Effects of intracellular pH on ATP-sensitive K^+ channels in mouse pancreatic β-cells

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1. The effects of intracellular pH (pH_i) on the ATP-sensitive K^+ channel (K^+_{ATP} channel) from mouse pancreatic β-cells were examined in inside-out patches exposed to symmetrical 140 mM K^+ solutions.

2. The relationship between channel activity and pH_i was described by the Hill equation with half-maximal inhibition (K_i) at pH_i 6·25 and a Hill coefficient of 3·7.

3. Following exposure to $pH_i < 6·8$, channel activity did not recover to its original level. Subsequent application of trypsin to the intracellular membrane surface restored channel activity to its initial level or above.

4. At −60 mV the relationship between pH_i and the single-channel current amplitude was described by a modified Hill equation with a Hill coefficient of 2·1, half-maximal inhibition at pH_i 6·48 and a maximum inhibition of 18·5 %.

5. A decrease in pH_i reduced the extent of channel inhibition by ATP: K_i was 18 μM at pH 7·2 and 33 μM at pH 6·4. The Hill coefficient was also reduced, being 1·65 at pH 7·2 and 1·17 at pH 6·4.

6. When channel activity was plotted as a function of ATP^{4-} (rather than total ATP) there was no effect of pH_i on the relationship. This suggests that ATP^{4-} is the inhibitory ion species and that the effects of reducing pH_i are due to the lowered concentration of ATP^{4-}.

7. Changes in external pH had little effect on either single-channel or whole-cell K^+_{ATP} currents.

8. The effects of pH_i do not support a role for H^+ in linking glucose metabolism to K^+_{ATP} channel inhibition in pancreatic β-cells.

Potassium channels closed by internal ATP, known as ATP-sensitive K^+ channels or K^+_{ATP} channels, have been identified in various tissues (for review see Ashcroft & Ashcroft, 1990). Their physiological function may be to couple K^+ fluxes and the electrical excitability of the cell to its metabolic state. The regulatory link between channel activity and cellular metabolism remains controversial but the most plausible candidates include ATP in β-cells and intracellular pH (pH_i) in cardiac and skeletal muscle.

The effects of pH_i on K^+_{ATP} channels in frog skeletal muscle have been well characterized (Davies, 1990; Davies, Standen & Stanfield, 1992). In this tissue, lowering pH_i from 7·2 to 5·7 produces a slight decrease in the unitary current amplitude and a slight increase in the channel open probability. The main action of reducing pH_i, however, is to lower the affinity of the channel for ATP, an effect which can be modelled by assuming that two protons compete with a single ATP molecule for the inhibitory site. The ATP concentration required for half-maximal inhibition (K_i) of channel activity is increased from 17 to 260 μM when pH_i is reduced from 7·2 to 6·3. At constant [ATP] a fall in pH_i will therefore result in an increase in channel activity. This may constitute an important regulatory mechanism for linking changes in muscle metabolism, which are accompanied by changes in pH_i, to K^+_{ATP} channel activity.

An increase in K^+_{ATP} channel activity on lowering pH_i in the presence of ATP is also found in ventricular myocytes (Lederer & Nichols 1989; Cuevas, Bassett, Cameron, Furukawa, Myerburg & Kimura, 1991; Koyano, Kakei, Nakashima, Yoshinaga, Matsuoka & Tanaka, 1993). However, the effect is less dramatic than in skeletal muscle, the K_i only increasing from 25 to 50 μM (Lederer & Nichols, 1989). The effect of pH_i on K^+_{ATP} channels in inside-out patches from β-cells is less well documented. A previous study (Misler, Gillis & Tabcharini, 1989) found that pH_i had little effect on channel activity until it was reduced below

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6·5, when the channels abruptly closed. In the presence of ATP, however, channel activity decreased more smoothly with decreasing pH_i.

In this paper we present a detailed investigation of the effects of pH_i on the K^+_{ATP} channel of the pancreatic β-cell. A preliminary report of some of these findings has been communicated to the Physiological Society (Proks, Takano & Ashcroft, 1993).

METHODS

Cell preparation

Pancreatic islets were isolated from NMRI mice by collagenase digestion and dispersed into single β-cells by low-Ca^{2+} treatment, as previously described (Rorsman & Trube, 1986). Mice were killed by cervical dislocation. Cells were plated onto Falcon Petri dishes and maintained for 1–5 days in RPMI tissue culture medium (Life Technologies Inc., Paisley, UK) supplemented with 10 % fetal calf serum, 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin and gassed with 95 % O_2–5 % CO_2.

Recording methods

Single K^+_{ATP} channel currents were studied in inside-out membrane patches using the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were pulled from 1·5 mm borosilicate glass capillaries (Hilgenberg, Malsfeld, FRG), coated with Sylgard to reduce their electrical capacitance and fire polished immediately before use. They had resistances of between 5 and 10 MΩ when filled with pipette solution. All experiments were carried out at room temperature (22–25 °C).

Solutions and chemicals

The pipette was filled with 140 mM K^+ external solution containing (mM): 140 KCl, 1·1 $MgCl_2$, 2·6 $CaCl_2$, 10 Hepes (*N*-[2-hydroxyethyl]piperazine-*N*'-2-ethanesulphonic acid, adjusted to pH 7·4 with NaOH). Before excising the patch, the bath contained standard extracellular solution comprising (mM): 140 NaCl, 5·6 KCl, 1·1 $MgCl_2$, 2·6 $CaCl_2$, 10 Hepes (adjusted to pH 7·4 with NaOH). The control intracellular (bath) solution contained (mM): 100 KCl, 40 KOH, 10 EDTA (ethylenediaminetetraacetic acid), 10 Hepes. The pH of the solution was adjusted to 7·2 by adding an appropriate value of HCl. The free concentrations of Ca^{2+} and Mg^{2+} were < 1 nM. Intracellular solutions of different pH were used as stated in the text; unless otherwise indicated 10 mM Hepes was used as the pH buffer at pH_i 6·9, 7·2 and 7·6, 10 mM Pipes (piperazine-*N*-*N*'-bis[2-ethanesulphonic acid]) at pH_i 6·4 and 6·6, 10 mM Mes (2-[*N*-morpholino]ethanesulphonic acid) at pH_i of 6·0 and 6·2, and 10 mM Ches (2-[*N*-cyclohexylamino]ethanesulphonic acid) at pH_i 9·0. ATP (sodium salt) was added to the intracellular solution at the concentrations indicated in the text and the pH was corrected as required with KOH. Trypsin (Sigma, Type IX) was applied at a concentration of 20 μg ml^{-1} for 5 min and was then removed.

Concentrations of free cations and ligands were calculated using the program PERTEM supplied by Dr Griffiths (Oxford). Binding constants were taken from Martell & Smith (1974).

In most experiments, an air-gate system was used to ensure a rapid exchange of solutions (Notsu, Takana, Takano & Noma, 1992). This was essentially the same as the oil-gate chamber described by Qin & Noma (1989) except that the gates were filled with air rather than oil. To prevent rupture of the patch membrane during transfer through the air-gate, a meniscus holder was used. This was formed by taking a 300 μm silver wire, bending it through 90 deg at its tip, flattening the tip and drilling a 100 μm small hole in it. After patch excision, the tip of the meniscus holder was positioned below the pipette tip. Transfer through the air-gate was effected by moving the chamber (using the microscope stage) to ensure that the pipette tip and mensicus holder remained in the same relationship to one another. A small bubble of solution of about 4 nl, in which the pipette tip was positioned, was transferred through the air-gate together with the patch. Solutions were perfused continuously through the recording chamber.

Data collection and analysis

Currents were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, FRG), digitized using an A–D converter (VR-10A, Instrutech Corp., Elmont, USA) and stored on videotape for later analysis. The reference potential for all measurements was the zero current potential of the pipette before establishment of the seal. No corrections have been made for liquid junction potentials (< 5 mV).

For analysis of single-channel currents, records were amplified (×10), low-pass filtered at 2 kHz using an 8-pole Bessel low-pass filter (Frequency Devices, Haverhill, MA, USA) and digitized at 5 kHz using an Axolab-1 A–D converter (Axon instruments Inc., Foster City, CA, USA) and pCLAMP software (Axon Instruments Inc.). Currents were analysed using a combination of pCLAMP and in-house software written by Dr P. Smith (Oxford). Single-channel current amplitudes were calculated from an all-points amplitude histogram. Channel activity (NP_o) was measured as the mean current (I) divided by the single-channel current amplitude (i), for segments of the current records of about 2 min duration. The data were plotted and further analysed using the program Sigmaplot. Measurements of open and closed times were confined to lifetimes within bursts of openings. Events were detected using a 50 % threshold level method.

RESULTS

Effect of pH_i on K^+_{ATP} channel activity

K^+_{ATP} channels were studied at a membrane potential of −60 mV, close to the resting potential of the β-cell, and with 140 mM K^+ solutions on either side of the membrane. Under these conditions, single K^+_{ATP} channel currents are inward. It is well established that K^+_{ATP} channel activity declines following excision in inside-out patches from both pancreatic β-cells (Findlay & Dunne, 1986; Ohno-Shosaku, Zünckler & Trube, 1987) and cardiac muscle (Trube & Hescheler, 1984; Takano, Qin & Noma, 1990). We therefore used a low-Mg^{2+} intracellular solution, which significantly reduces the rate of channel run-down (Kozlowski & Ashford, 1990), and to control for any residual run-down intracellular solutions of test pH_i were alternated with a control intracellular solution of pH_i 7·2.

Figure 1*A* shows a continuous recording of K^+_{ATP} currents from a multichannel patch in which the intracellular membrane surface was successively exposed to the control

solution of pH_i 7·2, to a test solution of pH_i 6·0 and then to control solution again. Decreasing pH_i produced a marked reduction in channel activity. This is seen more clearly in Fig. 1*B* where channel activity, defined as NP_o, the product of the number of active channels (N) and the channel open probability (P_o), is plotted as a function of time. In this patch, NP_o fell from 9·94 to 0·32 when pH_i was changed from 7·2 to 6·0 and was partially restored (5·87) after return to pH_i 7·2.

The relationship between channel activity and intracellular pH is examined in Fig. 2*A*. NP_o was expressed as a fraction of its value in the control solution (which had a pH 7·2) preceding the test solution. No effect of increasing pH_i to 7·6 was found but decreasing pH_i below about 6·7 produced a marked inhibition of channel activity, with complete block being observed at or below pH_i 6·0. The continuous curve is the best fit of the mean data to the following equation using a non-linear least-squares method:

$$\frac{NP_{test}}{NP_{7\cdot2}} = \frac{1 + 10^{(-7\cdot2 + pK)\,n_H}}{1 + 10^{(-pH_i + pK)\,n_H}}, \quad (1)$$

where NP_{test} is the channel activitity at the test pH_i, $NP_{7\cdot2}$ is the channel activity at pH_i 7·2 before exposure to the test pH_i, pK is the apparent dissociation constant and n_H is the Hill coefficient. We have used this modified form of the Hill equation since the proton concentration in our control solution, pH 7·2, is not zero but is 63 nM. However, the error is less than 1% if we use the standard Hill equation and assume that channel activity at a proton concentration of zero is the same as that at pH_i 7·2. The best fit to eqn (1) was obtained with $pK = 6\cdot27$ and $n_H = 3\cdot7$. This suggests that a proton-binding site with a pK of ~6·3 might be involved in the inhibition of K^+_{ATP} channel activity produced by H^+, and that more than one proton must bind to the channel to produce inhibition. The Hill coefficient of 3·7 also suggests that the co-operative binding of more than one proton is needed for channel inhibition.

Recovery of channel activity following exposure to low pH

Exposure to low pH_i also produced an irreversible inhibition of K^+_{ATP} channel activity, with both the rate and extent of recovery of NP_o decreasing as the pH_i of the test solution was reduced. Figure 2*B* plots the percentage recovery of channel activity as a function of pH_i. In these experiments, channel activity was measured at pH_i 7·2 both before and after exposure to a solution of test pH_i. The percentage recovery of activity was determined by expressing NP_o measured at pH_i 7·2 following exposure to the test pH_i as a percentage of channel activity at pH_i 7·2 before exposure to the test pH_i. As shown in Fig. 2*B*, full recovery of activity was observed only following solutions of pH_i above 6·4 and

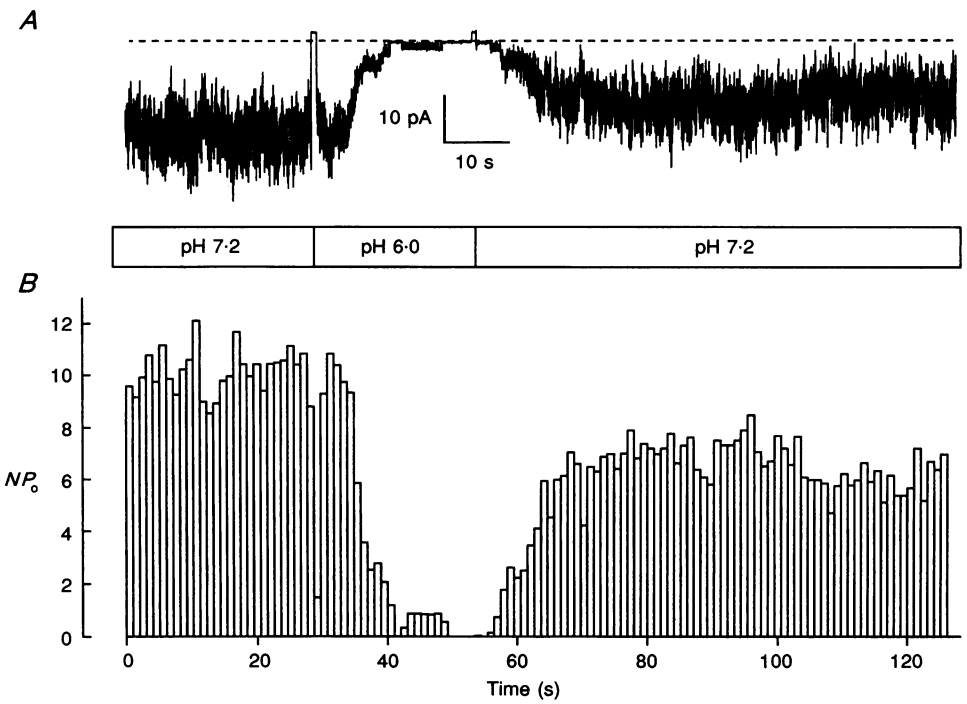

Figure 1. Effect of pH_i on the K^+_{ATP} channel in the absence of ATP

A, continuous recording of single K^+_{ATP} channel currents at a membrane potential of −60 mV in an inside-out patch. The pH of the internal solution was switched from the control value (7·2) to a test value of 6·0 during the period indicated by the bar. The dashed line represents the zero current level and the glitches in the current record indicate the period during which the patch was passed through the air gap. *B*, channel activity (NP_o) as a function of time for the record illustrated above. Each bar corresponds to a time interval of 1·62 s.

recovery was substantially reduced as the test pH_i was decreased below 6·4. The relationship between recovery and pH_i may be fitted by the equation:

$$\frac{NP_{before}}{NP_{after}} = \frac{1 + 10^{(-7.2 + pK)n_H}}{1 + 10^{(-pH_i + pK)n_H}}, \quad (2)$$

where NP_{before} and NP_{after} are the channel activities at pH_i 7·2 before and after exposure to the test pH_i respectively. The best fit was obtained with $pK = 6{\cdot}14$ and $n_H = 3{\cdot}3$.

Having defined the extent of the irreversible inhibition of channel activity produced by low pH_i, we can now estimate the reversible proton inhibition, using a modified form of the Hill equation to account for the fact that the proton concentration at pH 7·2 is not zero:

$$\frac{NP_{test}}{NP_{7.2}} = \frac{R_{pH_i}(1 + 10^{(-7.2 + pK)n_H})}{1 + 10^{(-pH_i + pK)n_H}}, \quad (1)$$

where $R_{pH_i} = NP_{before}/NP_{after}$. The best fit to eqn (3) was obtained with $pK = 6{\cdot}12$ and $n_H = 2{\cdot}4$.

As seen in Fig. 2*B*, there was considerable variability in the extent of recovery following low pH_i. This appears to be the result of the different condition of the patch prior to exposure, since we noticed that those patches in which channel activity was very high were less sensitive to permanent inhibition by low pH_i.

Trypsin effects on channel inhibition induced by low pH_i

One explanation for the finding that channel inhibition is only partially reversible is that it results from the titration of an amino acid residue which produces an irreversible conformational change in the K^+_{ATP} channel. An alternative possibility is that low pH_i promotes entry into the run-down or inactivated state. We have previously reported that brief exposure of the cytosolic surface of the membrane to trypsin can restore the activity of K^+_{ATP} channels after complete run-down (Proks & Ashcroft, 1993). We therefore tested whether trypsin was also able to restore channel activity inactivated by exposure to low pH_i.

Figure 3 is a continuous record of K^+_{ATP} channel activity from a multichannel patch. The top trace shows channel activity in control solution ($NP_o = 7{\cdot}54$), its complete inhibition at pH_i 6·0, and partial recovery to about 64 % of its original value on return to control solution. Subsequent application of trypsin (20 μg ml^{-1}) restored NP_o to a value of 8·73, which is greater than that observed initially. This result suggests the existence of at least two distinct mechanisms responsible for the effect of pH_i on K^+_{ATP} channel activity. One mechanism produces a reversible inhibition of the channel whilst the other appears to promote entry into an inactivated or closed state which can only be reversed by proteolysis.

We next tested the effect of pH_i on the trypsin-treated K^+_{ATP} channel (Fig. 3, lower two traces). Tryptic digestion did not prevent the inhibition of channel activity by low pH_i. However, in contrast to untrypsinized patches, channel activity recovered fully upon return to normal pH_i ($NP = 8{\cdot}82$). Furthermore, after trypsin treatment, the time course of the block by protons was very much slower, with a time constant about tenfold slower than that found for non-trypsinated channels (Fig. 3*B*). The single-channel current amplitude, however, decreased immediately upon changing to pH_i 6·0, unlike the change in the channel activity, suggesting that these two effects of protons may differ

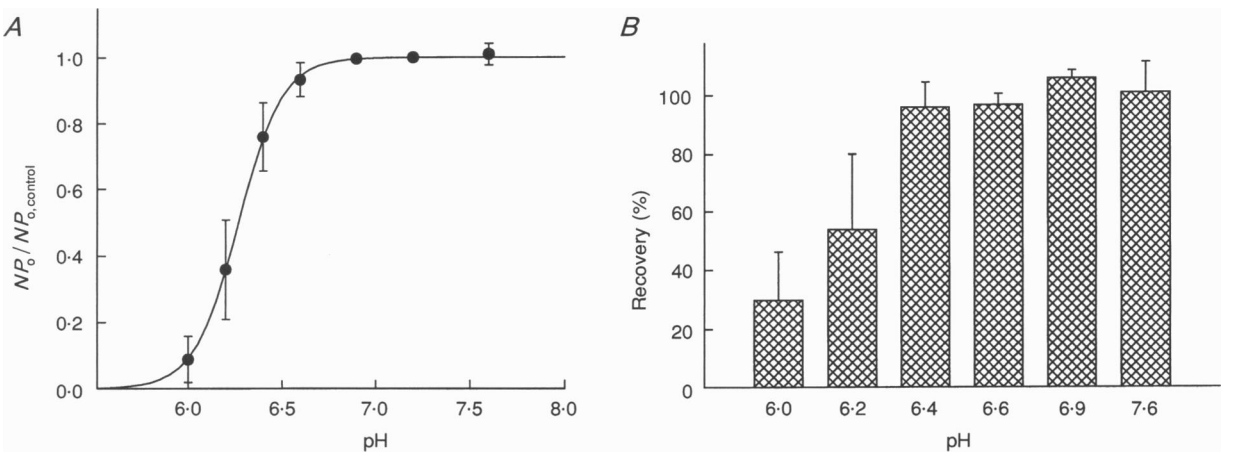

Figure 2. Inhibition of K^+_{ATP} channel activity by protons
A, mean relationship between channel activity (NP_o) and pH_i measured for 6 patches. Channel activity is expressed as a fraction of that measured at pH_i 7·2. The continuous line is the best fit of eqn (1) of the text to the data with $pK = 6{\cdot}27$ and $n_H = 3{\cdot}7$. Data lengths of 1 min were averaged to obtain NP_o. The vertical bars indicate ± 1 S.E.M. *B*, histogram of the percentage recovery of channel activity following exposure to lowered pH_i measured on the same 6 patches as in *A*. Channel activity was measured at pH_i 7·2 before and after exposure to a solution of test pH_i. The percentage recovery of activity was determined by expressing NP_o following exposure to the test pH_i as a percentage of NP_o before exposure the test pH_i. The total time of channel exposure to low pH_i was about 2 min. The vertical bars indicate ± 1 S.E.M.

mechanistically. Inspection of the current record reveals that following typsinization acidification appears to reduce the number of active channels in the patch, while leaving the open probability of the surviving channels unaltered.

Effect of pH_i on channel kinetics

At negative membrane potentials, K^+_{ATP} channel openings occur in bursts separated by relatively long closed intervals with at least one open state and two closed states being required to fit the lifetime distributions (Rorsman & Trube, 1985; Ashcroft, Ashcroft & Harrison, 1988). Our analysis of the effect of pH_i on the single-channel kinetics was complicated by the fact that in control solution patches in which only a single channel was active were rare. Moreover in these patches lowering pH_i invariably abolished channel activity. We have therefore confined our analysis to the open and closed times within the bursts by selecting sections of the current record in which only a single open level was observed. We have assumed that this represents the activity of a single channel. The effects of pH_i on K^+_{ATP} channel kinetics in a typical patch are shown in Fig. 4. Open and closed time distributions were determined from data stretches of 5 min. At pH_i 7·2, the open time distribution was described by a single exponential with a time constant of 2·61 ms and the closed time distribution was best fitted by an exponential with a time constant of 0·37 ms. These values are similar to those reported elsewhere for K^+_{ATP} channels in β-cells (Rorsman & Trube, 1985; Ashcroft *et al.* 1988). When pH_i was reduced to 6·4, both the mean open time (2·69 ms) and the intraburst closed time (0·48 ms) were unaffected. Thus protons must increase the long closed times between burst of openings and/or reduce the number of functional channels. When inhibition occurs more slowly, as after trypsin treatment (Fig. 3), it is evident that the number of channels is reduced, but that the kinetics of the surviving channels remain unaltered.

Effect of pH_i on the single-channel conductance

Figure 5 shows the effect of reducing pH_i from 7·2 (○) to 6·4 (●) on the amplitude of the single-channel current and on the current–voltage relationship. Although no cations other than K^+ are present in the intracellular solution, the current–voltage relationship recorded at pH_i 7·2 shows inward rectification, with outward currents at +60 mV being only 80 % of those at −60 mV. This phenomenon has been observed previously (Ashcroft & Kakei, 1989).

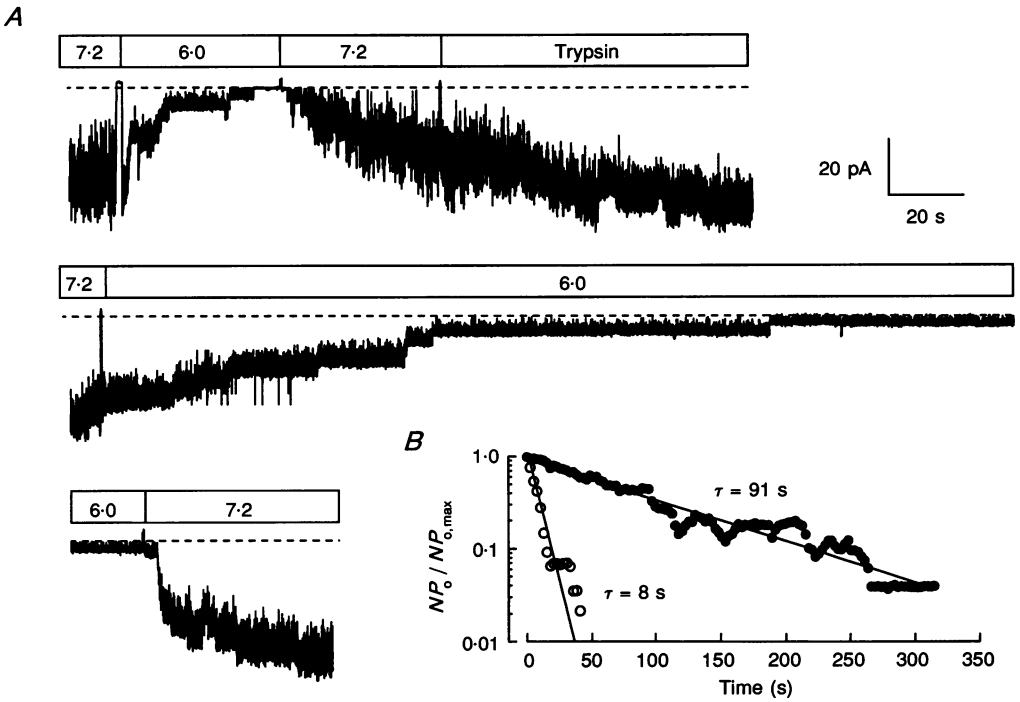

Figure 3. Effects of trypsin on the run-down of channel activity induced by low pH_i

A, continuous recording of single K^+_{ATP} channel currents at −60 mV from an inside out-patch. The pH_i of the internal solution was switched from 7·2 to 6·0 as indicated by the bar. Trypsin (Sigma, Type IX; 20 μg ml^{-1}) was added during the period indicated and was then removed. The dashed line represents the zero current level and the glitches in the current record indicate the period during which the patch was passed through the air gap. *B*, the time course of the effect of lowering pH_i on channel activity before (○) and after (●) trypsin treatment. Channel activity (NP_o) is expressed as a fraction of the maximum channel activity measured immediately after changing to the lower pH_i. Time zero is the time at which the patch entered a solution of pH_i 6·0. The data are fitted with the equation: $\log(NP_o/NP_{max}) = -t/\tau$, where t is time and the time constant, τ, is 8 s before, and 91 s after, trypsin treatment.

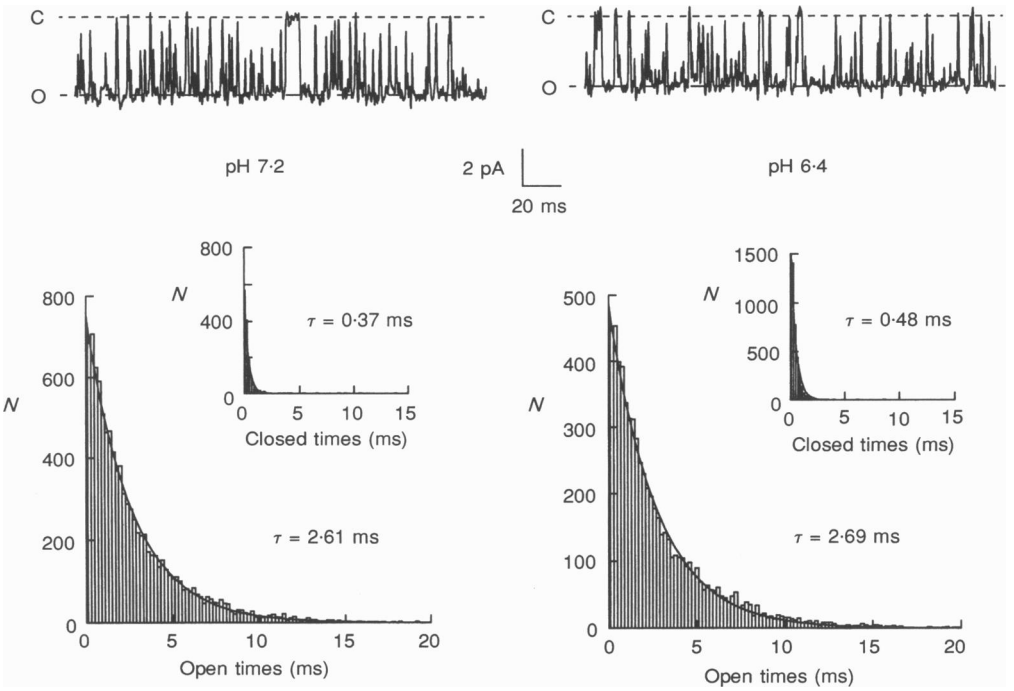

Figure 4. The effect of pH_i on K^+_{ATP} channel kinetics
Single-channel currents (above) and open and closed time distributions (below) recorded at −60 mV from the same patch at pH_i 7·2 (left) and at pH_i 6·4 (right). The dashed lines indicate the current levels; C, closed state; O, open state. Open and closed time distributions were determined for data stretches of 5 min. Open times and closed times were fitted with a single exponential: $f(t) = A\exp(-t/\tau)$, where t is time, A is the number of samples at $t = 0$ and τ is the time constant. Open times were fitted with $A = 756$ and $\tau = 2{\cdot}6$ ms for pH_i 7·2 and $A = 489$ and $\tau = 2{\cdot}7$ ms for pH_i 6·4. Closed times were fitted with $A = 594$, $\tau = 0{\cdot}37$ ms for $pH_i = 7{\cdot}2$ and $A = 1772$, $\tau = 0{\cdot}46$ ms for $pH_i = 6{\cdot}4$.

Decreasing intracellular pH_i reduced both the inward and outward currents without changing the reversal potential. The single-channel conductance, measured from the slope of the line between −100 and −10 mV, decreased from 73 to 66 pS. The reduction in the unitary current amplitude was not accompanied by an increase in the open channel noise (Fig. 5*A*), suggesting that protons do not act as open channel blockers.

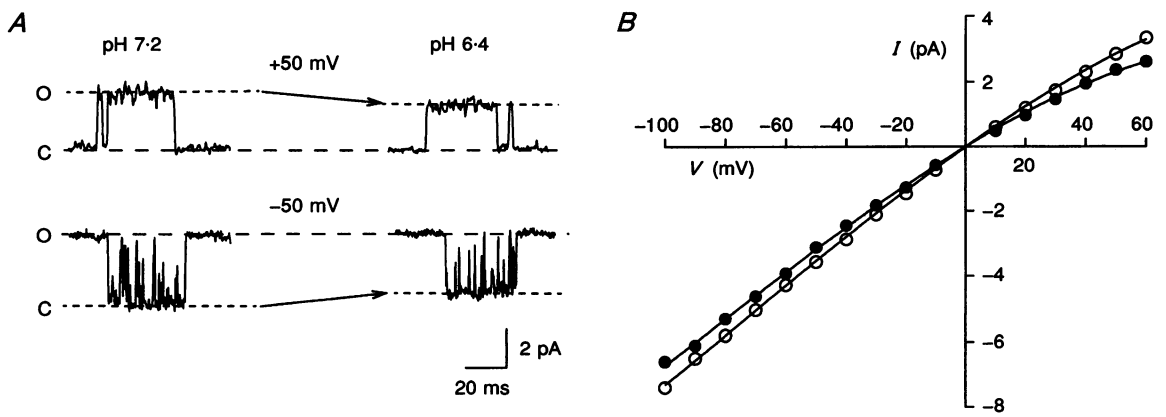

Figure 5. The effect of pH_i on the single-channel current–voltage relationship
A, single-channel currents recorded at a membrane potential of +50 mV (upper traces) and −50 mV (lower traces), first with a pH_i of 7·2 (left) and then a pH_i of 6·4 (right). The dashed lines indicate the current levels: C, closed state; O, open state. *B*, representative single-channel current–voltage relationship measured first at a pH_i of 7·2 (○) and subsequently at a pH_i of 6·4 (●). The lines are drawn through the points by eye.

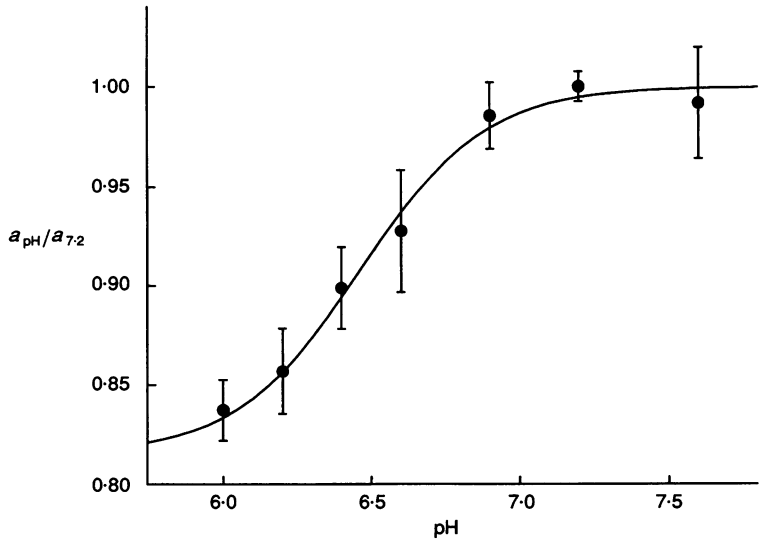

Figure 6. Effect of pH_i on the single-channel current amplitude
Relationship between single-channel current amplitude at −60 mV and pH_i measured in 10 patches. Current amplitude is expressed as a fraction of that measured in the same patch at pH_i 7·2 (control). The line is the best fit of eqn (6) of the text to the data with $K_i = 6{\cdot}48$ μM, $a = 0{\cdot}815$ and $n_H = 2{\cdot}1$. The vertical bars indicate ± 1 S.E.M.

The mean single-channel current amplitude at −60 mV is plotted as a function of pH_i in Fig. 6. For each patch, the unitary current was measured at a number of different pH_i levels and its amplitude expressed as a fraction of that measured at pH_i 7·2 (control solution). The current amplitude decreased with pH_i to a limiting value of 81·5 % of that in control solution (pH_i 7·2) at pH_i 6·0. We were unable to explore the effect of reducing pH_i below 6·0 because at these pH_i levels channel openings were so infrequent that the current amplitude could not be determined.

The data were fitted with the equation:

$$\frac{I_{pH_i} - I_{min}}{I_{max} - I_{min}} = \frac{1}{1 + 10^{(pH_i + pK)n_H}}, \quad (5)$$

where I_{min} is the minimum current and I_{max} is the maximum current. In order to take account of the fact that

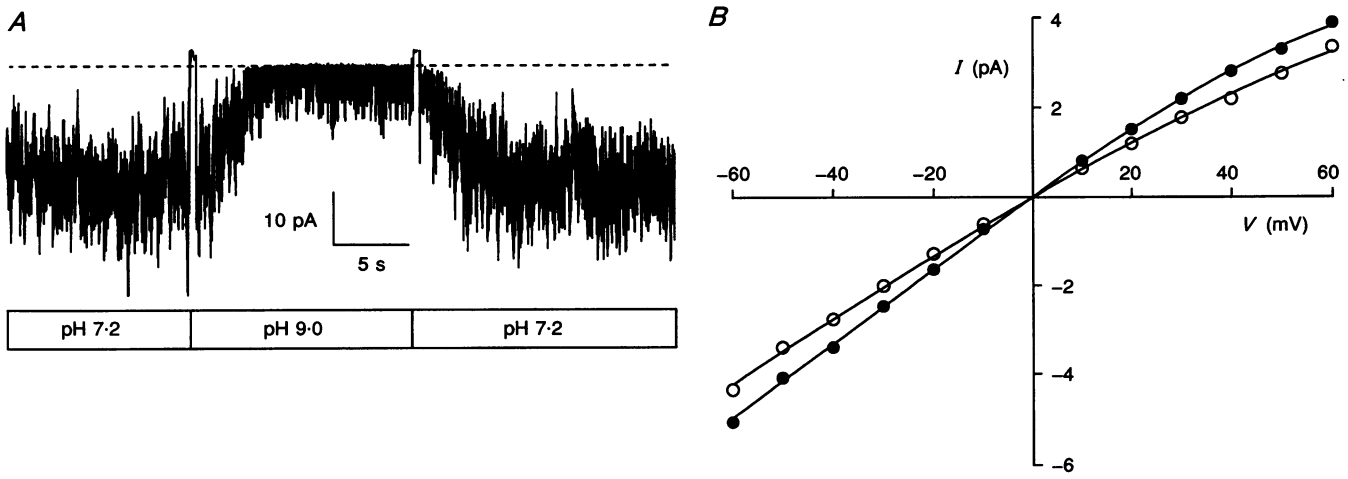

Figure 7. Effect of high pH_i on K^+_{ATP} channel currents
A, continuous recording of single K^+_{ATP} channel currents at a membrane potential of −60 mV in an inside-out patch. The pH_i of the internal solution was switched from 7·2 to 9·0 during the period indicated by the bar. The dashed line indicates the zero current level and the glitches in the current record indicate the time during which the patch was moved through the air gap. *B*, representative single-channel current–voltage relationships measured from the same inside-out patch first at pH_i 7·2 (○) and subsequently at pH_i 9·0 (●).

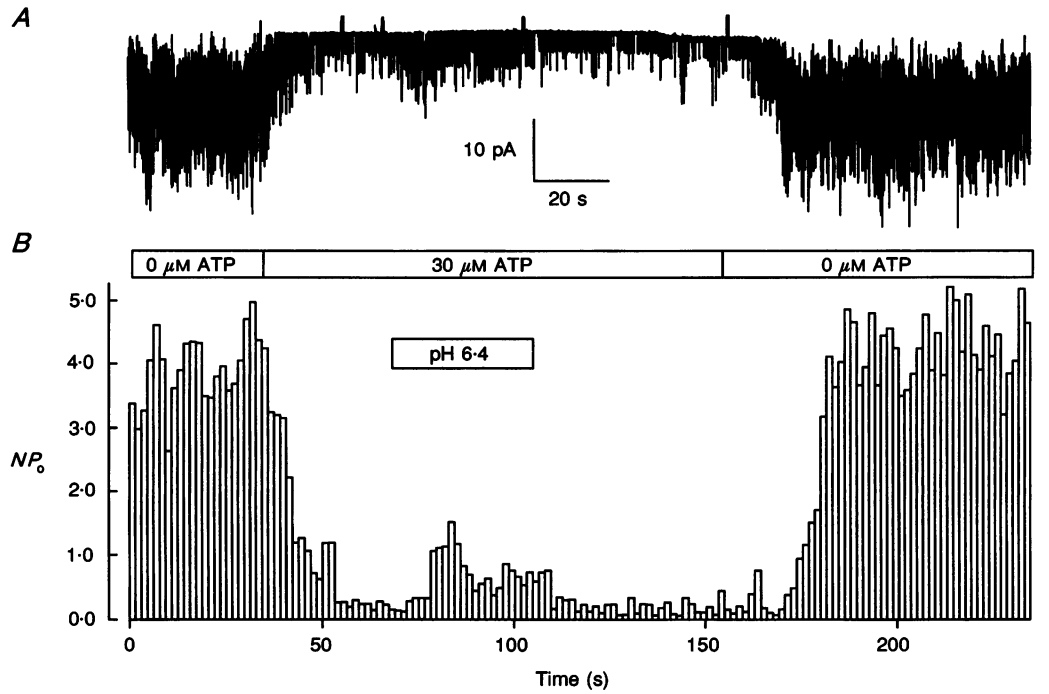

Figure 8. Effect of pH_i on the ATP-sensitive K^+ channel in the presence of ATP
A, continuous recording of single K^+_{ATP} channel currents at a membrane potential of −60 mV in an inside-out patch. The pH_i of the internal solution was switched from 7·2 to 6·0 during the period indicated by the bar, and the ATP concentration was increased from 0 to 30 μM as indicated. The glitches in the current record indicate the period during which the patch was passed through the air gap. *B*, channel activity (NP_o) as a function of time for the record illustrated above. Each bar corresponds to a time interval of 1 s.

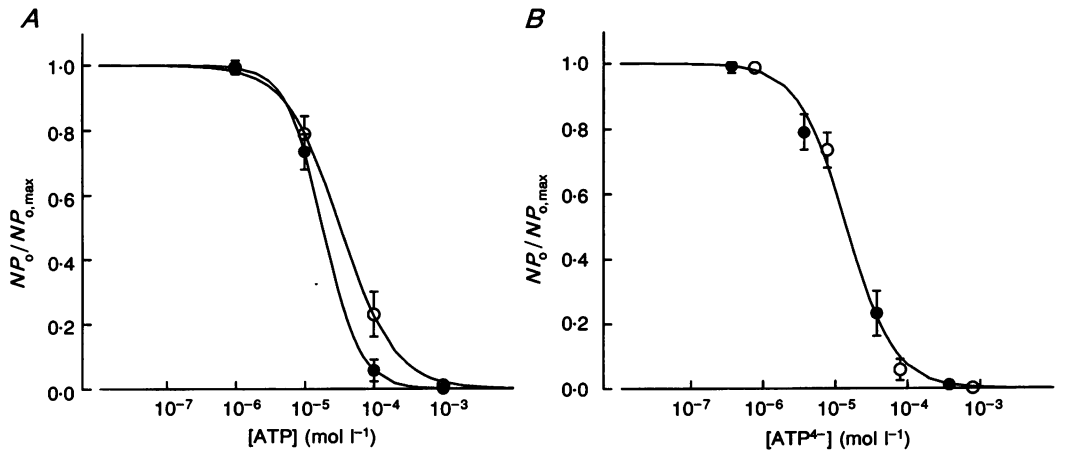

Figure 9. Protons shift the ATP sensitivity to higher ATP concentrations
A, mean relationship between the total ATP concentration and channel activity measured for 6 patches at pH_i 7·2 (●) and at pH_i 6·4 (○). Channel activity is expressed as a fraction of that in ATP-free solution. The lines are drawn to the Hill equation (eqn (7)) with a K_i of 18 μM and a Hill coefficient, n_H, of 1·65 in control solution of pH_i 7·2 and a K_i of 33 μM and n_H of 1·17 at pH_i 6·4. Data lengths of 1 min were averaged to obtain NP_o. The vertical bars indicate ± 1 S.E.M. *B*, mean relationship between the ATP^{4-} concentration and channel activity for the same data as in *A*. The line is drawn to the Hill equation (eqn (7)) with a K_i of 14 μM and n_H of 1·3. Data lengths of 1 min were averaged to obtain NP_o. The vertical bars indicate ± 1 S.E.M.

the proton concentration at pH_i 7·2 is not zero, this equation must be rewritten as:

$$\frac{I_{pH_i}}{I_{pH_i 7\cdot2}} = \frac{a + (1-a)/(1+10^{(-pH_i + pK)n_H})}{a + (1-a)/(1+10^{(-7\cdot2 + pK)n_H})}, \quad (6)$$

where $a = I_{min}/I_{max}$. The best fit to eqn (6) was obtained with $pK = 6\cdot48$, $n_H = 2\cdot1$ and $a = 0\cdot815$.

Effect of high pH_i

Since reducing pH_i decreased the single-channel current amplitude, we investigated whether increasing pH_i would increase the unitary current. This was indeed the case. Increasing pH_i from 7·2 to 9·0 increased the unitary current at −60 mV from 4·3 ± 0·1 to 5·0 ± 0·2 pA ($n = 3$). The effect on the single-channel current–voltage relationship is given in Fig. 7*B*: over the negative voltage range the slope conductance increased from 69 to 80 pS. There was also a marked decrease in channel activity, from 7·8 to 0·71, when pH_i was raised to 9·0 (Fig. 7*A*).

Effect of pH_i in the presence of ATP

In cardiac and skeletal muscle low pH_i, is known to decrease the ATP sensitivity of the K^+_{ATP} channel (Lederer & Nichols, 1989; Davies, 1990; Davies *et al.* 1992; Koyano *et al.* 1993). We next examined the effect of intracellular pH_i in the presence of ATP. In contrast to the inhibition of channel activity observed when pH_i was reduced in control solution, decreasing pH_i to 6·4 in the presence of 30 μM ATP resulted in an increase in NP_o from 0·2 to 0·7 (at pH_i 7·2 and 6·4, respectively; Fig. 8).

To investigate the mechanism by which pH_i increases channel activity in the presence of ATP, we measured the ATP sensitivity of the K^+_{ATP} channel at two pH_i levels (Fig. 9*A*), a control solution of pH 7·2 and a test solution of pH 6·4, which blocks channel activity by about 20 % in the absence of ATP. In each case the ATP dose–response curve was measured first at a pH_i of 7·2 and then at a pH_i of 6·4. Channel activity (NP_o) was expressed as a fraction of that in the absence of ATP (NP_{max}) and the normalized data were fitted by the Hill equation:

$$\frac{NP_o}{NP_{max}} = \frac{1}{1 + ([ATP]_i/K_i)^{n_H}}, \quad (7)$$

where K_i is the ATP concentration at which inhibition is half-maximal and n_H is the slope factor (Hill coefficient). There was a slight decrease in the ATP sensitivity of the channel and a slight increase in n_H when pH_i was increased. The best fit to the mean data was obtained with values for K_i of 18 and 33 μM and with Hill coefficients of 1·65 and 1·17, at pH_i 7·2 and 6·4, respectively.

In pancreatic β-cells, free ATP, rather than MgATP appears to act as the main inhibitory species (Ashcroft & Kakei, 1988; Dunne, West-Jordan, Abraham, Edwards & Petersen, 1988). In these earlier studies, the relative potencies of the different ATP complexes was not assessed. The relative proportions of the ATP complexes present in our solutions will vary with pH_i, with acidification increasing $ATPH^{3-}$ and decreasing ATP^{4-}. One explanation, therefore, for the shift in the ATP dose–response curve to higher concentrations produced by acidification, is that the K^+_{ATP} channel is primarily sensitive to ATP^{4-}. If this is the case, then there should be little effect of protons on the relationship between channel activity and ATP^{4-}. Figure 9*B* shows that this is indeed the case: when channel activity is plotted as a function of ATP^{4-}, 50 % inhibition occurs at 14 μM ATP^{4-} with a Hill coefficient of 1·3 and there is no effect of $[H^+]$ on the relationship. This is consistent with the idea that ATP^{4-} is the main modulator of channel activity and that $ATPH^{3-}$ has little inhibitory effect.

Effects of external pH on K^+_{ATP} currents

The effect of external acidification on K^+_{ATP} currents was examined both in outside-out patches and in perforated patch whole-cell recordings. Whole-cell K^+_{ATP} currents were

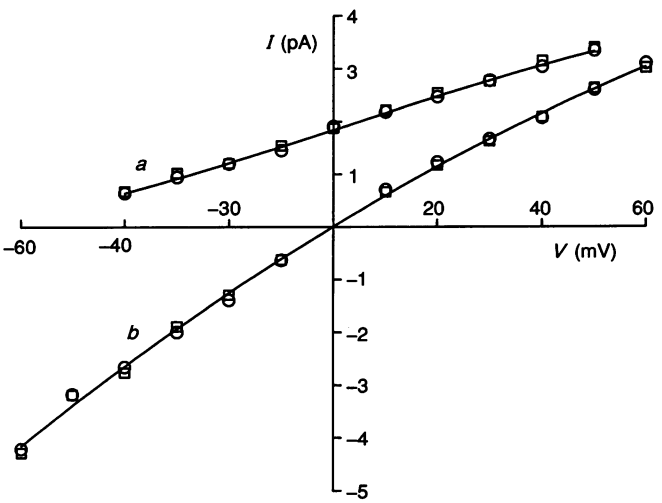

Figure 10. Effect of external pH on K^+_{ATP} currents
Representative single-channel current–voltage relationships measured from the same outside-out patch first at a pH_o of 7·2 (○) and subsequently at pH_o of 6·2 (□). *a*, standard extracellular solution (mM): 140 NaCl, 5·6 KCl, 1·1 $MgCl_2$, 2·6 $CaCl_2$, 10 Hepes. *b*, high-K^+, low-divalent cation extracellular solution (mM): 140 KCl, 4·6 $CaCl_2$ (free Ca^{2+}, 0·3 mM), 10 EDTA, 10 Hepes.

unaffected by lowering the external pH (pH_o) from 7·2 to 6·2 (data not shown). There was also no obvious effect of pH_o on channel activity measured in outside-out patches, or on the single-channel current–voltage relationship, whether measured with 140 or 5 mM $[K^+]_o$ (Fig. 10). Divalent cations were reduced in the 140 mM $[K^+]_o$ solution in order to prevent screening of external surface charges.

DISCUSSION

Inhibitory effects of intracellular protons on channel activity

An inhibitory effect of protons on K^+_{ATP} channel activity has been described qualitatively by Misler *et al.* (1989) for K^+_{ATP} channels in rat β-cells. In the present study, we have demonstrated that the relationship between pH_i and channel activity could be described by the Hill equation with a pK value of 6·25 and a Hill coefficient of 3·7. The latter suggests that closing of the K^+_{ATP} channel may involve the co-operative action of more than one proton. The pK value of 6·25 may suggest the involvement of a histidine residue since histidine has a pK value in this range. Alternatively, the pK of the inhibitory site may simply reflect the combined effects of a number of amino acid side groups with differing pK values.

Inactivation induced by acidification

Exposure of the patch to solutions of pH_i less than about 6·3 results in an irreversible inhibition of channel activity or a partial recovery to some reduced level. The finding that trypsin can restore channel activity inhibited by exposure to the low-pH_i solutions suggests that the irreversible part of channel inhibition produced by low pH_i involves sites which are accessible to the cytosol. These sites do not appear to lie within the pore itself because trypsin does not alter the single-channel current–voltage relationship (Proks & Ashcroft, 1992). The ability of trypsin to restore channel activity after its permanent inhibition by low pH_i is reminiscent of the ability of trypsin to restore channel activity after complete run-down (Proks & Ashcroft, 1992). One may therefore consider the run-down state as an inactivated state and protonation of the channel (or an associated control protein) as facilitating entry into this state.

Trypsin does not remove the ability of low pH_i to inhibit channel activity, but trypsinated channels always recover fully from acidification. Thus it appears that protons may interact with two sites, one of which which promotes entry into an inactivated (run-down) state and a second site which produces a reversible inhibition of the channel. It is not clear whether both sites are accessible under control conditions, or whether proteolysis exposes the latter site.

pH_i effects on the single-channel conductance

The inhibition of K^+_{ATP} channel activity at low pH_i is accompanied by a reduction in the amplitude of both the inward and outward single-channel currents. This decrease saturates with increasing proton concentration at around 18·5% inhibition. The pK of 6·48 for half-maximal inhibition of the unitary current suggests that a histidine residue may also be involved in this effect, but is not clear whether this residue is the same as that involved in the inhibition of channel activity by protons.

There are a number of mechanisms by which protons might reduce the conductance of the K^+_{ATP} channel. These include: (1) screening of negative membrane surface charges in the vicinity of the channel mouth, which might reduce the local concentration of K^+ ions, both by electrostatic repulsion or by displacement of K^+ bound to negative surface charges; (2) protonation of negative charges located on the cytoplasmic mouth of the channel, outside the membrane voltage field, which might allosterically alter the conformation of the pore and thereby its conductance; (3) binding of protons inside the pore, within the membrane voltage field, which might alter its conformation; (4) protons acting as a permeant blocker of K^+ fluxes with fast kinetics (Woodhull, 1973; Daumas & Andersen, 1993). The decrease in the unitary current amplitude is not accompanied by an increase in the open channel noise and shows no significant voltage dependence. Furthermore, the maximal inhibition of the unitary current is only 18·5% whereas complete inhibition at low pH_i is expected if protons enter the pore and block K^+ fluxes. This suggests that a fast block of the open channel by protons (mechanism (4) above) is not the cause of the reduction in the single-channel conductance. The binding of protons within the membrane voltage field is also excluded (mechanism (3) above). We were unable to accurately evaluate the surface charge on the intracellular side of the K^+_{ATP} channel because of a significant and variable amount of inward rectification which was present in most patches, even when the only internal cation was K^+. However, a simple surface charge effect cannot explain the reduction in the amplitude of both inward and outward currents. We therefore consider that the most likely reason for the decrease in the unitary curent is that proton binding to a site located outside the membrane voltage field produces a conformational change which alters the channel conductance pathway.

A reduction in the unitary current amplitude by protons was also observed for K^+_{ATP} channels in both skeletal (Davies, 1990; Davies *et al.* 1992) and cardiac (Koyano *et al.* 1993) muscle. Similar results have also been reported for the inward rectifier in cardiac myocytes (Ito, Vereecke & Carmeliet, 1992).

Effects of pH_i on the channel ATP sensitivity

In the presence of 30 μM ATP, acidification increased channel activity. This effect could be satisfactorily explained by the concommittant reduction in the concentration of ATP^{4-}, which appears to be the most potent inhibitory ATP species. There was no evidence that protons compete with ATP for the ATP-binding site, as has been shown in skeletal muscle (Davies *et al.* 1992).

Comparison of the effects of pH in other tissues

In pancreatic β-cells we found that protons markedly decrease K^+_{ATP} channel activity. By contrast, in skeletal

muscle pH_i has little effect on the K^+_{ATP} channel open probability over the range 5·6–8·0 (Davies *et al.* 1992). Providing that run-down of channel activity was prevented, a similar result was found in guinea-pig cardiac myocytes; however, when channel activity had partially run-down, acidification produced an increase in channel activity (Koyano *et al.* 1993). By contrast, Cuevas *et al.* (1991) report a decrease in K^+_{ATP} channel open probability on reducing pH_i in feline ventricular myocytes. These differences in the effects of protons in β-cells and in muscle suggest that the structure of the K^+_{ATP} channels in these tissues may be different.

The effect of intracellular pH on the ATP sensitivity of the K^+_{ATP} channel in β-cells resembles that reported for ventricular muscle, where a small shift in ATP sensitivity, from a K_i of 25 μM to one of 50 μM, was found on lowering pH_i from 7·25 to 6·25 (Lederer & Nichols, 1989). A similar shift, from 20 μM ($pH_i = 7·2$) to 40 μM ($pH_i = 6·4$) has also been reported by Koyano *et al.* (1993). By contrast, intracellular pH has a much greater effect on the channel sensitivity to ATP in skeletal muscle, where an increase in K_i from 17 to 260 μM was found on decreasing intracellular pH from 7·2 to 6·3 (Davies *et al.* 1992).

In β-cells, we found that acidifcation was without effect on the Hill coefficient for channel inhibition by ATP. This was also the case in skeletal muscle (Davies *et al.* 1992). In cardiac muscle, however, the steepness of the dose–inhibition curve for ATP has been variously reported to increase (Lederer & Nichols, 1989) or to remain unchanged (Koyano *et al.* 1993) when pH_i is reduced.

Physiological significance

Using the fluorescent indicator, BCECF, steady-state cytosolic pH values ranging between 6·7 and 7·4 have been reported for resting β-cells (Arkhammer, Berggren & Rorsman, 1986; Grappengeisser, Gylfe & Hellman, 1989; Lynch, Trebilcock, Tomlinson & Best, 1991) and somewhat higher values of around pH 7·2 (Lindstom & Sehlin, 1984) to 7·45 (Lindstom & Sehlin, 1986) have been reported from the accumulation of ^{14}C[DMO] (5,5-dimethyl [2-^{14}C]oxazolidine-2,4-dione. These data suggest that the K^+_{ATP} channel may be poised at the top of the pH dose–response curve in the resting β-cell.

If protons are to serve as coupling factor linking metabolism to channel activity then clearly intracellular pH must change in response to nutrient stimulation. Nutrient metabolism is accompanied by the generation of acidic metabolites such as lactic acid and CO_2 and at first sight it might be expected that increased metabolism would lead to an intracellular acidification. However, there has been considerable argument as to whether metabolism produces any change in β-cell cytoplasmic pH. Experiments using the fluorescent pH indicator, BCECF, suggest that 20 mM glucose induces an increase in pH_i of less than 0·05 of a pH unit (Arkhammer *et al.* 1986; Grappengeiser *et al.* 1989). These small changes in pH_i have little effect on K^+_{ATP} channel activity and they do not support the idea that metabolically induced changes in pH_i constitute the physiological regulator of the K^+_{ATP} channel. Furthermore, whereas glucose produces a slight increase in pH_i, another nutrient secretagogue, glyceraldehyde, causes a decrease in pH_i (Arkhammer *et al.* 1986). The fact that both nutrients close the K^+_{ATP} channel (Ashcroft, Harrison & Ashcroft, 1984; Rorsman & Trube, 1985; Dunne, Findlay, Petersen & Wollheim, 1986; Misler, Falke, Gillis & McDaniel, 1986) but have opposite effects on cytosolic pH is also inconsistent with a role for protons as a coupling factor linking metabolism to channel activity.

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