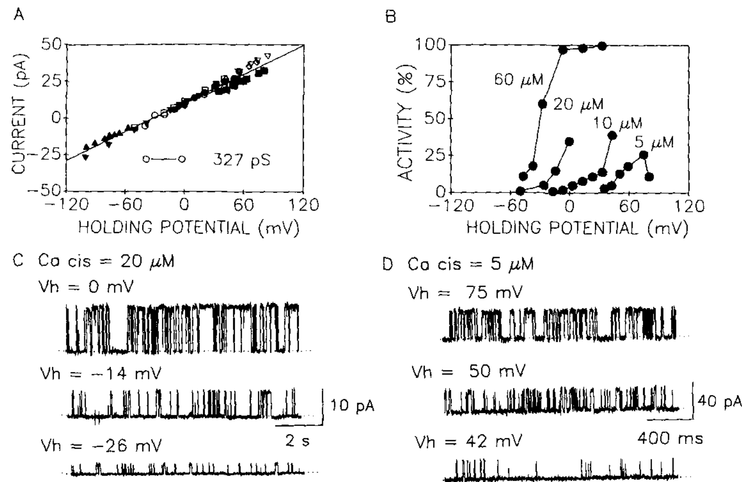

FIGURE 2. Properties of K(Ca) channels of rabbit *t*-tubules. (*A*) Current vs. voltage curve measured in *cis* 250 mM, *trans* 50 mM KCl. Mean slope conductance given by the solid line was 327 pS (SD = 10 pS, $n = 8$). Each symbol is for a separate experiment. (*B*) Voltage dependence of open probability at the indicated concentrations of *cis* free Ca. Po corresponds to the open probability of a single channel. (*C* and *D*) Records at indicated holding voltage in *cis* 20 μM and 5 μM Ca. Average fraction of open time in *C* was 35% at 0 mV, 15% at −14 mV, and 5% at −26 mV. Average fraction of open time in *D* was 25% at +75 mV, 19% at +50 mV, and 3% at +42 mV. Records were filtered at 1 kHz. Dotted line indicates baseline.

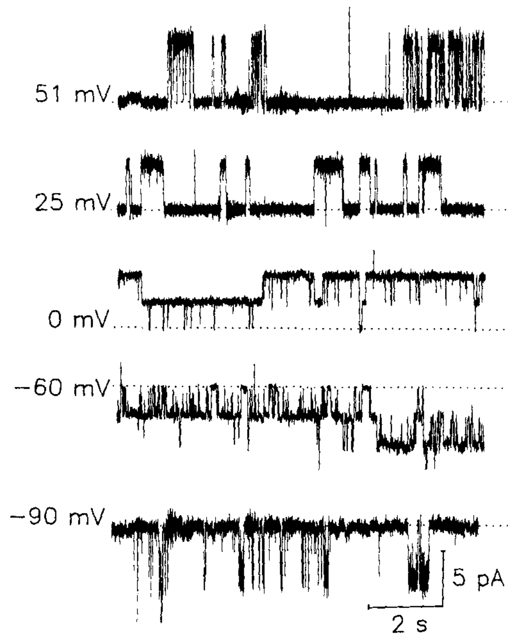

FIGURE 3. Records of K(ATP) channels of rabbit *t*-tubules. Unitary currents of K(ATP) channels measured in *cis* 260 mM; *trans* 60 mM KCl plus 1 mM K_2-EGTA on both sides are shown at five holding voltages. Average amplitudes were −4.5 pA at −90 mV and 5.7 pA at +50 mV. Records were filtered at 100 Hz.

Properties of K(ATP) Channels in Planar Bilayers

Fig. 3 shows records of K(ATP) channels in KCl solutions containing 1 mM EGTA (<10 nM free Ca^{2+}). Under these conditions, the open probability of K(Ca) was extremely low at all holding potentials, <1% open time. K(ATP) is characterized by a complex kinetics composed of bursts of opening lasting up to 2 s followed by closed periods of similar lengths. Openings occurred spontaneously at 0 mV and were not triggered by positive or negative potentials. Fig. 4 *A* shows that current amplitudes of K(ATP) are in the range of −4 pA at −90 mV to 5 pA at 50 mV with a mean slope conductance within this range of 67 pS (SD = 2, n = 59). The mean reversal potential was −33 mV (SD = 1 mV, n = 59), which indicated a cationic selectivity with a close-to-zero chloride permeability. Selectivity towards K^+ was confirmed using a high concentration of NaCl in the *trans* side. In an ionic gradient composed

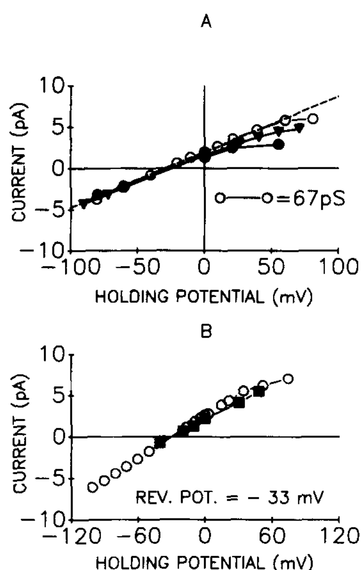

FIGURE 4. Current-voltage relationship of K(ATP) channels. (*A*) Current vs. voltage curves in *cis* 250 mM, *trans* 50 mM KCl in the presence of *cis* 1 mM K_2-EGTA and <10 nM free Ca^{2+} (*open circles*), or *cis* 10 μM free Ca^{2+} (*solid triangles*) or *cis* 10 mM Mg^{2+} (*solid circles*). The mean slope conductance at negative voltages was 67 pS (SD = 2, n = 59). *Cis* 10 mM Mg^{2+} decreases slope conductance at positive voltages (+75 mV) from 53 to 28 pS. (*B*) Current vs. voltage curve in *cis* 250 mM KCl, *trans* 50 mM KCl (*open circles*) and after addition of 171 mM NaCl to the *trans* side (*solid squares*). The reversal potential was interpolated to −33 mV (SD = 2 mV) in either case.

of *cis* 260 mM K^+, 0 mM Na^+, and *trans* 60 mM K^+, 171 mM Na^+ the reversal potential averaged −33 mV (Fig. 4 *B*, solid squares). This value was experimentally the same as that measured in the absence of *trans* NaCl (Fig. 4 *B*, open circles). Accordingly, the permeability ratio (PK/PNa) was estimated to be >50, considering an experimental error of ~ ±2 mV associated with the determination of reversal potential. Thus the K(ATP) channel in *t*-tubules is impermeable to Cl^- and Na^+ ions. In high Na^+, the current vs. voltage curve rectifies slightly at holding potentials more positive than +60 mV. The slope conductance at this potential is ~20 pS. We noticed that the direction of the rectification in Na^+ is opposite to that expected on the basis of the K^+ gradient alone. Horie et al. (1987) showed that millimolar internal Mg^{2+} is responsible for the rectification of the ATP-sensitive K channel of cardiac cells. In our preparation, bending of the current vs. voltage curve is observed

in the presence of *cis* 1 mM EGTA, that is, in nominally Ca^{2+}- and Mg^{2+}-free solutions (Fig. 4 *A*, open circles). Addition of *cis* 1 mM $CaCl_2$ in the presence of 1 mM EGTA, which resulted in 10 μM free Ca^{2+} (Fig. 4 *A*, solid triangles), produced a slight nonlinearity at positive potentials, whereas 10 mM $MgCl_2$ (9.8 mM free Mg^{2+}) (Fig. 4 *A*, solid circles) significantly reduced the conductance for outward currents. The effect of internal Mg^{2+} is similar to that reported by Findlay (1987) in RIN5mF cells, where a slight rectification of K(ATP) can be observed in 0.1 μM internal Mg^{2+}. However, in our hands, *cis* millimolar Mg^{2+} does not significantly decrease channel activity as it does in the case in RIN5mF cells (Findlay, 1987).

The fraction of open time of K(ATP) channels was found to vary widely in separate experiments, typically from 10 to 60%. Activity was always higher at the beginning of the recording and decreased thereafter, similar to K(ATP) channels of pancreatic-beta and heart cells after patch excision (Ohno-Shosaku et al., 1987). In Fig. 5 we plotted the time course of open probability in four bilayers (labeled 1–4) formed in the same solutions. *T*-tubule protein was added to the *cis* chamber at $t = 0$, and each new membrane was formed after breaking the previous one in the presence of the same suspension of *t*-tubules. Blanks separating bilayers 1–4 account for the time required to incorporate K(ATP) channels. Activity in all cases decreased to essentially zero within 6 min (SD = 1 min, n = 22). This phenomenon, herein referred to as channel rundown, was not a function of the incubation time of *t*-tubules in the *cis* chamber. As shown in the records in Fig. 5, rundown occurred in bilayer 1 (2-min incubation) and in bilayer 4 (40-min incubation) with approximately equal rates. Thus, rundown is unlikely to reflect a nonspecific denaturation of *t*-tubule protein when added to the *cis* chamber. This observation also suggests that

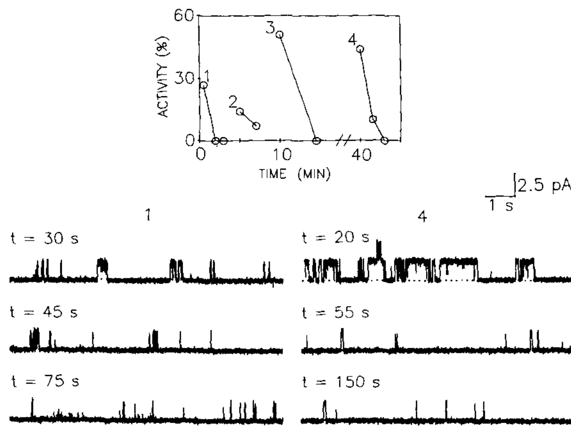

FIGURE 5. Spontaneous rundown of K(ATP) channels. Channel activity as a function of time is shown in four separate bilayers formed with the same suspension of *t*-tubules added to the *cis* solution at $t = 0$. Bilayers were formed sequentially (1–4). Single channels from bilayers 1 and 4 are shown in the bottom panel. Records of bilayer 1 correspond to 30, 45, and 75 s, after incorporation at $t = 0$. Records of bilayer 4 correspond to 20, 55, and 150 s after incorporation at $t = 0$. The number of observable channels was one in bilayers 1, 2, and 3; two in bilayer 4. Holding potential was 0 mV.

K(ATP) channels remain functional in the native *t*-tubule membrane for periods much longer than those observed after their incorporation into the planar bilayer. In addition, rundown did not seem to be a consequence of the membrane fractionation procedure because under similar conditions the activity of the K(Ca) channel remained constant in time (not shown). The fraction of open time immediately after incorporation and the rate of rundown varied widely among preparations. In the two most extreme preparations, the fraction of open time immediately after incorporation varied from 5% (SD = 3, n = 5) to 65% (SD = 10, n = 6), and the time until complete rundown varied from 2 min (SD = 1, n = 5) to 9 min (SD = 3, n = 6).

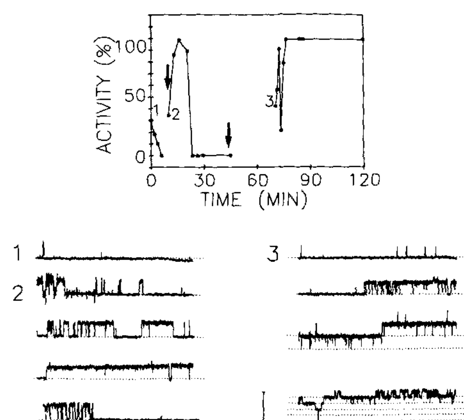

FIGURE 6. Rundown and reactivation of K(ATP) channels by GTP-y-S. Upper panel shows channel activity as a function of time. Bilayers were formed sequentially (1–3) after addition of *t*-tubules to the *cis* solution at t = 0. Channel of bilayer 1 (control) was recorded in the presence of *cis* 1 mM K_2-EGTA. Channel rundown was complete at 2 min. At t = 10 min (*arrow*), 100 μM GTP-y-S was added to the *cis* chamber immediately after channel incorporation into bilayer 2. At t = 45 min (*arrow*), 1 mM $MgCl_2$ and 1 mM $CaCl_2$ were added to the *cis* solution. *Cis* free Mg^{2+} was ~1 mM. Bilayer 3 was formed in presence of *cis* 100 μM GTP-y-S added at t = 10 min and *cis* 1 mM free Mg^{2+} added at t = 45 min. Records from each bilayer are shown in the lower panel. The number of observable channels was one in bilayers 1 and 2; five in bilayer 3. Calibration bars are 4 pA, 2 s for the first seven segments and 8 pA, 2 s for the last segment of bilayer 3.

Activation of K(ATP) Channels by GTP-y-S

In K(ATP) channels of pancreatic-beta cells, rundown has been attributed to a depletion of MgATP necessary to keep channels in a phosphorylated and therefore functional state. In these cells, perfusion of millimolar MgATP for a few minutes was sufficient to reactivate K(ATP) (Findlay and Dunne, 1986; Ohno-Shosaku et al., 1987). In our preparation, preincubation of membrane fractions with millimolar MgATP for 20–60 min did not prevent channel rundown. However, GTP-y-S had a profound effect on channel activity. Fig. 6 shows the time course of open probability and the corresponding channel traces in three separate bilayers (labeled 1–3). K(ATP) channels were incorporated into each bilayer using the same suspension of *t*-tubules added at t = 0 to the *cis* chamber. A control channel in bilayer 1 had an initial activity of ~32% and complete rundown occurred at 6 min. Addition of *cis* 100 μM GTP-y-S (arrow at 10 min) to a separate channel incorporated into bilayer 2 resulted in activity that rapidly increased to ~100% and decreased thereafter. After this transient activation, the fraction of open time remained zero (i.e., channel did

not reopen) for the next 25 min. In bilayer 3 a third set of channels was incorporated after addition of 1 mM Mg^{2+} to the *cis* solution (arrow at 45 min). In this case there were at least six channels activated which kept the overall percentage of activity close to 100% until bilayer breakdown. From this result we conclude that 100 μM GTP-y-S without added Mg^{2+} opens K(ATP) channels transiently but a sustained activation required millimolar Mg^{2+}.

The combined effect of GTP-y-S and Mg^{2+} was further explored in the time course of Fig. 7 and the corresponding channel traces. In this experiment four bilayers (labeled 1–4) were formed in the same *cis* and *trans* solutions and all channels were recorded from the same suspension of *t*-tubules added at $t = 0$. In bilayer 1 of this sequence (control) we show that activity in the absence of nucleotide

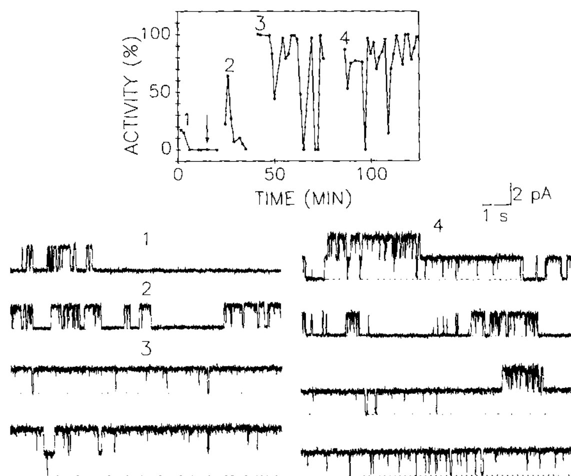

FIGURE 7. Sustained activation of K(ATP) channels. Bilayers 1–4 were formed sequentially in the same *cis*, *trans* solutions. Activity in bilayer 1 (control) was measured in presence of 1 mM K_2-EGTA and no added divalent cations. Channel rundown was complete at 3 min. At the time marked by the arrow ($t = 20$ min), 100 μM GTP-y-S, and 1 mM free Mg^{2+} (1 mM $MgCl_2$ plus 1 mM $CaCl_2$) were added simultaneously to the *cis* chamber. Channel activity was afterwards recorded for 2 h in three separate bilayers (2–4). Records from each bilayer are shown in the lower panel. The number of observable channels were one in bilayers 1 and 2, two in bilayers 3 and 4.

decreased to zero within 5 min. After complete rundown GTP-y-S and Mg^{2+} were added to bilayer 1 (arrow at 20 min). Clearly, this addition did not reactivate the channel of bilayer 1. However in bilayer 2, 12 min after the addition of nucleotide, a freshly incorporated channel had a peak activity higher than that observed in control. In bilayer 3, 27 min after the addition, the average fraction of open time of a third channel further increased to ~70%. Finally, incorporation of channels into bilayer 4 resulted in a sustained level of activity of ~90% which lasted 45 min. Thus, sustained activation by nucleotide and Mg^{2+} developed slowly in time. Activated channels alternated between intervals of high activity with numerous overlaps of openings and intervals of low activity where no more that one channel was open at any given time. Sustained activation of K(ATP) by GTP-y-S and Mg^{2+} was repro-

duced in 16 experiments from three different preparations even though the average level of activation varied significantly in each preparation. For instance the bar histograms in Fig. 8 *A* show that in three preparations, the average time until complete rundown was 2, 6, and 9 min before and 159, 27, and 69 min after incubation with GTP-y-S plus Mg^{2+}, respectively. Despite this variability, the sustained activation by GTP-y-S plus Mg^{2+} was nucleotide-specific and Mg^{2+}-dependent. In Fig. 8 *B* we computed the mean and standard deviation of the total recorded time in which channel activity was 10% or higher under various conditions. The nonhydrolyzable adenine nucleotide ATP-y-S (100 μM) plus Mg^{2+} (lane 4) or 100 μM GTP-y-S without Mg^{2+} (lane 3) were ineffective in activating channels above control levels (lane 1). Each of these combinations produced an average activity not significantly higher than that of control and ~10-fold lower than that measured in the presence of GTP-y-S plus Mg^{2+} (lane 2). Finally, Fig. 9 demonstrates that 100 μM GTP-y-S plus 1 mM Mg^{2+} had no significant effect on the single-channel current vs. voltage characteristics. This strongly suggested that only one channel type, the K(ATP) channel, was present in control and after nucleotide activation.

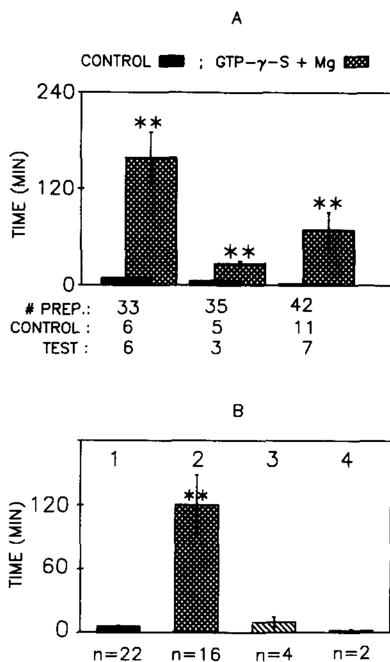

FIGURE 8. Time histograms of K(ATP) channel activity. (*A*) Total recording time before complete channel rundown in three preparations of *t*-tubules (preparation Nos. 33, 35, and 42) is compared in control (*solid bars*) and after activation by 100 μM GTP-y-S and 1 mM Mg^{2+} (*hatched bars*). In preparations 33, 35, and 42, control activity lasted 9 min, 6 min, and 2 min whereas the GTP-y-S plus Mg^{2+} activity lasted 159 min, 27 min, and 69 min. Number of experiments in control and activated (test) channels are indicated under each preparation. (*B*) Mean and SD of time during which channel activity was 10% or higher under four test conditions. (*Column 1*) Spontaneous activity in 1 mM K_2-EGTA (t = 6 min). (*Column 2*) Activation by 100 μM GTP-y-S plus 1 mM Mg^{2+} (t = 120 min). (*Column 3*) Activation by 100 μM GTP-y-S plus 1 mM K_2-EGTA (t = 10 min). (*Column 4*) Activation by 100 μM ATP-y-S plus 1 mM Mg^{2+} (t = 2 min).

Activation of K(ATP) Channels by AlF_4^-

The requirement for GTP-y-S and Mg^{2+} to elicit activation of K(ATP) strongly suggested the participation of a *t*-tubule G protein as a cofactor for opening the channel (Gilman, 1987; Birnbaumer et al., 1987). This possibility was supported independently by experiments in which we activated K(ATP) channels with AlF_4^- (Fig. 10). Millimolar AlF_4^- is thought to activate G proteins by mimicking the action of the gamma-phosphate of GTP on the alpha subunit (Bigay et al., 1985; Cockcroft, 1987;

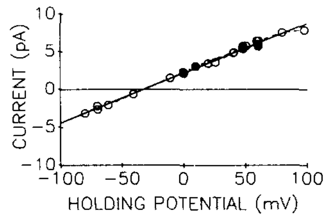

FIGURE 9. Current-voltage relationship of GTP-γ-S activated channels. Current vs. voltage curve measured first during the control period (bilayer 1 of Fig. 7, *solid circles*) and following the sustained activation phase (bilayers 2–4 of Fig. 7, *open circles*). Solid line is the best-fitted linear regression.

Gilman, 1987). *T*-tubule fractions were preincubated in presence of 4 mM AlF_4^- for 30 min at room temperature. Aliquots were then added into the *cis* chamber, where the large volume of solution lowered the actual concentration of AlF_4^- to the micromolar range. Channel activity in membranes treated with AlF_4^- was compared to the activity from the same preparation in control conditions. One set of such experiments is shown in Fig. 10. The control activity reached a maximum of 40% before total rundown at 10 min (no openings were seen in the following 40 min). In the channel treated with AlF_4^- there was a sustained activation which developed slowly and lasted until membrane breakdown at 40 min. Interestingly, this sustained activity was observed in spite of the fact that the actual concentration of AlF_4^- in the *cis* chamber was 25 μM, suggesting that the effect of AlF_4^- is essentially irreversible. Unlike what was observed with GTP-γ-S, the sustained activity elicited by AlF_4^- was not significantly different from the peak control activity seen shortly after channel incorporation. From a total of 112 control events (experiment shown in Fig.10 *A*) and 553 events collected after activation by AlF_4^- (experiment shown in Fig. 10 *B*) the mean lifetimes of channels were 53 and 68 ms, respectively. Thus it appears that AlF_4^- decreases rundown but it does not activate channels per se.

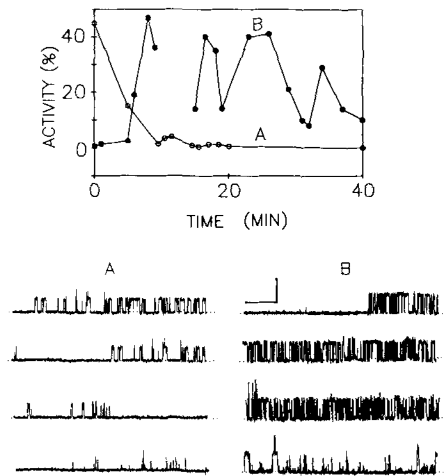

FIGURE 10. Activation of K(ATP) channels by AlF_4^-. Upper panel shows comparative time course of channels after 30 min of incubation in the absence (*A*) or presence of 4 mM AlF_4^- (*B*). *T*-tubules at a protein concentration of 5–10 mg/ml were incubated at room temperature in 4 mM AlF_4^-, 1 mM $MgCl_2$, 50 mM KCl, 25 mM Hepes, 10 mM KOH, pH 7.4. Control was the same without AlF_4^-. Aliquots were afterwards added to the *cis* chamber in solutions described in Fig. 3. In trace *B* the actual concentration of AlF_4^- in the *cis* chamber was 25 μM. Lower panel shows the corresponding records at 0 mV holding voltage. Calibration bars are 4 pA, 2 s. For each of the records in the lower panel, the fraction of open time was 30, 11, 5, 1% in (*A*); and 10, 30, 40, 8% in (*B*). Two bilayers were formed in (*B*) and a single bilayer in (*A*). Records were filtered at 100 Hz.

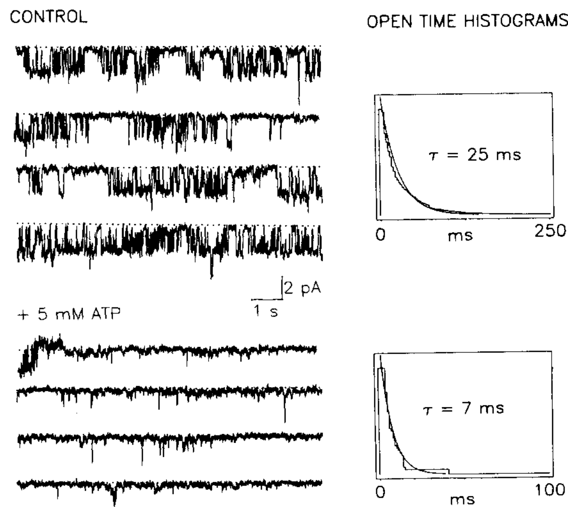

FIGURE 11. Inhibition of K(ATP) channels by ATP. Spontaneous channel activity measured at −60 mV before (*top*) and after (*bottom*) the addition of 5 mM ATP-KOH, pH 7.4, to the *cis* side. Dotted lines indicate baseline. Channel openings are downward deflections. Fraction of open time averaged 67% in control and 3% after 10 s following nucleotide addition. Calibration bars are 2 pA and 1 s. Histograms and best fitted times constants are for 393 events before and 13 events after addition of ATP.

Most Significant Fingerprint of the K(ATP) Channel: Inhibition by ATP

The most significant fingerprint characteristic of K(ATP) channels was the inhibitory effect of ATP. To separate the inhibition by ATP from the rundown, we investigated ATP blockade in experiments in which activity was >30% before the addition of ATP (Fig. 11) and in channels activated by GTP-y-S and Mg^{+2}, where rundown was a minimum (Fig. 12). In Fig. 11, channels were recorded at −60 mV for 1 min, and the fraction of open time during this period was ~67%. This was immediately followed by addition of 5 mM ATP (neutralized to pH 7.4 with KOH) to the *cis* solution. Channel activity decreased to ~4% within the mixing time of the chamber, which was ~10 s. On the average, addition of 1–5 mM ATP to the *cis* chamber

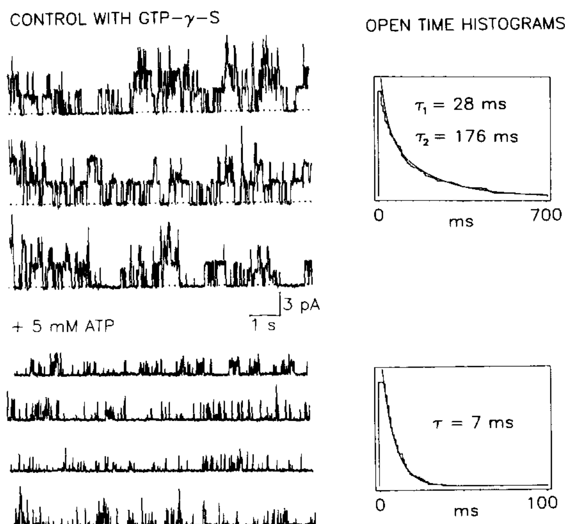

FIGURE 12. Inhibition of GTP-y-S activated K(ATP) channels by ATP. GTP-y-S-activated channels at +27 mV before (*top*) and after (*bottom*) addition of *cis* 5 mM ATP-KOH pH 7.4. Dotted lines indicate baseline. There were three active channels in the bilayer during the control period. Channel activity was 72% in control and 6% at 20 s after nucleotide addition. Calibration bars are 3 pA and 1 s. Histograms and best fitted time constants are for 250 events before, and 74 events after addition of ATP.

blocked 80–100% of channel activity within 30 s, whereas the spontaneous rundown at 30 s was ≤10% (see Fig. 5). Distribution of lifetimes for the same experiment are shown in the right top and bottom panel of Fig. 11. The mean open time decreased from 25 to 7 ms upon blockade by ATP. Averaged over several experiments, open lifetime was 30 ± 2 ms (n = 5, 1,370 events) in control, and 7 ± 1 ms (n = 3, 137 events) after addition of 5 mM ATP. In a second set of experiments shown in Fig. 12, 5 mM ATP blocked channels activated by GTP-y-S. As in the case of spontaneous activity, blockade occurred within 10–20 s. Thus the time course of inhibition by ATP is distinct from the time course of rundown. Lifetime distributions of Fig. 12 show that GTP-y-S activated two types of channels (or two open states). There are brief events of duration similar to that of control channels (shown in Fig. 11) and long events not seen in control (28 ms, 176 ms, n = 250 events). Evidently, ATP blocked both types of openings decreasing the lifetime to 7 ms (n = 74 events). Decrease in channel lifetime was also reported in K(ATP) of frog muscle (Spruce et al., 1985) and rat cultured central neurons (Ashford et al., 1988). This result strongly suggests that K(ATP) recorded in planar bilayers is a bona fide ATP-sensitive channel.

DISCUSSION

We investigated for the first time the properties of the reconstituted ATP-sensitive K^+ channel of the transverse tubular membrane of rabbit skeletal muscle. When measured in a gradient of 250 and 50 mM KCl without added divalents, the K(ATP) channel is described by a current vs. voltage curve with a mean slope conductance of 67 pS within −50 to +50 mV and a slight rectification at positive potentials (Fig. 4). This conductance is higher than that reported for the ATP-sensitive channel in frog skeletal muscle (42 pS, Spruce et al., 1987) but lower than that of cardiac cells (80 pS, Trube and Hescheler, 1984). The slope conductance for current in the outward direction decreased from 53 p to 28 pS in the presence of 10 mM free Mg^{2+}. Internal Mg^{2+} in the same concentration range also increased rectification of K(ATP) in cardiac cells (Horie et al., 1987) and in RIN5mF cells (Findlay, 1987). Unlike the K(ATP) channel in frog muscle (Spruce et al, 1987) we did not find a consistent relationship between channel activity and membrane potential, a conclusion also reached by Kakei and Noma (1984) for K(ATP) channels in the heart preparation. The sensitivity of the reconstituted channel to ATP is within the range previously reported in muscle cells. The IC_{50} for ATP varies from 10 μM in an insulin-secreting cell line CRI-G1 (Sturgess et al., 1986) to 500 μM in cardiac atrial cells (Kakei and Noma, 1984). In our hands, 3 mM ATP is required to decrease activity 10-fold (Fig. 4). Thus we estimate the IC_{50} to be ~400 μM ATP, assuming a simple isotherm binding for the nucleotide.

Findlay (1987) and Dunne and Petersen (1986) in the insulin-secreting cell line RIN5mF showed that perfusion of the inner face of the membrane patch with GTP-y-S in presence of millimolar Mg^{2+} led to the reactivation of some of the K(ATP) channels whose activity had previously decayed. These results lead us to test GTP-y-S in K(ATP) of rabbit t-tubules. When incorporated in planar bilayers the most obvious kinetic feature of K(ATP) is the spontaneous loss of channel activity and its

recovery after incubation with GTP-y-S and Mg^{2+}. The latency of activation by GTP-y-S (15–20 min) was consistent with results in vivo. In atrial K^+ channels, activation by GTP analogues varies from 3 to 15 min when applied in the absence of receptor occupancy by an agonist (Kurachi et al., 1986*a*; Kurachi et al., 1987; Breitwieser and Szabo, 1988). To explain our results we first considered the suggestion of Paiement (Paiement, 1984*a* and *b*) that GTP could facilitate fusion of membranes within cells. These studies showed that fusion between endoplasmic reticulum and nuclear membranes could be induced by 500 μM GTP. In our case, a fusion effect of GTP-y-S would have resulted in an increase in the number of channels incorporated into the planar membrane and an increase in the "observable" fraction of open time. In such a case, channel activity should have increased in a monotonic fashion as function of time as more channels are fused into the bilayer in the constant presence of *cis* nucleotide. Several observations clearly indicate that GTP-y-S does not induce fusion in our system. (*a*) In a few cases where the number of channels was estimated from binomial fit of n and p, the mean of n (number of active channels) was approximately the same before and shortly after the addition of nucleotide plus Mg^{2+}. (*b*) GTP-y-S plus Mg^{2+} does not increase the number of incorporated K(Ca) channels which are in the same vesicle population. (*c*)The effect of GTP-y-S was dependent on the activity of K(ATP), i.e., no activation is observed after complete rundown, an observation which would be difficult to reconcile with a fusion model.

We favored instead the model first proposed for atrial potassium channels and dihydropyridine calcium channels (Breitweiser and Szabo, 1985; Pfaffinger et al., 1985; Yatani et al., 1987*b*; Imoto et al., 1988) of direct gating of K(ATP) by a guanine nucleotide–binding protein or G protein. In such a model, GTP-y-S is believed to play two major roles (Gilman, 1987). In the absence of any receptor stimulation, GTP-y-S promotes the dissociation of the alpha subunit from the beta-gamma anchor of the G protein by displacing GDP from its binding site on the alpha subunit. Because of its nonhydrolyzable properties, GTP-y-S maintains the alpha subunit in the active state for a longer time than GTP. In the absence of Mg^{2+} the rate of dissociation of the GTP-y-S from the alpha subunit is relatively high (0.4 min^{-1} for Go and 0.2 min^{-1} for Gi; Gilman, 1987). However, in the presence of Mg^{2+} the complex formed by GTP-y-S and the alpha subunit is stabilized. Thus both are required for sustained channel activation in the absence of stimulation by agonists. Channel rundown could thus be a consequence of uncoupling of the G protein from the channel upon fusion of *t*-tubule vesicles into the artificial membrane. Once the G protein diffuses away from the channel, GTP-y-S plus Mg^{2+} would be rendered ineffective because the density of G proteins and channels in the planar bilayer is too low to permit reaggregation. Thus it appears that while the channels are in the native membrane and shortly after incorporation into the bilayer, the G protein, GTP-y-S, and the K(ATP) channel are intimately associated.

Of five G proteins described at the present time, Gs (stimulatory protein for adenylyl cyclase), Gi (inhibitory protein for adenylyl cyclase), Go (other G protein), Gt (transducin), and Gk (stimulatory protein for the atrial K^+ channel), probably Gs and Gi are present in transverse tubules (Scherer et al; 1987). G proteins are identified by their capacity to be ADP-ribosylated by a toxin from Bortedella pertussis (PTX) or Vibrio Cholera toxin (CTX). Gi and Go are specifically covalently modified

by PTX; Gs_1 and Gs_2 are substrates for CTX, whereas Gt is modified by both toxins. In *t*-tubule membranes, both cholera toxin and pertussis toxin labeling resolved two substrates that may indicate the presence of both Gs and Gi. The labeling by PTX and CTX substrates is higher in the transverse tubular membrane than in the sarcoplasmic reticulum membrane of skeletal muscle (Scherer et al; 1987). Gs has been recently shown to stimulate dihydropyridine-sensitive calcium channels in transverse tubules (Yatani et al., 1988), so it could also gate the K(ATP) channel. Our results in vitro suggest that the K(ATP) channel of skeletal muscle is intrinsically coupled to a guanine nucleotide signaling system.

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