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Dual effects of diazoxide on ATP-K^+ currents recorded from an insulin-secreting cell line

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1 The effects of diazoxide on ATP-K^+ channel currents, recorded from the insulin-secreting cell line, CRI-G1, were studied using patch-clamp techniques.

2 Under current-clamp recording conditions diazoxide (0.6 mM), inhibited action potential activity and hyperpolarized CRI-G1 cells with a concomitant increase in membrane conductance. Recordings from voltage-clamped whole-cells and isolated patches indicate that activation of ATP-K^+ channel currents underlie these effects.

3 Diazoxide elicited an activation of ATP-K^+ channels which had been partially inhibited by ATP, on application to either surface of the plasma membrane, although it was more effective when applied directly to the cytoplasmic side. Activation of the ATP-K^+ currents involves an increase in the single channel open-state probability and an apparent increase in the number of functional channels.

4 Activation was observed only when Mg-ATP was present in the cytoplasmic bathing solution. There was no activation of currents by diazoxide when ATP, in the absence of Mg^{2+} ions, or Mg-AMP-PNP was present to inhibit the ATP-K^+ channels.

5 In the absence of ATP and Mg^{2+} ions in the cytoplasmic bathing solution, diazoxide (0.6 mM) produced an inhibition of ATP-K^+ currents.

6 Cromakalim (BRL 34915) at 10 μM and 100 μM had no significant effects on ATP-K^+ currents.

7 It is concluded that diazoxide-induced activation of ATP-K^+ channel currents probably involves phosphorylation of the channel or some closely associated membrane protein.

Introduction

The adenosine 5′-triphosphate (ATP)-sensitive potassium (ATP-K^+) channel has been shown to exist in mammalian cardiac muscle, pancreatic β-cells and amphibian skeletal muscle (Ashcroft, 1988) and, more recently, mammalian central neurones (Ashford *et al*., 1987; 1988). In insulin-secreting cells, the ATP-K^+ channel current is a major influence on the resting electrical characteristics of the cell, even though the channels are approximately 99% inhibited (Cook *et al*., 1988a). Although the ATP-K^+ current will act to clamp the potential towards the potassium equilibrium potential (about -80 mV), the resting membrane potential of β-cells in low glucose (3–5 mM) is closer to -60 mV (Croghan *et al*., 1986) and in glucose-free solution is approximately -70 mV (Rorsman & Trube 1985; Ashcroft *et al*., 1988). Hence a counter-balancing depolarizing current must exist, although its identity has not yet been elucidated. Any significant increase or decrease induced in the ATP-K^+ current will therefore cause a hyperpolarization or depolarization respectively with concomitant changes in calcium entry and therefore insulin secretion (i.e. decrease and increase respectively).

Alterations in the concentrations of substrates able to be metabolized (e.g. glucose or leucine) extracellularly will produce such effects (Ashcroft *et al*., 1984; Trube & Hescheler, 1984; Misler *et al*., 1986; Sturgess *et al*., 1988). In addition, the sulphonylureas, tolbutamide and glibenclamide by inhibiting ATP-K^+ channels (Sturgess *et al*., 1985; Ashford *et al*., 1986; Trube *et al*., 1986) induce depolarization and insulin secretion (Sturgess *et al*., 1988), whereas the benzothiadiazine, diazoxide by activating

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ATP-K^+ channels (Trube *et al.*, 1986; Zunkler *et al.*, 1988; Dunne *et al.*, 1987; Sturgess *et al.*, 1988) induces hyperpolarization and inhibition of insulin release (Sturgess *et al.*, 1988).

It is likely that the sulphonylureas act directly on the ATP-K^+ channel or a closely associated protein by virtue of their potent inhibition of channel activity on isolated membrane patches (Sturgess *et al.*, 1985; Ashford *et al.*, 1986; Trube *et al.*, 1986; Sturgess *et al.*, 1988). However, the evidence is less clear with respect to the mechanism by which diazoxide activates ATP-K^+ conductance. Trube *et al.* (1986) demonstrated, using whole-cell recording and isolated patch techniques, that diazoxide activated the ATP-K^+ current and that this effect was related to the concentration of ATP within the cell (high levels of ATP prevented the action of diazoxide), and Sturgess *et al.* (1988) showed that diazoxide only activated ATP-K^+ channels under inhibition by ATP or tolbutamide (but not glibenclamide). Similar results were also obtained by Dunne *et al.* (1987) using the open cell-attached patch configuration. These authors concluded that diazoxide does not act as a 'genuine channel activator', but interferes with the inhibition of the ATP-K^+ channel produced by the ATP^{4-} ion. Recent evidence suggests that it is the ATP^{4-} ion and not Mg-ATP which acts to inhibit the opening of ATP-K^+ channels (Ashcroft & Kakei 1987a; Dunne *et al.*, 1987). We have re-examined whether diazoxide activation of ATP-K^+ channels is dependent upon ATP^{4-} ions or upon the presence of Mg-ATP, and suggest, contrary to the conclusion of Dunne *et al.* (1987), that the Mg-ATP complex is required for diazoxide-induced activation of the ATP-K^+ conductance. Furthermore, we show that diazoxide, in the absence of Mg-ATP, produces an inhibition of the ATP-K^+ current. The effectiveness of another putative K^+-channel activator (Hamilton *et al.*, 1986), cromakalim, was tested. This compound (also known as BRL 34915) is, like diazoxide, a potent antihypertensive agent and there are conflicting results on whether it reduces insulin secretion or not (Wilson *et al.*, 1988; Cook *et al.*, 1988b). In this study we show that cromakalim has no observable effect on the ATP-K^+ current recorded from this insulin-secreting cell line. Some of these results have been communicated to the Physiological Society (Ashford *et al.*, 1989).

Methods

Cell culture

Cells of the rat pancreatic islet cell line (CRI-G1) were cultured and passaged at 2–4 day intervals as previously described (Carrington *et al.*, 1986). Cells used for patch clamp experiments were plated onto 3.5 cm petridishes (Sterilin) at a density of 1.5×10^5 cells per dish. The cells were used 2–4 days (inclusive) after plating.

Electrical recording and analysis

This study employed both the cell-free and whole-cell configurations of the patch clamp recording technique, as described by Hamill *et al.* (1981). Recording electrodes were pulled from borosilicate glass capillaries, and when filled with electrolyte had resistances of 8–12 MΩ for isolated patch experiments, and 3–6 MΩ for whole-cell recording. Single channel events were detected using a Dagan 8900 patch clamp amplifier or an EPC-7 (List Electronics) and were stored on magnetic tape (Racal 4D tape recorder). Records used for illustrative purposes were replayed into a chart recorder (Gould 2200) which filtered the data at 140 Hz. The potential across the membrane is described following the usual sign convention for membrane potential (i.e. inside negative). Outward current (defined as the current flowing from the intra to extracellular side of the membrane) is shown as upward deflections on all traces. The single channel current analysis was determined off line using a programme that incorporated a 50% threshold crossing parameter to detect events (Dempster, 1988) and run on an IBM-AT microcomputer or an Apricot XEN-i286/45. Data segments between 30 and 90 s duration were replayed at the recorded speed and filtered at 1.0 kHz using a 8-pole Bessel filter and digitized at 5.0 kHz using a Data Translation 2801 interface. The open state probability was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time recorded; the number of channels observed in the patch was taken into consideration. To obtain whole cell currents, the cell was voltage clamped at a holding potential of -70 mV, and alternate ± 10 mV pulses of 200 ms duration were elicited at 2 s intervals as described previously (Trube *et al.*, 1986; Sturgess *et al.*, 1988). Drug effects were quantified by measuring the amplitudes of the current responses (I) during drug exposure and comparing them with those observed under control conditions (I_c) immediately preceding drug administration. Because of the transient activation induced by diazoxide, the value for I was taken at the peak of the current. As it was not possible to compensate for rundown and because the inhibitory effects of diazoxide were progressive and did not reach a maximum, the inhibition was quantified by taking a value 2.5 min after diazoxide application.

Solutions

Before use cells were washed thoroughly with solution A which consisted of (mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 10.0, pH 7.4 with NaOH. For whole-cell voltage clamp studies, cells were bathed in solution A and the pipette contained (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, K-EGTA 10.0, HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 20 nM and 0.65 mM respectively (solution B). When 0.1 mM ATP was added to the pipette solution the Mg^{2+} concentration was raised to 1.1 mM to allow for Mg^{2+} chelation by ATP, thus the free Mg^{2+} concentration was maintained at 0.65 mM. In whole-cell experiments where the cell was dialysed with a Mg^{2+}-free solution the pipette contained (mM): KCl 140.0, $CaCl_2$ 4.6, K-EDTA 10.0, HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 20 nM and <3.0 nM respectively (solution C). In some early experiments Ca^{2+} was omitted entirely from this solution and trace amounts chelated with EGTA 1.0 mM or EDTA 1.0 mM. This variation did not affect the results obtained.

In experiments on outside-out patches, the bath solution was A and the pipette solution was B, C or one composed of (mM): KCl 140.0, $MgCl_2$ 1.0, K-EGTA 1.0, ATP 0.1 and HEPES 10.0; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of <0.2 nM and 0.85 mM respectively (solution D). For inside-out patches the pipette contained solution E, composition (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH. The bath contained solution F, composition (mM): KCl 140.0, $CaCl_2$ 0.9, $MgCl_2$ 1.0, K-EGTA 1.0, HEPES 10.0; pH 7.4 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentrations of 0.4 μM and 1 mM respectively. When 0.1 mM ATP was included in solution F the concentration of $MgCl_2$ was raised to 1.1 mM, thus Mg^{2+} was kept constant. Experiments conducted in the absence of Mg^{2+} utilised solution G, composition (mM): KCl 140.0, $CaCl_2$ 0.9, K-EDTA 1.0, HEPES 10.0; pH 7.4 with KOH which resulted in free Mg^{2+} and Ca^{2+} concentrations of <75 nM and 0.1 μM respectively. The concentrations of free ATP and divalent cations were determined by using a programme for calculating metal ion/ligand binding 'METLIG' (England, P. & Denton, R., University of Bristol). The ATP^{4-} concentration was calculated by using a pK_a value of 6.80 (Perrin, 1979).

Solutions containing 0.6 mM diazoxide were prepared freshly before each experiment (from a 30–40 mM stock solution in 0.1 M KOH) and maintained at pH 7.4 due to its long term instability in solution. Changes in pH and potassium concentration were thus compensated for in the control solutions. Cromakalim (BRL 34915) was added from a 25 mM stock solution prepared in 70% ethanol/H_2O v/v. (In control experiments 0.3% ethanol, equivalent to 100 μM BRL 34915, was shown to have no effect on recordings.) K_2-ATP and Li_4-adenylyl- imidodiphosphate (AMP-PNP) were prepared as 20–30 mM stock solutions in H_2O. When AMP-PNP was included in either the pipette or bath solution the appropriate divalent cation adjustments were made as described above. The metal chelating properties of AMP-PNP were assumed to be similar to those of ATP (Yount, 1975).

Drugs were applied to membrane patches or whole-cells by superfusing the bath, by a gravity feed system, at a rate of approximately 0.5 ml s^{-1} which allowed complete solution exchange within 45 s. ATP (K^+ salt, vanadium free) and Li_4-AMP-PNP were obtained from Sigma (Poole, Dorset). Diazoxide was donated by Glaxo Pharmaceuticals (Greenford, England) and cromakalim (BRL 34915) by Beecham Pharmaceuticals (Harlow, Essex, England). All experiments were performed at room temperature, 22–25°C.

All data in text and figures are presented as mean values $\pm$ s.e.mean unless otherwise stated.

Results

On recording from CRI-G1 cells under current-clamp conditions, the initial resting membrane potential was -44.7 ± 1.8 mv ($n = 31$) and subsequently slowly became more negative as an outward K^+ current developed, until eventually it stabilized at a value (only measured occasionally so as not to interrupt the voltage-clamp experiments) of -72.2 ± 0.8 mV ($n = 6$), close to the equilibrium potential for potassium ions. It is generally accepted that this current develops as the cell interior (and hence the ATP) is slowly dialysed with pipette solution, which results in a progressive activation of ATP-K^+ channels. In order to test the action of diazoxide on these cells under current-clamp, 0.1 mM ATP was present in the pipette solution resulting in a much reduced and slower activation of this current. Under these conditions the CRI-G1 cells were occasionally observed to fire action potentials spontaneously. A typical example of such a current-clamp recording is shown in Figure 1a, where hyperpolarizing current pulses (10 pA and one second duration) were given every four seconds in order to assess changes in cellular conductance (an action potential was usually elicited at the termination of these pulses). Application of 0.6 mM diazoxide to the cell resulted in a cessation of action potential activity, and a progressive hyperpolarization of the cell

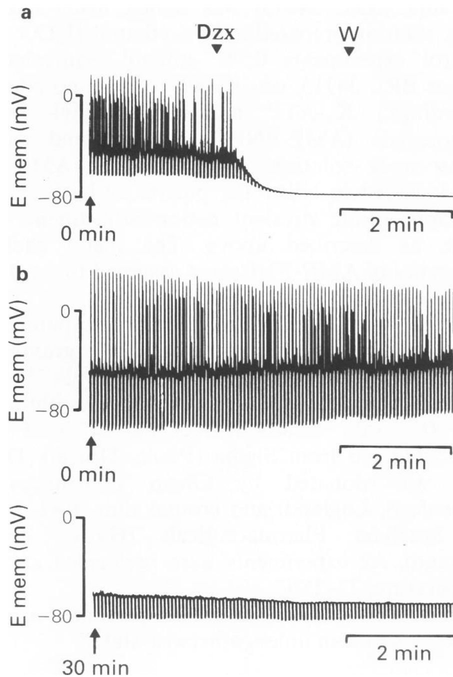

Figure 1 Whole cell current-clamp records from CRI-G1 cells, comparing the time course of hyperpolarization induced by diazoxide (a) with that by cell dialysis (b). Hyperpolarizing current pulses (10 pA and 1 s duration) were applied every 4 s, often resulting in anodal-break action potentials (for the first 5–10 min after formation of the whole cell configuration) although spontaneous action potentials were also evident. (a) The initial resting membrane potential was −41 mV. Bath application of 0.6 mM diazoxide (Dzx) produced an increase in membrane conductance, hyperpolarization (to a value of −74 mV) and inhibition of firing, effects not reversible on wash (W). Compare this with (b) which shows a current-clamp recording from a separate cell, having an initial resting potential of −51 mV. In this experiment no diazoxide was applied and it is apparent from the figure that the time course of increased membrane conductance, hyperpolarization (to a value of −74 mV) and inhibition of firing are much reduced, a steady membrane potential was not attained until 35 min after whole cell formation (lower trace). In both (a) and (b) the extracellular solution was A and the pipette contained solution B and 0.1 mM ATP. The zero minute mark (a) and (b) is equivalent to approximately 30 s elapsed time from the formation of the whole cell to achieving a stable current clamp.

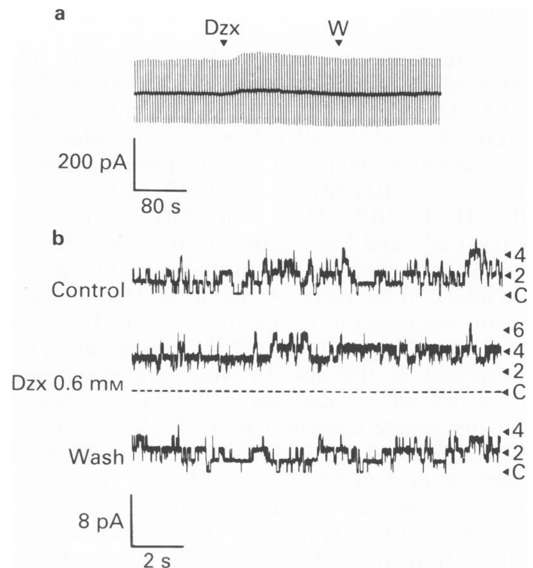

Figure 2 (a) Recording of whole-cell voltage-clamped ATP-K^+ currents. The cell membrane was clamped at −70 mV and the voltage alternately stepped by +10 and −10 mV. The current pulses are denoted by vertical lines. Diazoxide (Dzx, 0.6 mM) induced an increase in current which waned with time of exposure. (b) Activation of ATP-K^+ channels by 0.6 mM diazoxide applied to an outside-out membrane patch, held at a membrane potential of zero mV. Channel openings are denoted by upward deflections (outward currents). The closed state (C) and the number of channels open at a given time are indicated to the right of this and all subsequent isolated patch figures. The values of $N_f \cdot P_o$ were as follows: control 2.208; diazoxide (0.6 mM) 3.359; wash 2.336. In both (a) and (b) the extracellular solution was A and the pipette contained solution B and 0.1 mM ATP.

membrane potential with a concomitant increase in its resting conductance. We found that removal of the diazoxide rarely resulted in any recovery of the resting membrane potential or resting conductance, although, if no diazoxide was applied, at the equivalent time in control experiments (Figure 1b) the cell exhibited only a much slower hyperpolarization and increase in conductance, presumably due to washout of the ATP. In many experiments a transient depolarization preceded the slow hyperpolarization (Figure 1b), an effect not yet understood. The hyperpolarization and increased conductance induced by diazoxide are indicative of activation of a potassium current. This is illustrated more clearly under voltage clamp conditions, where the cell membrane potential is held at −70 mV and alternate ± 10 mV pulses (200 ms duration at 2 s intervals) are given in order to assess the magnitude of the ATP-K^+ current (Trube *et al.*, 1986; Sturgess *et al.*, 1988). As previously shown, the ATP-K^+ current increases slowly as the cell interior is dialyzed, until a peak is reached, thereafter the current declines slowly with time, due to a phenomenon known as channel run-

down (Trube *et al.*, 1986; Sturgess *et al.*, 1988). In the present study the effects of diazoxide were tested on the ATP-K$^+$ currents after the peak had occurred. Figure 2a shows a voltage-clamp recording from a cell where the pipette contained 100 μM ATP. Addition of 0.6 mM diazoxide produced an increase in the ATP-K$^+$ current which slowly declined with time and was not reversed on washing. This increase in K$^+$ current is due to an activation of ATP-K$^+$ channels. This is shown in Figure 2b where diazoxide (0.6 mM) applied to an outside-out membrane patch (with 0.1 mM ATP present in the electrode) produced a reversible increase in the ATP-K$^+$ channel activity. Hence, these data clearly indicate that diazoxide induces an increase in the ATP-K$^+$ current which results in a hyperpolarization of the cell and inhibition of action potential activity. The consequence of these actions would be an inhibition of insulin secretion and this has been observed to occur in these cells (Sturgess *et al.*, 1988).

Diazoxide is also capable of activating ATP-K$^+$ channels when applied to the cytoplasmic membrane surface, as shown in Figure 3a which is a recording from an inside-out membrane patch. Initially, in control conditions (no ATP present) at least four channels were observed to be active. Application of 0.1 mM ATP to the bath (in the presence of 1.0 mM Mg^{2+}) resulted in an inhibition of channel activity such that only two channels could now be discerned. But following the addition of 0.6 mM diazoxide in the presence of 0.1 mM ATP, there was an increase in the ATP-K$^+$ channel activity such that it was again possible to observe the opening of four channels simultaneously. This effect of diazoxide was partially reversed on substitution of the drug with the 0.1 mM ATP solution, although it was noticeable that channel activity did not immediately return to pre-exposure levels (Figure 3a). However, when this experiment was repeated using 0.1 mM ATP in the absence of Mg^{2+} ions (by EDTA chelation and omission of $MgCl_2$), it was found that the inhibition of the channels by 0.1 mM ATP was greater than when Mg^{2+} ions were present and that diazoxide did not produce an activation of channel activity (Figure 3b).

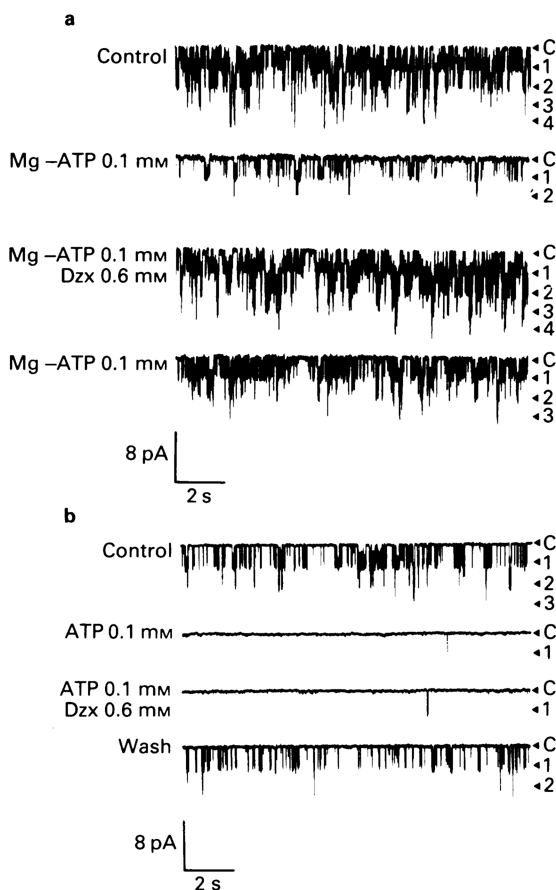

Figure 3 Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of −50 mV. Single channel openings are denoted by downward deflections (inward currents). (a) Activation of ATP-K$^+$ channel currents by diazoxide (Dzx, 0.6 mM) in the presence of Mg-ATP. The values of $N_f \cdot P_0$ were as follows: control 0.988; Mg-ATP 0.145; Mg-ATP and diazoxide 0.903; Mg-ATP 0.619. (b) Lack of activation of ATP-K$^+$ channels by diazoxide in the presence of ATP and no Mg^{2+}. Note the increased potency of the ATP producing channel inhibition compared to (a). The values of $N_f \cdot P_o$ were as follows: control 0.207; ATP 0.0005; ATP and diazoxide 0.001; wash 0.072. The electrode solution was E in both (a) and (b) and the bath solution was F in (a) and G in (b).

Involvement of Mg^{2+} ions versus ATP^{4-} for activation

There are two possible explanations for the results shown in Figure 3, that activation requires the presence of Mg^{2+} ions or that the lack of activation in Figure 3b was simply due to an enhanced level of the species ATP^{4-} producing a greater (and therefore non-reversible) degree of block. Dunne *et al.* (1987) have recently put forward evidence supporting the latter possibility and suggest that diazoxide in some way interferes directly or indirectly with the inhibitory effect of ATP^{4-}. The experiments shown in Figure 3 certainly do not distinguish between the possibilities as in Figure 3a, with Mg^{2+} present, the ATP^{4-} concentration (6 μM) was an order of magnitude lower than that for the patch shown in Figure

Table 1 The effects of diazoxide (0.6 mM) on whole-cell voltage-clamped ATP-K^+ currents

Pipette solution	*I/Ic*	
B + 0.1 mM ATP	1.27 ± 0.05	(7)
C + 100 μM ATP	0.87 ± 0.03	(6)
C + 10 μM ATP	0.84 ± 0.03	(4)
B	0.88 ± 0.05	(4)
C	0.75 ± 0.05	(7)

The results are expressed as the mean relative change in current ± s.e.mean, with the number of cells tested in parentheses.
Solution B contains Mg^{2+} ions whereas solution C is effectively Mg^{2+} free (see Methods).

3b (80 μM). Hence, it was decided to test the actions of diazoxide in conditions where the concentrations of ATP^{4-} in the absence or presence of Mg^{2+} ions were more comparable. This was achieved by reducing the total ATP level to 10 μM in the absence of Mg^{2+} ions resulting in an ATP^{4-} concentration of 8 μM.

However, even at this lowered ATP^{4-} concentration diazoxide did not produce an activation of the ATP-K^+ currents, when tested on whole cell currents (Figure 4a and Table 1) or on inside-out patches (Figure 4b and 6b). Replacement of the 10 μM Mg-free ATP with 0.1 mM Mg-ATP (ATP^{4-} = 6 μM) in the presence of diazoxide in the inside-out patch experiment resulted in an activation (Figure 4b). In addition, even with a lowered total ATP concentration of 50 μM ([ATP^{4-}] = 36 μM in Mg^{2+}-free and 4 μM in the presence of Mg^{2+}) activation only occurred if the Mg-ATP species was present (data not shown). Indeed, it appears that, if anything, diazoxide inhibits the currents under Mg-free conditions (Figure 4a,b and Table 1). Altering the levels of total ATP and the Mg^{2+} concentration with EDTA also has the effect of changing the concentration of calcium ions in the solutions. Thus, because the free calcium concentrations varied so widely in these experiments (from approximately 5×10^{-7} M to 2×10^{-8} M), we decided to determine whether diazoxide produced an activation of the ATP-K^+ current in an essentially calcium-free environment. This experiment was performed using outside-out membrane patches rather than whole cells, in an attempt to obviate possible contributions from intracellular storage/release sites. Figure 4c illustrates that diazoxide (0.6 mM) clearly produces an activation of ATP-K^+ channels in the virtual absence of calcium ions (free calcium $<10^{-9}$ M) but in the presence of Mg-ATP. Thus, from these data, it appears that diazoxide is not capable of activating ATP-K^+ channel currents in the absence of Mg^{2+}

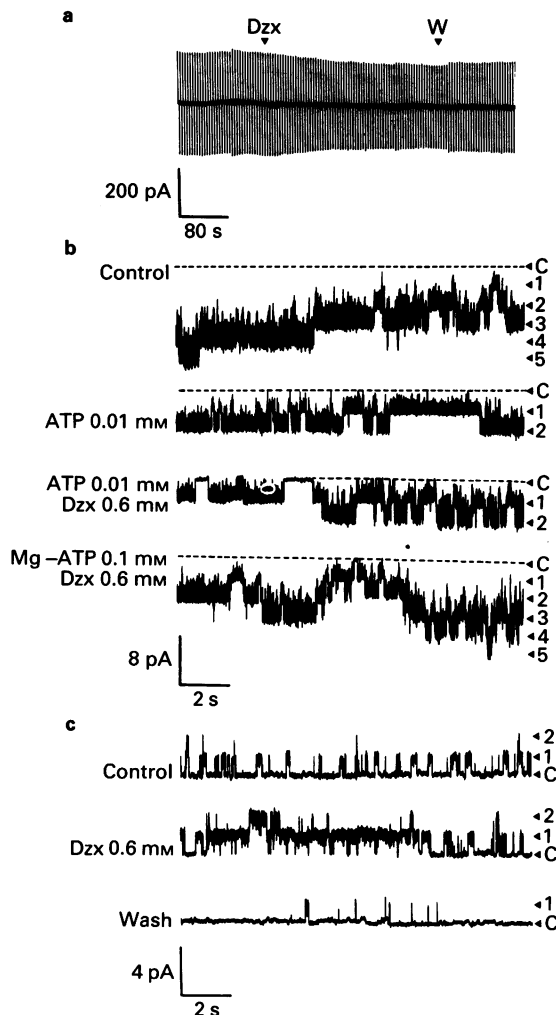

Figure 4 Diazoxide (Dzx) did not activate ATP-K^+ currents with 10 μM ATP and no Mg^{2+} in the 'intracellular' solution, either in (a) voltage-clamped cells where the extracellular solution was A and the solution in the electrode was C plus 10 μM ATP, or (b) an inside-out membrane patch held at a membrane potential of −50 mV (electrode solution was E and bath solution G for 10 μM ATP and F for 0.1 mM Mg^{2+}-ATP). Note that in (b) the ATP-K^+ channels could be activated by diazoxide if the 10 μM ATP was substituted with 0.1 mM Mg-ATP. The values for $N_f \cdot P_o$ in (b) were: control 3.778; ATP 2.202; ATP and diazoxide 1.142; Mg-ATP and diazoxide 1.921. ATP-K^+-channels could be activated by diazoxide in the virtual absence of calcium ions, and this is shown in (c) where an outside-out membrane patch and solution D plus 0.1 mM ATP in the electrode were used. The values for $N_f \cdot P_o$ were: control 0.201; diazoxide 0.394; wash 0.015. The membrane potential was zero mV and the bath solution was A.

ions, regardless of whether whole-cells or isolated patches are used.

The changes in the channel activity induced by ATP and diazoxide could be due to an alteration in the average open-state probability (P_0) of the channels and/or the number of functional channels (N_f). Unfortunately we could not obtain an isolated patch with only one active channel and so performed a binomial analysis of the average current recorded from an inside-out patch in order to try to determine which of these parameters was changing. The result of such an analysis is illustrated in Figure 5 where the probability that a given number of channels are open (P_n) is plotted against the number of channels open (n). Figure 5a shows that the channels open independently of one another (i.e. a good fit is observed to that predicted by the binomial theorem) in an ATP-free solution assuming five channels are active at a measured P_o of 0.196. In the presence of 0.1 mM ATP (Figure 5b), the distribution can now only be well-fitted if it is assumed that two channels are active and at a reduced P_o of 0.072. This is consistent with a reduction in the individual open-state probability and with some of the channels entering a long-lived closed state (an apparent reduction in N_f), as this effect of ATP is reversible on wash (data not shown). Diazoxide (0.6 mM) (in the presence of 0.1 mM ATP) partially reversed this action of ATP (Figure 5c), the distribution being well-fitted by four active channels at a P_o of 0.226. Thus a recovery of the open-state probability and of the number of functional channels is observed.

Therefore in order to present the single channel data quantitatively, and as no change in the single channel amplitude (i) was observed, $N_f \cdot P_o$ was used ($I = N_f \cdot P_o \cdot i$) as the above analysis indicates that both parameters appear to be affected (at least over the time periods used for data analysis). Figure 6 illustrates the combined data from outside-out membrane patches (Figure 6a) for activation of the ATP-K^+ current by diazoxide, and for inside-out membrane patches (Figure 6b) and shows the inhibition induced by 0.1 mM total ATP and 10 μM Mg-free ATP and the effect of diazoxide. It is clear from this figure that there is a marked contrast in the action of diazoxide depending upon the presence of Mg^{2+} ions. This is also evident from the whole-cell voltage-clamp data (Table 1).

Diazoxide and Mg^{2+} inhibit ATP-K^+ currents

The inhibition of the ATP-K^+ current by diazoxide observed in Figure 4 was investigated further in order to determine whether this was also Mg^{2+}- and/or nucleotide-dependent. In the absence of ATP, but with 1 mM Mg^{2+} still present in the solution bathing the cytoplasmic membrane surface, diazo-

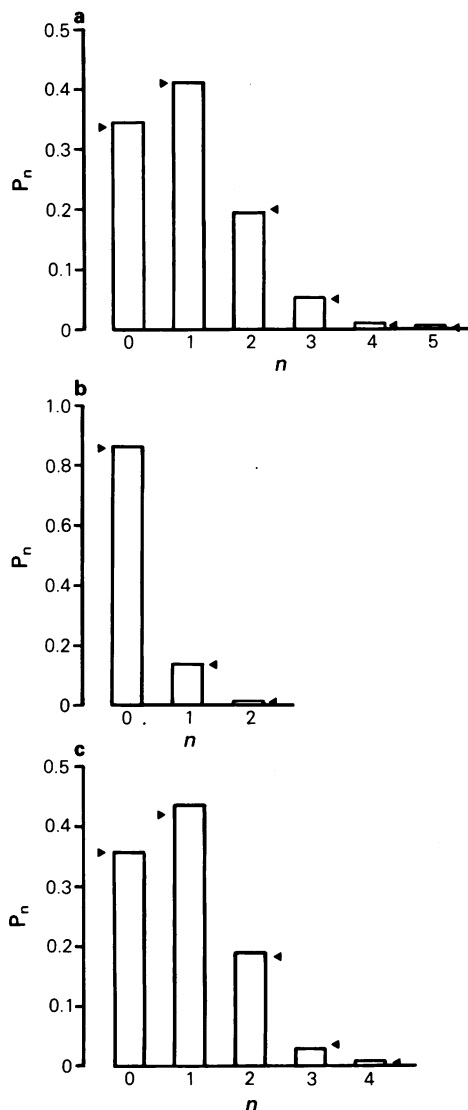

Figure 5 Binomial analysis of single channel data from the inside-out membrane patch shown in Figure 3a. Ordinate scales: probability that a given number of channels, n, are open (P_n). Abscissa scales: the number of channels open, n. The columns show the experimental probabilities obtained using the measured values for each unitary current level. The arrows denote the probabilities predicted by the binomial theorem by use of the measured open-state probability for each channel and the number of channels observed. The experimental probabilities were in (a) ATP-free solution, the arrows indicating the values predicted using a measured P_o = 0.196 and N_f = 5; (b) in a solution containing 0.1 mM Mg-ATP, measured P_o = 0.072 and N_f = 2; (c) in a solution containing 0.1 mM Mg-ATP and 0.6 mM diazoxide, measured P_o = 0.226 and N_f = 4.

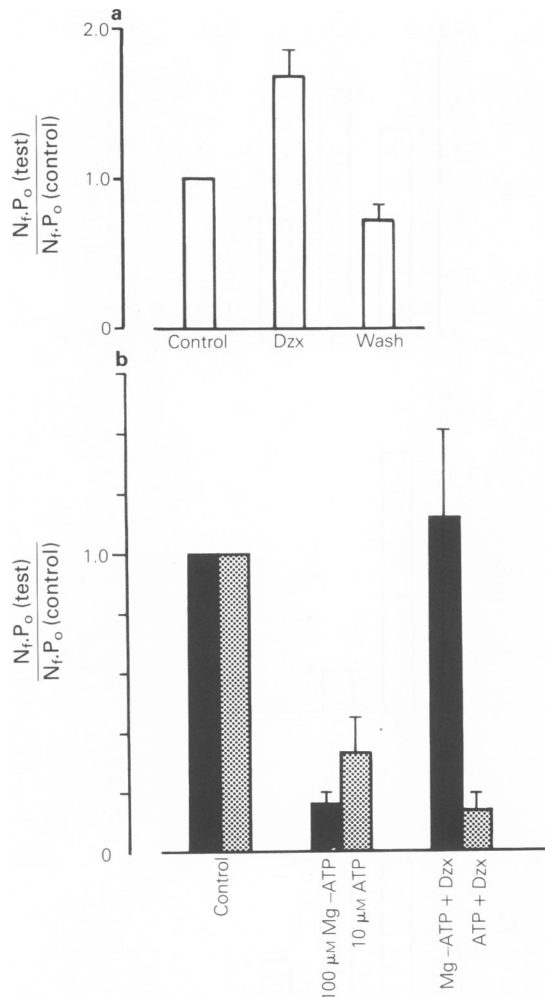

Figure 6 Quantitative effects of diazoxide (Dzx, 0.6 mM) on ATP-K^+ channels recorded from isolated membrane patches. (a) Outside-out membrane patches. The electrode solution was A and the membrane potential was zero mV. The value of $N_f \cdot P_o$ is plotted relative to the control value (in the presence of nucleotide) prior to addition of diazoxide. Values are means of five patches, and vertical lines indicate s.e. (b) Inside-out membrane patches. Electrode solution was E and bath solutions were G for 10 μM ATP and F for 0.1 mM Mg-ATP. The membrane potential was -50 mV. $N_f \cdot P_o$ is plotted relative to control value (i.e. in the absence of nucleotide). Solid columns refer to data from patches exposed to Mg-ATP ($n = 7$) whereas stippled columns refer to those exposed to ATP^{4-} only ($n = 4$). Vertical lines indicate s.e.mean.

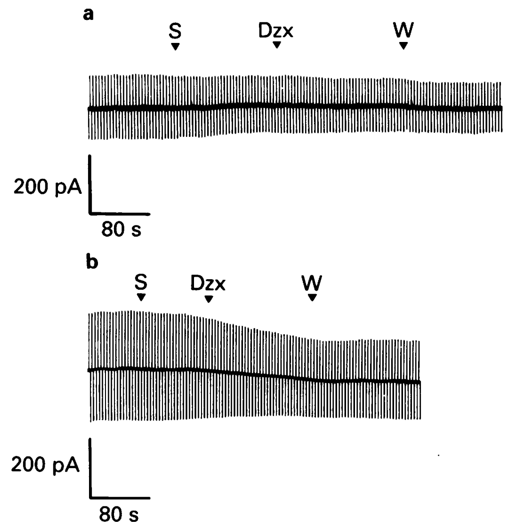

Figure 7 (a) Recording of whole-cell voltage-clamped ATP-K^+ currents (solution B in electrode (i.e. 1 mM Mg^{2+} and no ATP) and solution A in the bath). Diazoxide (Dzx, 0.6 mM) produced little effect on the currents. Inhibition of ATP-K^+ currents by diazoxide was more readily observed in the absence of Mg^{2+} ions and ATP. This is illustrated in (b) using whole-cell voltage-clamp recording (solution C in the electrode and A extracellularly).

xide (0.6 mM) produced little inhibition of the ATP-K^+ current recorded by whole cell clamp (Figure 7a and Table 1). Whereas on outside-out membrane patches the inhibition was seemingly greater (data not shown), the value of $N_f \cdot P_o$ (test)/$N_f \cdot P_o$ (control) being 0.45 ± 0.06 ($n = 5$), although reversibility was not always observed upon washout of the drug, indicating that a substantial run-down of channel activity had occurred during these experiments.

The inhibitory effects of diazoxide were more readily apparent when neither Mg^{2+} nor ATP was present in the 'intracellular' solution. This is shown on whole-cell clamp currents (Figure 7b and Table 1) and was also observed for inside-out patches (data not shown) where $N_f \cdot P_o$ (test)/$N_f \cdot P_o$ (control) was 0.33 ± 0.13 ($n = 4$) in the presence of diazoxide (0.6 mM). Although, again, there was no consistent reversal on removal of the drug indicating that a substantial run-down of channels may have also occurred. The presence of Mg^{2+} ions themselves also produced an inhibition of the ATP-K^+ single channel activity, when present in the solution bathing the cytoplasmic side of the membrane (inside-out patch, and this effect was reversible on wash, $n = 3$). A typical example produced values for

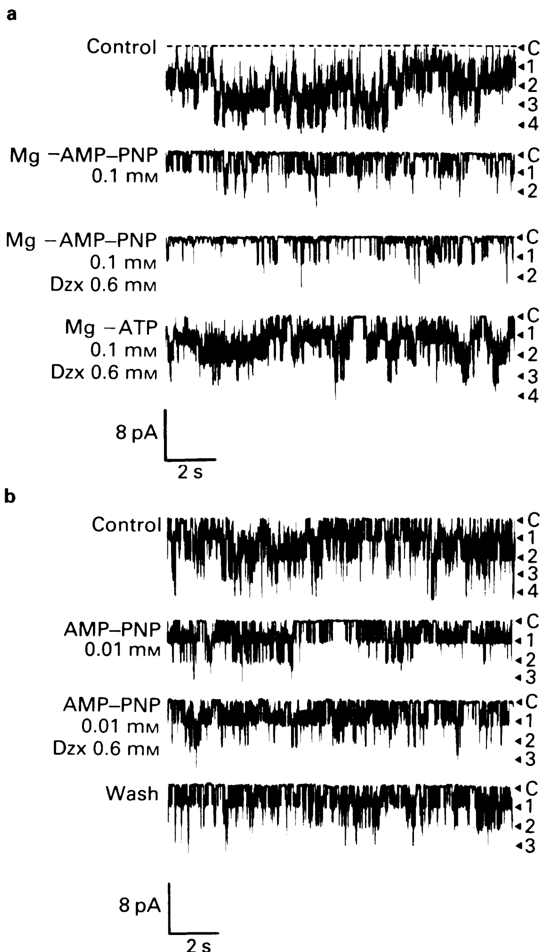

Figure 8 Single channel currents recorded from inside-out membrane patches exposed to symmetrical 140 mM KCl held at a membrane potential of −50 mV. (a) Li_4-adenylyl-imidodiphosphate (AMP-PNP) (0.1 mM) in the presence of Mg^{2+} ions inhibited the activity of ATP-K$^+$ channels. Addition of diazoxide did not activate the channels but caused further inhibition. Replacement of the Mg-AMP-PNP with an equivalent concentration of Mg-ATP in the presence of diazoxide did induce channel activation (solution E in the electrode and F in the bath). The values for $N_f \cdot P_o$ were as follows: control 2.285; Mg-AMP-PNP 0.204; Mg-AMP-PNP and diazoxide 0.109; Mg-ATP and diazoxide 0.503. (b) A similar experiment in which 10 μM AMP-PNP and no Mg^{2+} was used, clearly indicates that this ATP analogue also inhibits the ATP-K$^+$ channel activity and, in agreement with the ATP data (Figures 2, 3 and 5), diazoxide induces further inhibition. This effect was also not readily reversed on wash (solution E in the electrode and G in the bath). The values for $N_f \cdot P_o$ were: control 2.397; AMP-PNP 0.805; AMP-PNP and diazoxide 0.325; wash 0.368.

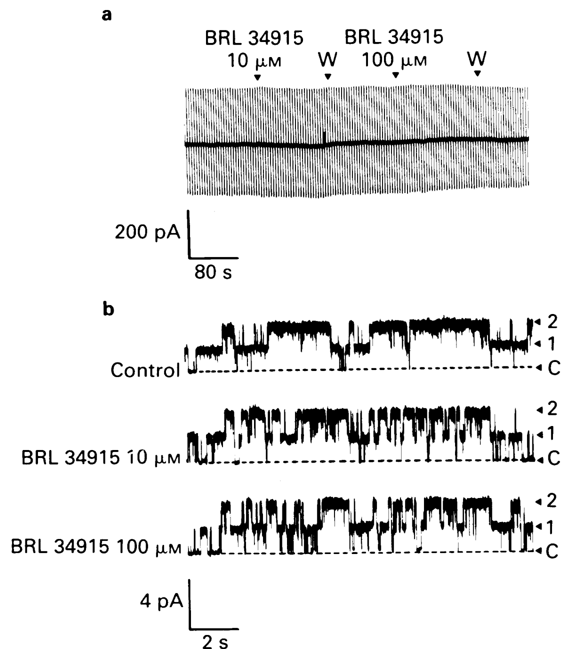

Figure 9 Cromakalim (BRL 34915), tested at concentrations of 10 μM and 100 μM, did not activate ATP-K$^+$ currents with 0.1 mM ATP present in the intracellular solution either in (a) voltage-clamped cells or (b) outside-out membrane patches (held at a membrane potential of zero mV). In both (a) and (b) the electrode solution was B plus 0.1 mM ATP and the extracellular solution was A. The values for $N_f \cdot P_o$ in (b) were: control 1.161; cromakalim 10 μM 0.875; 100 μM 0.885.

$N_f \cdot P_o$ of, control, 0.368; 1.0 mM Mg^{2+}, 0.049; and wash 0.153. Thus, diazoxide can not only activate ATP-K$^+$ currents when Mg-ATP is present, but can also produce a depression of these currents in the absence of this species and this is especially marked if neither ATP^{4-} nor Mg^{2+} is present.

Does activation of the ATP-K$^+$ current require hydrolysis of ATP?

The preceding data therefore show that the diazoxide-induced activation of ATP-K$^+$ channels is dependent upon the presence of Mg-ATP and cannot simply be ascribed to a displacement of ATP^{4-}. This raises the possibility that nuclectide hydrolysis is required for activation, diazoxide perhaps acting to stimulate an endogenous kinase (which would have to be closely associated with cytoplasmic membrane for the effect to be observed in isolated patches). The observation that diazoxide is more effective in producing activation when applied to the cytoplasmic side of isolated patches (Figure 6) supports this possibility.

Thus the non-hydrolysable analogue, AMP-PNP was substituted for ATP and the effect of diazoxide tested on inside-out membrane patches. This analogue inhibited the ATP-K^+ channels in a manner similar to ATP. Addition of diazoxide (0.6 mM) in the presence of either Mg-AMP-PNP or Mg-free AMP-PNP induced only a further inhibition of channel activity (Figure 8a,b). On replacement of the 100 μM Mg-AMP-PNP with 100 μM Mg-ATP, diazoxide was shown to induce ATP-K^+ channel activation (Figure 8a). Thus, these data suggest that magnesium-dependent hydrolysis of ATP may well be a prerequisite for diazoxide-induced ATP-K^+ channel activation.

Cromakalim does not affect the ATP-K^+ current

The putative K^+ channel activator cromakalim (BRL 34915) was also tested for its actions on the ATP-K^+ currents. This compound was chosen for comparison with diazoxide because certain of its vasorelaxant effects (Wilson *et al.*, 1988) can be inhibited by glibenclamide (a potent and, as far as has been shown, selective ATP-K^+ current inhibitor) and there are conflicting results regarding its effect on insulin and glucose levels *in vivo* (Wilson *et al.*, 1988; Cook *et al.*, 1988b). Application of cromakalim at concentrations of 10 μM and 100 μM to ATP-K^+ whole-cell channel currents, or to single channel currents recorded from outside-out membrane patches, was totally ineffective at inducing either an activation or a significant inhibition with 0.1 mM ATP present in the 'intracellular' environment (Figure 9a,b; Table 2). Similar data were obtained in the absence of Mg^{2+} and/or ATP in the electrode solutions (data not shown).

Discussion

Diazoxide, a sulphonamide derivative which is closely related to the hypoglycaemic sulphonylureas, produces hyperglycaemia in mammals. This increase

Table 2 The lack of effect of cromakalim (BRL 34915) on ATP-K^+ currents

[Cromakalim] (μM)	*Whole-cell* *I/Ic*	*Outside-out* $N_f \cdot P_o$ *(test)* / $N_f \cdot P_o$ *(control)*
10	0.99 ± 0.02 (6)	0.74 ± 0.13 (5)
100	0.90 ± 0.03 (6)	0.77 ± 0.26 (5)

Results are expressed as the mean relative change in current or $N_f \cdot P_o \pm$ s.e.mean. The number of cells/patches used are shown in parentheses. The electrode solution for both whole-cell and outside-out voltage clamp configurations was B plus 0.1 mM ATP, the extracellular solution was A.

in blood glucose results mainly from its action to suppress insulin release, although stimulation of catecholamine release and hepatic glycogenolysis contribute to its hyperglycaemic activity (Bowman & Rand, 1980). Recent studies have indicated that diazoxide reduces insulin release from freshly isolated β-cells and insulin-secreting cell lines by activating ATP-K^+ channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988; Zunkler *et al.*, 1988). It is argued that activation of this channel hyperpolarizes the cell membrane, thus making it less likely that the cell will fire action potentials in response to stimuli (e.g. nutrients, transmitters or hormones) and so reduce calcium entry and hence insulin secretion. We have shown in this study, using an insulin-secreting cell line, that under current-clamp whole-cell recording conditions, application of diazoxide causes the cessation of action potential activity and this was associated with an increase in resting membrane conductance and hyperpolarization. These effects were due to activation of a potassium current and, in agreement with the previous studies, this was shown to be an ATP-K^+ current.

Diazoxide activates the ATP-K^+ current when applied to either side of the membrane, apparently being more effective when applied directly to the cytoplasmic side. This is evident on comparing data from inside-out membrane patches (Figure 5b) to that of outside-out patches (Figure 5a) or voltage-clamp whole-cell recordings (Table 1). Diazoxide produced approximately a 7 fold increase in current in inside-out patches compared to less than a 2 fold increase when applied at the extracellular membrane (outside-out and whole-cell recording). Diazoxide also activates ATP-K^+ channels in cell-attached patches when applied to the bath solution (Trube *et al.*, 1986; Sturgess *et al.*, 1988). As there is no direct route to the channels due to the diffusion barrier created by the giga-ohm seal (Sakmann & Neher, 1984), diazoxide must partition into the membrane before diffusing to its site of action. Thus, the receptor site for diazoxide appears to be accessed more easily from the intracellular side of the membrane.

In previous studies, diazoxide-induced ATP-K^+ channel activation has been shown to depend upon the presence and concentration of ATP in the intracellular environment (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988). The ATP-K^+.channels were not activated in the absence of ATP or if high concentrations of ATP were present. Dunne *et al.* (1987) have suggested a mechanism for the action of diazoxide, namely that the drug interferes in some way with the inhibitory effect of ATP^{4-}, the purported active species of ATP (Ashcroft & Kakei 1987a; Dunne *et al.*, 1987). However, the data presented here do not concur with this suggestion. On changing the total ATP levels and maintaining the

ATP^{4-} concentration relatively constant, we showed that ATP-K^+ channel activation only occurs if the magnesium-ATP species is present, otherwise diazoxide has no effect or produces an inhibition (see below). These effects were observed regardless of whether isolated patches or voltage-clamped whole-cell recordings were used. Thus there is a requirement for the presence of magnesium ions for diazoxide to evoke an activation of ATP-K^+ channel activity. In addition, activation could not be elicited when the ATP was replaced by AMP-PNP either in the presence or absence of magnesium ions. These data indicate that a phosphorylation process may well underlie diazoxide-induced activation. This is different from nucleotide-induced inhibition of channel activity which can be mimicked by non-hydrolysable analogues and probably involves simple nucleotide binding not linked to a metabolic process (Cook & Hales, 1984; Ashcroft, 1988). It has been shown that, for isolated membrane patches, in order to maintain ATP-K^+ channel activity Mg-ATP must be present (Findlay & Dunne, 1986; Misler *et al.*, 1986; Ohno-Shozaku *et al.*, 1987) and that non-hydrolysable analogues are unable to mimic this effect. This suggests that phosphorylation of the ATP-K^+ channel may be required to maintain the channel in an excitable state. If this is the case then the protein kinase responsible must be closely associated with the cytoplasmic membrane for the activation to occur in isolated patches. Diazoxide may elicit activation by stimulating this kinase to increase the activity of the channels in a magnesium-dependent but calcium-independent manner.

The binomial analysis of the single channel data obtained from inside-out membrane patches indicate that diazoxide partially reverses the effects of ATP on the channels. Addition of ATP to inside-out membrane patches produced a reduction in the open state probability and an apparent decrease in the number of functional channels. It is likely that this effect of ATP is due to some channels entering a very long-lived closed state over the time period of our recordings, which manifests itself as an apparent reduction in the number of functional channels. This has also been observed for frog skeletal muscle ATP-K^+ channels (Spruce *et al.*, 1987). Diazoxide seemingly reverses this effect of ATP such that there is an increase both in the number of channels active and in the individual open state probability.

In the absence of Mg-ATP diazoxide was found to produce an inhibition of the ATP-K^+ channel currents and this was especially marked if neither ATP nor magnesium ions was present at the cytoplasmic aspect of the membrane. Thus it is possible that the activation produced by diazoxide may be somewhat underestimated or masked by inhibition. The inhibitory effect of diazoxide is similar to the effect of tolbutamide (a sulphonylurea), although much less potent. However, one difference from tolbutamide block is the lack of reversal of the diazoxide inhibition. This inhibition appears to be progressive (Figure 7b for example) and does not reach steady-state. On wash-out of diazoxide the current level remains relatively constant (but will decline slowly due to run-down). Hence, one explanation for this action of diazoxide is that it enhances the rate of channel run-down, removal of the drug allowing the previous run-down rate to be re-established. The inhibition of the ATP-K^+ channel by diazoxide may also be magnesium-dependent, as in the presence of magnesium ions this action was less noticeable. In agreement with other investigators, we also found that magnesium ions themselves decreased the single-channel activity when applied to the intracellular environment. This has been demonstrated for ATP-K^+ channels, in β-cells (Findlay, 1987a; Ashcroft & Kakei, 1987b) and cardiac muscle (Findlay, 1987b).

Cromakalim (BRL 34915) was found to have no significant effect on ATP-K^+ channels, producing neither an activation nor an inhibition. This agent is thought to produce its anti-hypertensive action by acting as a potassium channel activator. At present it is not clear which potassium channel this drug acts upon. However, from the data presented here it is unlikely that diazoxide and cromakalim act by a similar mechanism upon ATP-sensitive potassium channels in insulin-secreting cells. These data support the conclusions of Wilson *et al.* (1988) that cromakalim had no effect on plasma insulin levels.

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