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Effect of Adenosine and Intracellular GTP on K_{ATP} Channels of Mammalian Skeletal Muscle

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Abstract. We investigated the action of adenosine and GTP on K_{ATP} channels, using inside-out patch clamp recordings from dissociated single fibers of rat flexor digitorum brevis (FDB) skeletal muscle. In excised patches, K_{ATP} channels could be activated by a combination of an extracellular adenosine agonist and intracellular Mg^{2+}-ATP and GTP or GTP-γ-S. The activation required hydrolyzable ATP and could be partially reversed with Mg^{2+}, suggesting that it may involve a G-protein dependent phosphorylation of K_{ATP} channels. We found that K_{ATP} channels of the rat FDB could not be activated by Mg^{2+}-ATP alone or by Mg^{2+}-ATP in the presence of extracellular adenosine. Patches whose channel activity had been 'rundown' by Ca^{2+} could not be recovered by adenosine, GTP or Mg^{2+}-ATP. K_{ATP} channels activated by adenosine receptor agonists had a similar ATP sensitivity to those under control conditions; but adenosine appears to be able to switch these K_{ATP} channels from an inactive to an active mode.

Key words: Potassium channel — Adenosine — Adenosine triphosphate (ATP) — Skeletal muscle

Introduction

ATP-dependent potassium channels (K_{ATP} channels) occur in several tissues including muscle, pancreatic beta cells, some neurones and epithelia (Ashcroft & Ashcroft, 1990). In many of these tissues, it is unlikely that changes in intracellular ATP concentration ($[ATP]_i$) form the major regulator of channel activity under physiological conditions, since $[ATP]_i$ often changes little except under conditions of severe metabolic stress. Rather, ATP binding may set a low background open probability against which other regulators, including signalling systems linked to G-protein coupled receptors, serve to control channel activity. In skeletal muscle, ATP is especially well buffered by creatine phosphate and creatine kinase (Carlson & Siger, 1960), and it seems certain that regulators other than ATP are involved in the physiological control of K_{ATP} channel activity: one of these may be intracellular pH (Davies, Standen & Stanfield, 1992). It has also been suggested that adenosine released from active muscle fibers under conditions of systemic hypoxia opens K_{ATP} channels, to increase K^+ efflux and cause a rise in extracellular $[K^+]$ and so vasodilation (Marshall, Thomas & Turner, 1993; Comtois et al., 1994). Receptors for adenosine have been shown to be present in the sarcolemmal membrane of skeletal muscle fibers (Challiss, Richards & Budohoski, 1992) and the activation of K_{ATP} channels by adenosine has been demonstrated in both cardiac muscle and coronary arterial smooth muscle (Kirsch et al., 1990; Dart & Standen, 1993). In cardiac cells, the link between the receptor and the channel apparently occurs via G_i, since the application of $G_{\alpha i}$-subunits to the cytoplasmic face of the membrane activates K_{ATP} channels (Terzic et al., 1994).

Regulation of K_{ATP} channels by receptors coupled to G proteins has not so far been demonstrated in skeletal muscle, though GTP-γ-S has been reported to activate K_{ATP} channels reincorporated from t-tubule membrane into lipid bilayers (Parent & Coronado, 1989). We have previously reported that when membrane patches from rat skeletal muscle fibers were exposed to intracellular GTP, in the presence of extracellular adenosine and intracellular ATP, a modest increase in K_{ATP} channel activity was observed (Barrett-Jolley et al., 1995). We have found that in the presence of extracellular adenosine, the removal of intracellular ATP elicits a substantial increase in the subsequent K_{ATP} channel activity. A possible explanation for this is that ATP plays a dual role in the regulation of skeletal K_{ATP} channels, so that while

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the skeletal K_{ATP} channel is inhibited by intracellular ATP, there is also an ATP-dependent mechanism, such as phosphorylation, which acts to increase channel availability. This latter effect would generally be obscured by the dominant inhibitory action of ATP on these channels but would be revealed on removal of ATP and so, of channel inhibition. In mammalian insulinoma cells, Ribalet & Eddlestone (1995) have recently provided evidence that the G protein-mediated activation of K_{ATP} channels by somatostatin occurs via protein kinase-C dependent phosphorylation.

In the present paper, we have used the increase in channel activity seen on removal of ATP to investigate the activation of K_{ATP} channels by GTP and agonist. We find that intracellular GTP in the presence of an extracellular adenosine agonist can activate K_{ATP} channels, but requires the presence of hydrolyzable ATP. The activity of 'rundown' channels could not, however, be substantially recovered by this mechanism.

Materials and Methods

Preparation

Single muscle fibers were isolated from rat flexor digitorum brevis muscle (FDB) using collagenase as described previously (McKillen et al., 1994).

Solutions

The extracellular solution, used to fill the patch pipette, had the following composition (mM): KCl, 10; NaCl, 145; $CaCl_2$, 2; HEPES, 10, adjusted to pH 7.4 with NaOH. In some experiments adenosine (Sigma) or CCPA (2-chloro-N^6-cyclopentyladenosine; RBI, St. Albans, Herts) were added to the pipette solution. The internal solution contained 155 mM K^+ and was composed as follows (mM): KCl, 99; $MgCl_2$ (where stated) 1.4; EGTA, 5; K-gluconate, 40; HEPES, 10, adjusted to pH 7.4 with KOH. K-gluconate was included in the solution to minimize K_{ATP} channel 'rundown' (McKillen et al., 1994). The following chemicals: GTP (lithium salt), guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S; lithium salt) and ATP (dipotassium salt) were purchased from Sigma and added to the internal solution as indicated in the figure legends.

Recording Methods

K_{ATP} channel currents were recorded using patch clamp of excised, inside-out membrane patches (Hamill et al., 1981). Patch pipettes were pulled from thick-walled borosilicate tubing, coated with Sylgard resin, and fire polished. Pipette resistance was around 10 MΩ when filled with pipette solution. Following seal formation, patches were excised and placed in a switchable stream of intracellular solution. Currents were measured with a List EPC-7 amplifier and stored on videotape for offline analysis.

Analysis

Data analysis was performed by playing back records through an 8-pole Bessel filter at a cut-off frequency (-3dB) of 1–1.5 kHz and digitizing at 4 kHz using a TL-1 A-D interface (Axon Instruments) and a 486 PC. As a measure of channel activity we calculated NP_o, where N is the number of channels and P_o is the open probability. For patches containing up to 6 channels, we measured the times, t_j, spent at current levels corresponding to $j = 0, 1, 2 \ldots N$ channels open, and calculated NP_o, as

$$\left(\sum_{j=1}^{N} t_j j\right) \Big/ T$$

where the duration of the recording, T, was usually 60–90 sec. For patches with more channels we measured the mean current over 60–90s. 'Activation' was calculated as NP_o or mean current after treatment divided by the value found before treatment; no activation is thus defined as 1.0.

For display purposes, data were compressed by plotting every nth point (typically n would be 400). This procedure provides a convenient means of displaying long recordings, but leads to the apparent prolonging of open and closed times, since some brief events are deleted. All experiments were carried out at room temperature, 18–22°C, and results are given as means ± SE.

Results

In these experiments we have used inside-out patches excised from the sarcolemma of the rat FDB muscle. Patches were held at 0 mV, with 155 mM K^+ in the flow solution bathing the intracellular surface of the patch, and 10 mM extracellular (pipette) K^+. Under these conditions, ATP-dependent K^+ channels were the only channels active in most recordings (McKillen et al., 1994). Channel openings lead to outward currents with a unitary amplitude of about 2.0 pA, and channel identity was confirmed by inhibition by intracellular ATP.

Adenosine Agonists with GTP or GTP-γ-S, Together with Mg^{2+} and ATP Activate Channels

Many of the membrane patches which we excised from the rat FDB muscle showed very low initial K_{ATP} channel activity (Fig. 1). Patches showed variation in initial channel activity, and our only criterion for using a patch was that we saw activity of at least one channel after excision. Under these conditions, intracellular application of GTP (300 μM), Mg^{2+} (1.4 mM), and ATP(100 μM) for 2 min did not cause activation of K_{ATP} channels, either during or after the application ($n = 7$ patches, mean 1.0 ± 0.3-fold, Fig. 1*A*). Adenosine has been shown to activate K_{ATP} channels of both cardiac and arterial smooth muscle (Kirsch et al., 1990; Dart & Standen, 1993; Ito, Vereecke & Carmeliet, 1994), and we therefore investigated the effect of application of GTP together with Mg^{2+} and ATP when adenosine (3 μM or

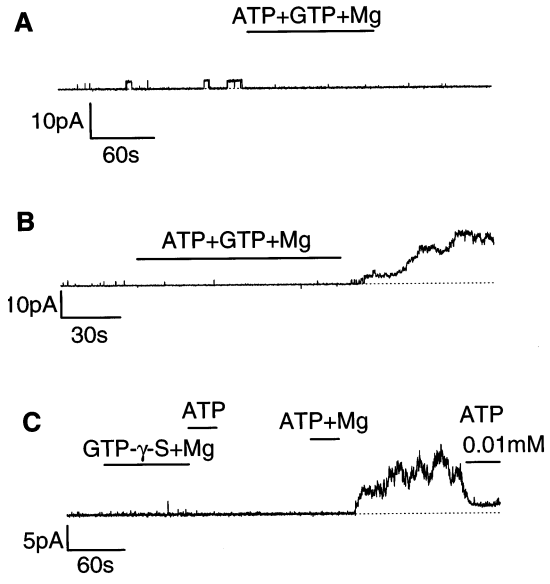

Fig. 1. ATP activation of K_{ATP} channels is G-protein dependent. (*A*) Recording from a patch that showed low channel activity. No agonist was present in the extracellular solution. Internal ATP (100 μM), Mg^{2+} (1.4 mM) and GTP (300 μM) were applied as indicated. Holding potential 0mV. (*B*) Recording from a patch in the presence of adenosine (100 μM) in the extracellular solution. Internal ATP, Mg^{2+}, and GTP were applied as indicated, and substantial channel activity was seen on their removal. (*C*) Recording in the presence of CCPA (10 μM) in the extracellular solution. Internal GTP-γ-S (200 μM), Mg^{2+} (1.4 mM) and ATP (100 μM) were applied as indicated. Channel activation was not seen until after the application of ATP-Mg^{2+}. Note, activations shown in *B* and *C* were the largest obtained, average values are illustrated in Fig. 2*B*.

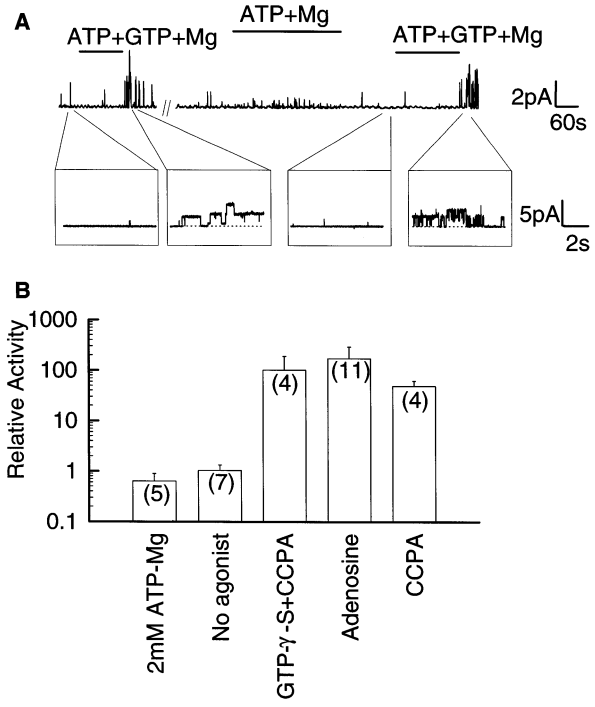

Fig. 2. Lack of 'reactivation' with ATP-Mg^{2+} alone. (*A*) Recording from a patch in the presence of CCPA (10 μM) in the extracellular solution. Intracellular GTP (300 μM), ATP (100 μM) and Mg^{2+} (1.4 mM) were applied as indicated. The insets show portions of the main record at higher resolution. The break in the record corresponds to 4 min. (*B*) Mean (±SEM) relative channel activity measured in excised patches after different treatments. For each patch relative activity was measured as NP_o following treatment divided by that before. The histogram bars correspond to experimental conditions as follows: 2 mM ATP-Mg, intracellular application of 2 mM ATP and 1.4 mM Mg^{2+} with no extracellular agonist. No agonist, intracellular application of 100 μM ATP, 300 μM GTP and 1.4 mM Mg^{2+} with no extracellular agonist. GTP-γ-S +CCPA, intracellular application of 100 μM ATP, 200 μM GTP-γ-S and 1.4 mM Mg^{2+} in the presence of extracellular CCPA. Adenosine, intracellular application of 100 μM ATP, 300 μM GTP and 1.4 mM Mg^{2+} in the presence of extracellular adenosine. CCPA, intracellular application of 100 μM ATP, 300 μM GTP and 1.4 mM Mg^{2+} in the presence of extracellular CCPA. The number of patches is given above each bar.

100 μM) was present in the extracellular (pipette) solution. We did not notice any systematic difference between the response when we used 3 μM, as opposed to 100 μM adenosine. These conditions should ensure activation of any G proteins linked to adenosine receptors. Although little K_{ATP} channel activation was seen while intracellular ATP was present, the removal of GTP, Mg^{2+} and ATP revealed substantial channel activation (Fig. 1*B*). It is likely that this activity was revealed because of removal of channel inhibition by ATP after channel activation by a G protein. The effect occurred in 11 out of 17 patches (6/8 with 3 μM and 5/9 with 100 μM adenosine), and the mean channel activation, measured as increase in NP_o was to 170 ± 115 times the control value before the application of nucleotides (Fig. 2*B*). In addition, 2-,chloro-N^6-cyclopentyladenosine (CCPA, 10 μM), a potent A_1-adenosine receptor agonist, mimicked the effect of adenosine in 4 out of 5 patches (mean activation 49 ± 11, Fig. 2*B*), suggesting that the activation of K_{ATP} channels may occur via stimulation of the A_1-receptor subtype.

To investigate further the mechanism of K_{ATP} channel activation, we used the nonhydrolyzable GTP analogue GTP-γ-S, which irreversibly activates G-protein α-subunits. Although GTP-γ-S does not require agonist binding to the receptor to activate G proteins, it acts more rapidly if an agonist is also present. For this reason we used CCPA (10 μM) in the pipette solution in experiments in which GTP-γ-S was applied to the cytoplasmic surface of patches. Figure 1*C*, shows an experiment in which GTP-γ-S was applied to the intracellular face of the membrane for 2 min at the beginning of the recording, to cause sustained activation of any G proteins present within the patch. The addition and removal of the GTP-γ-S alone was not associated with an increase in K_{ATP} channel activity, suggesting that G-protein activation in itself is not sufficient to elicit channel opening.

Similarly, after the GTP-γ-S pretreatment, application of ATP (0.1 mM) without Mg^{2+} failed to activate channels. Following this, however, the application and removal of a solution containing both Mg^{2+} (1.4 mM) and ATP (100 μM) resulted in a substantial increase in K_{ATP} channel activity (Fig. 1*C*), which was subsequently inhibited by the application of 10 μM ATP. Similar activation occurred in 4/8 patches, with a mean increase in channel activity of 102 ± 75 (Fig. 2*B*). Thus both G-protein activation and Mg^{2+}-ATP appear to be necessary for channel activation.

MgATP Alone Does not Cause Activation

We observed consistently that adenosine and CCPA activated the largest K_{ATP} currents in patches which had low background channel activity. In patches from cardiac muscle and from insulinoma cells, channel activity that has 'rundown' after patch excision can be recovered by cytoplasmic application of Mg^{2+}-ATP (Furukawa et al., 1994; Ribalet & Eddlestone, 1995). In patches from skeletal muscle, however, we found that Mg^{2+}-ATP alone was unable to cause channel activation. Figure 2*A* illustrates such an experiment. In the presence of CCPA in the pipette solution, brief application of GTP, Mg^{2+}, and ATP led on its removal to subsequent activation of K_{ATP} channels, which were then inhibited by ATP (*not shown*). Following this, application of Mg^{2+} and ATP without GTP and its removal was without effect, although subsequent application and removal of Mg^{2+}, ATP, and GTP was again able to cause activation. This finding was confirmed in 5 patches.

Calcium 'Rundown' Patches

Patches with low initial channel activity cannot therefore be activated by Mg^{2+}-ATP alone. In our experiments, spontaneous 'rundown' of channel activity was minimized by using gluconate. However, in both cardiac and skeletal muscle, K_{ATP} channels can be run down by application of millimolar Ca^{2+} to the cytoplasmic face of excised patches (Furukawa et al., 1994; Hussain & Wareham, 1994). We therefore applied 1 mM Ca^{2+} to patches with relatively high initial activity to induce 'rundown' and so test the effect of Mg^{2+}, ATP, and GTP on channels 'rundown' by Ca^{2+}. Figure 3*A* shows a membrane patch in which exposure to 1 mM intracellular Ca^{2+} abolished channel activity which was initially at a high level. Removal of the intracellular Ca^{2+} or application of 2 mM Mg^{2+}-ATP led to very little recovery of channel activity. This finding agrees with that reported by Hussain & Wareham (1994) in mouse skeletal muscle, but differs from the results reported by Furukawa et al. (1994) in cardiac muscle, where Mg^{2+}-ATP

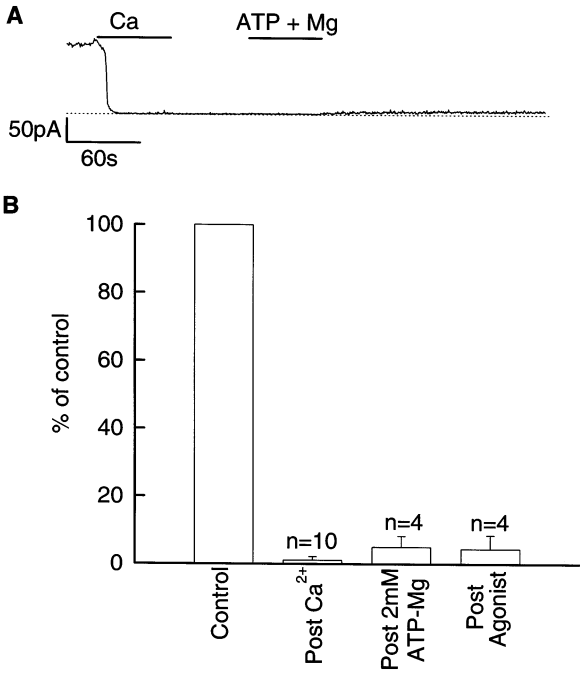

Fig. 3. Activity is not recovered from patches 'rundown' with Ca^{2+}. (*A*) Recording from a patch that showed high channel activity to clearly illustrate Ca^{2+}-induced 'rundown'. 1 mM intracellular Ca^{2+} was applied for 1 min as indicated to cause 'rundown'. Subsequent application of 2 mM ATP + 1.4 mM Mg^{2+} failed to elicit recovery (no agonist in pipette). (*B*) Mean (±SEM) channel recovery following 'rundown' induced by 1 mM Ca^{2+} after different subsequent treatments. The results are expressed as the mean NP_0 following treatment relative to that before induction of 'rundown'. The histogram bars correspond to experimental conditions as follows: Control, activity before addition of Ca^{2+}, defined as 100%; Post Ca^{2+}, removal of 1 mM intracellular Ca^{2+}; Post 2 mM ATP-Mg, intracellular application of 2 mM ATP and 1.4 mM Mg^{2+}; Post agonist, intracellular application of 100 μM ATP, 300 μM GTP and 1.4 mM Mg^{2+} in the presence of extracellular adenosine. The number of patches is given above each bar.

caused almost complete recovery. We obtained similar results in three other patches (Fig. 3*B*).

We also tested the effects of GTP, Mg^{2+}, and ATP, in the presence of an extracellular agonist, on patches whose activity had been 'rundown' by 1 mM Ca^{2+}. In the four patches tested we found very little recovery of channel activity under these conditions (Fig. 3*B*). This suggests that channels 'rundown' by Ca^{2+} are in a different state to those in patches that show low channel activity on excision, in that they cannot be reactivated by a combination of G-protein activation and Mg^{2+}-ATP.

Channel Activation Requires Hydrolyzable ATP

The results described above suggest that K_{ATP} channels can be activated in membrane patches from rat skeletal muscle by G-protein activation with extracellular aden-

osine/intracellular GTP, together with Mg^{2+} and ATP. In mammalian insulinoma cells, Ribalet and Eddlestone (1995) have proposed that the G-protein mediated activation of K_{ATP} by somatostatin occurs by way of ATP-dependent phosphorylation of these channels. To test whether a similar mechanism may exist in skeletal muscle cells, we repeated some of our earlier experiments replacing ATP with the nonhydrolyzable analogue adenylylimidodiphosphate (AMP-PNP). Like ATP, AMP-PNP inhibits K_{ATP} channels (Spruce, Standen & Stanfield, 1987); however, unlike ATP, AMP-PNP cannot be hydrolyzed to ADP + Pi, and therefore cannot act as a substrate in phosphorylation reactions. Thus, AMP-PNP is unable to support channel phosphorylation. In these experiments, we made use of the observation that the application of 1.4 mM Mg^{2+} alone inhibits K_{ATP} channels in two phases; the first phase, while Mg^{2+} remains present, reflects channel block (Davies et al. 1996). The second phase appears to be a partial, but substantial reversal of K_{ATP} channel activation. Figure 4*A* shows an experiment in which we activated K_{ATP} channels upon removal of GTP, Mg^{2+}, and ATP in the presence of extracellular adenosine, as described above. After activation, the current was rapidly reduced by the application of 1.4 mM Mg^{2+}, showing slight recovery on the removal of Mg^{2+}. The addition of AMP-PNP (2 mM), in conjunction with GTP and Mg^{2+}, abolished channel activity, as is expected since AMP-PNP is known to substitute for ATP in blocking K_{ATP} channels. Removal of the AMP-PNP solution, however, failed to induce the substantial activation of K_{ATP} channels observed upon the removal of ATP, Mg^{2+}, and GTP. To show that channels could still be activated, we reapplied the latter solution again. As at the beginning of the experiment, its subsequent removal resulted in substantial channel activation, and channel activity was inhibited by 0.1 mM ATP. We found similar results in four experiments; the data are summarized in Fig. 4*B*.

K_{ATP} Channel ATP Affinity

Several activators of K_{ATP} channels appear to act by reducing channel inhibition by ATP, and Ito et al. (1994) have reported that adenosine has such an effect in cardiac muscle. In contrast, Ribalet and Eddlestone (1995) found that G-protein-mediated stimulation of K_{ATP} channels in insulinoma cells did not involve a change in sensitivity to ATP. We compared the ATP-sensitivity of K_{ATP} channels activated by adenosine and G-protein stimulation to that of nonactivated channels. The results are shown in Fig. 4*C*. The mean of the K_Ds was 14 ± 5 μM (n = 11 measurements) for adenosine-activated channels and 19 ± 4 μM (n = 51) for control channels (P = 0.42, t-test assuming unequal variances). Thus the K_{ATP} channel activation by adenosine that we observe in rat skeletal muscle does not appear to involve a decrease in channel sensitivity to inhibition by ATP.

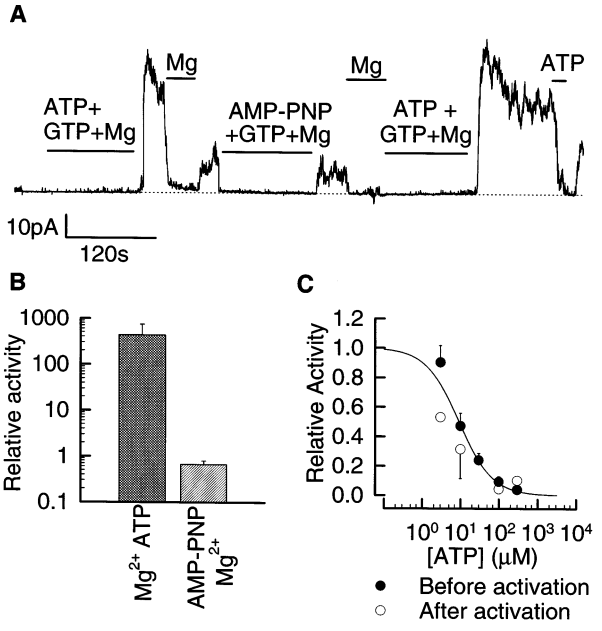

Fig. 4. Channel activation requires ATP hydrolysis. (*A*) Record from a patch showing channel activation in response to intracellular ATP, Mg^{2+}, and GTP, but a lack of response when AMP-PNP was substituted for ATP. 3 μM extracellular adenosine was present throughout. (*B*) The mean response from four patches in experiments as shown in *A*. In each case channel activity after nucleotide, Mg^{2+}, any GTP was expressed relative to its value before exposure. (*C*) The relation between channel activity and $[ATP]_i$ for patches measured before and after activation by adenosine. The solid line shows a least squares fit to all the data points, with the equation *Relative activity* = 1 − [ATP]/([ATP] + K_D). The K_D for the best fit line through all the data points is 9.2 μM, slightly lower than the values given in the text which were obtained from the mean of the K_Ds calculated from each individual K_D measurement.

Discussion

Potassium released from skeletal muscle during exercise acts as a local vasodilator, and, through a rise in arterial $[K^+]$, may also contribute to respiratory drive (Davies, Standen & Stanfield, 1991). Recent evidence suggests that adenosine contributes to hypoxic vasodilation in skeletal muscle by activating K_{ATP} channels to cause release of K^+ from skeletal muscle, and that this mechanism may also be important in exercising muscle (Marshall et al., 1993). Our results support this idea by providing evidence that K_{ATP} channels of mammalian skeletal muscle can be activated by adenosine. This activation requires intracellular GTP, consistent with the involvement of a G protein. K_{ATP} channels from tubular membranes of mammalian skeletal muscle which have been reincorporated into lipid bilayers can be activated by the application of GTP-γ-S (Parent & Coronado,

1989); our results suggest that a G-protein-dependent modulation also occurs in the native surface membrane of mammalian skeletal muscle.

In addition, our results suggest that hydrolysis of ATP is a prerequisite for the G-protein-mediated activation of K_{ATP} channels by adenosine, since ATP is effective only in the presence of Mg^{2+} and since the nonhydrolyzable ATP analogue AMP-PNP cannot support the modulatory effect, even though it mimics the channel closing action of ATP. Thus activation may involve channel phosphorylation, though the protein kinase involved has yet to be identified. Reversal of activation by Mg^{2+} (shown in Fig. 4) is consistent with such an hypothesis, since the cation activates certain protein phosphatases, for example PrP-2C (Hunter 1995). The situation is complicated by the fact that any ATP-dependent activation of the channels will always be masked by the dominant inhibitory effect of ATP. This would explain why in our experiments substantial G-protein-mediated channel activation could be observed only following the removal of ATP.

Previous reports of K_{ATP} channel modulation have proposed two types of mechanism. First, in K_{ATP} channels reincorporated from t-tubule membranes and in the activation of cardiac K_{ATP} channels by adenosine (Parent & Coronado, 1989; Kirsch et al., 1990; Ito et al., 1994), the G protein appears to act directly on the channel protein to reduce its sensitivity to inhibition by ATP. The second mechanism has been proposed by Ribalet and Eddlestone (1995) to explain the action of somatostatin in cultured insulinoma cells. Here, the agonist activates a G-protein which in turn opens K_{ATP} channels. In this case, however, activation occurs via phosphorylation and Ribalet & Eddlestone (1995) propose that the G-protein α-subunit enhances phospholipase C activity, so stimulating protein kinase-C and inducing channel phosphorylation which increases its activity. Thus, ATP is required as a substrate for phosphorylation, but the activation does not involve a shift in the ATP affinity of the K_{ATP} channel.

Our results suggest that adenosine activation of K_{ATP} channels of mammalian skeletal muscle also involves phosphorylation, since it requires hydrolyzable ATP. Further work will be necessary to establish the nature of the ATP-requiring step in K_{ATP} channel regulation in skeletal muscle.

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