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ATP-SENSITIVE K^+ CHANNELS IN RAT PANCREATIC β-CELLS: MODULATION BY ATP AND Mg^{2+} IONS

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SUMMARY

1. The inside-out configuration of the patch-clamp method was used to study the effects of MgATP, free ATP and Mg^{2+} on single ATP-sensitive K^+ channel currents in rat pancreatic β-cells.

2. Magnesium ions caused a marked reduction of channel activity: 5 mM-free Mg^{2+} produced a 50% reduction in the activity of inward currents recorded at -60 mV in symmetrical K^+ concentrations.

3. Inhibition of channel activity by MgATP does not involve phosphorylation as both free ATP (i.e. ATP in the absence of divalent cations) and non-hydrolysable ATP analogues were effective inhibitors.

4. Magnesium ions produced a striking reduction in the ability of ATP (total) to inhibit channel activity. When channel activity was plotted as a function of the total ATP concentration, the K_i for channel inhibition was 4 μM in Mg^{2+}-free solution, compared to a K_i of 26 μM in the presence of 2 mM-Mg^{2+}. The shape of the relationship between channel activity and the total ATP concentration was not changed by Mg^{2+}. When channel activity was plotted as a function of the free ATP concentration, however, Mg^{2+} had little effect on K_i. This suggests that free ATP is the more potent inhibitor of channel activity and that MgATP has little inhibitory effect.

5. ATP analogues that dissociate only as far as the tribasic form were also able to inhibit channel activity. This suggests that both ATP^{4-} and $ATPH^{3-}$ can block the channel.

6. Like ATP, ADP was more effective at inhibiting channel activity in the absence of Mg^{2+}, that is as the free base. The non-hydrolysable ATP analogues AMP-PNP and AMP-PCP, however, were more effective in the presence of Mg^{2+}.

7. It is suggested that (1) the potency of inhibition is related to the amount of negative charge carried by the ion and (2) the intracellular concentration of free ATP will be an important modulator of channel activity in the intact β-cell.

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INTRODUCTION

Glucose metabolism stimulates electrical activity and insulin secretion in pancreatic β-cells by closing ATP-sensitive K^+ channels (for review see Ashcroft, 1988). It is widely believed that changes in adenine nucleotide concentrations constitute the link between glucose metabolism and channel activity.

ATP influences channel activity by acting at two pharmacologically distinct sites on the channel or associated control proteins; interaction with one site leads to a decrease in channel activity (Cook & Hales, 1984; Rorsman & Trube, 1985) whereas interaction with the other site leads to an increase in channel availability (Findlay & Dunne, 1986; Misler, Falke, Gillis & McDaniel, 1986; Ohno-Shosaku, Zunckler & Trube, 1987). Phosphorylation is not associated with the inhibitory action of ATP since non-hydrolysable analogues are effective and ATP inhibits in the absence of Mg^{2+}. A number of other nucleotides also interact with this site, to differing extents, and lead to channel inhibition (Cook & Hales, 1984; Ohno-Shosaku *et al.* 1987; Findlay, 1987*a*). In addition to its inhibitory action, ATP is required to maintain channel activity. Several studies have shown that the run-down of channel activity that occurs in excised patches can be prevented or reversed by exposure to solutions containing MgATP. Unlike channel inhibition, this increase in channel availability may involve a phosphorylation step because Mg^{2+} is also required and non-hydrolysable analogues are unable to substitute for ATP (Findlay & Dunne, 1986; Misler *et al.* 1986; Ohno-Shosaku *et al.* 1987).

In this paper we examine further the properties of the inhibitory site at which ATP acts. We also investigate the role of Mg^{2+} in the regulation of channel activity. Some of these results have been presented briefly already (Ashcroft & Kakei, 1987).

METHODS

Preparation

Experiments were carried out on single pancreatic β-cells isolated from the islets of Langerhans of normal adult rats and maintained for up to 5 days in short-term tissue culture. These methods have been described in detail previously (Ashcroft, Ashcroft & Harrison, 1988). Briefly, rats were killed by cervical dislocation, islets were isolated by collagenase digestion and further dissociated into single cells by collagenase digestion and mechanical dispersion.

Recording methods

The patch-clamp technique was used to study single-channel activity in inside-out membrane patches (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Patch pipettes were pulled from 1·5 mm borosilicate glass (Boralex, Rochester Scientific), coated with Sylgard close to their tips to reduce their electrical capacitance and fire-polished immediately before use. They had resistances between 5–10 MΩ when filled with 140 mM-KCl. Seals were made in 5 mM-K^+ external solution and the bath solution was then exchanged for an intracellular solution before excising the patch. Seal resistances were greater than 5 GΩ.

The reference potential for all measurements was the zero-current potential of the pipette before establishment of the seal. In most cases the pipette potential was held constant at +60 mV (−60 mV membrane potential). In this paper we use the usual sign conventions, i.e. membrane potentials are expressed as inside relative to outside and inward membrane currents are negative and plotted downwards.

Solutions

The pipette was filled with a 140 mM-K^+ external solution containing (mM): 140 KCl, 5 $CaCl_2$, 5 $MgCl_2$, 5 HEPES (buffered to pH 7·4 with NaOH). Before excising the patch the bath contained a 5 mM-K^+ external solution (containing (mM): 5 KCl, 135 NaCl, 5 $CaCl_2$, 5 $MgCl_2$, 5 HEPES; adjusted to pH 7·4 with NaOH). For most experiments the intracellular (bath) solution contained

TABLE 1. Concentrations of free cations and ligands and their complexes in intracellular solutions

Solution	ATP^{4-}	$ATPH^{3-}$	ADP^{3-}	$ADPH^{2-}$	MgATP	MgADP	Mg^{2+}	KATP	KADP
0·1 mM-ATP	$3·7e^{-5}$	$1·0e^{-5}$	—	—	$1·2e^{-9}$	—	$2·8e^{-9}$	$5·2e^{-5}$	—
0·1 mM-ADP	—	—	$4·8e^{-5}$	$1·0e^{-5}$	—	$1·8e^{-10}$	$2·8e^{-9}$	—	$4·2e^{-5}$
0·1 mM-ATP, 2 mM-Mg^{2+}	$5·9e^{-6}$	$1·6e^{-6}$	—	—	$8·4e^{-5}$	—	$1·3e^{-3}$	$8·3e^{-6}$	—
0·1 mM-ADP, 2 mM-Mg^{2+}	—	—	$2·6e^{-5}$	$5·4e^{-6}$	—	$4·6e^{-5}$	$1·3e^{-3}$	—	$2·3e^{-5}$
0·1 mM-ATP, 0·1 mM-ADP, 2 mM-Mg^{2+}	$3·9e^{-6}$	$1·1e^{-6}$	$2·8e^{-5}$	$5·8e^{-6}$	$5·5e^{-5}$	$4·7e^{-5}$	$1·3e^{-3}$	$3·9e^{-5}$	$1·9e^{-5}$

Concentrations of free metals and ligands and of the metal–ligand complexes were calculated using the binding constants of Martell & Smith (1974). The balance of Mg^{2+} in magnesium-containing solutions is complexed to EGTA. The small amounts of other metal–ligand complexes (e.g. CaATP, $ATPH_2^{2-}$) have been excluded for clarity.

(mM): 107 KCl (adjusted as required to give a final K^+ concentration of 140 mM), 11 EGTA, 1 $CaCl_2$ (free Ca^{2+} about 0·02 μM), 11 HEPES (buffered to pH 7·2 with KOH). The magnesium concentration ($MgSO_4$) is given in the text. In some experiments a Mg^{2+}-free solution was used and in this solution EDTA (11 mM) replaced EGTA as the divalent cation buffer. No corrections have been made for liquid junction potentials (< 5 mV).

Nucleotides were added to the intracellular solutions at the concentrations indicated in the text and the pH adjusted if necessary. K^+ concentrations were only adjusted if nucleotide addition was calculated to change the final K^+ concentration by more than 1 mM. The nucleotides were: ATP (adenosine triphosphate: disodium salt, Boehringer or Sigma); ADP (adenosine diphosphate: dipotassium salt, Boehringer or Sigma); AMP-PNP (adenylimidophosphate: tetralithium salt, Boehringer); AMP-PCP (adenyl (β-γ-methylene) diphosphate: tetralithium salt, Boehringer); ATP-γ-S (adenosine 5′ thiotriphosphate: tetralithium salt, Boehringer); A5pA (P^1, P^5, diadenosine-5′ pentaphosphate: sodium salt, Sigma); the trimethylamine salts of ATP-γ-F (adenosine 5′ (3-fluoro) triphosphate) and ATP-γ-anilino (adenosine 5′ (3-anilino) triphosphate) were the generous gift of Dr N. Cusack, Kings College, London.

The concentrations of free cations and ligands and of their complexes in the intracellular solutions were calculated (Table 1) using an IBM-AT microcomputer and a program PERTEM supplied by Dr P. Griffiths. Binding constants were taken from Martell & Smith (1974).

Solutions were exchanged using a rapid microflow perfusion system (Kakei & Ashcroft, 1987). Test solutions were applied in random order and in most cases were repeated several times on the same patch. All experiments were carried out at room temperature (18–22 °C).

Data collection and analysis

Recordings were made using an EPC5 patch-clamp amplifier (List Electronic, Darmstadt, FRG) and stored on FM tape for later analysis (usual tape speed 15 in/s; −1dB down at 10 kHz, Store 4DS, Racal Recorders, Hythe). Records were subsequently amplified, replayed through an 8-pole Bessel low-pass filter (Frequency devices, Haverhill, MA, USA) at 1 kHz, digitized at 5 kHz using a 12-bit A-D converter (Indec systems, CA, USA) and analysed using a PDP 11-73 microcomputer (Digital Equipment Corporation, USA).

Probability density functions were constructed by forming histograms of all the data points

(baseline and open level) and expressing the number in each histogram bin as a fraction of the total number of data points sampled. Dose–response curves for nucleotide or cation inhibition were constructed by measuring the mean current (I) and expressing this as a fraction of its amplitude in the control solution (I_c). I was computed for data lengths of 15–30 s by integrating the current record after subtraction of the baseline current. The baseline current (i.e. the current level when

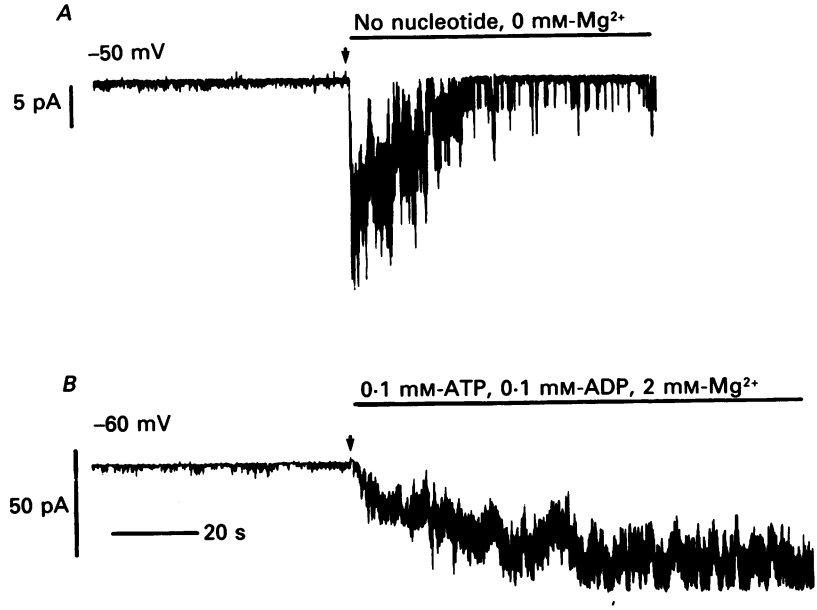

Fig. 1. Run-down of ATP-sensitive K^+ currents is decreased by solutions which contain MgATP. Single-channel currents recorded in the cell-attached configuration and after (arrowed) excision to the inside-out configuration. The bath contained intracellular solution without nucleotides (A) or with the addition of 0·1 mM-ATP, 0·1 mM-ADP, 2 mM-Mg^{2+} (B). The patch membrane potential is indicated above the trace: −50 mV in A, −60 mV in B. Since the β-cell resting potential will be very close to 0 mV in 140 mM-K^+ solution, the patch membrane potential is unlikely to change significantly on excision. Traces redisplayed after replaying taped data onto a chart recorder.

all channels are closed) was either measured directly (when channel activity was low) or, in the case of patches with high activity, by exposing the patch to 1 mM-ATP. The density of ATP-sensitive K^+ channels in our patches was usually so high (up to forty channels) that single-channel currents could not be resolved in nucleotide-free solutions. The mean current, I, is a product of i, the single-channel current, N, the number of functional channels and p, the probability of the channel being in the open state; i.e. ($I = N \times p \times i$). Providing that i is unaffected by nucleotides or Mg^{2+}, I is directly proportional to $N \times p$. In this paper $N \times p$ is defined as channel activity. Precautions taken to avoid run-down of channel activity are described in the text.

Some traces displayed in this paper have been photographed from records obtained by replaying taped data through a chart recorder (Gould 2200; bandwidth DC to 100 Hz.) Results are expressed as mean ± S.E. of the mean.

RESULTS

A difficulty in working with the ATP-sensitive K^+ channel in patches excised from pancreatic β-cells is that channel activity is generally not stable but runs down with time. This phenomenon is illustrated in Fig. 1*A*. At the beginning of the record the pipette is in the cell-attached configuration and a low level of channel activity is seen. The cell is bathed in intracellular solution so that its resting potential will be close

to 0 mV and the pipette potential is held at +50 mV to observe inward currents. Immediately following patch excision (arrowed) channel activity transiently increases indicating that ATP-sensitive K^+ channels are subject to resting inhibition in normal rat β-cells. Over the course of the next minute channel activity in this patch declined substantially when it was exposed to an intracellular solution lacking Mg^{2+} or nucleotides as in mouse β-cells (Misler *et al.* 1986, Ohno-Shosaku *et al.* 1987) and β-cell lines (Findlay & Dunne, 1986). The rate of this run-down in channel activity is extremely variable, with a few patches showing little run-down over 20 min and others becoming quiescent in less than 30 s.

Run-down can be prevented, and the channels reactivated, by exposure of the patch to a phosphorylating intracellular solution containing ATP and Mg^{2+} (Findlay & Dunne, 1986; Ohno-Shosaku *et al.* 1987). We used an ATP pulse protocol similar to that described by Ohno-Shosaku *et al.* (1987) to control for run-down, which involved alternating the test solutions with a 1 min exposure to a reactivating solution containing 0·1 mM-ATP, 0·1 mM-ADP and 2 mM-Mg^{2+}. Our solution also contained ADP because as we have shown before (Kakei, Kelly, Ashcroft & Ashcroft, 1986) channel activity in ATP solutions is increased in the presence of ADP. The ability of this solution to maintain channel activity following patch excision is illustrated in Fig. 1*B*.

Although channel activity is substantially greater in reactivating solutions containing 0·1 mM-ADP in addition to 0·1 mM-ATP and 2 mM-Mg^{2+}, this solution did not produce reactivation additional to that produced by MgATP alone when the patch was subsequently returned to nucleotide-free solution. As Fig. 2 shows, the current recorded following a 30 s exposure to reactivating solutions with or without ADP was the same. At present we are not certain that full channel activation is achieved by 30 s exposure to 0·1 mM-MgATP. Therefore we cannot determine whether 0·1 mM-ADP is also capable of sustaining channel reactivation, because the channels may already be fully activated by 0·1 mM-ATP. The advantage of using a phosphorylating solution containing ADP is that in this solution channel activity is sufficiently high for it to be used as a reference to check for run-down.

For these experiments we also used patches containing a large number of channels; this is readily achieved using standard size patch pipettes because of the high density of ATP-sensitive K^+ channels. Since the patches contained such a large number of channels long periods of recording were not required for determining the mean current and constructing the dose–response curves for Mg^{2+} and nucleotide inhibition. Normally 20 s of data were used.

Channel inhibition by magnesium ions

It is clear from the amplitude histograms illustrated in Fig. 3*A* that millimolar concentrations of magnesium ions cause a marked reduction of channel activity at −60 mV, as has been found in both RINm5F cells (Findlay, 1987*a*) and in cardiac cells (Findlay, 1987*b*). There is no significant effect of Mg^{2+} on the amplitude of the single-channel current at this membrane potential (Fig. 3A and *B*), although, as has already been reported (Horie, Irisawa & Noma, 1987; Findlay, 1987*a*; Ciani & Ribalet, 1988), outward currents are reduced. We have therefore only investigated the effects of Mg^{2+} on channel activity at −60 mV, a potential close to the resting potential of the β-cell.

The experimental protocol used to investigate the effects of Mg^{2+} on channel activity is shown in Fig. 3*C*. The membrane potential was held constant at −60 mV and the patch exposed to a series of intracellular solutions. Each test sequence consisted of a 10–15 s exposure to a control Mg^{2+}-free intracellular solution followed

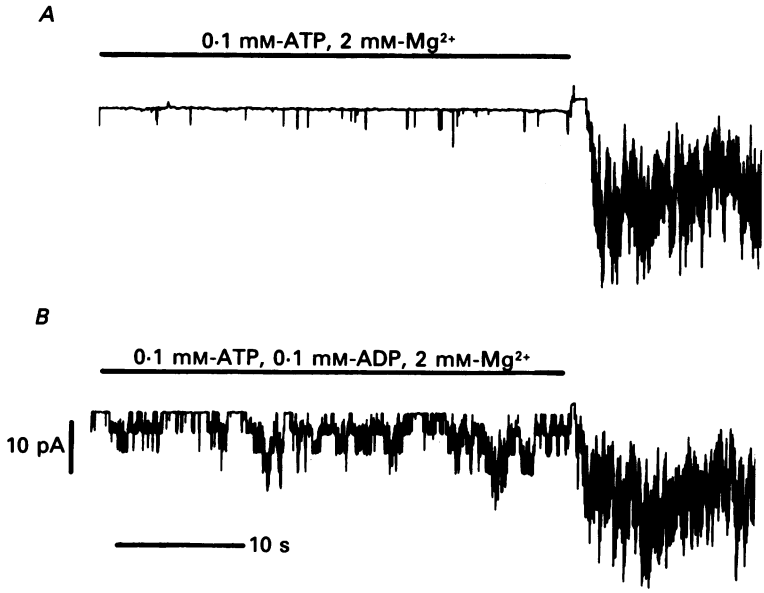

Fig. 2. Reactivation of ATP-sensitive K^+ channels by adenine nucleotides. Channels were activated during the period indicated by the bar with a solution containing 0·1 mM-ATP, 2 mM-Mg^{2+} (*A*) or 0·1 mM-ATP, 0·1 mM-ADP, 2 mM-Mg^{2+} (*B*), and then exposed to nucleotide- and Mg^{2+}-free solution. Currents recorded at −60 mV from the same patch. Chart recorder records of taped data. The glitches in the current record are artifacts associated with the solution exchange (Kakei & Ashcroft, 1987).

by a 20–30 s exposure to a solution containing Mg^{2+} at the test concentration and then by the control solution once again. Test sequences were alternated with the reactivating solution; in this way channel activity was maintained at a constant level in the reactivating solution throughout the experiment (Fig. 3*C*). Some run-down, however, does appear to take place during exposure to test solutions containing more than 0·1 mM-Mg^{2+} (nucleotide-free) since the mean current in control solution was smaller following exposure to these solutions.

Figure 4 shows the dose–response curve for the inhibitory effect of Mg^{2+} on the mean current. The mean current is expressed as a fraction of that in the control solution; in order to take account of any remaining channel run-down, the average of the control solutions which bracketed each test solution was used. The line is fitted to the Hill equation:

$$I/I_c = 1/\{1+([X]/K_i)^n\}, \quad (1)$$

where [X] is the Mg^{2+} concentration. The Hill coefficient (n) was 1·9, which suggests that more than one magnesium ion binds to the channel to induce closure, and the K_i, which indicates the free Mg^{2+} concentration at which the mean current is reduced to

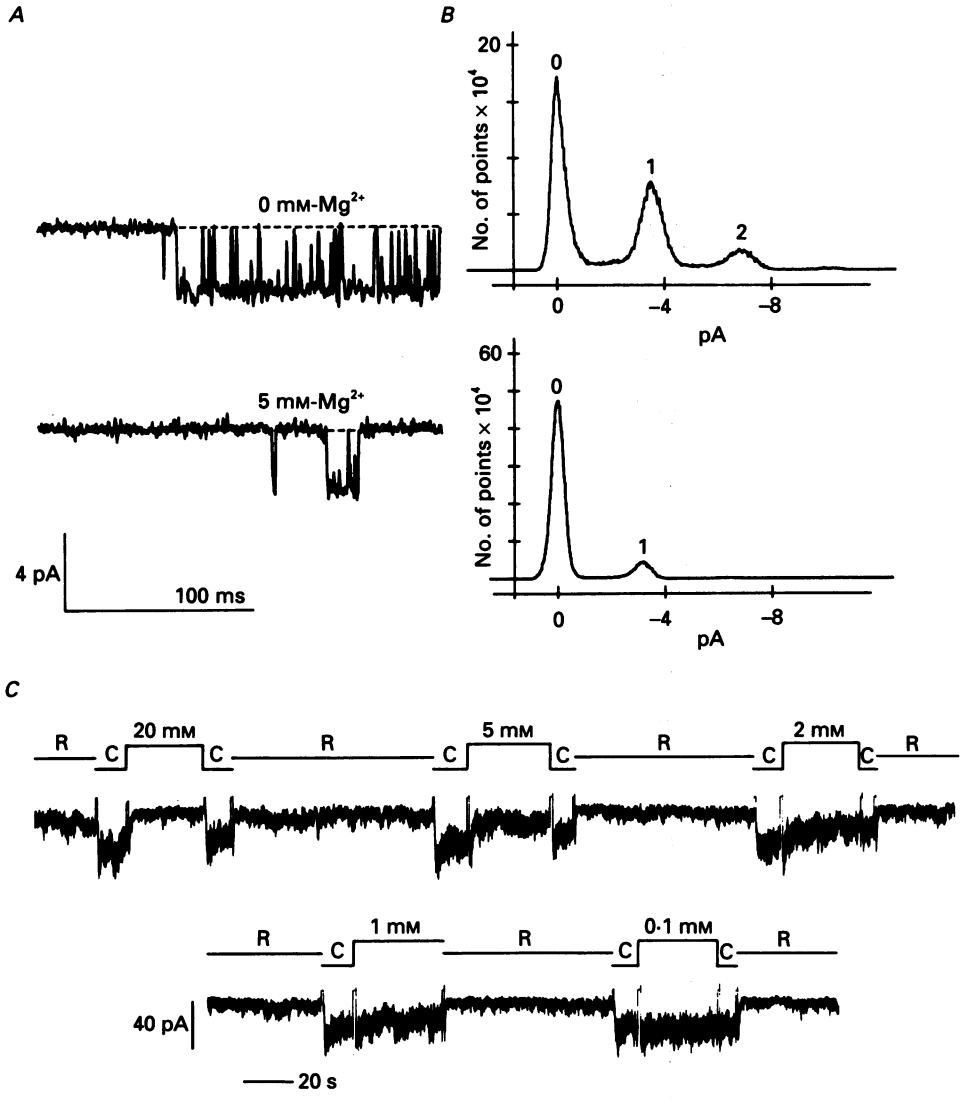

Fig. 3. Effect of Mg^{2+} ions on the ATP-sensitive K^+ channel. Single-channel currents (*A*) and amplitude histograms (*B*) recorded from the same patch at −60 mV in absence (above) and presence (below) of 5 mM-Mg^{2+}. Filter frequency, 1 kHz; sample rate, 5 kHz. The dashed line indicates the channel closed state. Amplitude histograms were constructed from recordings of 39 s (0 mM-Mg^{2+}) and 36 s (5 mM-Mg^{2+}). The number above each peak indicates the current level at which 0, 1, 2, ... channels are open. *C*, protocol used to construct the dose–response curve for the inhibitory effect of Mg^{2+}. Above, sequence of solutions. The reactivating solution (R) contained 0·1 mM-ATP, 0·1 mM ADP, 2 mM-Mg^{2+}. Control solution (C) was Mg^{2+}- and nucleotide-free. Test solutions contained no nucleotides and the Mg^{2+} concentration indicated. Below, currents recorded at a membrane potential of −60 mV in the solutions indicated above. The traces are a continuous recording from the same patch. Chart recorder records of taped data.

50%, was 5·4 mM-Mg^{2+}. For our intracellular solution, this corresponds to a total magnesium concentration of around 7 mM. As Fig. 4 also shows, channel activity was consistently higher in the presence of 0·1 mM-Mg^{2+} than in the absence of Mg^{2+}; we have no explanation for this observation.

One additional patch in which Mg^{2+} had no effect on the ATP-sensitive K^+ channel at any concentration has been excluded from the data; this patch was ATP sensitive.

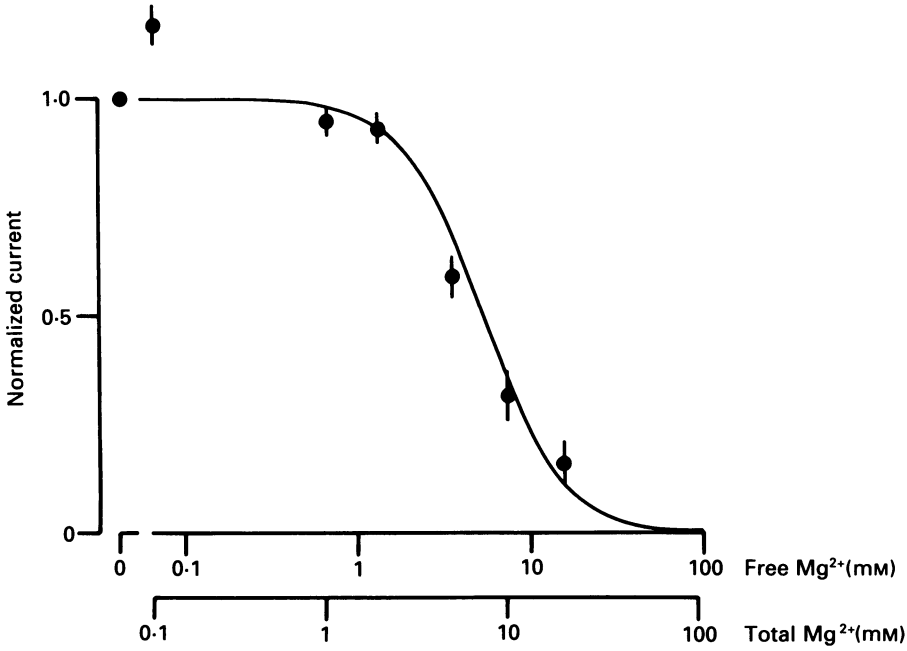

Fig. 4. Relationship between channel activity and Mg^{2+} concentration for four patches. Ordinate, mean current expressed relative to its value in Mg^{2+}-free solution. Abscissa, calculated free Mg^{2+} and total Mg^{2+}. The continuous line is drawn to eqn (1) of the text with a K_i of 5·4 mM (free Mg^{2+}) and a Hill coefficient of 1·9.

Channel inhibition induced by ATP

Several groups have reported that ATP can inhibit channel activity in divalent cation-free solutions indicating that free ATP is an effective channel inhibitor (Cook & Hales, 1984; Ashcroft & Kakei, 1987; Dunne, Ilott & Petersen, 1987; Dunne, West-Jordan, Abraham, Edwards & Petersen, 1988). Figure 5*A* and *B* demonstrates that addition of 2 mM-Mg^{2+} produces a striking reduction in the ability of ATP to inhibit the channel. We investigated the relative ability of free ATP and the MgATP complex to inhibit the channel. In solutions containing MgATP, channel activity will be determined by a balance between the activating and inhibitory effects of ATP. In order to evaluate the relative roles of free ATP and MgATP it is therefore important to maintain channel availability at a constant level throughout the experiment. To do this we used the experimental protocol illustrated in Fig. 5*C* in which each test solution was alternated with a 1 min exposure to the reactivating solution. For each

patch, we used two series of test solutions, one in which all solutions were Mg^{2+}-free and one in which all solutions contained 2 mM-Mg^{2+}. We chose this Mg^{2+} concentration as it is the highest one which has little direct blocking effect (Fig. 4). Each test solution consisted of intracellular solution containing ATP at the test

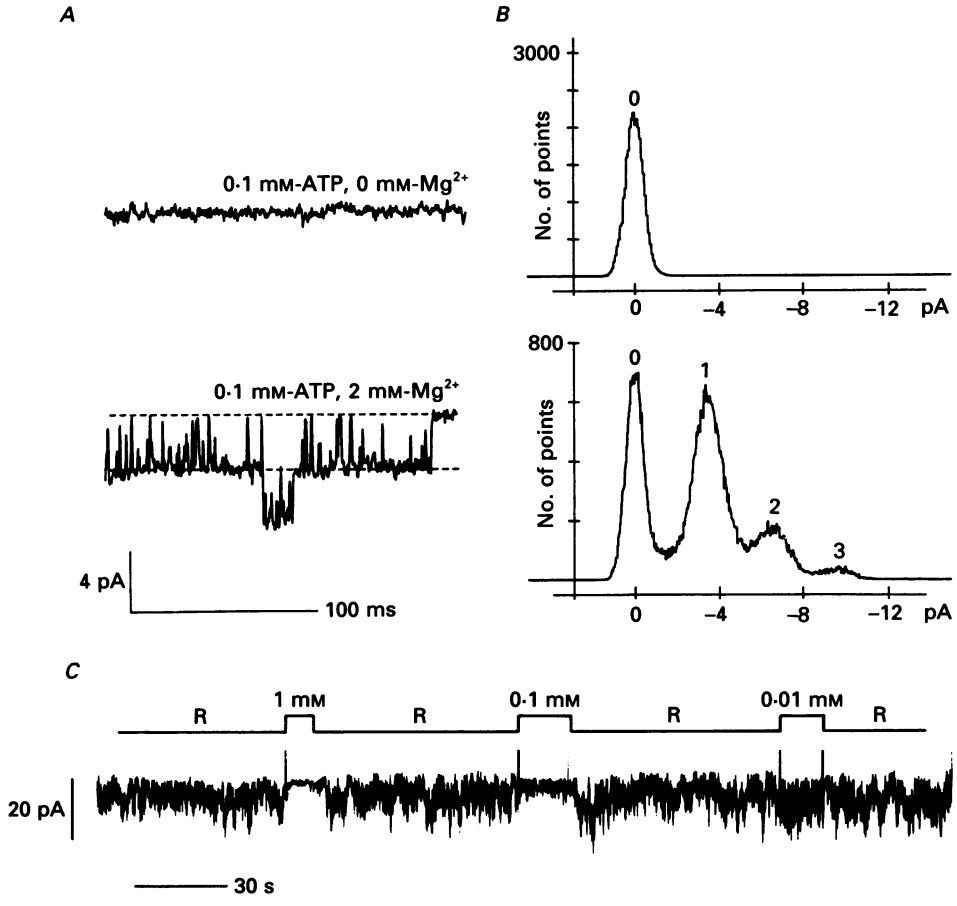

Fig. 5. The effect of Mg^{2+} on channel inhibition by ATP. Single-channel recordings (*A*) and amplitude histograms (*B*) recorded before (above) and after (below) the addition of 2 mM-Mg^{2+} to a 0·1 mM-ATP solution. Filter, 1 kHz; sample rate, 5 kHz. Pipette potential, −60 mV. Amplitude histograms were constructed from recordings of 11 s (above) and 15 s (below). The current level at which 0, 1, 2... channels are open is indicated above the trace. *C*, protocol used to construct the dose–response curve for the inhibitory effect of ATP. Currents recorded at −60 mV in the solutions indicated. The reactivating solution (R) contained 0·1 mM-ATP, 0·1 mM-ADP, 2 mM-Mg^{2+}. Test solutions were Mg^{2+}-free and contained the ATP concentration indicated. Chart recorder records of taped data.

concentration. In these experiments, the mean current during the test solution was expressed as a fraction of that in the reactivating solution which preceded it. These values were then normalized to those obtained in 0·1 μM-ATP.

Following exposure to ATP-containing solutions channel activity rapidly declines (Fig. 6*B*). We have assumed that this decline represents only a block by ATP and that there is no contribution from run-down. If substantial run-down did occur, the

mean current measured would be underestimated. Figure 6*A* shows that the error from run-down is likely to be small as there was usually little decrease in channel activity when the patch was exposed to nucleotide-free solutions for only short periods. Our protocol was to measure the mean current over the last 15 s of a 20 s

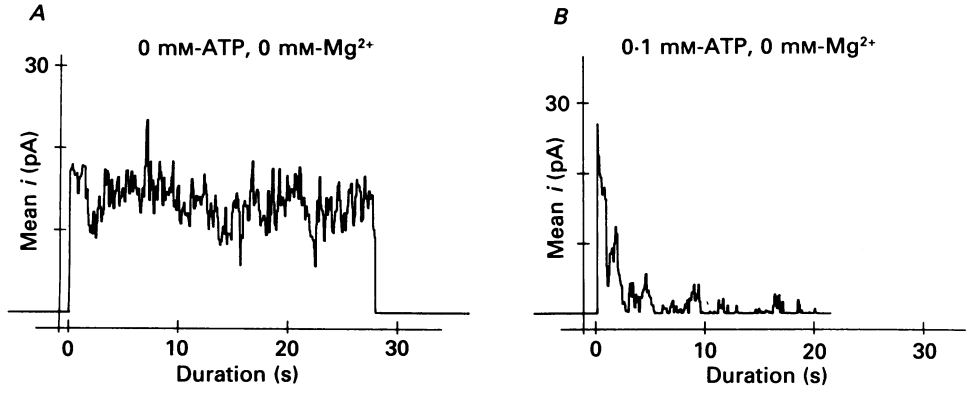

Fig. 6. Channel activity recorded at −60 mV as a function of time after removal from reactivating solution. *A*, in nucleotide- and Mg^{2+}-free solution. *B*, in the presence of Mg^{2+}-free solution containing 0·1 mM-ATP.

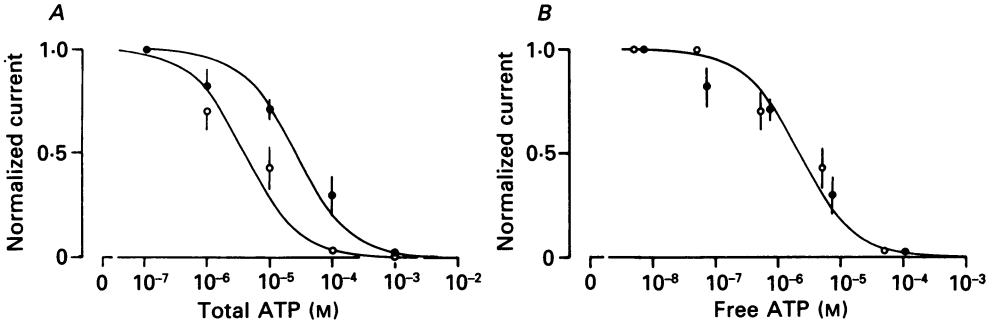

Fig. 7. Dose–response relationship for the inhibitory effect of ATP on channel activity at −60 mV measured in Mg^{2+}-free (○) and 2 mM-Mg^{2+} (●) solution. Ordinate, corrected mean current relative to its value in 0·1 μM-ATP. *A*, abscissa: total ATP (i.e. that added to the solution). The continuous line is drawn to eqn (1) of the text with a Hill coefficient of 1 and K_i values of 4 μM and of 26 μM in 0 mM-Mg^{2+} and 2 mM-Mg^{2+} respectively. Data represent mean ± S.E.M. of four or five patches (all patches were measured in both 0 mM-Mg^{2+} and 2 mM-Mg^{2+}). *B*, abscissa: calculated free ATP. Same data as in *A*. The continuous line (eqn (1) of text) has a Hill coefficient of 1 and a K_i of 2 μM.

exposure to ATP. However, if a significant decline in channel activity was observed in the test solution, we chose the peak current level during the test solution as being the best representation of the level of channel activity.

The effect of Mg^{2+} on the dose–response curve for inhibition by ATP is shown in Fig. 7*A*. The mean current, relative to its value in 0·1 μM-ATP solution, is plotted as a function of the total ATP concentration, that is the actual amount of ATP added

to the solution. Since the mean current was consistently higher in the presence of 0·1 μM-ATP and more stable than that in ATP-free solution, we have normalized the mean current to that found in 0·1 μM-ATP solution. The lines are drawn to eqn (1) of the text using a Hill coefficient of 1 in both Mg^{2+}-free and Mg^{2+}-containing solutions, consistent with a 1:1 binding between ATP and the channel. Addition of 2 mM-Mg^{2+} shifted the half-maximal inhibition from 4 to 26 μM (total ATP concentrations).

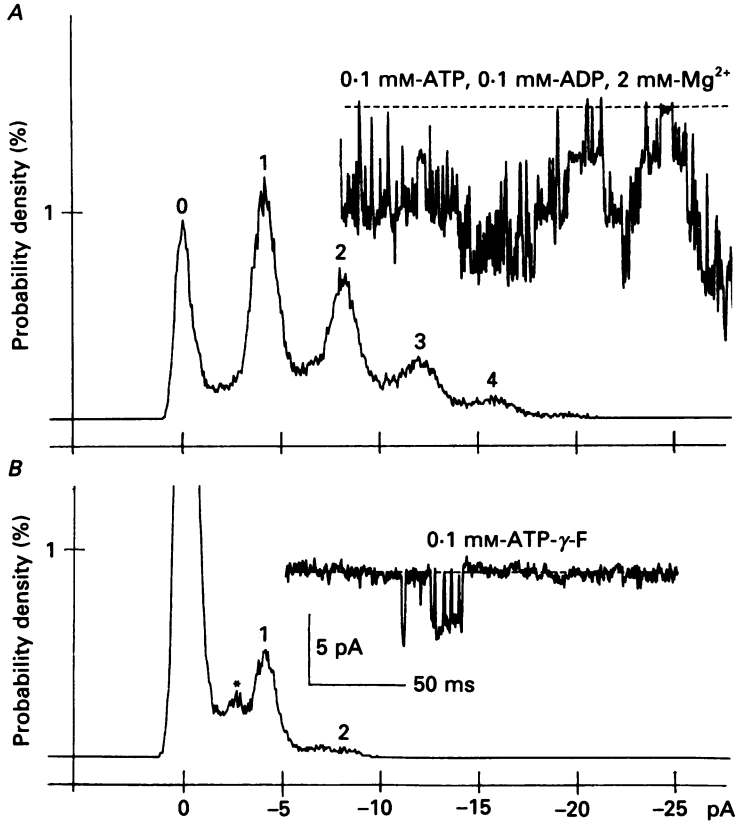

Fig. 8. Probability density histograms and single-channel currents (inset) recorded at −60 mV from the same patch in (*A*) re-activating solution (0·1 mM-ATP, 0·1 mM-ADP, 2 mM-Mg^{2+}) and (*B*) Mg^{2+}-free solution containing 0·1 mM-ATP-γ-F. Filter frequency, 1 kHz; sample rate, 5 kHz. The number above each peak indicates the current level at which 0, 1, 2, ... channels are open. The asterisked peak in the lower histogram represents current flow through a small conductance channel that became active late in this recording.

One explanation for the ability of Mg^{2+} to shift the ATP dose–response curves to higher concentrations is that Mg^{2+} reduces free ATP (ATP^{4-} and $ATPH^{3-}$) levels by forming the MgATP complex. If this is the case, then there should be little effect of Mg^{2+} on the relationship between channel activity and free ATP. Figure 7*B* shows that this is indeed the case: when channel activity is plotted against free ATP (ATP^{4-} + $ATPH^{3-}$) 50% inhibition occurs at 2 μM-free ATP and there is no significant effect of Mg^{2+} on the relationship. This is consistent with the idea that free

ATP is the more potent channel inhibitor and that MgATP has little if any blocking effect.

Effects of ATP analogues

In Mg^{2+}-free solution ATP exists as both the ATP^{4-} ion and the tribasic ion, $ATPH^{3-}$. To determine whether $ATPH^{3-}$ is inhibitory we tested two ATP analogues

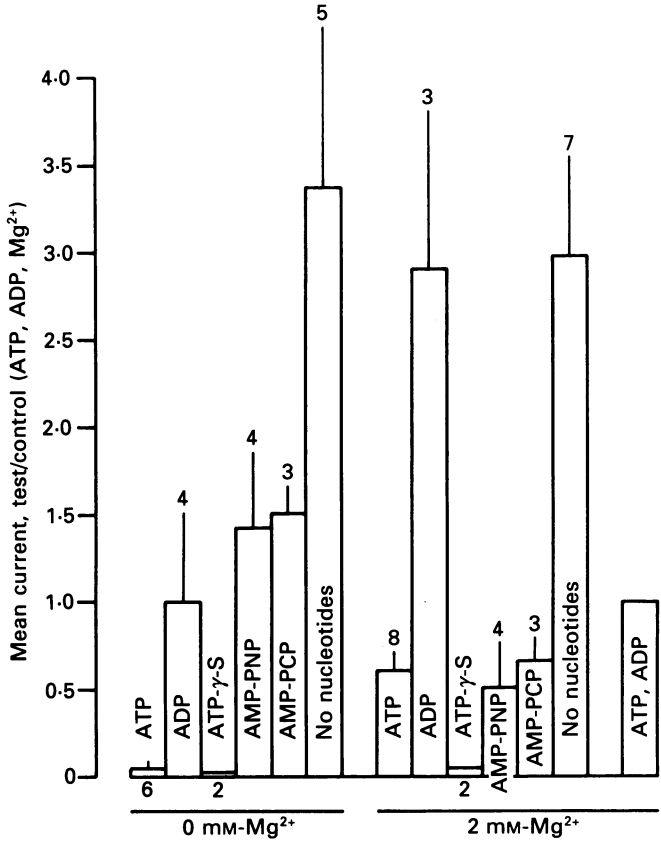

Fig. 9. Effects of ADP and ATP analogues on channel activity at −60 mV measured in Mg^{2+}-free solution (left) and 2 mM-Mg^{2+} solution (right). In each case the test substance was applied at 0·1 mM and the mean current recorded (during 20 s) is expressed relative to its value in control solution containing 0·1 mM-ATP, 0·1 mM-ADP, 2 mM-Mg^{2+}. The vertical bars indicate S.E.M. and the number of samples is given next to the bar (or below the histogram bin).

that dissociate only as far as the tribasic acid; ATP-γ-F and ATP-γ-anilino. These substances carry an uncharged fluorine or aniline group on the terminal phosphate instead of a negatively charged oxygen. Figure 8 shows that ATP-γ-anilino is an effective inhibitor of channel activity but has little effect on single-channel current amplitude. Indeed, at 0·1 mM concentration, both these compounds inhibited channel activity in Mg^{2+}-free solution in two patches tested. The mean currents measured were 55, 49, 666% and 19, 8, 224% of those recorded in reactivating solution for ATP-γ-F, ATP-γ-anilino and nucleotide-free solutions respectively.

Thus ATP-γ-F and ATP-γ-anilino are about equally as effective as blockers. Substantially greater inhibition (< 3% of the activity in reactivating solution), however, was found in both patches on exposure to a solution containing 2 mM-Mg^{2+} and 1 mM-ATP, in which the calculated free $ATPH^{3-}$ concentration was 23 μM, lower than that of either analogue (100 μM). Thus, either the tribasic ATP analogues are not good substitutes for the $ATPH^{3-}$ ion or ATP^{4-} can also inhibit the channel.

As shown in Table 1, a significant amount of ATP is complexed to K^+ ions. We have not attempted to assess whether KATP acts as a channel inhibitor; however, we point out that ATP can also block channel activity when Rb^+ replaces K^+ as the intracellular ion (Ashcroft, Kakei & Kelly, 1989).

The effect of the non-hydrolysable ATP analogues ATP-γ-S, AMP-PNP and AMP-PCP on channel activity is illustrated in Fig. 9. These compounds were inhibitory in both the absence and presence of 2 mM-Mg^{2+}, the order of potency being ATP-γ-S > AMP-PNP > AMP-PCP. These results confirm previous reports that non-hydrolysable ATP analogues are effective inhibitors and support the idea that phosphorylation is not required for channel inhibition (Cook & Hales, 1984; Ohno-Shosaku *et al.* 1987). As is the case for ATP, in both of the patches in which 0·1 mM-ATP-γ-S was tested the mean current increased in Mg^{2+} solution. AMP-PNP and AMP-PCP, however, were significantly less potent in the absence of Mg^{2+} (the data illustrated in Fig. 8 were obtained on the same patches).

We also found AMP-PCP and AMP-PNP to be less effective inhibitors in Mg^{2+}-free solutions in a number of preliminary experiments in which we did not control for run-down as described above. In these experiments channel activity was low and single-channel currents were easily resolved. Channel activity in two patches was reduced by 0·1 mM-AMP-PNP or AMP-PCP and subsequently decreased even further when 5 mM-Mg^{2+} was added to the solution. In the presence of 5 mM-Mg^{2+}, 1 mM-AMP-PNP was about as effective an inhibitor as 1 mM-ATP ($n = 3$).

The effect of the myokinase inhibitor A5pA, which inhibits the formation of ATP from ADP, was tested in a few patches. Structurally, this molecule consists of ATP linked to ADP. At 0·5 mM it blocked the channel in both the presence ($n = 2$) and absence of 5 mM-Mg^{2+} ($n = 1$) and in three out of five patches 0·1 mM-A5pA also blocked. There was little difference between the inhibition produced by 0·5 mM-ADP plus 5 mM-Mg^{2+} in either the presence or absence of 0·1 mM-A5pA.

Effects of ADP

Like ATP, ADP is a more potent inhibitor in Mg^{2+}-free solution, indicating that free ADP is an effective channel inhibitor (Fig. 9). At 0·1 mM concentration, free ATP is approximately 20 times more potent than free ADP. Because ADP binds less strongly to Mg^{2+}, free ADP concentrations in solutions containing Mg^{2+} are higher than those of free ATP (~ 32 μM as compared to ~ 8 μM; Table 1) which might account for the fact that in the 2 mM-Mg^{2+} solution ATP is only 5 times more potent than ADP.

We have already reported that ADP at a concentration of 1 mM is able to decrease the ATP sensitivity of the channel in solutions containing Mg^{2+} ions (Kakei *et al.* 1986). Figure 8 shows that this is also true for lower ADP concentrations; the mean current in 0·1 mM-ATP plus 2 mM-Mg^{2+} solution is only 61 ± 11% ($n = 8$) of its value

in the reactivating solution which, in addition to ATP and Mg^{2+}, contains 0·1 mM-ADP. Although ADP will bind to Mg^{2+} and thereby slightly reduce the free ATP level in the control solution (Table 1) this is insufficient to account for the increase in channel activity. Furthermore, GTP is unable to substitute for ADP yet might also be expected to reduce the free ATP concentration (Kakei *et al.* 1986).

DISCUSSION

Effects of Mg^{2+}

The results demonstrate that free Mg^{2+} concentrations greater than 1 mM reduce channel activity. A similar finding has been reported for the insulin-secreting RINm5F cell line (Findlay, 1987*a*) and for ventricular myocytes (Findlay, 1987*b*) although in these cells the channel appears somewhat more sensitive to Mg^{2+}.

Resting levels of free Mg^{2+} in pancreatic β-cells have not been reported but several methods have been used to estimate the cytosolic free Mg^{2+} concentration in other tissues. Nuclear magnetic resonance of the ratio of complexed and free forms of Mg^{2+} ligands in cardiac and skeletal muscle yields values of between 0·6 and 2·5 mM (depending on the value taken for the *in situ* dissociation constant for Mg^{2+}–ATP; Gupta & Moore, 1980; Wu, Pieper, Salhany & Elliot, 1981). Similar values (0·2–1·2 mM) have been also obtained in skeletal muscle using Mg^{2+}-selective microelectrodes (Alvarez-Leefmans, Gamino, Giraldez & Gonzalez-Serratos, 1986). In rat hepatocytes, cytosolic free Mg^{2+}, determined using a null point titration technique, is around 0·4 mM (Corkey, Dusynski, Rich, Matchinsky & Williamson, 1986). Providing that cytosolic Mg^{2+} lies below 1 mM in β-cells, a direct effect of Mg^{2+} on the activity of ATP-sensitive K^+ channels is not indicated.

Although glucose does not affect the total Mg^{2+} content of isolated islets, as measured by atomic absorbtion spectroscopy (Anderson, Berggren, Gylfe & Hellman, 1982), it remains possible that the cytosolic free Mg^{2+} may be altered as a consequence of changes in adenine nucleotide concentrations. Thus we cannot yet discount a modulatory effect of changes in $[Mg^{2+}]_i$ in response to glucose stimulation of the β-cell.

In addition to the inhibition of channel activity we describe here for inward currents, Mg^{2+} has been reported to produce a voltage-dependent block of outward currents through ATP-sensitive K^+ channels (Horie *et al.* 1987; Findlay, 1987*a*, *b*). Magnesium ions also influence channel activity by complexing with ATP. Thus, MgATP maintains channel activity (Findlay & Dunne, 1986; Misler *et al.* 1986; Ohno-Shosaku *et al.* 1987) and, as discussed below, Mg^{2+} reduces the blocking effect of ATP by decreasing free ATP levels.

Inhibition by ATP

The results presented in this paper strongly suggest that free ATP rather than MgATP mediates the inhibitory action of ATP on channel activity. Two pieces of evidence support this idea. First, ATP is effective in the absence of Mg^{2+} ions. Secondly, Mg^{2+} does not alter the relationship between free ATP and channel activity, although it might be expected to shift it to lower concentrations if MgATP were also inhibitory. Conversely a shift of the dose–response curve to higher

concentrations when Mg^{2+} is increased is seen, if channel activity is plotted as a function of total ATP. This is to be expected if free ATP is the main inhibitor since increasing Mg^{2+} reduces free ATP.

An alternative hypothesis we have considered is that the addition of Mg^{2+} to the ATP solution causes the channels to be more fully activated. For example, if channel activity were not maximal in the control solution, higher MgATP concentrations might further increase channel activity. We consider this explanation cannot fully account for our results for the following reasons. (1) In several experiments, exposures of more than 60 s to our reactivating solution did not consistently result in greater activation. (2) The reactivating solution contained 0·1 mM-ATP plus 2 mM-Mg^{2+}. Subsequent exposure to a solution containing a lower ATP concentration (and Mg^{2+}) would therefore not be expected to elicit more activation, yet there is clearly greater inhibition in the absence of Mg^{2+} (see Figs 5 and 6). At ATP concentrations greater than 0·1 mM, the channel is blocked in both the presence and absence of Mg^{2+}. (3) Run-down of channel activity in Mg^{2+}-free solutions is not significant providing the length of the exposures is short (< 30 s, Fig. 6).

Effects of analogues

Several ATP analogues inhibit the channel, the order of potency being ATP = ATP-γ-S > AMP-PNP = AMP-PCP > ADP > AMP. The replacement of an electronegative oxygen by an electropositive carboxyl group means that AMP-PCP is not as fully ionized as ATP at physiological pH ($pK_a = 8{\cdot}5$ as compared to $pK_a = 7$): likewise AMP-PCP is less dissociated at physiological pH ($pK_a = 7{\cdot}8$). Indeed these analogues are often good substrates for enzymes that use $ATPH^{3-}$ (Yount, 1975). It is possible that the lower net charge on these analogues may account for their lower potency, a view which is supported by the strong inhibitory action of the thiophosphate analogue, ATP-γ-S, which is more negatively charged than ATP at physiological pH ($pK_a = 5{\cdot}8$; Neumann, Steinberg & Katchalski, 1965). Furthermore, in one experiment we found that AMP-PNP blocked more effectively at pH 7·6 than at pH 6·8. Since this analogue is more fully dissociated at high pH values, this finding is in agreement with the idea that inhibition is related to the amount of negative charge carried by the ion.

Unlike ATP, the analogues AMP-PNP and AMP-PCP appear to be more potent in the presence of Mg^{2+}. Therefore it appears that the Mg^{2+}-bound complex of these analogues may also interact with the channel and indeed may be more potent.

We considered the possibility that ADP is converted to ATP by myokinases present in the patch membrane. It seems unlikely that the major inhibitory effect of ADP occurs by this route since free ADP exerts a strong inhibition. Furthermore, the myokinase inhibitor, A5pA, tested in Mg^{2+} solutions did not relieve the ADP inhibition. Indeed, this compound blocked channel activity.

Mechanism of nucleotide inhibition

Our results allow us to make some predictions about the site on the channel or associated control proteins at which nucleotides bind to promote channel inhibition. The potency of inhibition appears to depend both on the number of negatively charged groups and on the three-dimensional structure of the nucleotide. We

therefore suggest that the more strongly the nucleotide binds to the inhibitory site the more inhibition is apparent, and that the extent of binding is determined by the amount of negative charge, that is by the number of free oxygens.

Let us consider first the position in the absence of Mg^{2+} ions. We suggest that the reason ATP is more potent than ADP may be because it carries more negative charge – a maximum of four charges (ATP) compared to that of three charges (ADP). Furthermore, in our solutions, ATP is about 75% dissociated, being primarily in the ATP^{4-} and $ATPH^{3-}$ forms, in the approximate ratio of 3:1 (Table 1), whereas ADP is less dissociated. AMP, which bears a maximum of two negative charges is correspondingly even less effective.

The ability of ATP analogues to inhibit channel activity may also be related to the amount of negative charge they carry. At physiological pH values, AMP-PNP and AMP-PCP are not as fully ionized as ATP, being mainly in the H^{3-} form, and they are less effective as inhibitors. Indeed, they are about equipotent with ADP. In the case of AMP-PCP, another important difference is that the bond angle between the β- and γ-phosphates is significantly smaller than that of ATP (Yount, 1975) and this may also contribute to its lower efficacy. The strong inhibitory effect of ATP-γ-S may reflect the close structural similarity of this analogue to ATP and the fact that it is *more* negatively charged than ATP in our solutions (Yount, 1975). The analogues ATP-γ-anilino and ATP-γ-F, are tribasic and correspondingly less potent than ATP. It is possible that the difference between the efficacy of these tribasic analogues and ADP is related to differences in the extent of dissociation of the nucleotides, but we cannot ignore a possible alteration in the three-dimensional structure of the molecule.

Our results suggest that MgATP and MgADP have little inhibitory effect on the channel. This can also be explained as a consequence of a reduction in the effective negative charge carried by the molecule since Mg^{2+} complexes with oxygens carried on the β- and γ-phosphate groups. In addition, Mg^{2+} binding may alter the three-dimensional structure of the ATP molecule in such a way that it can no longer fit into the binding site. Our model does not explain why the Mg^{2+}-forms of AMP-PNP and AMP-PCP are more potent than the free analogues. We can only speculate that Mg^{2+} binding alters the bond angle between the phosphate groups and facilitates binding.

Physiological significance

Our results suggest that in the intact β-cell the ATP-sensitive K^+channel will be modulated by ATP in a complex way, activity being increased by MgATP and decreased by free ATP. The sensitivity of the channel to ATP measured in the inside-out patch ($\sim 10\ \mu M$; Ashcroft, 1988) is greater than that calculated for the intact cell ($\sim 800\ \mu M$; Schmid-Antomarchi, De Weille, Fosset & Lazdunski, 1987). At least part of this difference may be attributable to the presence of intracellular ADP, which is known to reduce the ATP sensitivity of the channel (Kakei *et al.* 1986; Dunne & Petersen, 1986; Misler *et al.* 1986). Since measurements of intracellular ATP indicate the total ATP rather than the free ATP concentration, our results may also help to resolve this difference; free ATP levels are likely to be considerably lower than the total ATP content. It is difficult to quantify this effect, however. First,

because we do not know what the cytosolic free ATP level is nor, more importantly, what it is close to the membrane. Secondly, calculations of free ATP depend critically on the intracellular Mg^{2+} concentration (which is undetermined) and on the value taken for the Mg^{2+}–ATP dissociation constants.

Comparison with other systems

The results presented in this paper contrast with those reported for the ATP-sensitive K^+ channel of rat ventricular myocytes where ATP inhibition is increased by the addition of Mg^{2+} (Findlay, 1988). Thus in cardiac cells it appears that MgATP is a more potent inhibitor than free ATP. Interestingly, the analogues AMP-PNP and AMP-PCP were also more effective on cardiac muscle in the presence of Mg^{2+}, as we describe for the β-cell.

The pharmacological properties of the mast cell ATP^{4-} receptor seem rather different to those of the inhibitory site for free ATP on the ATP-sensitive K^+ channel. At concentrations at which ATP itself was maximally active (10 μM), the mast cell receptor is only activated by the phosphorothioates ATP-γ-S and ATP-β-S, and by those analogues having substitutes on the 2-position of the adenine ring (Cockcroft & Gomperts, 1980; Tatham, Cusack & Gomperts, 1987). By contrast, the β-cell ATP-sensitive K^+ channel is also blocked by several other ATP analogues (Fig. 8), including those having a maximum of three negative charges, by ADP and weakly by AMP and GTP (Ashcroft, 1988). The affinity of the site associated with the channel for free ATP is, however, similar, as the ATP^{4-} receptor of the mast cell has a K_i of 1·2 μM.

ATP also acts on purinergic receptors in vascular and visceral smooth muscle, which may be either excitatory, P_1, or inhibitory, P_2, (Burnstock, 1978). The pharmacological properties of the ATP-sensitive K^+ channel do not resemble either of these receptor subtypes.

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