Activation of ATP-dependent K^+ channels by metabolic poisoning in adult mouse skeletal muscle: role of intracellular Mg^{2+} and pH

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1. The effects of metabolic poisoning, intracellular Mg^{2+} and pH on ATP-dependent K^+ (K^+_{ATP}) channels were examined in adult mouse isolated skeletal muscle fibres using the patch clamp technique.

2. In cell-attached membrane patches, while openings of one kind of channel could only rarely be detected under control conditions, cell poisoning with fluorodinitrobenzene (FDNB), dinitrophenol (DNP) and cyanide (CN) induced a strong and partially reversible increase in channel activity.

3. Slope conductance and glibenclamide sensitivity of this outward current indicated that the channel activated during poisoning was the K^+_{ATP} channel.

4. Single channel current amplitude was reduced during poisoning, but remained unchanged when activation of the K^+_{ATP} channel was induced by cromakalim.

5. In inside-out membrane patches, in the absence of intracellular ATP, intracellular application of Mg^{2+} decreased channel activity and single channel current amplitude. Inhibition of K^+_{ATP} channels by ATP was also reduced.

6. In the absence of intracellular ATP, a decrease in intracellular pH induced a reduction in channel activity and single channel current amplitude. Inhibition of K^+_{ATP} channels by ATP was also reduced.

7. The reduction of single channel current amplitude during poisoning was attributed to an increase in intracellular Mg^{2+} concentration caused by a fall in intracellular ATP concentration. These results also show that metabolic poisoning causes direct activation of K^+_{ATP} channels in skeletal muscle, and that this activation is at least partially mediated through an increase in intracellular Mg^{2+} concentration and a decrease in intracellular pH.

Since their discovery in heart cells (Noma, 1983), ATP-dependent K^+ channels (K^+_{ATP}) have been identified in endocrine cells, smooth muscle, neurones and skeletal muscle (for reviews see De Weille & Lazdunski, 1989; Davies, Standen & Stanfield, 1991). In skeletal muscle, these channels have been described in excised patches from sarcolemmal vesicles (Spruce, Standen & Stanfield, 1985) and blebs of frog and human muscles (Burton, Dörstelmann & Hutter, 1988; Vivaudou, Arnoult & Villaz, 1991), and from transverse tubules (Parent & Coronado, 1989) and sarcolemma of adult mammalian skeletal muscle (Woll, Lönnendonker & Neumcke, 1989). The high density of these channels in skeletal muscle makes it likely that they play a role under physiological conditions, particularly in exercising muscle (Spruce *et al.* 1985). It has been suggested that K^+_{ATP} channels are responsible, at least partially, for the well-documented K^+ conductance increase which accompanies exhaustion of contractile activity (Davies *et al.* 1991). However, until now, only indirect evidence supported this hypothesis. Early experiments performed by Lüttgau's group (Fink & Lüttgau, 1976; Fink, Hase, Lüttgau & Wettwer, 1983) have shown that metabolic exhaustion triggered by poisoning gives rise to an increase in K^+ conductance in frog skeletal muscle fibres. Additionally, Castle & Haylett (1987) reported that metabolic exhaustion leads to an increase in K^+ and Rb^+ efflux from frog skeletal muscle which can be partially inhibited by the K^+_{ATP} channel blockers tolbutamide and glibenclamide. It was suggested that this enhanced K^+ permeability may be due predominantly to activation of K^+_{ATP} channels. Recent

investigations into the effect of glibenclamide on action potential and tetanic force in frog muscle provided evidence for the contribution of K^+_{ATP} channels to the repolarization phase of action potentials (Light, Comtois & Renaud, 1994). The delayed recovery of tetanic force observed in glibenclamide-treated muscles supported the notion that K^+_{ATP} channels play a protective role during the development of fatigue.

A class of pharmacological agents known as K^+ channel openers has been shown to restore the membrane potential in diseased human skeletal muscle (Spuler, Lehmann-Horn & Grafe, 1989) and denervated mouse muscle (Hong & Chang, 1992), reduce the amplitude of the twitch in frog muscle (Sauviat, Ecault, Faivre & Findlay, 1991), and enhance Rb^+ efflux from frog skeletal muscle (Benton & Haylett, 1992). The finding in these different studies, that this evoked K^+ permeability was inhibited by sulphonylureas, led to the conclusion that the underlying mechanism responsible was probably activation of K^+_{ATP} channels. However, Weselcouch, Sargent, Wilde & Smith (1993) found that K^+ channel openers did not affect the twitch force of rat muscle during normoxia, but accelerated the loss of function of anoxic muscle. Cromakalim was reported not to prevent acute ischaemia-induced skeletal muscle fatigue (Trezise, Drew, Roach, Watts & Weston, 1993).

To our knowledge, direct evidence for a physiological role of these channels during exercise is not available. The obvious difficulty in using the patch clamp technique on a working muscle fibre prevents clear demonstration of the activation of the K^+_{ATP} channel during exercise leading to fatigue. One way of overcoming this technical obstacle in skeletal muscle is to study the behaviour of K^+ channels in cell-attached patches in response to metabolic poisoning which may simulate the fatigue phenomenon. In the first part of this paper, using the cell-attached configuration of the patch clamp technique, the effect of different metabolic poisons on the K^+ permeability of adult mouse skeletal muscle fibres was investigated. It was demonstrated that these poisons activated K^+_{ATP} channels and that this activation was associated with a significant reduction of the channel conductance. In the second part of the paper, using the inside-out configuration, we examined the effects of intracellular Mg^{2+} concentration ($[Mg^{2+}]_i$) and intracellular pH (pH_i) on the K^+_{ATP} channel conductance and activity which may account, at least in part, for the modifications observed during metabolic exhaustion.

METHODS

Isolation of skeletal muscle fibres

Mice were killed by decapitation. Flexor digitorum brevis and interosseal muscles were removed and incubated at 37 °C in Tyrode solution containing collagenase (2 mg ml^{-1}, Type I; Sigma) for 1·5 h. After enzyme treatment, muscles were rinsed and stored in Tyrode solution (for composition see Solutions and chemicals) at 5 °C until use. Before each experiment, whole muscles were transferred into disposable 35 mm tissue culture dishes (Corning) and intact skeletal muscle fibres were separated from the muscle mass by gently triturating the muscle with a plastic Pasteur pipette.

Electrophysiology

Single channel currents were recorded at 0 mV from cell-attached or inside-out membrane patches at room temperature using a voltage clamp amplifier (model RK 300; Bio-Logic, Claix, France). Currents flowing into the pipette were considered to be positive. Currents were displayed on a chart recorder (model BD 7; Kipp & Zonen, Delft, Holland; cut-off frequency 5 Hz), and at the same time digitized using a PCM converter (model 701; Sony) and stored on videotape for further analysis using Bio-Patch software (Bio-Logic). Channel activity was determined from the average current (I) as $NP_o = I/i$ in each patch, where i is the single channel current, N is the number of open channels and P_o the open-state probability. NP_o was measured over 1 min recording periods after filtering at 100 Hz and sampling at 500 Hz.

Single channel current amplitudes in cell-attached experiments were statistically analysed using Student's unpaired t test. Values were considered significant when $P < 0·05$.

Solutions and chemicals

In cell-attached experiments, pipettes were filled with Tyrode solution containing (mM): 140 NaCl, 5 KCl, 2·5 $CaCl_2$, 1 $MgCl_2$, 10 Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), adjusted to pH 7·5 with NaOH; or, as in Fig. 2*B*, with K^+-rich solution containing (mM): 140 KCl, 2·5 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, adjusted to pH 7·5 with KOH.

The fibres were bathed in K^+-rich solution. For inside-out and $[Mg^{2+}]_i$ experiments, pipettes were filled with Tyrode solution and membrane patches were exposed to a solution containing (mM): 140 KCl, 1 EGTA, 10 Hepes–KOH and various concentrations of Mg^{2+}. The proportion of free Mg^{2+} and free ATP were determined using the dissociation constants of EGTA and ATP from Fabiato & Fabiato (1979). For inside-out and pH_i experiments, pipettes were filled with Tyrode solution and membrane patches were exposed to a solution containing (mM): 100 KCl, 10 EGTA, 10 Hepes (for pH 7·5) or 10 Pipes (piperazine-*N*-*N'*-bis(2-ethanesulphonic acid); for pH 7 and 6·5) or 10 Mes (2-(*N*-morpholino)ethane sulphonic acid; for pH 6), adjusted with KOH. KCl was added in each solution to bring $[K^+]$ to 150 mM.

Isolated fibres for cell-attached experiments or membrane patches for inside-out experiments were exposed to different solutions by placing them in the mouth of a perfusion tube from which the rapidly exchanged solutions flowed.

ATP (potassium salt; Sigma) was buffered at pH 7 (adjusted with KOH). Glibenclamide (Sigma) and cromakalim (Leo Pharmaceutical) were dissolved in dimethyl sulphoxide both at a concentration of 100 mM. 2,4-Fluorodinitrobenzene (FDNB; Aldrich) and 2,4-dinitrophenol (DNP; Merck) were dissolved in dimethyl sulphoxide both at a concentration of 1 M. KCN (Merck) and iodoacetate (IA; Aldrich) were dissolved in water at concentrations of 100 mM and 1 M, respectively; and KCN was buffered at pH 7 (adjusted with HCl). All drugs were diluted to the required concentrations in the perfused solutions.

RESULTS

Effect of metabolic poisons on channel activity in the cell-attached configuration

In these experiments, isolated skeletal muscle cells were bathed and superfused with K^+-rich medium (150 mM K^+) in order to clamp the fibre near 0 mV. Membrane currents were recorded at 0 mV in cell-attached patches using a pipette filled with Tyrode solution (5 mM K^+). Under control conditions, brief openings of one type of outward current were only rarely detected. Addition of 1 mM FDNB, an inhibitor of creatine kinase, induced a large increase in channel activity within 20 s (Fig. 1*A*). In this patch, at least thirteen channel levels were revealed after exposure to the drug, whereas only one channel level was detected in the control. Such an increase in channel activity was observed in thirty of thirty-two patches tested with concentrations of FDNB ranging from 200 μM to 1 mM. This activation by FDNB was partially reversible 2 min after removal of the drug, and a second application of FDNB gave rise instantaneously to reactivation of the channels, indicating that the contact between the pipette and the cell had not been lost.

The effects of DNP, a classical uncoupler of phosphorylative oxidation, were then tested. As described previously, opening of the channel was only rarely seen under control conditions (Fig. 1*B*). Thirty seconds after exposure of the fibre to 500 μM DNP, a dramatic increase in channel activity

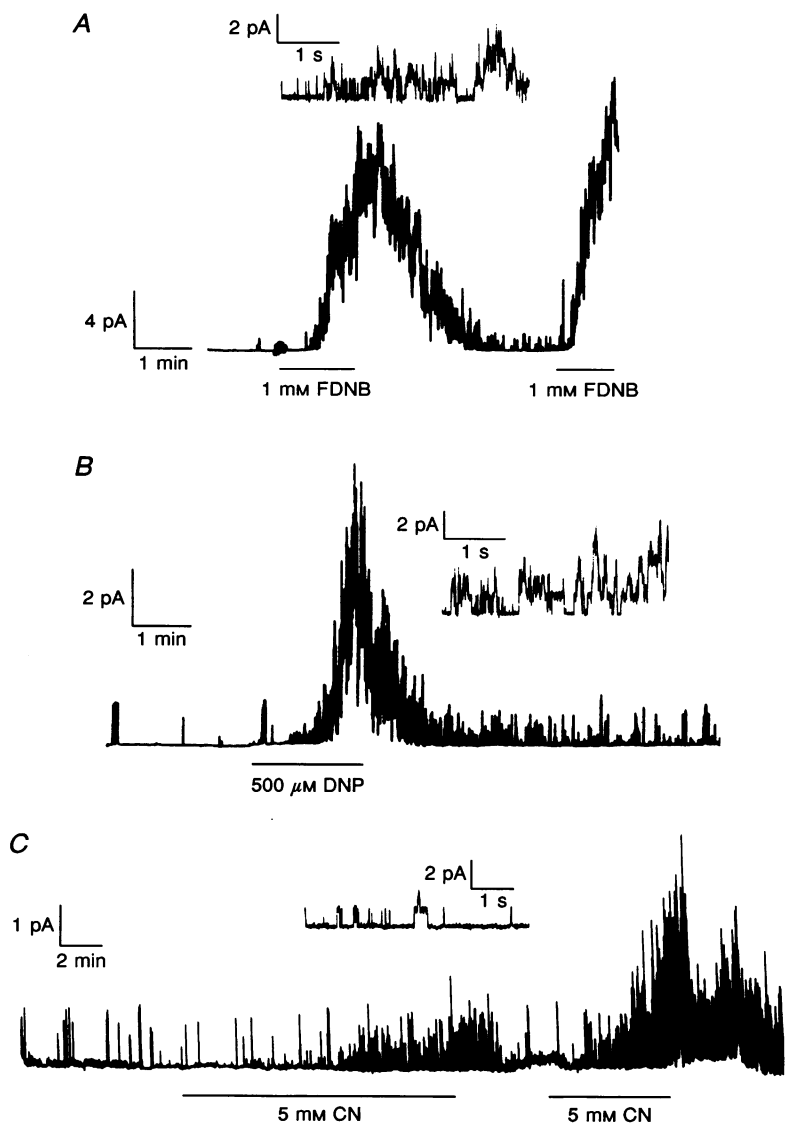

Figure 1. Single channel currents recorded from cell-attached patches in isolated skeletal muscle fibre during metabolic poisoning

The metabolic poisons, FDNB (*A*), DNP (*B*) and CN (*C*), were added to the bath solution for the periods indicated by the bars. Insets show short segments of the main traces during poisoning on an expanded scale.

was observed. In this patch, after 2 min of application, at least seven channels were active. Four minutes after wash out of the drug, a low channel open probability could again be observed. DNP-evoked channel activation was detected in sixteen of twenty-three patches.

Figure 1*C* shows that enhancement of channel activity could also be induced when the fibre was exposed to 5 mM CN, an inhibitor of phosphorylative oxidation. In contrast to DNP and FDNB, a delay of 4 min 30 s was observed before channel activation occurred. In this patch, two channels were active after CN treatment. A partial reversibility could be obtained 2 min after withdrawal of the drug. A second exposure to CN irreversibly reactivated the channels within 2 min.

In ten patches tested, 2 mM IA, an inhibitor of glycolysis, did not give rise to channel activation. This result is illustrated in Fig. 3*A*, where exposure to IA for 5 min 30 s was unable to enhance channel activity. In the continued presence of IA, subsequent addition of 5 mM CN caused progressive channel activation after a delay of 5 min. Channel activation in the presence of 5 mM CN or 2 mM IA + 5 mM CN was observed in all tested patches ($n = 15$).

Characterization of the channel activated by metabolic poisoning

Ion channel current–voltage relationships

In Fig. 2*A*, membrane current was recorded in a cell-attached patch using a pipette filled with Tyrode solution, the cell was bathed with K^+-rich solution containing 1 mM FDNB. Under these conditions, only outwardly directed channel currents were recorded. A typical current–voltage relationship is illustrated, the slope being 16 pS. In Fig. 2*B*, the pipette was filled with K^+-rich solution and the cell was bathed with K^+-rich solution containing 1 mM FDNB. Under these conditions, the current–voltage relationship displayed inward rectification at positive voltages and a reversal potential at 0 mV, the presumed K^+ equilibrium potential under symmetrical K^+ concentrations of 150–160 mM. The conductance of the channel was 67 pS calculated from the slope in the negative voltage region.

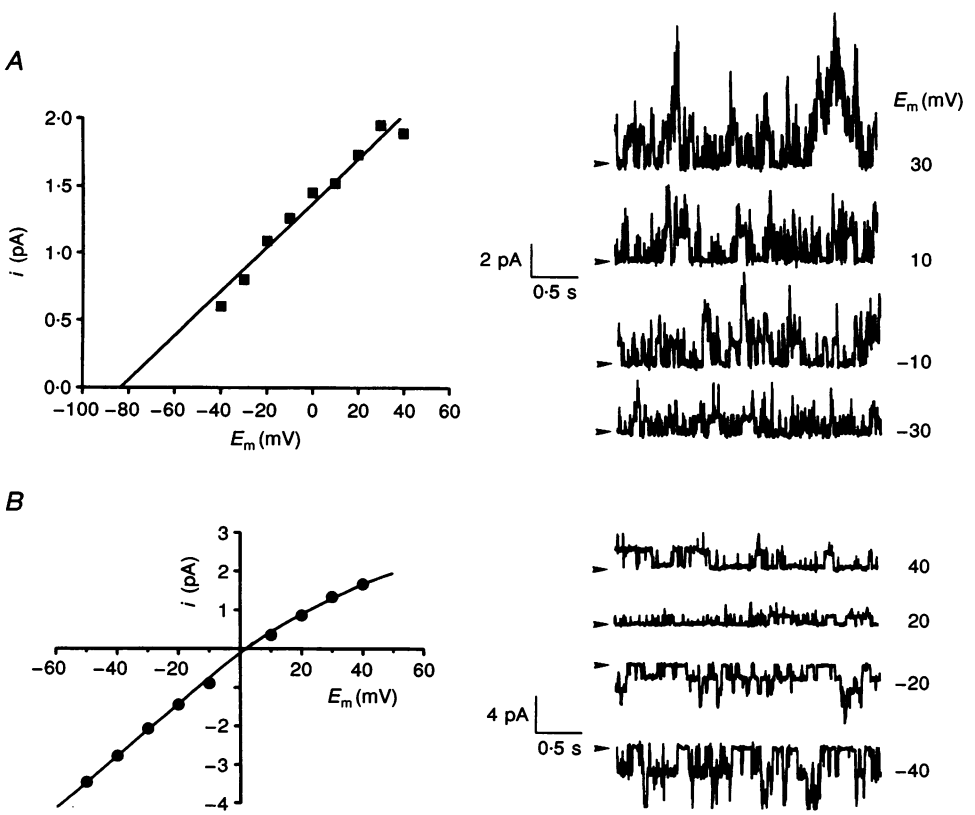

Figure 2. Conductance properties of the channel activated by metabolic poisoning in cell-attached patches

In *A*, the pipette was filled with Tyrode solution and the cell was bathed in K^+-rich solution containing 1 mM FDNB. In *B*, the pipette was filled with K^+-rich solution and the cell was bathed in K^+-rich solution containing 1 mM FDNB. The left panels present the current–voltage relationships and the right panels show examples of channel currents at the various membrane potentials indicated. The arrows to the left of the current traces indicate the zero current level.

Effect of glibenclamide on channel activation induced by metabolic poisoning

During these experiments, the pipette was filled with Tyrode solution and the cells were bathed with K^+-rich solution. Figure 3 shows the effect of 10 μM glibenclamide added to the perfused external solution on channel activation triggered by treatment of the fibre with 2 mM IA + 5 mM CN (Fig. 3*A*), 1 mM FDNB (Fig. 3*B*) and 500 μM DNP (Fig. 3*C*). NP_o was 1·3 in the presence of IA + CN. Addition of 10 μM glibenclamide transiently reduced NP_o to 0·2. It must be mentioned that in seven patches in which glibenclamide was applied after IA + CN treatment, channel opening was poorly and transiently blocked by the drug used at concentrations of up to 30 μM. Glibenclamide inhibited channel activity induced by FDNB from a NP_o of 0·7 to 0·08. In the case of DNP, a decrease in NP_o from 0·4 to 0·02 was observed in the presence of glibenclamide.

Effect of cromakalim on channel activity

The effects of the K^+ channel openers have been extensively studied in excised patches from mouse skeletal muscle (Allard & Lazdunski, 1993). We show here that, in cell-attached patches, activation of this outward current can also be produced by addition of the K^+ channel opener cromakalim to the external perfusion solution at concentrations ranging from 100 to 300 μM. A typical experiment is shown in Fig. 4. Very rare openings were observed in the control. On exposure of the cell to 300 μM cromakalim, three channels were activated. This effect was partially reversible on removal of the drug. Such activation was observed in ten cells.

Effect of metabolic poisoning and cromakalim on single channel current amplitude

In forty-one patches in which the open duration in control was long enough to allow measurement, the unitary current amplitude was 1·4 ± 0·015 pA. Figure 5*A* (upper panels) shows short segments of current recordings from the experiment presented in Fig. 3*B*. Corresponding amplitude histograms (lower panels) indicate that, under control conditions, single channel current amplitude was 1·32 pA, while in the presence of 1 mM FDNB, single channel current

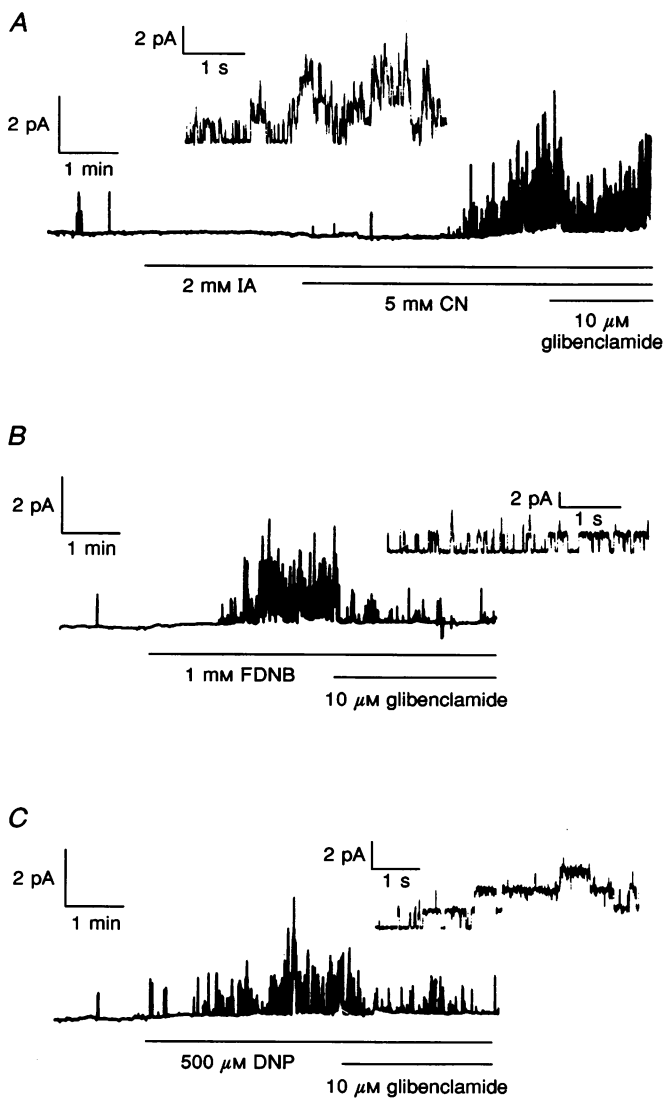

Figure 3. Effect of glibenclamide on single channel currents activated by IA + CN (*A*), FDNB (*B*) and DNP (*C*) in cell-attached membrane patches

Metabolic poisons and glibenclamide were added to the bath solution for the periods indicated by the bars. Insets show short segments of the main traces during poisoning on an expanded scale.

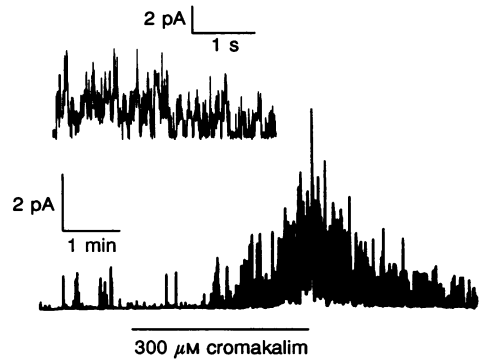

Figure 4. Single channel currents recorded from cell-attached patches in a muscle fibre exposed to the K^+ channel opener cromakalim

Cromakalim was added to K^+-rich bath solution for the period indicated by the bar. The inset shows a short segment of the main trace on an expanded scale.

amplitude was slightly decreased to 1·21 pA. Figure 5*B* presents the mean unitary current amplitudes both under control conditions and in the presence of the different metabolic inhibitors. During metabolic poisoning, unitary current amplitudes were significantly reduced from $1·4 \pm 0·02$ to $1·27 \pm 0·03$ pA ($n = 17$) by FDNB treatment, from $1·39 \pm 0·03$ to $1·25 \pm 0·05$ pA ($n = 10$) by DNP treatment and from $1·44 \pm 0·02$ to $1·34 \pm 0·03$ pA ($n = 13$) by IA + CN treatment. Figure 5*B* also shows that in the ten cells in which channel activation was induced by cromakalim, single channel current amplitude was not significantly modified ($1·39 \pm 0·04$ pA in control compared with $1·41 \pm 0·04$ pA in the presence of cromakalim).

Under physiological ion gradients (Na^+ pipette/K^+ bath) the current activated by metabolic poisoning was reversed near −85 mV, the predicted K^+ equilibrium under these conditions. In the presence of 150 mM K^+ in the pipette (K^+ bath), the current reversed at 0 mV, the presumed K^+ equilibrium potential under symmetrical K^+ concentrations. We can assume that the outward current induced by poisoning is a K^+ current. Since metabolic poisoning is assumed to induce severe intracellular ATP depletion, and because the channels activated by metabolic poisoning are inhibited by glibenclamide, it is highly probable that these channels belong to the K^+_{ATP} class of channels. For an intracellular concentration of K^+ estimated to be 160 mM,

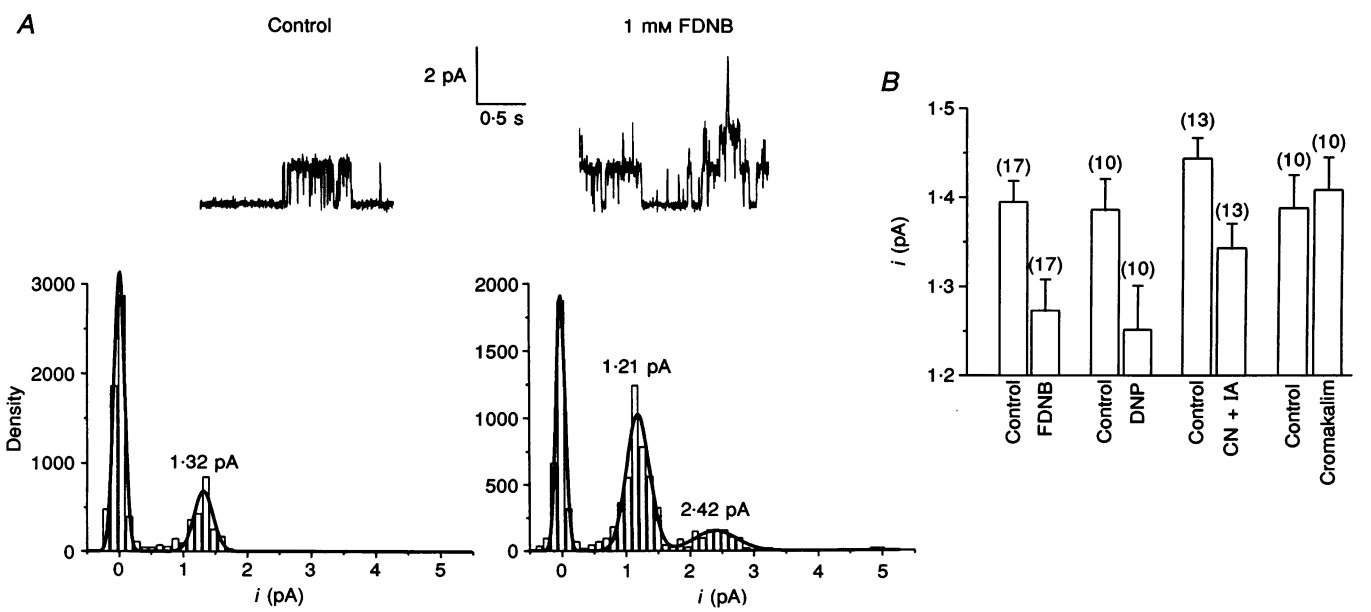

Figure 5. Effect of metabolic poisoning and cromakalim on unitary current amplitude

A, single channel current recordings and corresponding amplitude histograms obtained from cell-attached patches under control conditions and in the presence of 1 mM FDNB. The recordings correspond to short segments of the main traces presented in Fig. 3*B*. *B*, comparison of the mean unitary current amplitudes under control conditions and in the presence of different metabolic poisons and cromakalim. The single channel current amplitudes were determined using amplitude histograms established in each patch for each condition. Numbers above the columns denote the number of patches for which a comparison could be made and bars indicate S.E.M. The mean of single channel current amplitude was significantly reduced in the presence of FDNB, DNP and CN for which P was 0·007, 0·038 and 0·017, respectively. Single channel current amplitude was not significantly changed by cromakalim ($P = 0·66$).

mean unitary current amplitudes for control at 0 mV, with Tyrode solution in the pipette, provide a channel conductance of about 16 pS. Current–voltage relationships in the presence of poison indicated a conductance of 16 pS with Tyrode solution in the pipette and 67 pS with K^+-rich solution in the pipette. These values are close to those found for the K^+_{ATP} channel in excised patches under similar ionic conditions (Woll *et al.* 1989; Allard & Lazdunski, 1992). So, it is likely that the channel recorded in control and during poisoning is the K^+_{ATP} channel.

Effect of $[Mg^{2+}]_i$ in the inside-out configuration

$[Mg^{2+}]_i$ has been shown to increase during fatigue and metabolic inhibition (Westerblad & Allen, 1992), and ATP breakdown is likely to be the source. The increase in $[Mg^{2+}]_i$ has been shown to depress the amplitude of single K^+_{ATP} channels in frog skeletal muscle cells (Forestier & Vivaudou, 1993). The following part of this paper will be devoted to analysis of the effects of increasing concentrations of intracellular Mg^{2+} in inside-out patches on activity and conductance properties of the mammalian K^+_{ATP} channel. The purpose is to try to establish whether such intracellular modifications can account for the reduced unitary current amplitude and the increased activity of K^+_{ATP} channels observed during metabolic inhibition.

Effect of $[Mg^{2+}]_i$ on channel activity in the absence of intracellular ATP

The effects of $[Mg^{2+}]_i$ were first studied in inside-out membrane patches clamped at 0 mV in the absence of nucleotide at the cytoplasmic face. Tyrode solution was present in the pipette and the cytoplasmic face was perfused with an internal solution containing 1 mM EGTA. Figure 6*A* shows that, on excision, when Mg^{2+} was absent from the internal solution, K^+_{ATP} channels were spontaneously highly active. In this patch, NP_o was 7·3 in control. On increasing Mg^{2+} concentrations at the cytoplasmic face, channel opening was reduced. NP_o was 1·9 in the presence of 3 mM free Mg^{2+} and 0·6 in the presence of 10 mM free Mg^{2+}. On removal of Mg^{2+}, activity was partially restored. In addition, it was observed that the presence of intracellular Mg^{2+} induced an apparent change in channel gating. This result is illustrated in Fig. 6*B*. This particular patch displayed a low activity, thus changes in channel gating could be more easily detected. In control, the cytoplasmic face was exposed to a solution containing 1 mM free Mg^{2+}. Three channels were active and the current rapidly stepped from one level to the other. Removal of Mg^{2+} from the cytoplasmic face induced an increase in the time the channels spent in the open state and NP_o increased from 2·4 in the presence of Mg^{2+} to 4·3 in its absence. Re-addition of Mg^{2+} in the internal perfused medium restored initial gating and activity. Figure 6*C* shows the dose–response curve for inhibition of K^+_{ATP} channels by free Mg^{2+}. Half-maximal inhibition of K^+_{ATP} channel activity was observed at about 1·25 mM Mg^{2+}.

Effect of $[Mg^{2+}]_i$ on single channel current amplitude

Figure 7*A* (left panels) presents short segments of current recordings obtained at 0 mV in the presence of 0, 1 and 3 mM free Mg^{2+}. Corresponding amplitude histograms (right panels) indicate a unitary current amplitude of 2·2 pA in the absence of Mg^{2+}, and 1·7 and 1·1 pA in the presence of 1 and 3 mM free Mg^{2+}, respectively. Figure 7*B* shows the relationship between single K^+_{ATP} channel amplitude and Mg^{2+} concentration.

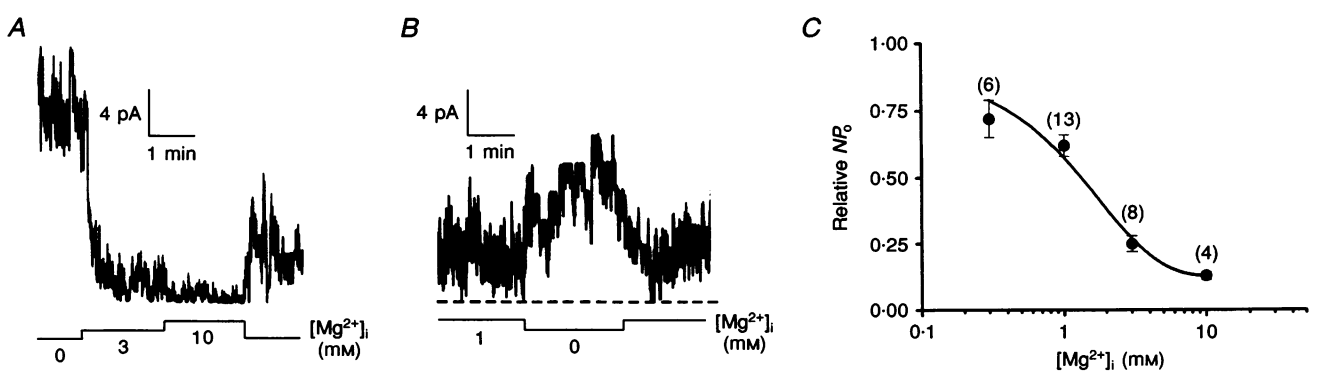

Figure 6. Effect of $[Mg^{2+}]_i$ on K^+_{ATP} channel activity in inside-out patches

A, effect of increasing $[Mg^{2+}]_i$. *B*, increase in channel activity induced by removal of intracellular Mg^{2+}. Note the increase in the time the channels spent in an open state in the absence of intracellular Mg^{2+}. The indicated concentrations of Mg^{2+} are given as free Mg^{2+} and were added for the periods indicated by the bars. *C*, dose–response relationship between channel activity and $[Mg^{2+}]_i$. Values were normalized to the value obtained in the absence of Mg^{2+}. Mean values of these normalized values were again normalized and plotted. Here and in subsequent figures, bars indicate S.E.M. and numbers of measurements are given in parentheses. The curve was drawn by eye.

Effect of $[Mg^{2+}]_i$ on channel activity in the presence of intracellular ATP

Although Mg^{2+} behaves as an inhibitor of K^+_{ATP} channel activity in the absence of intracellular ATP, we found that Mg^{2+} reduced the inhibition of channel activity by ATP. As shown in Fig. 8*A*, in experiments in which patches were first excised in the absence of Mg^{2+} at the cytoplasmic face, the addition of 200 μM intracellular ATP decreased channel activity and NP_o was reduced from 17·1 in control to 0·13 in the presence of ATP. In the continued presence of ATP, 400 μM total Mg^{2+} was added. Under these conditions, in which the majority of ATP was complexed to Mg^{2+}, a significant increase in channel activity was observed. NP_o increased from 0·13 in the presence of 200 μM free ATP to 1·53 in the presence of 152 μM Mg-ATP and 48 μM free ATP. This effect was reversible on removal of Mg^{2+} (NP_o, 0·38). When ATP was washed out, activity was transiently increased above control (NP_o, 24). This potentiation of channel activity may be interpreted as the well-described 'refreshment' phenomenon observed after washout of Mg-ATP, previously described in insulin-secreting cells (Findlay & Dunne, 1986) and in rat ventricular myocytes (Findlay, 1987*a*). Similar effects were observed when ATP was used at a concentration of 1 mM. Figure 8*B* shows that the channel activity was completely abolished by addition of 1 mM ATP in the absence of intracellular Mg^{2+}. This inhibition was relieved on removal of ATP. Subsequently, addition of 1 mM free Mg^{2+} reduced activity as described above. ATP (1 mM) and a total of 2 mM Mg^{2+} were then added in order to attain 0·932 mM Mg-ATP (0·068 mM free ATP) and to keep free $[Mg^{2+}]$ constant (1 mM). Under these conditions, ATP inhibition was much less effective and NP_o was 0·4, which corresponds to 3% of the control activity. When ATP was removed (and free Mg^{2+} kept at 1 mM), the control activity was restored. Quantitative results from additional experiments are presented in Fig. 8*C*. NP_o was

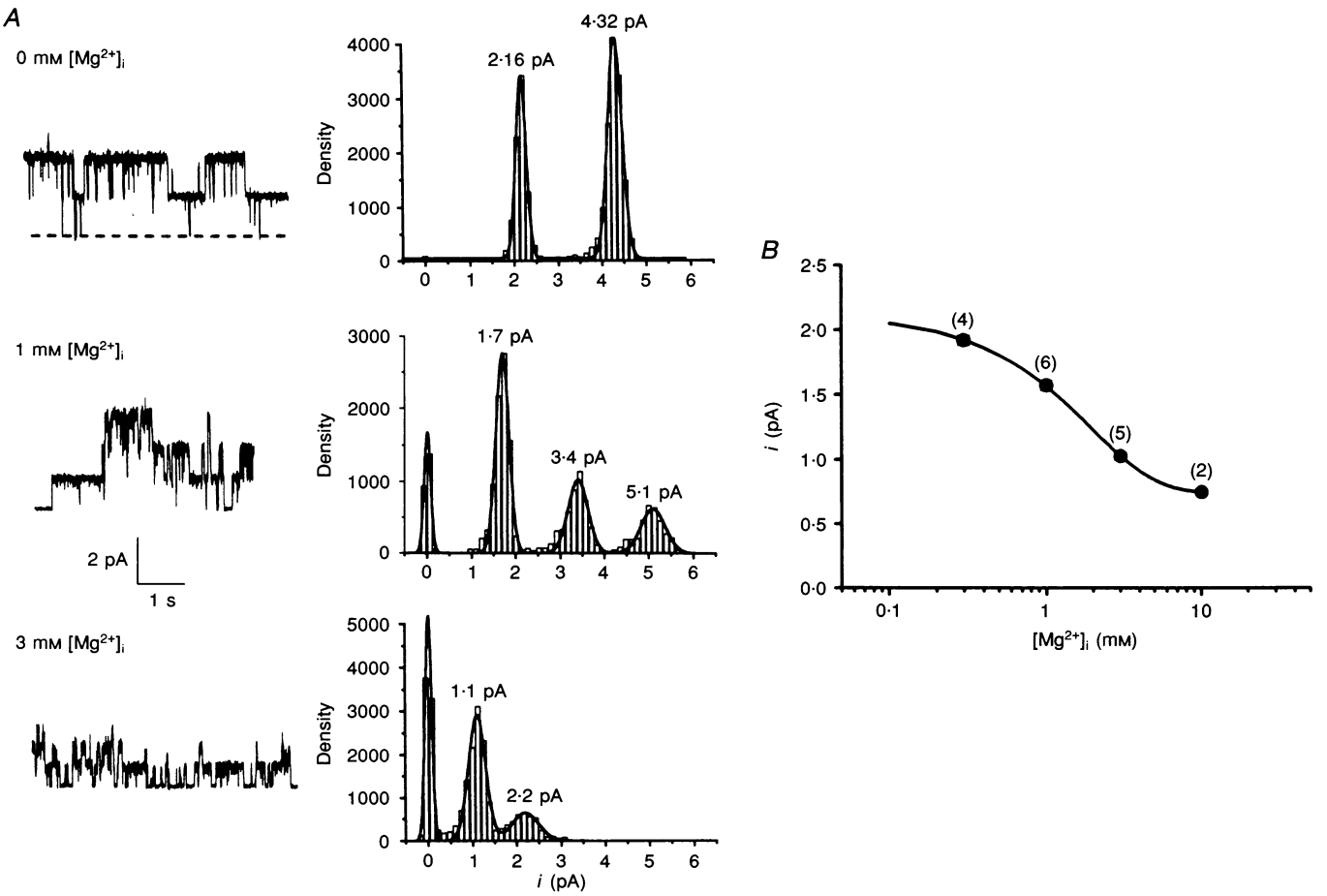

Figure 7. Effect of $[Mg^{2+}]_i$ on the amplitude of single K^+_{ATP} channel currents in inside-out patches

A, short segments of single channel currents, and corresponding amplitude histograms, recorded in the presence of various concentrations of intracellular free Mg^{2+}. *B*, the dose–response relationship between single channel current amplitude and free Mg^{2+} concentration. Single channel current amplitudes were determined using amplitude histograms established in each patch for each condition. The curve was drawn by eye. Mean values are plotted, bars indicate S.E.M., the number of measurements is given in parentheses.

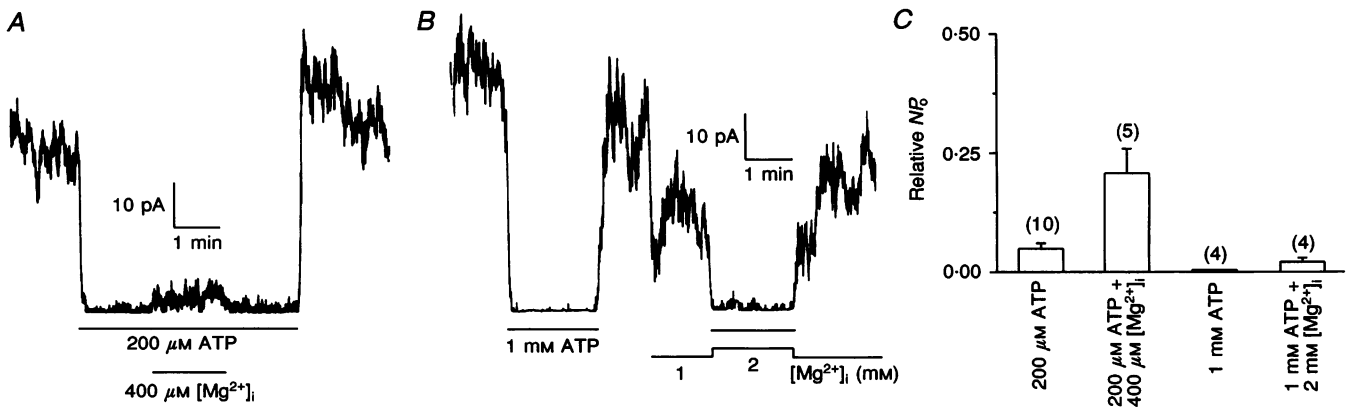

Figure 8. Effect of $[Mg^{2+}]_i$ on K^+_{ATP} channel activity in the presence of 200 μM (*A*) and 1 mM intracellular ATP (*B*) in inside-out patches

The indicated ATP and Mg^{2+} concentrations were added during the periods indicated by the bars. The concentrations of Mg^{2+} and ATP are given as total Mg^{2+} and total ATP. *C*, comparison of the degree of inhibition by free ATP and Mg-ATP. Values were normalized to the values obtained under control conditions in the absence of ATP. Means of these normalized values were again normalized and plotted.

reduced to 5 ± 1·2% ($n = 10$) of control by 200 μM free ATP, whereas 152 μM Mg-ATP and 48 μM free ATP reduced NP_o to 21 ± 5·2% ($n = 5$) of control. In the presence of 1 mM ATP NP_o was 0, while 2 ± 0·9% ($n = 4$) of control activity was maintained in the presence of 0·932 mM Mg-ATP. These data suggest that, in mammalian skeletal muscle, free ATP is a much more potent inhibitor of K^+_{ATP} channels than Mg-ATP.

Effect of pH_i in the inside-out configuration

It is well known that fatigue is associated with a fall in pH_i (Davies *et al.* 1991). This fall in pH_i has been shown to depress the amplitude of single K^+_{ATP} channels and to reduce the inhibitory effect of ATP in frog skeletal muscle cells (Davies, 1990). In this section, we investigate, in inside-out patches, the effects of a decrease in pH_i on activity and conductance properties of mammalian K^+_{ATP}

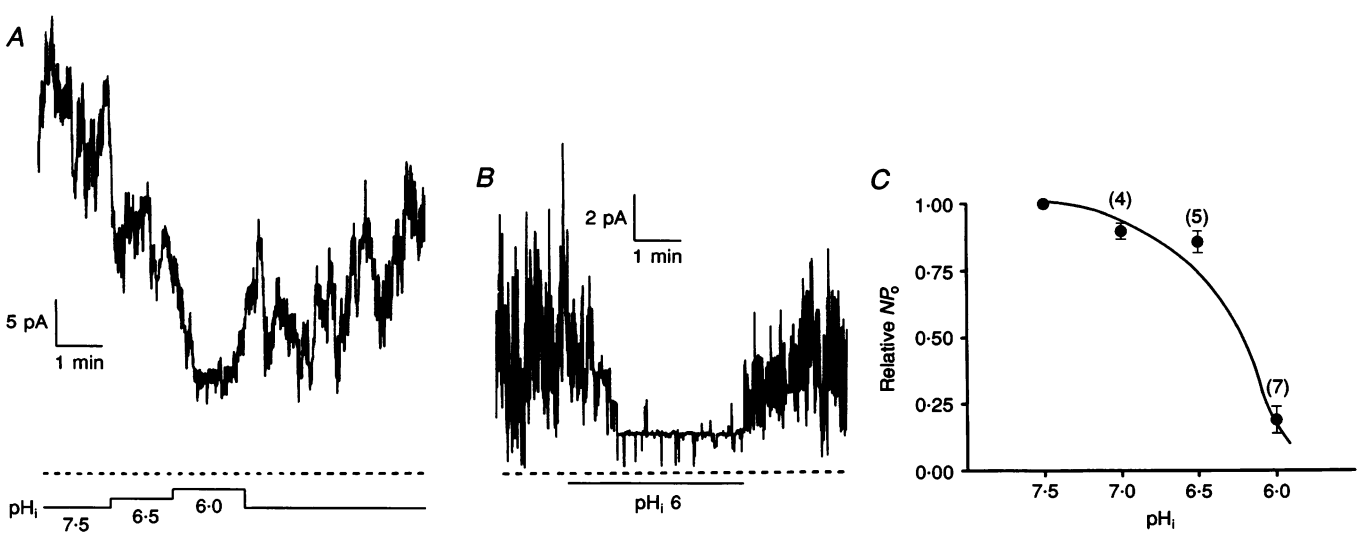

Figure 9. Effect of a decrease in pH_i on K^+_{ATP} channel activity in inside-out patches

A, continuous recording of single K^+_{ATP} channel currents at various values of pH_i in a patch displaying high activity. The pH_i was switched from 7·5 to 6·5 and 6 during the periods indicated by the bars. *B*, effect of a decrease of pH_i from 7·5 to 6 (during period indicated by bar) on channel gating in a patch displaying lower channel activity. *C*, pH_i–response relationship. Values were normalized to that obtained at pH 7·5. Means of these normalized values were again normalized and plotted. The curve was fitted by eye.

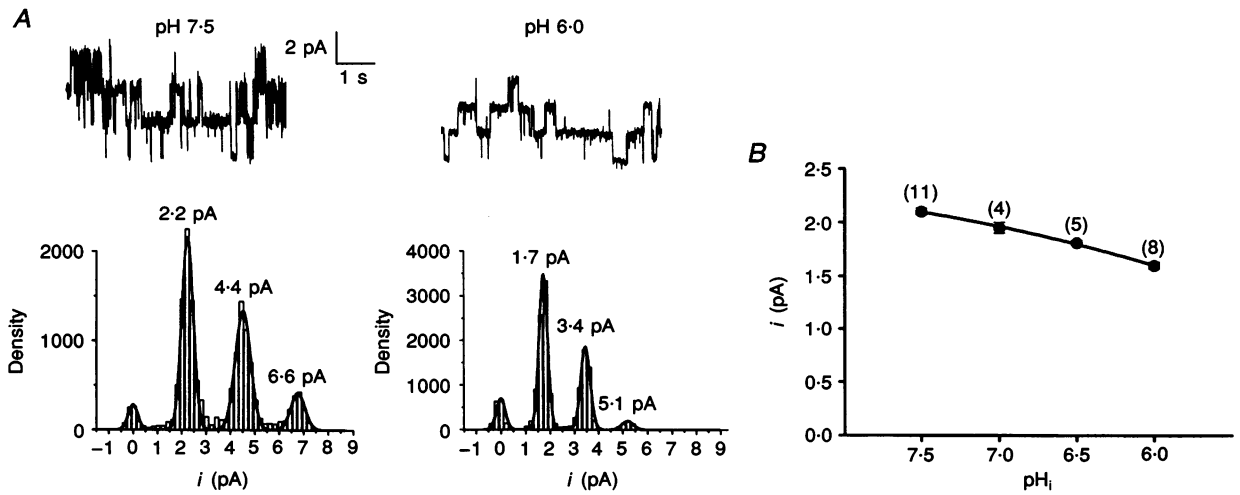

Figure 10. Effect of a decrease in pH_i on the amplitude of single K^+_{ATP} channel currents in inside-out patches

A, segments of single channel currents recorded at pH 7·5 and 6 and corresponding amplitude histograms. *B*, relationship between single channel current amplitude and pH_i. The single channel current amplitudes were determined with amplitude histograms established in each patch for each condition. The curve was fitted by eye.

channels and evaluate, as for $[Mg^{2+}]_i$, whether these effects could explain the reduced single channel current amplitude and the increase in channel activity observed during metabolic poisoning.

Effect of a decrease in pH_i on channel activity in the absence of intracellular ATP

Figure 9*A* shows a continuous recording of K^+_{ATP} currents from an inside-out patch the cytoplasmic face of which was exposed to decreasing pH_i. Acidification from pH 7·5 to pH 6·5 and 6·0 produced a reduction in NP_o from 16 to 12·8 and 4·6, respectively. When pH_i was returned to 7·5, NP_o was partially restored after several minutes. In another experiment shown in Fig. 9*B*, where less channels were active in the patch, it could be seen that reducing pH from 7·5 to 6·0 led also to a change in channel gating. Whereas seven channels were active at pH 7·5, only two channel levels could be detected after 2 min exposure to pH 6·0, with one channel remaining almost continually open. On return to pH 7·5, the initial gating was restored and five channels were again active. The relationship between channel activity and pH_i is given in Fig. 9*C*. Apparent half-inhibition of channel activity was observed at pH 6·3.

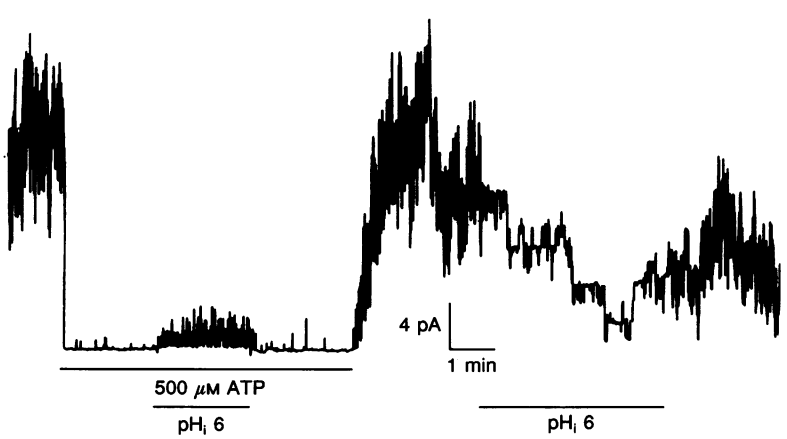

Figure 11. Effect of a decrease in pH_i on K^+_{ATP} channel activity in the presence of intracellular ATP and in its absence in the same inside-out patch

The pH_i was switched from 7·5 to 6 during the periods indicated by the bars. ATP was increased from 0 to 500 μM as indicated.

Effect of a decrease in pH_i on single channel current amplitude

Figure 10*A* shows current recordings of K^+_{ATP} channels obtained at pH 7·5 and 6·0. The corresponding amplitude histograms indicate that unitary amplitude was decreased from 2·2 pA at pH 7·5 to 1·7 pA at pH 6·0. The mean single channel current amplitude is plotted as a function of pH_i in Fig. 10*B*.

Effect of a decrease in pH_i on channel activity in the presence of intracellular ATP

We next studied the effects of intracellular acidification after inhibition of channel activity by ATP. Patches were first exposed to an intracellular solution buffered at pH 7·5. Figure 11 shows that addition of 500 μM ATP reduced activity so that openings were rare. In the continued presence of ATP, decreasing pH_i from 7·5 to 6·0 restored substantial channel activity and NP_o was increased to 0·3 at pH 6·0. This effect was reversible on return to the control pH_i. On ATP removal, the initial activity was restored. Subsequently, it can be seen that inhibition of channel activity could still be attained when pH_i was decreased in the absence of ATP at the cytoplasmic face. As described above, a decrease in pH_i from 7·5 to 6·0 led to a reduction in channel activity and blocking of the remaining channels in an open state. This effect was also partially reversible on return to pH 7·5. In four patches, in the presence of 500 μM ATP, NP_o was increased from 0 to 6 $\pm$ 0·03% of control when pH_i was decreased from pH 7·5 to 6·0.

DISCUSSION

Activation of K^+_{ATP} channels by metabolic poisoning

The purpose of this paper was to analyse the effects of metabolic poisoning on the K^+ permeability of adult mouse isolated skeletal muscle fibre. In cell-attached patches, only one type of K^+ channel could be observed in control conditions and during metabolic poisoning. Because of the low open probability of these K^+ channels in the control, it was not possible to characterize them adequately using pharmacological tools. However, the very similar values for single channel current amplitude measurements in control and during poisoning, and the fact that opening of these channels by metabolic poisoning is inhibited by glibenclamide, strongly suggest that these channels are K^+_{ATP} channels in both conditions. The reduced inhibiting effect of glibenclamide when channel opening was induced by IA + CN may be reliant on some loss of glibenclamide sensitivity of the K^+_{ATP} channel during metabolic exhaustion as demonstrated in cardiac cells (Findlay, 1993). Metabolic poisoning did not produce activation of any other type of K^+ channel, for example, the Ca^{2+}-activated K^+ channel the amplitude and gating of which are unmistakably different from those of K^+_{ATP} channels. In skeletal muscle, the intracellular concentration of ATP is known to be maintained at 5–10 mM at rest and even during vigorous activity. However, as already indicated, it was observed that the open probability of K^+_{ATP} channels, though very low, was not zero in control resting muscle cells. This observation might support the notion that ATP is compartmentalized, and that the subsarcolemmal ATP concentration differs from the intracellular bulk concentration. Three different types of metabolic poisons which alter different metabolic pathways have been used. FDNB, an inhibitor of creatine kinase, is assumed to strongly reduce the buffering action of creatine phosphate, which represents the main source of ATP regeneration in skeletal muscle, thus leading to a rapid fall in intracellular ATP concentration and opening of the K^+_{ATP} channels. We did indeed observe that K^+_{ATP} channels activate during FDNB treatment of resting fibres. This activation was reversible, indicating that the creatine kinase reaction was still functional after washout of the poison. Inhibition of oxydative phosphorylation by DNP and CN also induced activation of the K^+_{ATP} channels. In the presence of DNP, activation of K^+_{ATP} channels occurred within the first minute, while in the presence of CN, 5 min of perfusion were necessary for activation to occur. It can be suggested that in the presence of CN, which blocks ATP synthesis at the level of the phosphorylative chain, a slow rate of ATP depletion occurs due to basal ATP consumption by the resting cell. During uncoupling of phosphorylative oxidation with DNP, it is expected that depletion of ATP is faster due to an additive ATP breakdown through activation of mitochondrial ATPase. Also the possibility that DNP reaches mitochondria more rapidly than CN cannot be excluded. The intracellular ADP concentration is known to increase during metabolic exhaustion and high intensity exercise (Westerblad, Lee, Lännergren & Allen, 1991). Allard & Lazdunski (1992) reported that ADP behaves as a potent activator of the K^+_{ATP} channel. It can be proposed that channel activation is mediated, at least in part, by an increase in ADP. Furthermore, the relatively fast recovery (about 2 min) from drug-evoked channel activation may imply that ATP is rapidly rephosphorylated after washout of the drugs, suggesting that ADP, rather than AMP, is the major product formed from ATP hydrolysis. The inability of IA to activate K^+_{ATP} channels was striking at first sight. IA is known to inhibit glycolysis at the level of glyceraldehyde phosphate dehydrogenase. However, in muscle, glycolysis is not a major pathway for energy supply at rest. Free fatty acids (FFA) are the primary bioenergetic substrates. Therefore, it is likely that during inhibition of glycolysis, ATP production by the oxidative metabolism, and from creatine phosphate, is high enough to meet the energy demand and to keep K^+_{ATP} channels closed.

Reduction of single channel current amplitude during metabolic poisoning

The observation that the amplitude of single K^+_{ATP} channel current was reduced during metabolic inhibition is an interesting result. Inside-out experiments have shown that the decrease in channel current amplitude could be attributed to an increase in $[Mg^{2+}]_i$ and/or to a fall in pH_i. It has been demonstrated in this study that, in inside-out patches, intracellular Mg^{2+} decreases single K^+_{ATP} channel current amplitude in a dose-dependent manner. A similar reduction of channel current amplitude was described for K^+_{ATP} channels in cardiac cells (Findlay, 1987*a*), in pancreatic β-cells (Findlay, 1987*b*), and in frog skeletal muscle (Vivaudou *et al.* 1991). Using the relationship between K^+_{ATP} channel current amplitude and Mg^{2+} concentration (Fig. 7*B*), we estimate an intracellular concentration of about 1·4 mM free Mg^{2+} in resting fibres for an average current amplitude of 1·4 pA, under control conditions. This value is in the same range as those measured with Mg^{2+}-sensitive microelectrodes or fluorescent Mg^{2+} indicators (for reviews see Blatter, 1990; Westerblad & Allen, 1992). Quantitatively, ATP is the most important binding molecule for Mg^{2+} at rest, and free Mg^{2+} is a reliable index for the concentrations of intracellular ATP (Westerblad & Allen, 1992). In fatigued isolated mouse skeletal muscle cells, Mg^{2+} concentration was found to be twice as high as that at rest, while during metabolic blockade an increase from 0·78 to 2·08 mM was observed (Westerblad & Allen, 1992). It seems reasonable to assume that the reduction in K^+_{ATP} channel current amplitude observed in this study during metabolic poisoning was caused, at least partly, by the fall in intracellular ATP concentration, which results in an elevation of the $[Mg^{2+}]_i$. The fact that the channel current amplitude was not modified during cromakalim-evoked activation, which does not change ATP concentrations, reinforces this hypothesis. A current decrease from 1·4 to 1·3 pA was observed during metabolic poisoning. This could be interpreted as being due to an increase in $[Mg^{2+}]_i$ of about 400 μM during metabolic blockade. This change in Mg^{2+} would be somewhat lower than values reported by Westerblad & Allen (1992). However, it must be noted that metabolic blockade experiments carried out by these authors were conducted on a larger time scale leading to complete and irreversible exhaustion. Moreover, in their experiments, the fibre was stimulated to produce a tetanus every minute, implying that the solicited metabolic pathways are different from those involved in our own experiments in which the muscle cells remained at rest. Finally K^+_{ATP} channels probably 'monitor' the subsarcolemmal concentration of Mg^{2+}, which might be different from the bulk intracellular Mg^{2+} concentration. The threshold for K^+_{ATP} channel opening in intact cell-attached patches might precede the appearance of the fatigue phenomenon.

Effect of $[Mg^{2+}]_i$

In this study it was observed that, in the absence of intracellular ATP, intracellular Mg^{2+} inhibited channel opening in a dose-dependent manner. These results are consistent with an apparent modification by Mg^{2+} of the time the channels spend in the open state. There is a clear difference between frog and mammalian muscle since, in frog skeletal muscle, Forestier & Vivaudou (1993) reported that Mg^{2+} did not affect channel activity. However, the behaviour of mammalian skeletal muscle resembles that observed for cardiac muscle and pancreatic β-cells (Findlay, 1987*a*, *b*), in which blockade of K^+_{ATP} channels was observed in the same range of $[Mg^{2+}]_i$. On the other hand, Mg^{2+} lowered ATP inhibition of K^+_{ATP} channels as in pancreatic β-cells (Findlay, 1987*b*), but unlike results in cardiac cells in which Mg-ATP was found to be a more effective channel blocker than free ATP (Findlay, 1988). Previous results obtained with skeletal muscle are controversial. Vivaudou *et al.* (1991) reported that free ATP was a much more potent inhibitor than Mg-ATP in frog skeletal muscle, whereas Spruce, Standen & Stanfield (1987) and Davies (1990) working on the same system found that Mg^{2+} did not affect channel inhibition by ATP. In mouse skeletal muscle, Weik & Neumcke (1989) indicated that ATP inhibition was reduced when no EDTA was added to the intracellular solution to minimize the effect of the presence of divalent ions. The fact that Mg^{2+} lowers ATP inhibition has obvious physiological significance since, as indicated above, the breakdown of Mg-ATP leads to a rise in $[Mg^{2+}]$ thus lowering the threshold for activation of K^+_{ATP} channels.

Effect of pH_i

In human skeletal muscle, the pH_i has been shown to fall to 6·1 at the end of fatiguing exercise (Pan, Hamm, Rothman & Shulman, 1988). It was found in this study that reducing pH_i to less than 6·5 in the absence of intracellular ATP resulted in inhibition of K^+_{ATP} channel activity. Similar observations have been made recently with pancreatic β-cells (Proks, Takano & Ashcroft, 1994). In contrast Koyano, Kakei, Nakashima, Yoshinaga, Matsuoka & Tanaka (1993) reported that intracellular protons were able to reactivate run-down channels in cardiac ventricular cells. Lowering pH_i in frog skeletal muscle produced a slight increase in the channel open probability (Davies, Standen & Stanfield, 1992). Clearly, as for Mg^{2+}, K^+_{ATP} channels in mammalian skeletal muscle and in frog skeletal muscle seem to behave differently. Decreasing pH_i also led to a decrease in the single channel current amplitude. This effect was also found in pancreatic β-cells (Proks *et al.* 1994), cardiac muscle (Koyano *et al.* 1993) and frog skeletal muscle (Davies, 1990; Davies *et al.* 1992). This reduction in channel current amplitude by lowering pH_i could account, at least partially, for the decrease in channel current amplitude observed during metabolic poisoning. In the presence of ATP, acidification increases K^+_{ATP} channel activity. This result

confirms data obtained in frog skeletal muscle, in which the main effect of reducing pH_i was to considerably lower the affinity of the channel for ATP. A 15-fold increase in the inhibitor constant (K_i) for ATP was observed when pH_i was decreased from 7·25 to 6·25 (Davies *et al.* 1992). In cardiac cells, reduction of pH_i also lowered the affinity of the channel for ATP but not to such an extent as in skeletal muscle (Koyano *et al.* 1993). In pancreatic β-cells, both a decrease (Misler, Gillis & Tabacharani, 1989) and an increase in ATP sensitivity (Proks *et al.* 1994) have been demonstrated. As previously noted by Davies *et al.* (1992), it is likely that metabolically induced changes in pH_i constitute a potent physiological regulator of K^+_{ATP} channel activity in skeletal muscle.

Physiological significance

It has long been known that fatigue in mammalian muscle is associated with a loss of intracellular K^+ (for review see Fitts, 1994). Juel (1986) observed an approximate doubling of extracellular K^+ following muscle stimulation. Metabolic poisoning and mechanical exhaustion of frog skeletal muscle fibres have long been known to promote the activation of K^+ conductance (Fink & Lüttgau, 1976), and pharmacological investigations have suggested that this K^+ conductance could be associated with activation of the K^+_{ATP} channel (Castle & Haylett, 1987; Light *et al.* 1994). We have shown that the open probability of K^+_{ATP} channels in cell-attached patches is very low at rest in mammalian skeletal muscle and that these channels open during metabolic exhaustion. Does such activation occur in physiological conditions? The ATP concentration in skeletal muscle rarely drops below 60–70% of the pre-exercise level even in cases of extensive fatigue (for review see Fitts, 1994). Then intracellular ATP in fatigued muscle would remain at a concentration which totally blocks K^+_{ATP} channels in excised patches. It is likely that metabolic factors other than ATP participate in the activation of the channel. ADP behaves as a K^+_{ATP} channel activator in muscle (Allard & Lazdunski, 1992), and is known to increase in concentration from a few micromolar to about 200 μM in working muscle (Westerblad *et al.* 1991). ADP is a likely candidate for channel activation in fatigued muscle. This paper shows that the increase in $[Mg^{2+}]_i$ and the fall in pH_i, which are associated with ATP breakdown during exercise, constitute other activatory factors which, when added up, may trigger K^+_{ATP} channel opening.

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Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (CNRS). Thanks are due to P. Mégas and V. Jacquemond for fruitful discussions.

Received 27 September 1994; accepted 21 November 1994.