The effect of intracellular anions on ATP-dependent potassium channels of rat skeletal muscle

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1. We have used excised inside-out patches to study the effects of anions bathing the cytoplasmic surface of the membrane on ATP-dependent K^+ channels of rat flexor digitorum brevis muscle. Channels were closed by ATP applied to the cytoplasmic face of the patch with a concentration for half-closure (K_i) of 14 μM, were highly selective for K^+ and had unitary conductances of 62 pS in symmetrical 155 mM K^+ and 27 pS in 5 mM $[K^+]_o$.

2. In 139 mM Cl^- internal solution channel activity declined rapidly after excision of the patch. Inclusion of 40 mM potassium gluconate (substituted for KCl) in the solution both restored channel activity and greatly slowed its subsequent run-down.

3. The action of gluconate was concentration dependent. The effect did not involve a change in ATP binding, since the K_i for ATP was not significantly changed by gluconate, and was specific for the cytoplasmic face of the patch.

4. The anions pyruvate, lactate and acetate were all able to restore channel activity after run-down, though less well than gluconate, while sulphate and methylsulphate were without effect.

5. Analysis of single channel kinetics showed that gluconate did not affect mean open lifetime, but led to a decrease in the number and duration of long closings.

6. Anions are most likely to act by stabilizing the structure of the channel protein. Changes in the intracellular concentration of certain anions may play a role in regulating channel activity.

ATP-dependent K^+ channels (K^+_{ATP} channels) occur in muscle tissues, in pancreatic β-cells, and in neurones (for reviews see Ashcroft & Ashcroft, 1990; Davies, Standen & Stanfield, 1991). They have been described in skeletal muscle from frog, human and mouse (Spruce, Standen & Stanfield, 1985; Burton, Dörstelmann & Hutter, 1988; Woll, Lönnendonker & Neumcke, 1989). In cardiac muscle, pancreatic β-cells and insulinoma cell lines K^+_{ATP} channel activity has been observed to decline rapidly after patch excision into the inside-out configuration, a phenomenon called run-down (Trube & Hescheler, 1984; Misler, Falke, Gillis & McDaniel, 1986; Findlay & Dunne, 1986). In these tissues, activity is restored, at least partially, after exposure to solutions containing MgATP. Since this reactivation is Mg^{2+} dependent, and does not occur with non-hydrolysable analogues of ATP, it has been proposed that run-down involves channel dephosphorylation, and that rephosphorylation returns the channel to its active state. In addition, it has been proposed that Ca^{2+} in the cytoplasmic solution promotes run-down in cardiac muscle by activating Ca^{2+}-dependent phosphatases (Furukawa, Fan, Sawanobori & Hiraoka, 1993).

Run-down of activity in K^+_{ATP} channels from skeletal muscle seems to depend to some extent on the preparation used. Thus run-down in skeletal muscle from frog and sometimes mouse appears slight (Spruce, Standen & Stanfield, 1987; Woll *et al.* 1989), though in mouse muscle run-down has been reported to be increased by internal Ca^{2+} (Hussain & Wareham, 1993). Further, MgATP produced little reactivation of K^+_{ATP} channels in mouse muscle after spontaneous run-down (Hussain & Wareham, 1993). Here, we report studies of K^+_{ATP} channels from rat skeletal muscle, and show that their activity shows rapid run-down. This can be reversed, and activity maintained, by certain anions applied to the intracellular surface of excised membrane patches. A brief report of some of these findings has been communicated to the Physiological Society (McKillen, Davies, Beirão, Standen & Stanfield, 1993).

METHODS

Preparation

Adult Wistar rats were killed by cervical dislocation and the flexor digitorum brevis muscle dissected out. To dissociate single muscle fibres, we used a method modified from that of Gillespie & Ribchester (1988). Muscles were incubated in 0·3% collagenase (Sigma, Type 1) made up in Ringer solution containing (mM): NaCl, 146·3; KCl, 4·75; $CaCl_2$, 1; Ca_2HPO_4, 0·95; $MgCl_2$, 0·5; Hepes, 9·5, adjusted to pH 7·4 with NaOH. Muscles were placed in this solution for 30 min at 4 °C to allow the solution to diffuse into the muscle, and then incubated for 90 min at 37 °C. Single fibres were isolated in Ringer solution without collagenase by trituration with a fire-polished Pasteur pipette. Measurements of seventeen isolated fibres gave a mean length of 871 ± 297 μm and diameter of 34 ± 11 μm. The isolated fibres were then placed in a 155 mM K^+ solution described below. In this solution sarcolemmal vesicles formed on the surface of the muscle fibres, and we normally excised membrane patches from these vesicles into the same solution.

Recording methods

Currents through single K^+_{ATP} channels were recorded using the inside-out configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were made from 1·5 mm borosilicate capillaries (GC150F, Clark Electromedical Ltd, Pangbourne, UK) pulled on a Kopf puller, coated either with Sylgard resin or dental wax to reduce background noise and fire-polished. Their resistances were 5–20 MΩ when filled with electrolyte solution. After seal formation the patch was excised and placed in the outlet stream of a perfusion pipette consisting of a common outlet connected to six or eight different reservoirs. Unitary currents were recorded with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA, USA) and stored on a modified Sony digital audio tape (DAT) recorder. Membrane potentials are expressed as inside of the membrane relative to outside, and outward currents are defined as positive and plotted upwards.

Solutions

For most experiments the solution in the patch pipette, which bathed the extracellular side of the membrane, contained 10 mM K^+, and was composed of (mM): KCl, 10; NaCl, 145; $CaCl_2$, 2; Hepes, 10, adjusted to pH 7·4 with NaOH. In some cases we used pipette solutions with 5, 100 or 155 mM K^+, made by substituting KCl for NaCl or vice versa in the above solution. Intracellular solutions consisted of a 155 mM K^+ solution containing (mM): KCl, 139; EGTA, 5; Hepes, 10, adjusted to pH 7·4 with KOH; or solutions in which a proportion of the Cl^- was replaced by gluconate, lactate, pyruvate, acetate, sulphate or methylsulphate by replacing KCl by an equivalent amount of the appropriate potassium salt. Formulae for these anions are given in Fig. 7. ATP (dipotassium salt), which was weighed and dissolved just before an experiment, was added to the intracellular solutions and the pH readjusted to 7·4. We measured the liquid junction potentials that occurred between pipette and bath solution and sometimes bath and flow solution. The junction potential between 5 mM K^+ solution and 155 mM K^+ solution was 4 mV, while that between intracellular solution with 40 mM gluconate and control (139 mM Cl^-) intracellular solution was −5 mV. We corrected the reversal potentials used to estimate channel selectivity (Fig. 2*D*) for the measured junction potential, but otherwise the voltages given here are uncorrected.

Analysis

For single channel analysis digital audio tapes were replayed through an 8-pole Bessel filter at a cut-off frequency (−3 dB) between 1·9 and 3·9 kHz, and digitized at 20 kHz using a TL-125 labmaster A–D interface (Axon Instruments) and a Dell 325 microcomputer. For measurement of channel activity as NP_{open} (see below), records were filtered at 1 kHz and digitized at 4 kHz. A suite of programs developed using the AxoBASIC library (Davies, 1993) was used both to apply voltage-clamp command potentials and for analysis of currents.

Measurement of P_{open}. Measurement of P_{open} was performed on patches containing up to six channels by measuring the times, t_j, spent at current levels corresponding to $j = 0, 1, 2 \ldots N$ channels open. The overall P_{open} was then obtained using:

$$P_{open} = \left(\sum_{j=1}^{N} t_j j \right) / TN, \quad (1)$$

where the duration of the recording, T, was usually 30–80 s. The maximum number of channels in a patch, N, was taken as the maximum number of simultaneously open events seen under control (ATP-free) conditions. Some patches contained as many as thirty active channels. For such patches we obtained the concentration dependence of inhibition by ATP by averaging the current remaining under different ATP concentrations and normalizing this to the value obtained in the absence of ATP.

Single channel kinetics. Open and closed events were detected using a 50% threshold, with the estimated times of crossing given by linear regressions joining the pairs of data points on either side of the threshold. A minimum resolution of 100 μs was imposed on the data. Events were then log-binned at 25 bins per $\log_{10}$ unit using the method described by Sigworth & Sine (1987). The binned data were fitted using maximum likelihood to probability density functions of the form:

$$f(t) = \sum_{j=1}^{m} (a_j/\tau_j) \exp(-t/\tau_j), \quad (2)$$

where a_j is the area of component j, τ_j is the time constant of component j and m is the number of components. The number of components in a particular distribution was estimated from log-likelihood ratios (see Horn, 1987). Mean open times were corrected for brief closings by multiplying by the proportion of closed events detected, obtained by integrating the fitted closed time distribution between the minimum resolution (100 μs) and infinity.

Bursts were defined as openings or groups of openings separated by a closed time shorter than a critical time, t_c. This was calculated by equalizing the proportion of short closings misclassified as long and of long closings misclassified as short (Colquhoun & Sakmann, 1985). The number of open time components should be the same as the number of components in the distribution of total open time/burst (Colquhoun & Hawkes, 1982). This latter distribution is relatively little affected by missed events and was thus used to give a more accurate estimation of the number of open states.

Experiments were done at room temperature, 18–25 °C, and results are given as means ± S.E.M.

RESULTS

Properties of K^+_{ATP} channels from the rat FDB muscle

Inhibition by ATP

Many of the inside-out patches that we excised from rat muscle contained K^+_{ATP} channels, though channel activity usually declined rapidly after patch excision. In some patches, however, a number of channels remained active, allowing their inhibition by ATP to be studied. Figure 1*A* shows recordings made from a patch in which a large number of channels were active. The pipette solution contained 10 mM K^+, giving an equilibrium potential for K^+ of −69 mV, and the patch was held at 0 mV. Under these conditions channel openings gave outward unitary currents with an amplitude of 1·76 pA. Application of 1 mM ATP to the cytoplasmic face of the patch inhibited channel activity, and activity showed partial recovery on removal of ATP (Fig. 1*A*).

To measure the concentration dependence of channel inhibition by ATP we applied ATP at concentrations between 3 and 1000 μM, and measured NP_{open} over a

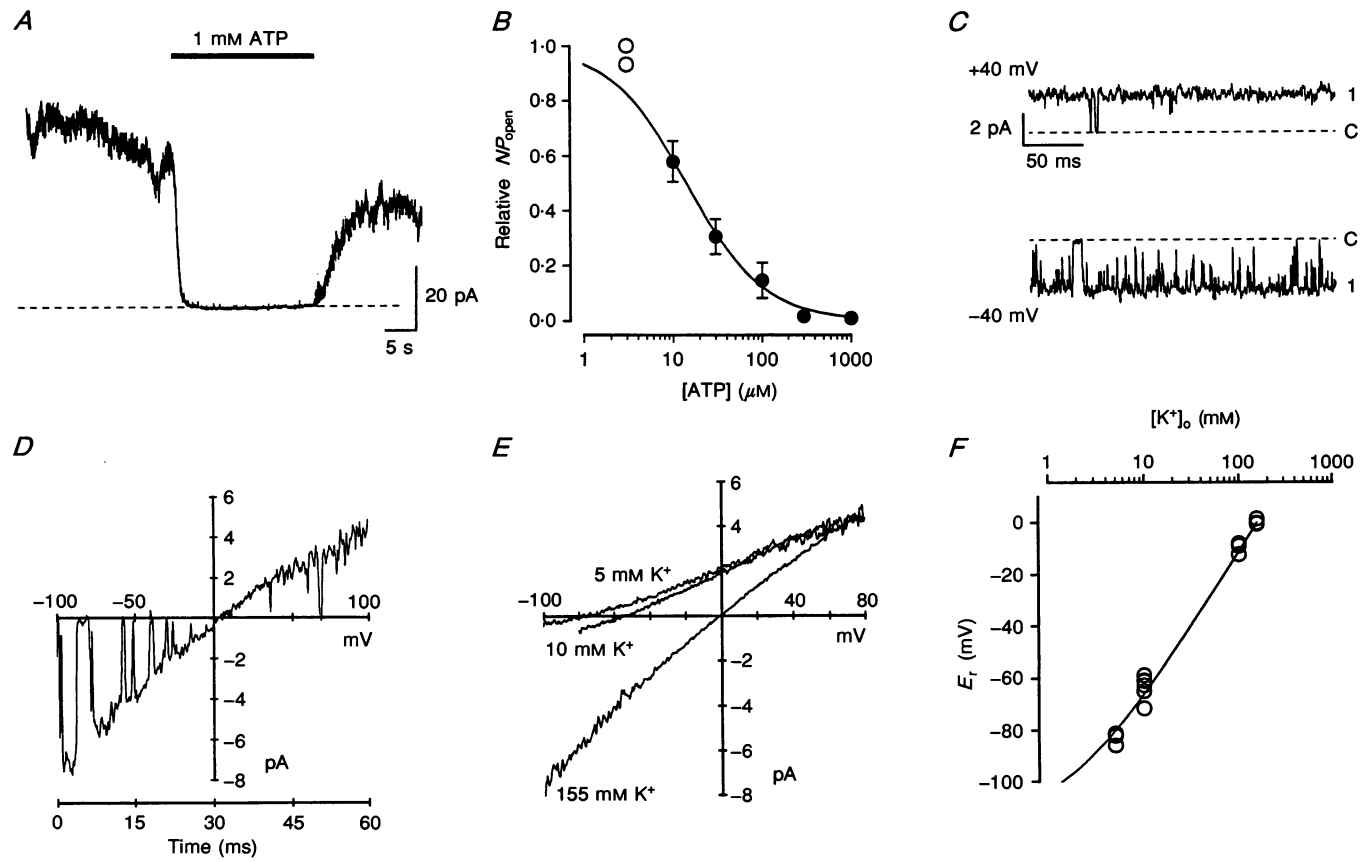

Figure 1. Properties of ATP-dependent K^+ channels in rat skeletal muscle
A, recording of current from an inside-out patch held at 0 mV and with an external (pipette) $[K^+]$ of 10 mM. The solution perfusing the cytoplasmic face of the patch contained 155 mM K^+ and 139 mM Cl^-. The solution bathing the inside of the patch was changed to a solution with 1 mM ATP as indicated, and the broken line indicates the zero current level. The record was filtered at 1 kHz. *B*, concentration–effect curve for channel inhibition by ATP. ●, means ± S.E.M., where larger than the symbol, of measurements from 6, 11, 4, 11 and 4 patches at 10, 30, 100, 300, and 1000 μM ATP respectively; ○, two individual measurements at 3 μM. The line is the least squares fit to eqn (3) with a K_i of 14 μM. *C*, single channel records from an inside-out patch in symmetrical 155 mM K^+ held at +40 mV and −40 mV. C and 1 mark the closed and open levels in each case. *D*, current recorded in response to a linear voltage ramp from +100 to −100 mV from a patch under the same conditions as in *C*. *E*, mean *I–V* relations measured using voltage ramps in external $[K^+]$ of 5, 10 and 155 mM. *F*, dependence of the reversal potential, E_r, on $[K^+]_o$. Each point shows the measurement from an individual patch. The line is drawn to:

$$E_r = \frac{RT}{F} \ln \frac{[K^+]_o + \alpha[Na^+]_o}{155 \text{ mM}},$$

with $\alpha = P_{Na}/P_K = 0{\cdot}01$. *R*, *T* and *F* have their usual thermodynamic meanings.

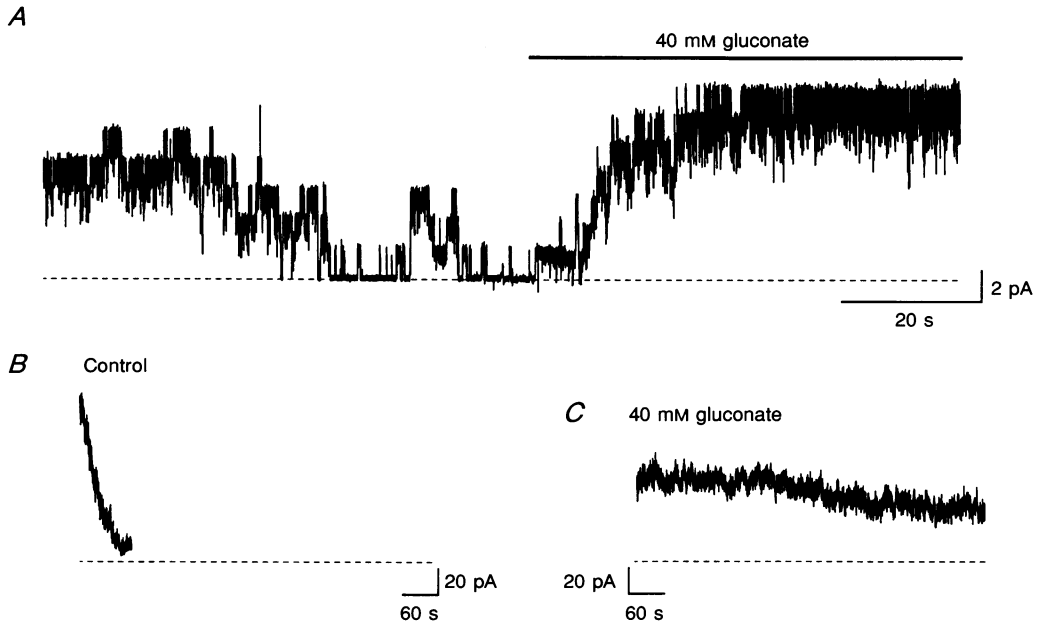

Figure 2. Gluconate restores channel activity
A, recording from an inside-out patch held at 0 mV with $[K^+]_o = 10$ mM. The recording began immediately after patch excision into 139 mM Cl^- solution. The solution bathing the inside of the patch was changed to 40 mM gluconate solution as indicated, and the dashed line indicates the zero current level. *B*, the decline of channel activity in a patch exposed to control (139 mM Cl^-) internal solution. The decline was fitted by a single exponential with a time constant of 32·6 s. The recording conditions were as in *A*, above. *C*, decline in channel activity in a patch exposed to 40 mM gluconate internal solution throughout, shown on the same time and current scale as *B*.

period of at least 30 s under each condition. Channel inhibition by ATP did not appear to be affected by run-down, though any such effect would be very hard to study quantitatively. To allow for effects of run-down in ATP-free solution, applications of ATP were bracketed by exposure to ATP-free solution, and NP_{open} in ATP was expressed relative to the mean of the values measured in ATP-free solution before and after the application of ATP. Figure 1*B* shows the concentration–effect curve for channel inhibition by ATP. The experimental results were fitted by the expression:

$$\text{Relative } NP_{open} = 1 - \frac{[ATP]^n}{[ATP]^n + K_i^n}, \qquad (3)$$

with an integer Hill coefficient, n, of 1. The line in Fig. 1*B* shows the best fit to all the individual values of Relative NP_{open} and gives an ATP concentration for half

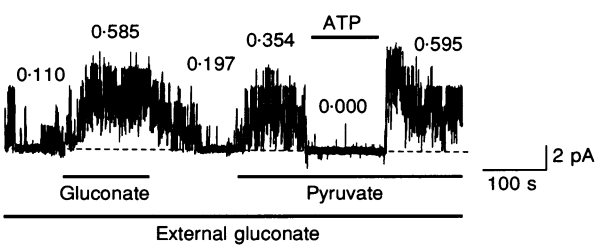

Figure 3. Lack of effect of extracellular gluconate
Recording from an inside-out patch held at 0 mV. The external (pipette) solution contained 10 mM K^+ and 40 mM sodium gluconate, substituted for NaCl. The bars show periods when the solution bathing the internal face of the patch was switched from 139 mM Cl^- solution to 40 mM gluconate solution, 40 mM pyruvate solution or 40 mM pyruvate solution with 300 μM ATP, as indicated. The numbers above the recording give the P_{open} values calculated from eqn (1), assuming that there were 5 channels in the patch, under each condition.

inhibition, K_i of 14 μM. This K_i is quite similar to the values of 12 μM measured in K^+_{ATP} channels from skeletal muscle of mouse (Neumcke & Weik, 1991) and 17 μM in frog (Davies, Standen & Stanfield, 1992).

Unitary conductance and selectivity

To establish that the ATP-dependent channels of rat muscle were K^+ selective, we varied $[K^+]_o$ by using pipette (external) K^+ concentrations of 5, 10, 100 or 155 mM. In 155 mM $[K^+]_o$ channel openings led to outward currents at +40 mV and to inward currents at −40 mV (Fig. 1*C*), consistent with a reversal potential close to the calculated E_K of 0 mV. Unitary current–voltage relations were measured using voltage ramps. Figure 1*D* shows the current recorded in response to a 60 ms voltage ramp from +100 to −100 mV from a patch in symmetrical 155 mM K^+ solutions with one active channel. Current–voltage relations were constructed by averaging the current recorded in response to many such ramps, but including only those segments of the current record during which the channel was open. Figure 1*E* shows such unitary *I–V* relations in 5, 10 and 155 mM $[K^+]_o$.

Figure 1*F* shows a plot of the reversal potential for the single channel current measured from *I–V* relations like those of Fig. 1*E*, and corrected for junction potentials, against external $[K^+]$. The results are well fitted by a line drawn assuming that the K^+_{ATP} channel selects for K^+ over Na^+ with a permeability ratio P_{Na}/P_K of 0·01. Single

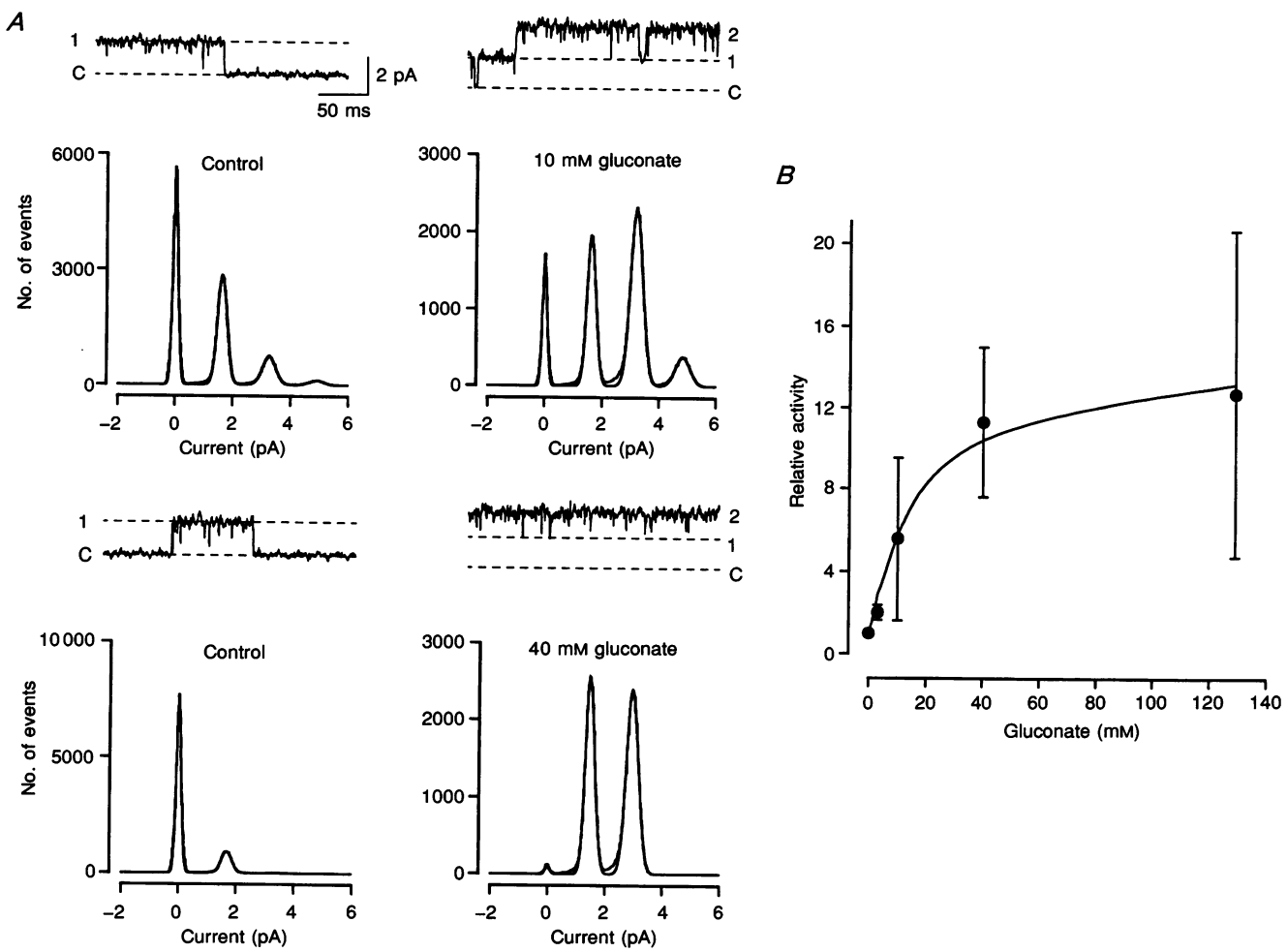

Figure 4. Concentration dependence of the effect of gluconate
A, all-points histograms of current amplitude made from the same patch under control conditions and in the presence of 10 and 40 mM gluconate. In each case a segment of current recording is shown above. The recordings were made in the sequence control, 10 mM gluconate, control, 40 mM gluconate, and the histograms were formed from 80, 80, 60 and 70 s of recording, respectively. The pipette solution contained 10 mM K^+ and the patch was held at 0 mV throughout. *B*, channel activation as a function of gluconate concentration. Relative activity, calculated as described in the text, is plotted against [gluconate]. The points show means ± S.E.M. of results from 5, 8, 10 and 4 patches at 3, 10, 40 and 129 mM gluconate. The line is a least-squares fit to eqn (4), with K_D of 14 mM, relative activity of 19·4, and Hill coefficient, n, of 1.

channel slope conductances measured at the reversal potential in each $[K^+]$ were: 5 mM $[K^+]_o$, 27 ± 1 pS (3 patches); 10 mM $[K^+]_o$, 27 ± 1 pS (7 patches); 100 mM $[K^+]_o$, 57 ± 1 pS (3 patches); 155 mM $[K^+]_o$, 62 ± 1 pS (3 patches).

The effect of gluconate on channel run-down

When the cytoplasmic face of excised patches was perfused with control (139 mM Cl^-) solution, the solution into which we routinely excised patches, channel activity declined rapidly with time after excision. This can be seen in Fig. 2*A*, which shows a recording that begins at the time of patch excision into control solution. Initially up to five active channels were seen, but activity declined rapidly over a period of about 1 min, so that at times all channels were closed. Figure 2*A* also shows the effect of changing the solution bathing the cytoplasmic face of the patch from control solution to one that contained 40 mM gluconate, substituted for Cl^-. This solution increased channel activity, often restoring NP_{open} to the level seen before run-down. In some patches which showed no activity after excision, application of 40 mM gluconate solution also caused channel activation. Switching the perfusing solution back to control solution led to a repetition of the rapid run-down seen before the application of gluconate. Gluconate always reversed run-down in patches in which channel activity declined after excision (22 patches). However, gluconate did not increase activity in six patches in which channel activity remained stable after excision into 139 mM Cl^- solution.

Figure 2*B* shows that channel activity in 139 mM Cl^- declined exponentially, with a time constant in this patch of 32·6 s. The mean time constant under these conditions was 36 ± 7 s (7 patches). In some patches channel activity apparently declined to zero, while in others a low level of activity remained after run-down. The rapid run-down of channel activity that we observe did not appear to depend on intracellular Ca^{2+}, since all our cytoplasmic solutions contained 5 mM EGTA, which should reduce free Ca^{2+} to $< 0{\cdot}6$ nM, assuming an initial contamination of 50 μM Ca^{2+}.

Channel activity also showed some decline with time in the presence of 40 mM gluconate, but this was very much slower than that seen without gluconate (Fig. 2*C*). This decline could not readily be fitted with an exponential, but the decline in activity was generally $< 10\%$ over the initial 60 s of perfusion with gluconate. In addition to increasing channel activity, gluconate also caused a small reduction in unitary current amplitude. At 0 mV with 10 mM $[K^+]_o$, the measured unitary current was reduced from $1{\cdot}76 \pm 0{\cdot}01$ pA ($n = 42$) in control solution to $1{\cdot}65 \pm 0{\cdot}01$ pA ($n = 14$) in the presence of 40 mM gluconate. This is most likely to be caused by the change in the real holding potential because of the extra junction potential in the gluconate solution, which we estimate to be about −5 mV.

Gluconate caused channel activation only when applied

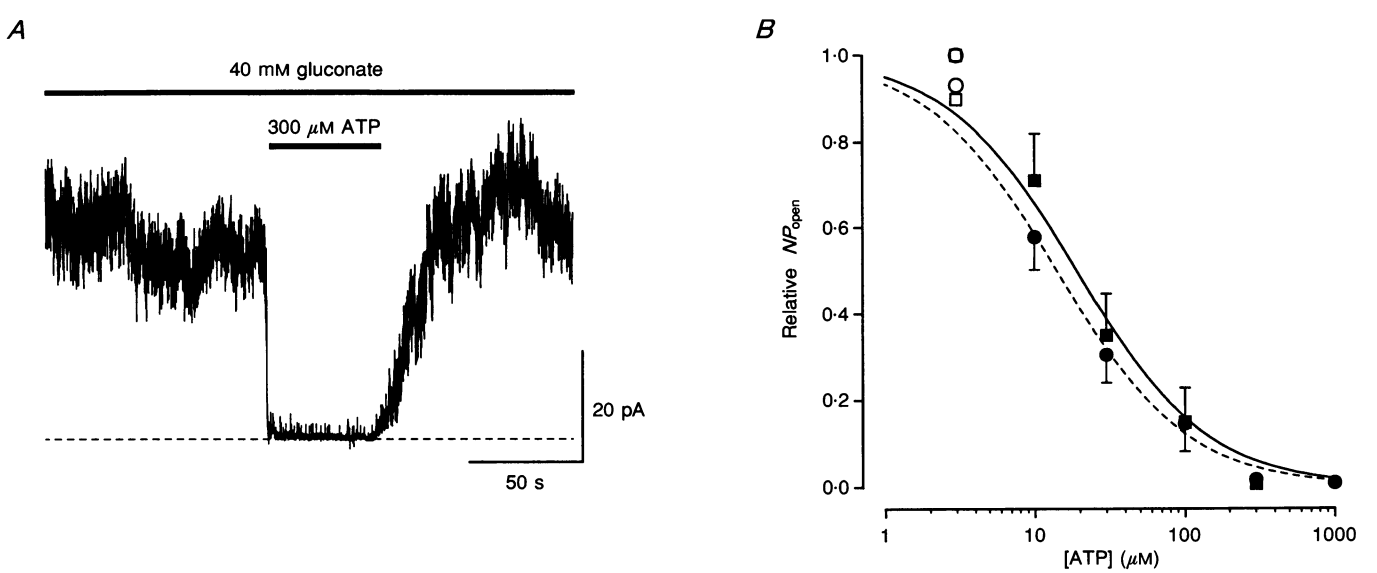

Figure 5. Gluconate does not affect channel closure by ATP
A, recording from a multi-channel patch held at 0 mV, and with an external $[K^+]$ of 10 mM. The solution perfusing the cytoplasmic face of the patch contained 40 mM gluconate, which was switched to 40 mM gluconate solution containing 300 μM ATP as indicated. The dashed line indicates the zero current level. *B*, concentration–effect curve for channel inhibition by ATP, measured in the presence of 40 mM gluconate. ■, means and S.E.M. where larger than the symbol, of measurements from 6, 7, 5 and 6 patches at 10, 30, 100 and 300 μM ATP, respectively; □, individual measurements from two patches at 3 μM ATP. The continuous line is drawn to eqn (3) with $K_i = 19$ μM. The dashed line and the circles show results in 139 mM Cl^- solution for comparison, replotted from Fig. 1*B*.

to the cytoplasmic surface of the patch. Figure 3 shows an example of an experiment in which 40 mM gluconate was applied to the external surface of the patch throughout the recording by inclusion in the 10 mM K^+ pipette solution (40 mM sodium gluconate replaced NaCl). Under these conditions, channel activity ran down rapidly with 139 mM Cl^- internal solution, as it does without external gluconate. Application of internal gluconate or pyruvate was able to restore channel activity (Fig. 3). In three other patches to which we applied external gluconate, we were also unable to detect any channel activation.

Concentration dependence

The activating effect of intracellular gluconate was concentration dependent. Channel activity was measured as P_{open} in patches exposed to control solution (139 mM Cl^-) and to solutions containing gluconate at 3, 10, 40 and 129 mM, substituted for Cl^- in each case. Exposures to gluconate solutions were bracketed by measurements in control solution, and P_{open} values were expressed relative to the mean of the values measured in control solution before and after exposure to gluconate to give a value for relative activity. Figure 4*A* shows recordings of channel activity and corresponding amplitude histograms measured from long periods of recording under control conditions and in the presence of 10 and 40 mM gluconate. The concentration–effect curve for the action of gluconate is shown in Fig. 4*B*. The results can be well fitted by a saturating function of the

$$\text{Relative activity} = 1 + \frac{\overline{\text{relative activity}}}{1 + (K_D/[\text{gluconate}])^n}, \quad (4)$$

with $\overline{\text{relative activity}}$, the maximal value of relative activity, of 19·4; K_D, the gluconate concentration for half-maximal activation, of 14 mM; and Hill coefficient, n, of 1.

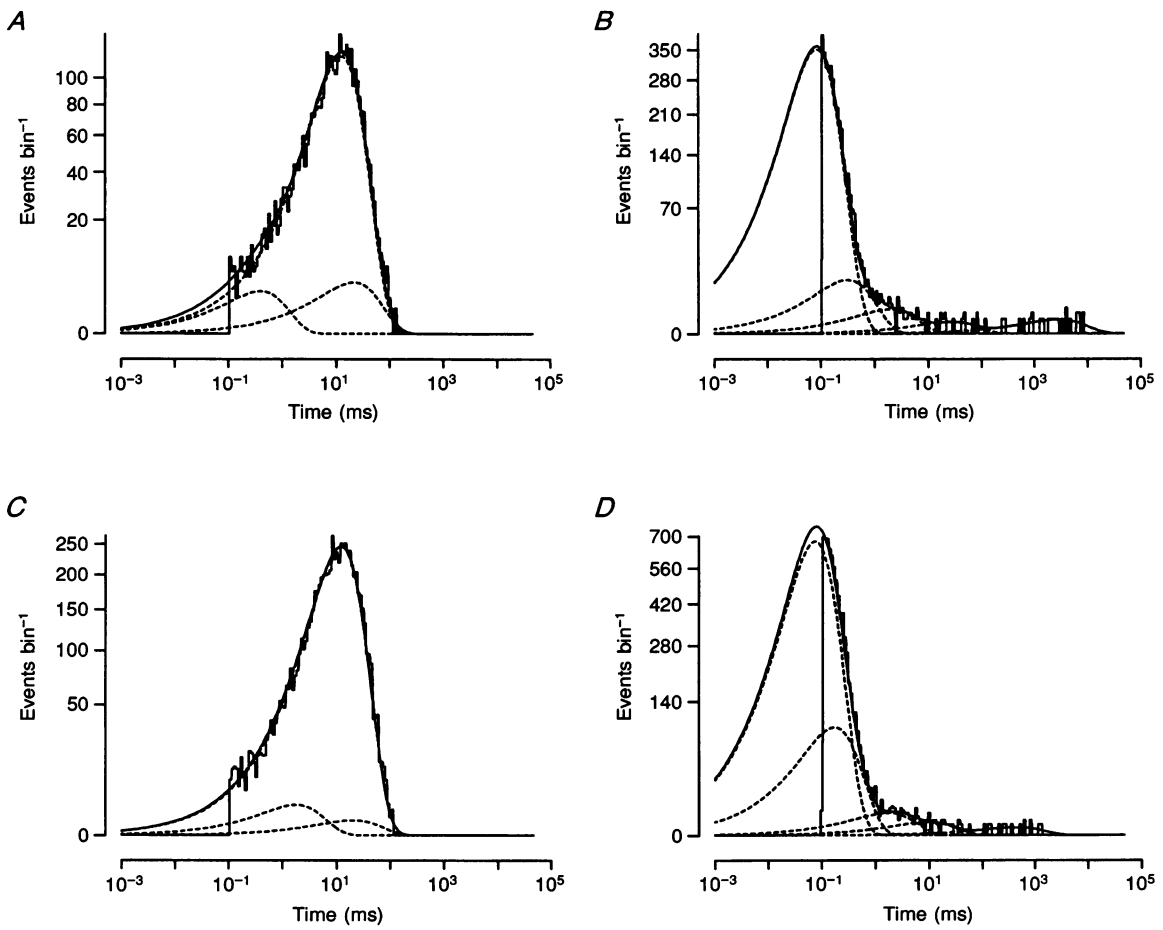

Figure 6. The effect of gluconate on open and closed times
A–D, distributions of open and closed times from the same patch, which contained one active channel. *A*, open time distribution in control 139 mM Cl^- solution. *B*, corresponding closed time distribution. *C*, open time distribution in 40 mM gluconate. *D*, corresponding closed time distribution. The distributions were fitted to eqn (2), with $j = 3$ components for open times and $j = 5$ components for closed times. The means, τ_j, (and relative areas, a_j) were as follows: *A*, 0·40 ms (0·02), 12·55 ms (0·95), 21·7 ms (0·03). *B*, 0·09 ms (0·95), 0·32 ms (0·03), 1·98 ms (0·01), 27·1 ms (0·002), 2874 ms (0·002). *C*, 1·82 ms (0·01), 12·10 ms (0·99), 20·5 ms (0·003). *D*, 0·07 ms (0·87), 0·18 ms (0·12), 1·73 ms (0·005), 9·7 ms (0·002), 453 ms (0·001).

The doubling of channel activity seen with 3 mM gluconate suggests that the activating effect is caused by gluconate itself, rather than the reduction in [Cl^-], which only changes by 2·2% under these conditions. This suggestion is confirmed by the observation that only certain anions are able to cause channel activation, as we describe below.

Gluconate does not affect channel inhibition by ATP

A number of agents that activate K^+_{ATP} channels in skeletal muscle or other tissues have been shown to act by modulating channel inhibition by ATP so as to shift the ATP inhibition curve to higher [ATP] values. For example, internal H^+ in frog skeletal muscle, internal vanadate in mouse skeletal muscle and the K^+ channel opener RP 49356 in cardiac muscle have all been reported to act in this way (Thuringer & Escande, 1989; Davies, 1990; Neumcke & Weik, 1991; Davies *et al.* 1992). In contrast, intracellular gluconate caused channel activation without affecting channel inhibition by ATP. Figure 5*A* shows that ATP was highly effective in inhibiting channel activity in 40 mM gluconate solution: in this multichannel patch 300 μM ATP virtually abolished channel activity. The concentration–effect curve for ATP in 40 mM gluconate is shown by the squares and continuous line in Fig. 5*B*, and was not significantly shifted from that measured in 139 Cl^- solution (circles and dashed line), a least-squares fit to eqn (3) giving a K_i of 19 μM with a Hill coefficient of 1.

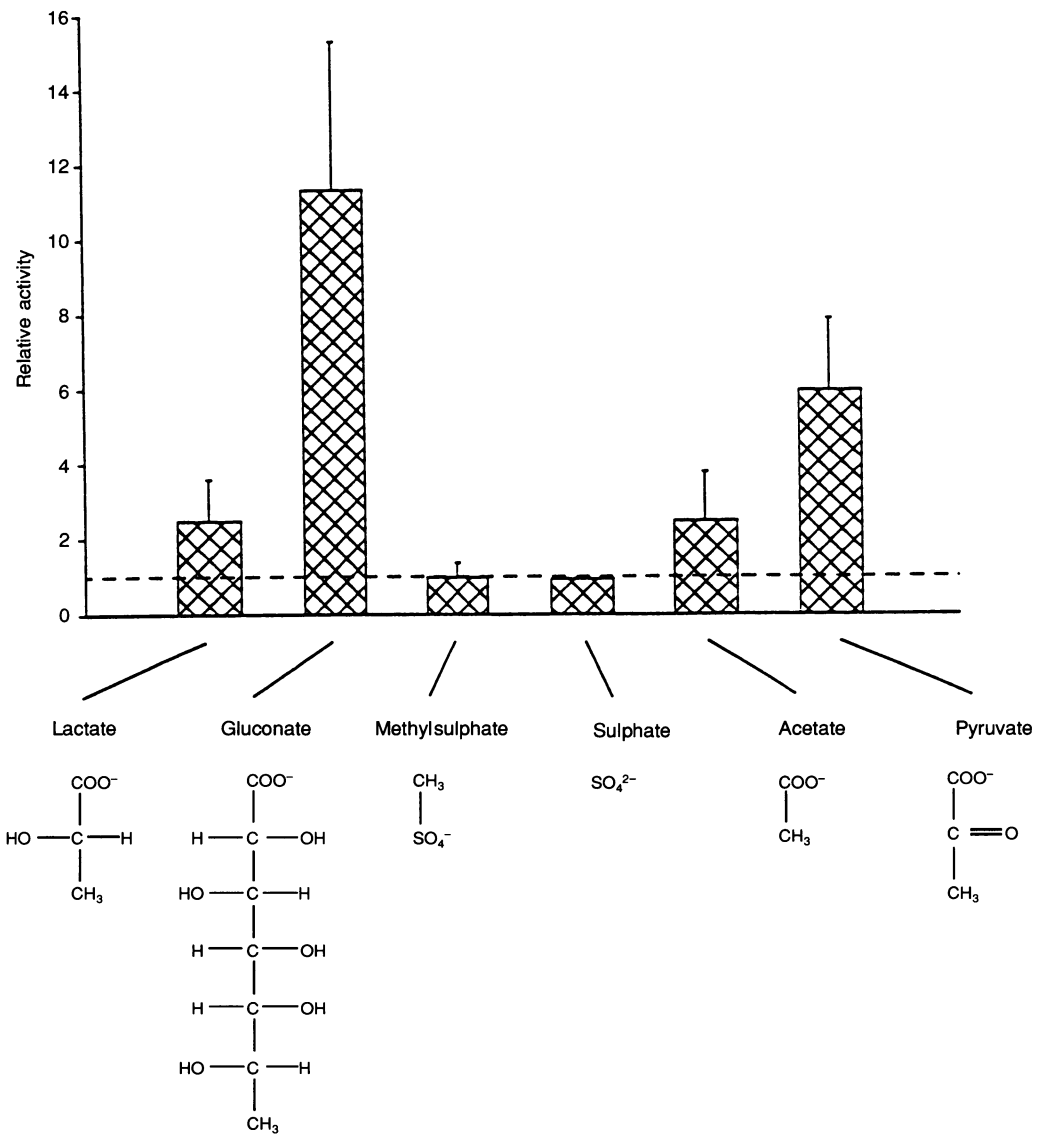

Figure 7. The effect of different anions in causing channel activation
The histogram shows values of channel activity in the presence of 40 mM of the anions shown relative to its value in control (139 mM Cl^-) solution. The bars show S.E.M. and the number of patches tested in each anion was as follows: lactate, 6; gluconate, 10; methylsulphate, 3; sulphate, 3; acetate, 3; and pyruvate, 3. The structure of the anions is given below.

Table 1. Values for the K_i for ATP in the presence of different internal anions

Anion	K_i	Number
Chloride	14 ± 2	41 (30)
Gluconate	19 ± 4	26 (11)
Acetate	9 ± 3	17 (5)
Lactate	6 ± 1	27 (10)
Pyruvate	16 ± 3	25 (7)

In each case 40 mM of the anion was substituted for Cl^-. The mean ± S.E.M. K_i was calculated using a least-squares fitting routine to fit the relationship between relative P_{open} and [ATP] in the presence of ATP to eqn (3), with every measurement of relative P_{open} plotted individually. The number given is the number of measurements, with the number of patches on which these were made in brackets.

The effect of gluconate on single channel kinetics

A comparison of the kinetics of K^+_{ATP} channels under control conditions and in gluconate suggests that the major effect of gluconate was to reduce the frequency of entering a long-lived shut state, and also to shorten its duration. Figure 6 shows the distribution of open and closed events obtained from a patch exposed to control and 40 mM gluconate solutions. Open time distributions were fitted with three components (see Methods) and closed times with five. The corrected mean open time in this and three other patches were similar, being 3·55 ± 0·36 and 3·99 ± 0·16 ms in control and 40 mM gluconate, respectively. The mean closed times were markedly different, being 54·4 ± 37·2 and 2·26 ± 1·30 ms in control and gluconate, respectively. This difference is mainly caused by the reduced occupancy and shorter mean dwell time of the longest lived closed state (Fig. 6*B* and *D*).

The effects of other anions

We investigated the effectiveness of other internal anions in causing K^+_{ATP} channel activation. Anions were applied to the cytoplasmic face of excised patches at 40 mM (substituted for Cl^-), and channel activity was measured as described above for gluconate and expressed relative to its control value (in 139 mM Cl^- solution). Figure 7 shows a histogram of the relative activity values obtained with different anions. Gluconate was the most effective channel activator, while pyruvate, lactate and acetate were all able to cause some channel activation. Both sulphate and methylsulphate were without effect on activity, showing that the effect of anions is not merely due to a reduction in internal Cl^-.

Concentration–effect curves for channel inhibition by ATP were measured in the presence of each of the different anions at 40 mM. The results from each patch were fitted with eqn (3) using a least-squares method, and the mean K_i values for ATP in the different anion solutions are shown in Table 1. We did not detect a significant change in the affinity for ATP in the presence of any of the anions we tested apart from lactate. The K_i for ATP in the presence of 40 mM lactate of 6 ± 1 μM was significantly lower ($P = 0·004$) than that measured in control solution.

DISCUSSION

Unitary properties of K^+_{ATP} channels of skeletal muscle

The properties of single K^+_{ATP} channels reported from skeletal muscle preparations seem quite consistent. The unitary conductance of 62 pS in 155 mM symmetrical K^+ we have measured in channels from rat FDB muscle is comparable to the reported values of 60 pS in 140 mM symmetrical K^+ in human muscle (Burton *et al.* 1988) and 74 pS in 160 mM symmetrical K^+ in mouse skeletal muscle (Woll *et al.* 1989). The hundred-fold selectivity for K^+ over Na^+ is a little higher than that reported in frog muscle ($P_K/P_{Na} = 67$; Spruce, Standen & Stanfield, 1987), while Parent & Coronado (1989) estimated $P_K/P_{Na} > 50$ in K^+_{ATP} channels reconstituted from rabbit T-tubular membrane into lipid bilayers. The sensitivity of the channel to ATP also appears similar in different skeletal muscle preparations. Thus the K^+_{ATP} channels so far reported in skeletal muscle seem likely either to represent a single type or a closely related group of channels.

Mechanisms of channel run-down and reactivation

We found that K^+_{ATP} channel activity in patches from rat skeletal muscle displayed profound and rapid run-down upon patch excision (Fig. 2). Such rapid spontaneous run-down did not occur in frog muscle (Spruce *et al.* 1987) and was not reported when patches were excised from mouse muscle into solution containing 2 mM Ca^{2+} (Weik & Neumcke, 1989; Woll *et al.* 1989). However Hussain & Wareham (1993) have reported that run-down is enhanced by Ca^{2+} in patches excised from mouse muscle. Ca^{2+} also enhances run-down in cardiac muscle (Findlay, 1988*a*; Furukawa *et al.* 1993). Tryptic digestion can prevent this Ca^{2+}-dependent run-down of cardiac K^+_{ATP} channels, and this has led to the suggestion that run-down is caused, at least in part, by an enzyme-mediated dephosphorylation of the channel (Furukawa *et al.* 1993). However, Findlay (1988*a*) found that run-down was unaffected by phosphatase inhibitors. MgATP is thought to restore activity after run-down in cardiac muscle and insulin-secreting cells by causing channel phosphorylation (Ohno-Shosaku, Zunkler & Trube, 1987; Furukawa *et al.* 1993), while ADP has also been reported to reverse run-down in these tissues (Findlay, 1988*b*; Bokvist, Ämmälä, Ashcroft, Bergrren, Larsson & Rorsman, 1991). Tung & Kurachi (1991) found that such channel activation by ADP did not change the channel inhibition by ATP.

An alternative mechanism has been proposed to explain the Ca^{2+}-dependent run-down of voltage-dependent Ca^{2+} channels from *Limnaea* neurones and NMDA channels in cultured hippocampal neurones which can be reversed by

MgATP (Johnson & Byerly, 1993; Rosenmund & Westbrook, 1993). Both these groups found no evidence to suggest that dephosphorylation of the channel was involved in run-down. However, their results suggest that run-down may be due to the disruption of links between the channel and the cytoskeleton, as agents which disrupt the cytoskeleton (e.g. Ca^{2+} and cytochalasin) enhanced run-down and agents which act as cytoskeletal stabilizers (MgATP and phalloidin), reduced run-down.

The run-down of K^+_{ATP} channel activity in rat muscle we describe here does not appear to be Ca^{2+} dependent as our internal solutions contained 5 mM EGTA with no added Ca^{2+}, giving a calculated free $[Ca^{2+}] < 0{\cdot}6$ nM. Application of 2 mM MgATP only produced a small and transient reactivation of channel activity in six out of sixteen patches (unpublished observations of H.-C. McKillen & Z. Fan). However the inclusion of certain anions, of which gluconate was the most effective, in the internal solution caused restoration of channel activity and greatly slowed subsequent run-down. Internal gluconate has also been reported to activate K^+_{ATP} channels in the insulinoma cell line RINm5F under whole-cell conditions (de Weille & Lazdunski, 1990). It was suggested that in these cells gluconate acts by uncoupling oxidative phosphorylation, reducing $[ATP]_i$; however, gluconate was not tested in inside-out patches. Trube, Hescheler & Schröter (1989), in the same cell line, have shown that internal glutamate activates K^+_{ATP} channels recorded in inside-out patches, without changing the inhibition by ATP. In contrast to these results, internal gluconate did not affect the run-down of whole-cell K^+_{ATP} currents in another insulinoma cell line, CRI-G1 (Kozlowski & Ashford, 1990). Whether this indicates a difference in regulation of K^+_{ATP} channels in different cell lines is unclear. In ventricular myocytes Keung & Li (1991) found that lactate included in the intracellular solution activated whole-cell K^+_{ATP} currents, while fluoride has been reported to stabilize K^+_{ATP} channel activity for several hours in patches excised from cardiac cells (Zilberter, Burnashev, Papin, Portnov & Khodorov, 1988).

Possible mechanisms for the action of anions

The anions that we have found to activate K^+_{ATP} channels are all quite effective ligands for Ca^{2+}. It seems very unlikely, though, that they restore channel activity by reducing free $[Ca^{2+}]$ at the cytoplasmic face of the channel, since this is already at an extremely low level in all our solutions, as we discuss above. Similarly, an effect on $[Mg^{2+}]$ seems unlikely as all our internal solutions were Mg^{2+} free. The quite wide range of anions that are effective, and the relatively high concentrations at which channel activation occurs (K_D, the concentration for half-maximal activation for gluconate of about 14 mM), also argues against a highly specific binding site on the channel protein through which anions cause channel activation. We think it more likely that the action of anions is caused by an effect on the structure of the channel protein. For many years anions have been known to have profound effects on protein stability (reviewed by Collins & Washabaugh, 1985). In particular, Cl^- is known to destabilize the coiled structure of myosin (Stafford, 1985). This is better maintained in the presence of organic anions such as propionate or acetate, an effect which may be attributed to these anions binding less strongly to charged side chains on the protein than does Cl^- (Bartels, Cooke, Elliot & Hughes, 1993).

Anions in the intact muscle fibre

Although we, in common with many others, have used Cl^- as the major anion in solutions bathing the cytoplasmic face of excised membrane patches, in the intact muscle cell the internal face of the K^+_{ATP} channel will experience a $[Cl^-]$ of only a few millimolar, the main intracellular anions being phosphate, bicarbonate, and organic anions such as phosphocreatine, ATP and negatively charged groups on proteins (Conway, 1957). Solutions with anions other than chloride may therefore maintain the channel in a state closer to its normal physiological condition. The intracellular concentration of certain anions may also vary with the metabolic state of muscle during exercise. For example, muscle lactate may rise from less than 1 to over 20 mM in exercise (Saltin, Bangsbo, Graham & Johansen, 1992). It is therefore possible that changes in intracellular anions may play a physiological role in the activation of K^+_{ATP} channels. Keung & Li (1991) have suggested that lactate may play such a role in cardiac muscle during myocardial ischaemia, contributing to the activation of K^+_{ATP} channels and so to the shortening of the cardiac action potential seen under those conditions.

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