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Rundown and Reactivation of ATP-sensitive Potassium Channels (K_{ATP}) in Mouse Skeletal Muscle

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Abstract. Dissociated single fibers from the mouse flexor digitorum brevis (FDB) muscle were used in patch clamp experiments to investigate the mechanisms of activation and inactivation of K_{ATP} in mammalian skeletal muscle. Spontaneous rundown of channel activity, in many excised patches, occurred gradually over a period of 10–20 min. Application of 1.0 mM free-Ca^{2+} to the cytoplasmic side of the patch caused irreversible inactivation of K_{ATP} within 15 sec. Ca^{2+}-induced rundown was not prevented by the presence of 1.0 μM okadaic acid or 2.0 mg ml^{-1} of an inhibitor of calcium-activated neutral proteases, a result consistent with the conclusion that phosphatases or calcium-activated neutral proteases were not involved in the rundown process. Application of 1.0 mM Mg.ATP to Ca^{2+}-inactivated K_{ATP} caused inhibition of residual activity but little or no reactivation of the channels upon washout of ATP, even in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase (10 U ml^{-1}). Mg.ATP also failed to reactivate K_{ATP}, even after only partial spontaneous rundown, despite the presence of channels that could be activated by the potassium channel opener BRL 38227. Nucleotide diphosphates (500 μM; CDP, UDP, GDP and IDP) caused immediate and reversible opening of Ca^{2+}- inactivated K_{ATP}. Reactivation of K_{ATP} by ADP (100 μM) increased further upon removal of the nucleotide. In contrast to K_{ATP} from cardiac and pancreatic cells, there was no evidence for phosphorylation of K_{ATP} from the surface sarcolemma of dissociated single fibers from mouse skeletal muscle. The small degree of activation occasionally observed following application of 10 μM or 1.0 mM Mg.ATP could have been due to the generation of ADP from ATP hydrolysis and not through phosphorylation. Data are consistent with the suggestion that Ca^{2+} inactivation of K_{ATP} involves a gating mechanism that can be reopened by nucleotide diphosphates.

Key words: ATP-sensitive potassium channels — Rundown — Skeletal muscle — Single fibers

Introduction

K_{ATP} are inhibited by the application of micromolar concentrations of ATP to the cytoplasmic side of the sarcolemma. High densities of K_{ATP} have been found in cardiac (Noma, 1983), skeletal (Spruce, Standen & Stanfield, 1987) and smooth muscle cells (Standen et al., 1989) as well as neurons (Ashford et al., 1987) and pancreatic beta cells (Ashcroft & Kakei, 1989). Kinetic properties of muscle K_{ATP} and their modulation by physiological and pharmacological factors have been the subject of several recent reviews (Davies, Standen & Stanfield, 1991; Dunne & Petersen, 1991; Nichols & Lederer, 1991; Noma & Takano 1991).

In cardiac and pancreatic cells, spontaneous decay or rundown of K_{ATP} occurs within seconds or minutes of excising the patch to the inside-out configuration (Trube & Heschler, 1984). Application of Ca^{2+} to the cytoplasmic side of excised patches facilitated the rate of rundown of K_{ATP} in rabbit atrioventricular nodal cells (Kakei & Noma, 1984), rat ventricular myocytes (Findlay, 1988) and frog skeletal muscle fibers (Krippeit-Drews & Lönnendonker, 1992). In some cases, rundown can be prevented or partly reversed by exposure of the patch to Mg.ATP but not free ATP or the nonhydrolyzable analogues of ATP such as AMP-PNP or AMP-PCP (Findlay & Dunne, 1986; Ohno-

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Shosaku, Zunkler & Trube, 1987; Takano, Qin & Noma, 1990). This has led to suggestions that Mg- dependent phosphorylation may be necessary to sustain K_{ATP} in an active form.

More recent experiments have shown that spontaneous or Ca^{2+}-induced rundown can also be reversed by proteolysis of the cytoplasmic side of the patch with trypsin, carboxypeptidase A or papain (Furukawa et al., 1993; Proks & Ashcroft, 1993). The mechanism for this action is unknown but may involve proteolytic cleavage of an inactivation gate. Kirsch et al. (1990) have suggested that G proteins may be involved in reactivation of cardiac K_{ATP}. This has been supported by the demonstration that, in the presence of Mg at the cytoplasmic side, GTPγS could reactivate K_{ATP} from rabbit T tubules (Parent & Coronado, 1989) and that AlF^{4-}, an activator of GTP-binding proteins could mimic the effects of GTPγS.

Despite these studies, the mechanism of rundown remains poorly understood and the decay of channel activity continues to pose a practical limitation to experiments with excised patches. In the present study we have investigated the nature of rundown and the mechanisms of subsequent reactivation of K_{ATP} in single fibers from mammalian skeletal muscle. Skeletal K_{ATP} have been described in amphibian (Spruce et al., 1987; Vivaudou, Arnoult & Villaz, 1991) and mammalian (Burton, Dörstelmann & Hutter, 1988; Rowe, Wareham & Whittle, 1990) sarcolemmal vesicles, as well as dissociated single fibers (Weik & Neumcke, 1989; Woll, Lönnendonker & Neumcke, 1989; Hussain, Wareham & Head, 1994). K_{ATP} from these preparations are generally more stable and less susceptible to rundown, both in frog (Spruce et al., 1987) and mammalian tissues (Hussain et al., 1994). We were therefore interested in determining whether rundown of K_{ATP} in mouse skeletal muscle was facilitated by Ca^{2+} and also whether inactivated channels could be reactivated via rephosphorylation with Mg.ATP or other known activators of the channel. Results are discussed in terms of an inactivation gate being responsible for spontaneous or Ca^{2+}-induced rundown.

Materials and Methods

Dissociated Single Fibers

Adult C57BL/6J mice (2–3-months old) of either sex were killed by ether overdose and the flexor digitorum brevis muscles ablated from the hind limbs into prefiltered (0.2 μm filter, Gelman Sciences, Ann Arbor, MI) HEPES-Tyrodes buffer. Single fibers were obtained essentially as described previously (Woll et al., 1989). Muscles were incubated for 1–1.5 hr at 37°C in HEPES-Tyrodes buffer containing 3.0 mg ml^{-1} collagenase (Type I; Sigma, UK) and 1.0 mg ml^{-1} soyabean trypsin inhibitor (Type II-S; Sigma, UK). Upon subsequent transfer to enzyme-free Tyrodes, digested muscles were gently triturated to cause dispersion into single fibers. Aliquots of dissociated fibers were transferred to the patch clamp chamber where they adhered spontaneously to the methanol-cleaned glass base. Fibers were maintained at room temperature and used within 2–3 hr of preparation.

The dissociation procedure was also used for other skeletal muscle preparations, including extensor digitorum longus (EDL), soleus, extensor halucis longus and the diaphragm. The presence of soyabean trypsin inhibitor proved useful in minimizing the degree of fragmentation and achieving high yields of intact fibers, particularly for larger muscle preparations such as EDL. The greatest number of intact fibers was obtained from FDB muscles, where mechanical damage was considerably reduced due to small fiber size. Diaphragm fibers were the most difficult to disperse into intact single fibers. Dissociated fibers that twitched in response to square wave field stimulation pulses were considered to be intact and viable.

Patch Clamp

Experiments were performed in the inside-out configuration using conventional patch clamp techniques (Hamill et al., 1981). Patch pipettes were double-pulled from borosilicate capillaries (hard glass; GC150F: Clark Electromedical, UK) using a vertical List microelectrode puller (model L/M-3P-A; List-Electronic, Darmstadt, Germany). Microelectrode tip resistance was typically 5 MΩ after the pull and increased to 10–15 MΩ after fire-polishing the tip. Seal formation was carried out within 2–4 min of exchanging the bathing Tyrodes solution with high KCl (*see below* for composition). Depolarization with high KCl caused swelling of the fibers with concomitant production of sarcolemmal vesicles (*see* Fig. 1*A*). For the sake of consistency, except where otherwise indicated, experiments throughout the present study were carried out after patching the surface of the fiber rather than the vesicles. Vesicles were produced only from intact single fibers, indicating that the mechanical strain imposed on the plasmalemma, due to osmotic swelling in response to KCl loading, was the mechanism for the production of vesicles.

Solutions and Drugs

Muscle fibers were incubated and stored in Tyrodes of the following composition (mM): NaCl, 120; KCl, 5; $MgCl_2.6H_2O$, 1.0; $NaHPO_4$, 0.5; $CaCl_2$, 2.5; HEPES, 10.0; and glucose 11.0, adjusted to pH 7.3 with NaOH. The high KCl solution contained (mM): KCl, 120; EGTA, 1.0; $MgCl_2$, 1.0 and HEPES, 10.0 adjusted to pH 7.3 with KOH and was also used as the pipette (extracellular) solution. The following nucleotide diphosphates were used in the present study: Adenosine 5′-diphosphate (ADP), cytidine 5′-diphosphate (CDP), inosine 5′-diphosphate (IDP) and uridine 5′-diphosphate (UDP). ATP and nucleotide diphosphates (all Sigma, UK) were prepared in this high KCl solution, and the pH was readjusted to 7.3 as necessary. The cytoplasmic side of detached patches was exposed to test substances flowing (1.0 ml min^{-1}) down a glass capillary tube connected, by a manifold, to a series of reservoirs containing different solutions. This is a simple and inexpensive way of achieving relatively rapid exchange of solutions without having to realign the patch pipette with the delivery tube as suggested by Vivaudou et al. (1991).

Data Acquisition and Analysis

Ionic currents were measured using a List patch clamp amplifier (L/M-EPC 7; 10 kHz internal filtering) and recorded on FM videotape using a modified Sony PCM-701ES digital audio processor (Lamb, 1985). Off-line analysis was carried out by replaying data through an 8-pole Bessel filter at 1 kHz. Segments of data were either played back to a TDM PAR 1000 pen recorder (Tape Services,

UK) or acquired at 5 kHz into the Fetchex series of pCLAMP programs (pCLAMP 5.5; Axon Instruments, Foster City, CA) using a TL-1 interface linked to an Opus PCV 386 computer. Open probabilities (NP_{open}) for multi-channel patches were calculated using custom-made software provided by Dr. M. Hunter at the University of Leeds, UK. Average NP_{open} for 5 sec segments of data was calculated according to the following algorithm:

$$NP_{open} = \Sigma (n \cdot t_n)/T$$

where n = number of simultaneously active channels (0 = no channels open, 1 = 1 channel open, etc), t_n = length of time the channel is in state n, T = total time of the recording. NP_{open} values were normalized and presented as percent changes, as shown in the figures.

Results

K_{ATP} were inactive in the cell-attached configuration, both in intact single fibers and sarcolemmal vesicles produced by KCl depolarization (Fig. 1*A*). Excising the patch into Ca^{2+}-free high KCl bathing solution resulted in the opening of K_{ATP}, presumably through alleviation of the inhibitory effects of $[ATP]_i$. In some cases, patch excision resulted in the formation of a vesicle at the tip of the pipette and no channel activity could be observed until this was disrupted, either by a brief exposure of the patch to air, or by superfusion of the patch pipette with high KCl buffer (Fig. 1*B*). Channel activity due to K_{ATP} could then be repeatedly and reversibly inhibited by the application of ATP to the cytoplasmic side of the membrane (arrow in Fig. 1*B*). Slope conductance (69 pS), sensitivity to ATP and activation by BRL 38227 (K-channel opener) were generally similar for K_{ATP} recorded from sarcolemmal vesicles and those from the surface sarcolemma of intact fibers (*results not shown*). This was not surprising in view of the results of Milton and Caldwell (1990), where patching the fiber surface was shown to involve formation of a vesicle. The number of active channels varied considerably between patches, depending on the pipette resistance and the amount of suction applied during seal formation. Such patch-to-patch variations in the number of active channels may reflect variations in the area of the membrane sucked into the pipette as well as differences in channel distribution across the surface of the fiber. Patches containing large numbers of active channels were particularly common during experiments with sarcolemmal vesicles and proved more useful for qualitative experiments probing the mechanisms of channel regulation.

Figure 1*B* also shows that, in contrast to cardiac and pancreatic cells, the rate of rundown of K_{ATP} in many patches from skeletal muscle was much slower, so that considerable channel activity was often observed after 20 min of superfusion with high KCl. However, this was not a consistent observation and channel activity in some patches declined rapidly within seconds or minutes of excising the patch. Low concentrations of

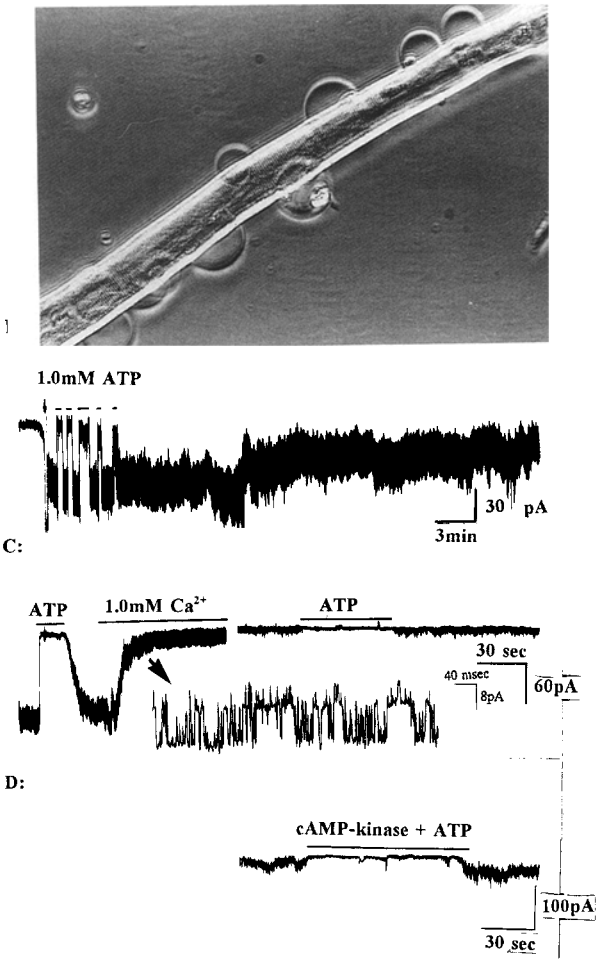

Fig. 1. Effect of Mg.ATP on Ca^{2+}-inactivated K_{ATP} in mammalian skeletal muscle. (*A*) Phase-contrast photomicrograph taken 30 min after KCl depolarization. K_{ATP} and other channels could be recorded from intact single fibers or from sarcolemmal vesicles (calibration bar = 50 μm). (*B*) A multichannel patch where activity of K_{ATP} was stable over the duration of the recording. ATP (1.0 mM) caused repeated and reversible inhibition of K_{ATP} as shown by the bold lines in the tracing. (*C*) Application of 1.0 mM Ca^{2+} to the cytoplasmic side of the membrane caused irreversible inactivation of K_{ATP} that could not be reversed by 1.0 mM Mg.ATP even in the presence of cAMP-kinase (*D*). Tracing (and inset) in *C* also shows that exposure of the cytoplasmic side of the membrane to Ca^{2+}-stimulated calcium-activated potassium channels where the latter were also present in the same patch. Membrane potential in this and all subsequent figures was −40 mV.

Mg.ATP have been found to reverse rundown in cardiac and pancreatic cells and are often used to sustain K_{ATP} in an active form (Findlay & Dunne, 1986; Ohno-Shosaku et al., 1987). Application of 5 or 10 μM Mg.ATP to K_{ATP} from mammalian skeletal muscle did not reverse spontaneous rundown (Hussain et al., 1994). Studies into reactivation of K_{ATP} in skeletal muscle

were complicated because rundown was often a slow process and prolonged superfusion frequently caused seal breakdown before mechanisms of reactivation could be studied. Experiments were therefore undertaken to try and facilitate the rate of rundown with Ca^{2+}.

Inactivation of K_{ATP} by Ca^{2+} and Subsequent Reactivation by ATP

Figure 1*C* shows that superfusion of the cytoplasmic side of a patch with high KCl solution containing 1.0 mM free-Ca^{2+} caused rapid and irreversible inactivation of K_{ATP}. Lower concentrations of Ca^{2+} (7 μM) produced slower but less consistent inactivation of K_{ATP} (*not shown*). In some patches, containing both calcium-activated potassium channels ($K_{Ca^{2+}}$) and K_{ATP}, inactivation of K_{ATP} was accompanied by simultaneous activation of $K_{Ca^{2+}}$ (Fig. 1*C*). Only $K_{Ca^{2+}}$ were therefore detectable after complete inactivation of K_{ATP}. After removal of Ca^{2+}, only the residual activity of K_{ATP} channels that had not yet been inactivated by Ca^{2+} was seen.

Exposure of the patch to 1.0 mM ATP, in the presence of Mg^{2+}, inhibited residual K_{ATP} activity but did not restore the Ca^{2+}-inactivated channels (Fig. 1*C*). This result is in marked contrast to the situation reported for cardiac cells where ATP rapidly and fully restored Ca^{2+}- inactivated K_{ATP} channels (Findlay, 1988; Furukawa et al., 1993). Reactivation of cardiac K_{ATP} by 1.0 mM ATP has been suggested to result from rephosphorylation of the channel or associated proteins (Findlay, 1988). The lack of reactivation demonstrated in Fig. 1*C* could have been due to loss of enzymes required for the phosphorylation process. However, inclusion of 10 U ml^{-1} of the catalytic subunit of the cyclic AMP-dependent protein kinase A (cAMP kinase) with ATP did not facilitate rephosphorylation of the Ca^{2+}-inactivated K_{ATP} in skeletal muscle (Fig. 1*D*). The small increase in channel activity occasionally observed following exposure of the patch to ATP was probably due to contaminant ADP rather than rephosphorylation (*see below*).

The mechanism of inactivation by Ca^{2+} is unknown. Exposure to Ca^{2+} was followed by a short delay before the channels began to inactivate. NP_{open} then declined to less than 10% of its original value within 10 sec. Figure 2*A* shows that Ca^{2+}-induced inactivation followed a mono-exponential decay with a time constant of 4 sec. Pre-exposure of the cytoplasmic side of the membrane to 1 μM okadaic acid (a potent inhibitor of serine and threonine phosphatases types 1 and 2A) did not prevent or retard the inactivation by 1.0 mM Ca^{2+}. Similarly, inhibition of Ca^{2+}-activated neutral proteases (2 mg ml^{-1}) was also without effect on the Ca^{2+}-evoked inactivation process. These results are consistent with an earlier study where neither leupeptin (a protease inhibitor) nor fluoride ions (phosphatase inhibitor) blocked the inactivation of cardiac K_{ATP} by Ca^{2+} (Findlay, 1988).

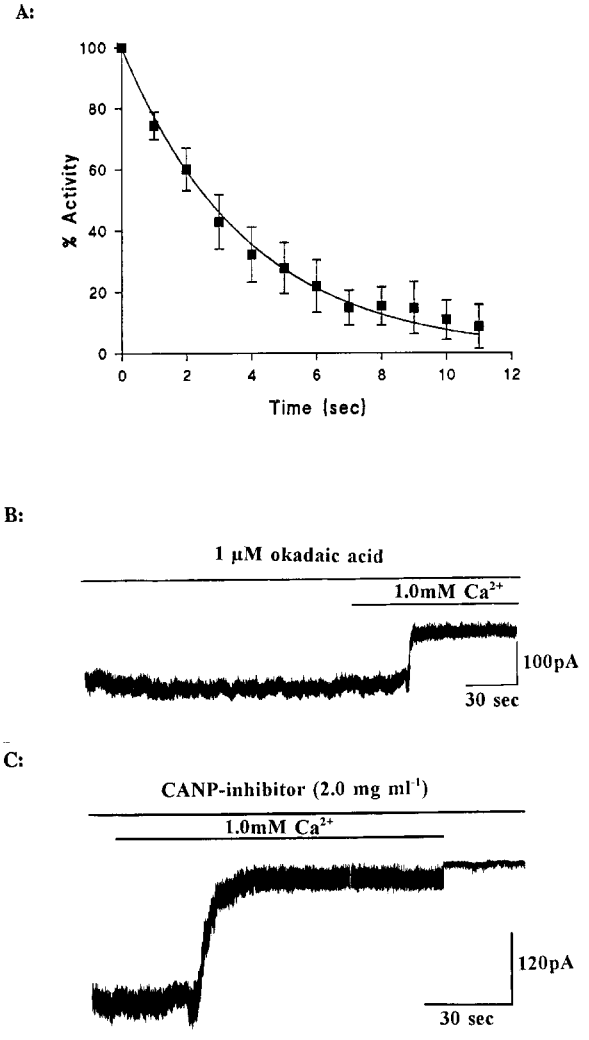

Fig. 2. Rate of Ca^{2+} inactivation of K_{ATP} (*A*) and effects of okadaic acid (*B*) and an inhibitor of calcium-activated neutral proteases (*C*; CANP-inhibitor). (*A*) Decline of channel activity due to K_{ATP}, in the presence of 1.0 mM Ca^{2+} is normalized to the level of activity immediately prior to the onset of inactivation ($n = 7$). Okadaic acid and CANP-inhibitor were applied to the patch for periods of 0.5 to 4 min prior to the application of Ca^{2+}. Tracings are typical of five experiments with okadaic acid and nine with the CANP-inhibitor.

In a previous study we demonstrated that low concentrations of Mg.ATP (5–10 μM) failed to reactivate K_{ATP} after spontaneous rundown (Hussain et al., 1994). In the same study we also showed that BRL 38227, a potassium channel opener, caused reactivation after partial, but not complete, rundown. Hence, ATP was unable to reactivate the channels despite the presence of "activatable channels" in the patch. Similar results were obtained during experiments in the present study where 1.0 mM Mg.ATP was unable to reactivate K_{ATP} after partial spontaneous rundown (*not shown*).

Reactivation of Ca^{2+}-inactivated K_{ATP} by Nucleotide Diphosphates

Unlike ATP, application of nucleotide diphosphates to the cytoplasmic side of patches containing Ca^{2+}-inactivated K_{ATP} caused marked and rapid reactivation of K_{ATP} (Fig. 3). The effects of nucleotide diphosphates were entirely reversible and sensitive to inhibition by 1.0 mM ATP (Fig. 3*A–C*), thereby confirming K_{ATP} to be responsible for the increased current.

Figure 3*D* shows a quantitative comparison of the effects of the NDPs and Mg.ATP on Ca^{2+}- inactivated K_{ATP}. The mean NP_{open}, after 1.0 mM Ca^{2+}, was reduced to 5.7 ± 1.0% (n = 14) of the original control level (normalized to 100%) and was reduced further to 1.0 ± 0.9% (n = 4) by 1.0 mM ATP. Washout of ATP increased activity only to 7.9 ± 3.8% (n = 4). In experiments where cAMP-kinase was co-applied with ATP, activity upon washout was only increased to 11.1% (n = 3), a value not significantly different from that observed upon washout of ATP alone. By comparison, the nucleotide diphosphates UDP, GDP, CDP and IDP (all 500 μM) caused up to 75.8, 58.5, 57.3 and 53.1% reactivation, respectively.

Effects of ADP are complicated by the fact that ADP can also bind to the inhibitory site of K_{ATP} in insulin-secreting cells (Dunne & Petersen, 1986), cardiac myocytes (Lederer & Nichols, 1989) and skeletal muscle fibers (Vivaudou et al., 1991; Allard & Lazdunski, 1992; Forestier & Vivaudou, 1993). The inhibitory effect of ADP is usually more pronounced, at high concentrations of the nucleotide (>100 μM), and therefore masks any activation. Accordingly, K_{ATP} in the present study were reversibly inhibited by 500 μM ADP. The mean activity of K_{ATP} after 1.0 mM Ca^{2+} was 15.3% in two separate patches. This decreased further to 7.5% following application of 500 μM ADP but increased to 26.4% upon washout of ADP. Figure 4 shows the effects of lower concentrations of ADP (50 and 100 μM) on Ca^{2+}- inactivated K_{ATP}. Although a small degree of activation can be seen in the presence of 100 μM ADP (activity increased from 2.5 to 16.0%), more activity (43.7%) became apparent upon switching to ADP-free control solutions. This increase in activity may result from a faster dissociation of ADP from the inhibitory site than from the activation site of the K_{ATP} channel. Alleviation of inhibition therefore allows the activation effects of the nucleotide to become apparent.

Effects of GTP and GTPγS

Data from Kirsch et al. (1990) and Parent and Coronado (1989) have implicated G proteins in regulating K_{ATP} from cardiac cells and T tubules of skeletal muscle, respectively. Figure 5 shows that K_{ATP} from the surface sarcolemma of skeletal muscle could also be stimulated by GTP or GTPγS (both 200 μM). However, due to rapid spontaneous rundown in these patches, we were unable to standardize these effects to the control activity observed upon patch excision. This may be important because as Fig. 5*B* shows, both GTP and GTPγS caused only 14.0 and 23.4% recovery of Ca^{2+}-inactivated K_{ATP}, respectively. Effects of both GTP and GTPγS were reversible in both cases. This may argue against the involvement of G proteins because GTPγS, the slowly hydrolyzable analogue of GTP, would be expected to maintain stimulation of the GTPase if G proteins were involved. Further experiments are necessary to clarify the mechanisms involved in the effects of GTP and GTPγS.

Discussion

Potassium efflux through K_{ATP} is believed to regulate insulin secretion in the β cell and provide cardioprotection in ischemic heart cells. In skeletal muscle, metabolic exhaustion during muscle fatigue may open K_{ATP} to reduce excitability and cause local vasodilation of the surrounding vascular smooth muscle (Castle & Haylett, 1987). However, $[ATP]_i$ is in the millimolar range in muscle cells and falls only gradually even during vigorous muscle activity. Cellular mechanisms must therefore exist to activate K_{ATP} while $[ATP]_i$ is high. Alternatively, $[ATP]_i$ is compartmentalized into localized subsarcolemmal pools which may modulate K_{ATP}. Such pools may be subject to depletion during metabolic stress. ATP sensitivity of K_{ATP} may also be attenuated by other intracellular modulators such as ADP (Dunne & Petersen, 1986; Lederer & Nichols, 1989; Vivaudou et al., 1991) and protons (Davies et al., 1992). Both [ADP] and $[H^+]$ increase during ischemia and could act to regulate K_{ATP} in the intact cells. Modulation of K_{ATP} in inside-out patches may provide insights into the mechanisms regulating K^+ efflux in the intact cell.

Results in the present study demonstrated that in the absence of ATP, K_{ATP} in mouse skeletal muscle could be rapidly inactivated by the application of 1.0 mM Ca^{2+} to the cytoplasmic side of the membrane. The mechanism of inactivation by Ca^{2+} did not appear to involve a serine/threonine phosphatase or a calcium-activated neutral protease because inhibitors of these enzymes did not prevent inactivation by Ca^{2+}. Rapid inactivation of K_{ATP} by 1.0 mM Ca^{2+} poses the question as to why Woll et al. (1989) and we (Hussain et al., 1994) have previously been able to measure *some* K_{ATP} after excising patches into HEPES-Tyrodes buffer containing up to 2.5 mM Ca^{2+}. Immediate replacement of the bathing Tyrodes with Ca^{2+}-free high KCl resulted in a gradual increase in channel activity. Such a post-wash increase in channel activity has been described previously (Bodewei, Hehl & Neumcke, 1992) and was suggested to be due to partial alleviation of Ca^{2+} inac-

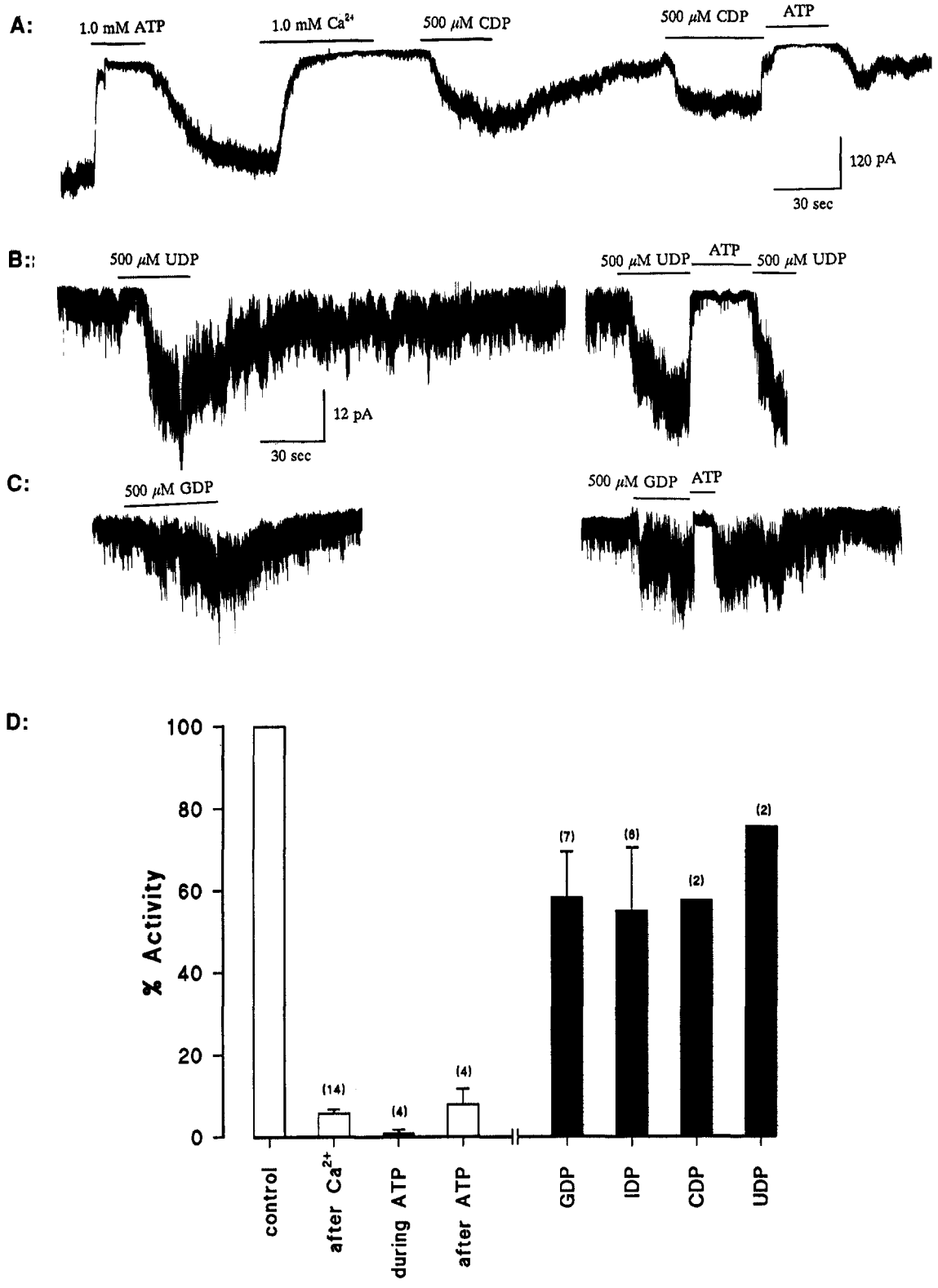

Fig. 3. Reactivation of Ca^{2+}-inactivated K_{ATP} by nucleotide diphosphates, and comparison to the effects of Mg.ATP. Figure shows tracings from three separate experiments illustrating the reactivation of K_{ATP} by 500 μM CDP (*A*), UDP (*B*) and GDP (*C*). In *B* and *C*, UDP and GDP were added after K_{ATP} activity inactivated by Ca^{2+} to less than 10% of the original activity observed upon patch excision. Nucleotide diphosphate-evoked responses were reversible and inhibited by 1.0 mM ATP in each case. Calibrations and applications of nucleotide diphosphates were as shown by the bold lines in each tracing. Calibration in *B* also applies to tracings in *C*. (*D*) Quantitative comparison of data showing reactivation of Ca^{2+}-inactivated K_{ATP} by ATP, and nucleotide diphosphates. Bars represent means ± SE from separate patches as indicated in parentheses above each bar.

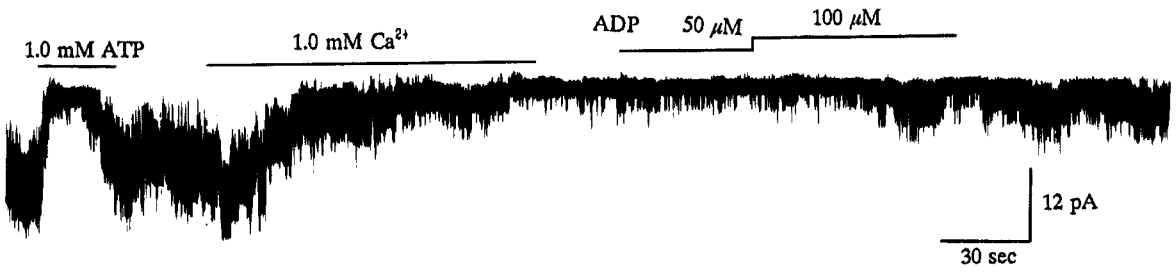

Fig. 4. Effects of ADP on Ca^{2+}-inactivated K_{ATP} from mouse skeletal muscle. The tracing shows the level of ATP-inhibited activity at the start of the experiment and then after Ca^{2+} inactivation. Application of 50 or 100 μM ADP caused a noticeable increase in channel activity which became more apparent upon removal of the nucleotide.

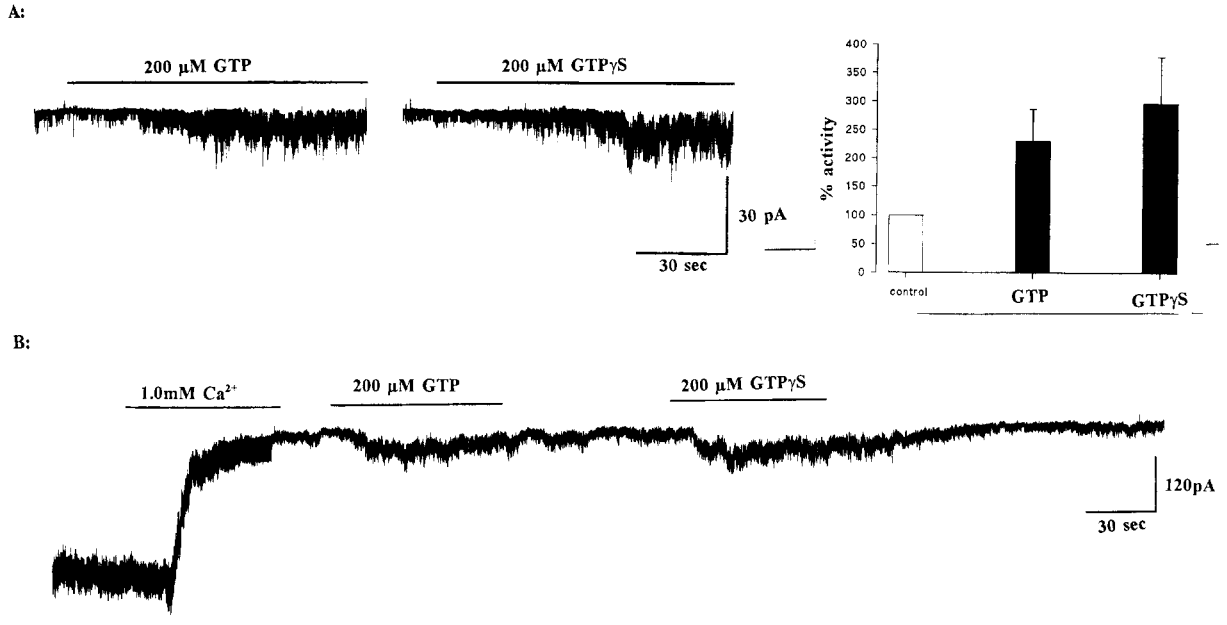

Fig. 5. Effects of GTP and GTPγS on K_{ATP} after spontaneous rundown and Ca^{2+}-induced inactivation. In patches showing rapid rundown (*A*), both GTP and GTPγS caused two- to threefold increases in channel activity. (*A*) Mean ± SEM GTP- and GTPγS-evoked increases in K_{ATP} from three separate patches after spontaneous rundown. K_{ATP} after Ca^{2+} inactivation were apparently less susceptible to reactivation (*B*) when compared to nucleotide diphosphates (Fig. 3).

tivation. In our experiments, alleviation of Ca^{2+} inactivation only occurred if K_{ATP} had not been fully inactivated by Ca^{2+}. It is therefore possible to detect some activity due to K_{ATP} even after patch excision was initially carried out in Ca^{2+}-containing bathing solutions, providing that the latter was exchanged for a Ca^{2+}-free solution within seconds of excision. Such activity, however, reflects only a fraction of the total number of channels in the patch. Comparison of current-voltage relations in high KCl to those in Tyrodes indicated a complete block of the outward current when the latter bathed the cytoplasmic side. This has also been described by Woll et al. (1989) and attributed to block of the channel pore by Na^+ at the cytoplasmic side.

K_{ATP} in the present study could not be activated by low or high concentrations of Mg.ATP to the degree described for other cells, despite the presence of channels that could be activated by the application of nucleotide diphosphates (Fig. 3) or the K-channel opener BRL 38227 (*not shown*). The latter is known to activate skeletal K_{ATP} through a Mg- dependent binding site (Hussain et al., 1994) in a similar manner to nucleotide diphosphates (Tung & Kurachi, 1991; Allard & Lazdunski, 1992). The inability of Mg.ATP to cause any significant reactivation of skeletal K_{ATP}, after spontaneous or Ca^{2+}-induced rundown, is in marked contrast to the results from cardiac and pancreatic cells and implies that additional regulatory molecules required for phosphorylation may be absent from excised patches in skeletal muscle. However, addition of Mg.ATP in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase failed to reactivate Ca^{2+}-inactivated K_{ATP} (Fig. 1*D*), indicating that the conditions for phosphorylation were probably not limiting.

Krippeit-Drews and Lönnendonker (1992) were also unable to reactivate K_{ATP} from frog skeletal muscle.

One possible reason for this difference between tissues may be that cytoskeletal elements are involved in regulating K_{ATP} (Dunne & Petersen, 1991). In skeletal muscle, such cytoskeletal elements may be more firmly associated with the contractile apparatus and therefore less easily obtained in excised patches. A role of cytoskeletal elements has also been proposed in relation to rundown of the Ca^{2+} channels in *Lymnaea* neurons which were also inactivated by Ca^{2+} but not reactivated by phosphorylation (Johnson & Byerly, 1993).

The small increase in activity of K_{ATP} occasionally observed after exposure of the patch to ATP may have been due to contaminant ADP generated from the hydrolysis of ATP. The lack of reactivation following application of AMP-PNP would substantiate this view. Indeed, ATP left standing at room temperature for several hours was more effective than fresh ATP at activating K_{ATP}. Low concentrations of ADP (<100 μM) could stimulate skeletal K_{ATP} after Ca^{2+}-induced (Fig. 4) or spontaneous (Forestier et al., 1993) rundown.

More direct attempts to fully restore Ca^{2+}-inactivated K_{ATP} with GTP and GTPγS were also unsuccessful (Fig. 5*B*), whereas both GTP and GTPγS could stimulate K_{ATP} after spontaneous rundown (Fig. 5*A*). The site of action of Ca^{2+} may therefore be spatially or functionally closer to the gating mechanism than the GTP-binding site. This would explain why the channels closed by Ca^{2+}-induced rundown can no longer be fully reopened by GTP or GTPγS. However, whether K_{ATP} after spontaneous rundown are more susceptible to activation than after Ca^{2+} inactivation is unclear. Experiments where reactivation by GTP and GTPγS is normalized to the control activity observed upon patch excision are necessary to answer this question.

In contrast to nucleotide triphosphates (ATP, GTP and GTPγS), nucleotide diphosphates (GDP, CDP, UDP and IDP) could all activate K_{ATP} after Ca^{2+}-induced (Fig. 3) or spontaneous (Allard & Lazdunski, 1991) rundown. This ability of nucleotide diphosphates to reactivate Ca^{2+}-inactivated skeletal K_{ATP} suggests that Ca^{2+}-induced changes are reversible through occupation of the Mg-dependent activation site and that K_{ATP} do not undergo extensive conformational derangements during inactivation by Ca^{2+} but merely close. Proteases prevent or reverse spontaneous or Ca^{2+}-induced rundown (Furukawa et al., 1993; Proks & Ashcroft, 1993) perhaps by cleaving the inactivation gate that is modulated by Ca^{2+}. Trypsin has also been shown to abolish the inhibitory action of the sulfonylurea, glibenclamide (Nichols and Lopatin, 1993). The latter and Ca^{2+} may therefore modulate a common gating mechanism. Proteolytic modification does not abolish the inhibition of K_{ATP} by ATP, as would be expected if the same gate was also responsible for mediating the inhibitory effect of ATP. A second inhibitory gating mechanism is therefore likely to be modulated by ATP. Further experimentation with interactions between different modifiers of the channel are necessary to elucidate the precise nature of the molecular mechanisms controlling channel function.

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