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Nucleotide-dependent activation of K_{ATP} channels by diazoxide in CRI-G1 insulin-secreting cells

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1 Patch-clamp recording techniques were used, to examine the effects of diazoxide on K_{ATP} currents in CRI-G1 insulin-secreting cells in the presence of non-hydrolysable nucleotides.

2 In the presence of non- or slowly-hydrolyzed ATP analogues, bathing the intracellular aspect of cell-free membrane patches diazoxide inhibited K_{ATP} channel activity.

3 Under whole-cell recording conditions, with various non-hydrolysable nucleotides present intracellularly (after dialysis), diazoxide induced K_{ATP} current activation. The largest activation occurred with Mg-adenylyl-(β,γ-methylene) diphosphate (Mg-AMP-PCP) present in the dialysing solution. This activation was diazoxide- and nucleotide-concentration-dependent.

4 In the absence of Mg^{2+}, or in the presence of manganese (Mn^{2+}) ions intracellularly, diazoxide did not induce K_{ATP} current activation, regardless of the species of nucleotide present in the pipette.

5 Intracellularly applied trypsin prevented the activation of K_{ATP} currents by diazoxide in the presence of Mg-AMP-PCP, an effect reversed by co-application of intracellular polymethylsulphonyl fluoride with the trypsin.

6 The application, by dialysis, of a CRI-G1 cell lysate, with negligible Mg-ATP, resulted in a substantial activation of the K_{ATP} current by diazoxide.

7 It is concluded that diazoxide can activate K_{ATP} channel currents by two separate pathways, one requiring a phosphorylation process, the other the presence of an intracellular protein coupled with a Mg-purine nucleotide.

Keywords: Diazoxide; K_{ATP} channels; non-hydrolysable nucleotides; CRI-G1 cells; insulin-secretion

Introduction

Adenosine-5′-triphosphate (ATP)-sensitive (K_{ATP}) channels have been described in a variety of tissues including cardiac muscle, skeletal muscle, vascular and arterial smooth muscle, central neurones, pancreatic β-cells and a range of insulin-secreting cell types (see reviews by Ashcroft, 1988; Ashcroft & Ashcroft, 1990; Ashcroft & Rorsman, 1991). Due to the involvement of these channels in the control of cell excitability, their pharmacological modulation has considerable therapeutic potential. For example, the sulphonylureas, tolbutamide and glibenclamide, are useful in the treatment of Type II diabetes since they mimic the effect of glucose by inhibiting K_{ATP} channel opening, albeit by a different mechanism (Sturgess *et al.*, 1985; Ashford, 1990). Closure of these channels induces β-cell depolarization and triggers Ca^{2+} entry through voltage-sensitive Ca^{2+} channels, an effect which ultimately results in insulin secretion (Ashcroft & Rorsman, 1991). In contrast, the sulphonamide diazoxide hyperpolarizes β-cells (Henquin & Meissner, 1982) thereby inhibiting the secretion of insulin and inducing hyperglycemia. This action is believed to be due to the activation of K_{ATP} channels (Trube *et al.*, 1986; Kozlowski *et al.*, 1989).

Initial studies with mouse pancreatic β-cells (Trube *et al.*, 1986) indicated that the presence of ATP was required at the intracellular surface of cell-free patches before diazoxide could activate K_{ATP} channels. Sturgess *et al.* (1988) reported a similar finding with CRI-G1 insulin-secreting cells and Dunne *et al.* (1987), using RINm5F cells, suggested that diazoxide acts by displacing the ATP^{4-} molecule from its binding site thereby reversing the K_{ATP} channel inhibition induced by the nucleotide. However, using isolated inside-out membrane patches from CRI-G1 insulin-secreting cells Kozlowski *et al.* (1989) demonstrated that activation was observed only when Mg-ATP was present in the cytoplasmic bathing solution. There was no activation when ATP, in the absence of Mg^{2+} ions (i.e. ATP^{4-}), or the non-hydrolysable analogue, Mg-adenylylimidodiphosphate (Mg-AMP-PNP) was present to inhibit the K_{ATP} channels. It was therefore concluded that diazoxide-induced activation of K_{ATP} currents involves phosphorylation of the channel or some closely associated membrane protein (Kozlowski *et al.*, 1989). This hypothesis was also found to be applicable to the K_{ATP} channel-activating action of diazoxide observed in RINm5F cells (Dunne, 1989).

However, following additional experiments designed to investigate the nature of the kinase responsible for this putative phosphorylation, it became apparent that under whole-cell recording conditions, diazoxide also activates K_{ATP} channel currents in the presence of non-hydrolysable ATP analogues. Thus we examined further the interactions of diazoxide and Mg-nucleotide complexes on both single channel and whole-cell K_{ATP} currents recorded from CRI-G1 cells.

Methods

Cell culture

Cells of the rat pancreatic islet cell-line (CRI-G1) were cultured and passaged at 3 to 7 day intervals as previously described (Carrington *et al.*, 1986). Cells used for patch-clamp experimentation were plated onto 3.5 cm petri dishes (Sterilin) at a density of approximately 1.5×10^5 cells per dish. Cells were used between 2 and 4 days after plating.

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Electrical recording and analysis

Cell-free and whole-cell recordings of K_{ATP} channel currents were obtained by standard procedures (Hamill *et al.*, 1981). Recording electrodes were pulled from borosilicate glass capillaries and, when filled with electrolyte, had resistances of 8 to 12MΩ for isolated patch experiments and 3 to 5 MΩ for whole-cell recordings. Ionic currents (single channel and whole-cell) were detected with an EPC-7 patch-clamp amplifier (List Electronics) and were recorded onto magnetic tape with a bandwidth (3db down) of 1250 Hz (Racal 4D tape recorder) for off-line analysis at a later date. Records used for illustrative purposes were replaced into a chart recorder (Gould 2200) which filtered the data at ~140 Hz. The potential across the membrane patch is described following the usual sign convention for membrane potential (i.e., inside negative). Outward current (defined as current flowing from the inside to the extracellular side of the membrane) is shown as upward deflections in all traces. Single channel current analysis was determined off-line by use of a programme that incorporates a 50% threshold crossing parameter to detect events (Dempster, 1988) and run on an Apricot XEN-Xi 286/45 microcomputer. Data segments between 30 and 60s were replayed at the recorded speed and filtered at 1.0 kHz (− 3bd; 8-pole Bessel) and digitally sampled at 5.0 kHz with a Data Translation 2801A interface The average channel activity ($N_f.P_o$) where N_f is the number of functional channels in the patch and P_o is 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 (Kozlowski *et al.*, 1989; Kozlowski & Ashford, 1990). Changes in $N_f.P_o$ as a result of drug effects are expressed as a percentage of control.

To obtain whole-cell K_{ATP} currents, the cell was voltage-clamped at a holding potential of − 70mV and alternate ± 10mV pulses of 200 ms duration were applied every 2 s (Trube *et al.*, 1986; Kozlowski *et al.*, 1989; Kozlowski & Ashford, 1990). The effects of diazoxide were quantified by measuring the amplitude of the current response during exposure to the drug (I_D) and comparing those recorded under control conditions (I_C) immediately preceding drug application. Because the effect of diazoxide was transient the value for I_D was taken at the peak of the response. Due to the presence of current run-down the inhibitory effects of diazoxide were quantified by taking a value 2.5 min after diazoxide application (Kozlowski *et al.*, 1989).

Solutions

Before use, cells were washed with normal bath saline which contained (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, N-2-hydroxyethypiperazine-N'-2-ethane-sulphonic acid (HEPES) 10; pH 7.4 with NaOH (solution A) and this solution was also used to bathe the cells during whole-cell current and outside-out single channel current recordings. For both these configurations the recording pipette contained solution B which consisted of (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 2, K^+-ethyleneglycol-*bis* (β-aminoethyl-ether)-N,N'-tetraacetic acid (KEGTA) 10, HEPES 10; pH 7.2 with KOH resulting in a free calcium and magnesium concentration of ~20 nM and 0.65 mM, respectively. In the presence of nucleotides in the pipette, the concentration of Mg^{2+} was increased to compensate for chelation by the nucleotides, thereby maintaining the free Mg^{2+} concentration at 0.65 mM. Thus for triphosphate nucleotides at concentrations of 0.1, 0.3 and 1.0 mM the concentration of $MgCl_2$ was increased to 1.1, 1.3 and 1.8 mM, respectively. Similarly when 0.1 mM tripolyphosphate or adenosine tetraphosphate was added to solution B the concentration of Mg^{2+} was increased to 1.1 mM, and for 0.1 mM adenosine 5'-diphosphate (ADP), it was raised to 1.05 mM. It was assumed that the affinities of Mg^{2+} for adenylyl-(β,γ-methylene) diphosphate (AMP-PCP), adenosine 5'-o-[3-thioitriphosphate] (ATPγS), guanosine 5'-o-[3-thioitriphosphate] (GTPγS), cytosine 5'-triphosphate (CTP), AMP-PNP and ATP were identical (Yount, 1975). No adjustment of Mg^{2+} concentration was made when adenosine 5'-monophosphate (AMP) or adenosine was added to this solution. When examining the effects of diazoxide in the presence of manganese (Mn^{2+}) ions and AMP-PCP (0.1 mM) intracellularly the 1.1 mM $MgCl_2$ present in solution B was replaced by 1.1 mM $MnCl_2$. In some experiments solution B was replaced with solution C consisting of (mM): KCl 140, $CaCl_2$ 4.6, KEGTA 10 and HEPES 10; pH 7.2 with KOH which resulted in free Ca^{2+} and Mg^{2+} concentration of ~25 nM and < 5.0 nM respectively.

For inside-out patches the extracellular surface was bathed in solution D which contained (in mM): KCl 140, $MgCl_2$ 1, $CaCl_2$ 1 and HEPES 10; pH 7.4 with KOH, whilst the intracellular surface was bathed in solution E, composition (mM): KCl 140, $CaCl_2$ 0.9, $MgCl_2$ 1.0, KEGTA 1.0 and HEPES 10.0; pH 7.4 with KOH which resulted in free Mg^{2+} and Ca^{2+} concentrations of 1.0 mM and < 0.4 μM, respectively. In some experiments triphosphate nucleotides at a concentration of 0.1 mM were present in solution E, and therefore the concentration of Mg^{2+} was increased to 1.1 mM to maintain the free Mg^{2+} concentration of 1.0 mM. The concentration of free divalent cations was determined by use of 'METLIG' a programme for calculating metal ion/ligand binding (England, P. & Denton, R., University of Bristol).

Solutions containing diazoxide were prepared freshly before each experiment (from a 30 to 40 mM stock solution in 0.1 M KOH). Changes in pH due to addition of diazoxide and its vehicle were compensated for in all solutions. All nucleotides were prepared as 20 and 30 mM stock solutions in water. Drugs were applied to cell-free patches or whole-cells by exchanging the bath solution by a gravity feed system, at a rate of approximately 0.5 ml s^{-1}. This allowed complete solution exchange within 45 s (cells were not continuously superfused by the bathing solution). ATP (K^+ salt), AMP-PNP (Li^+ salt), ADP (K^+ salt), AMP (Na^+ salt), adenosine, sodium tripolyphosphate and adenosine tetraphosphate (Na^+ salt), GTPγS (Li^+ salt), CTP (Na^+ salt) and polymethylsulphonylfluoride (PMSF) were obtained from Sigma (Poole, Dorset). ATPγS (Li^+ salt) and AMP-PCP (Li^+ salt) were obtained from Boehringer (Mannheim, Germany). Diazoxide was donated by Glaxo Pharmaceuticals (Greenford, England). All experiments were conducted at room temperature (22–25°C).

All data in text, tables and figures are presented as mean values ± s.e.mean unless otherwise stated. The statistical significance between experimental groups was assessed by Student's *t* test for unpaired data.

Preparation of cell lysate

CRI-G1 cells were grown in 50 ml tissue culture flasks (Nunclon Ltd.), until 80% confluent. Cells were then taken up in 10 ml of solution containing trypsin (1 mg ml^{-1}), NaCl (137 mM), KCl (5 mM), NaH_2PO_4 (1.1 mM), KH_2PO_4 (1.1 mM), glucose (6 mM) and phenol red (0.001%); pH 7.4. Subsequently, 10 ml of culture medium (Dulbecco's modified Eagle's medium; Gibco) containing 10% foetal calf serum (Gibco), glucose (10 mM), penicillin (50 mg ml^{-1}; Gibco) and streptomycin (50 mg ml^{-1}; Gibco) was added. Following centrifugation at 185 *g* for 5 min the supernatant was discarded and the pellet re-suspended in 10 ml of culture medium and the cells counted. This centrifugation procedure was repeated and the pellet washed in 20 ml of solution A and centrifuged for a third time. The resultant pellet was re-suspended in 1 to 1.5 ml of distilled water and allowed to lyse on ice for 15 min. The lysate was centrifuged at 2950 *g* for 5 min, the pellet discarded and an equal volume of solution B (2 × concentrated) added to the supernatant. On occasion 0.2 mM ATP was added to solution B (2 × concentrated) which under these conditions contained 2.2 mM $MgCl_2$. Thus, the free Mg^{2+} concentration in the diluted lysate was main-

tained at 0.65 mM. Prior to filling of electrodes the diluted lysate was passed through a 0.45 μm syringe filter (Millex-GA). This electrode-filling solution contained lysate from a mean of 5.3 ± 0.07 million cells ml^{-1} ($n = 5$) and had a protein concentration of 0.12 to 0.44 mg ml^{-1} determined according to the method of Bradford (1976) using a protein assay kit (Bio-Rad Laboratories GmbH). The ATP content within the cell lysate was assayed with a luciferase bioluminescence assay kit (Boehringer Mannheim Ltd.), based on the method described by Deluca (1969). Luminescence from the samples was recorded with a luminometer (Canberra Packard, Pico-lite). In the experiments where exogenous ATP at a final concentration of 0.1 mM had been added to the lysate (see above), the measured ATP concentration ranged from 0.4 to 15 μM, presumably as a result of its rapid breakdown by endogenous ATPases present within the lysate.

Results

Single K_{ATP} channel studies

In our previous study, using inside-out membrane patches isolated from CRI-G1 cells, we demonstrated that suppression of K_{ATP} single channel current activity with Mg-AMP-PNP or Mg-free ATP resulted in the inability of diazoxide to induce activation (Kozlowski *et al.*, 1989). We have extended these observations to include other nucleotides and have shown that, in agreement with our earlier study, diazoxide is unable to activate K_{ATP} channel currents recorded from isolated membrane patches in the presence of Mg^{2+} complexes of other non-hydrolysable adenine nucleotides. For example, application of diazoxide (0.6 mM) to the extracellular aspect of outside-out patches was unable to elicit activation of K_{ATP} channel currents in the presence of 0.1 mM Mg-AMP-PCP in the electrode solution (Figure 1a). In fact under these conditions, diazoxide induced a $74.7 \pm 16.5\%$ ($n = 4$) reduction in K_{ATP} channel activity relative to control. Upon washout of the drug, little or no reversal was observed. A similar decrease ($75.6 \pm 9.0\%$; $n = 4$) in K_{ATP} channel activity by extracellularly applied diazoxide (0.6 mM) was observed when 0.1 mM Mg-ATPγS was present in the pipette solution. The application of diazoxide (0.6 mM) to the intracellular membrane surface of inside-out patches, where it is more effective in causing K_{ATP} channel activation (Kozlowski *et al.*, 1989), was also unable to reverse near-maximal inhibition or submaximal inhibition induced by 0.1 mM or 0.01 mM Mg-ATPγS, respectively (Figure 1b,c). For example, the application of Mg-ATPγS (0.01 mM) reduced K_{ATP} channel activity by $83.9 \pm 4.5\%$ ($n = 4$) with a slight decrease ($82.6 \pm 9.7\%$; $n = 4$) being observed in the presence of diazoxide.

Whole-cell K_{ATP} currents

On repeating these experiments with non-hydrolysable analogues on whole-cell K_{ATP} currents a completely different result was obtained. The effects of diazoxide (0.6 mM) were examined on whole-cell K_{ATP} currents using a range of nucleotides and related compounds added to solution B at the concentration (0.1 mM) used in cell-free patch experiments. Diazoxide was tested on these currents only after the peak whole-cell K_{ATP} current had been reached (Trube *et al.*, 1986; Kozlowski *et al.*, 1989). In direct contrast to the isolated patch data, application of diazoxide produced a marked activation of whole-cell K_{ATP} currents when Mg-AMP-PNP dialysed the cell interior (Figure 2a). This effect declined rapidly with time in the continued presence of diazoxide and its time course was unaffected by washout of the drug. Subsequent re-application of diazoxide (at least 4 min after washout of the first application of the drug, throughout) resulted in a significant inhibition of the whole-

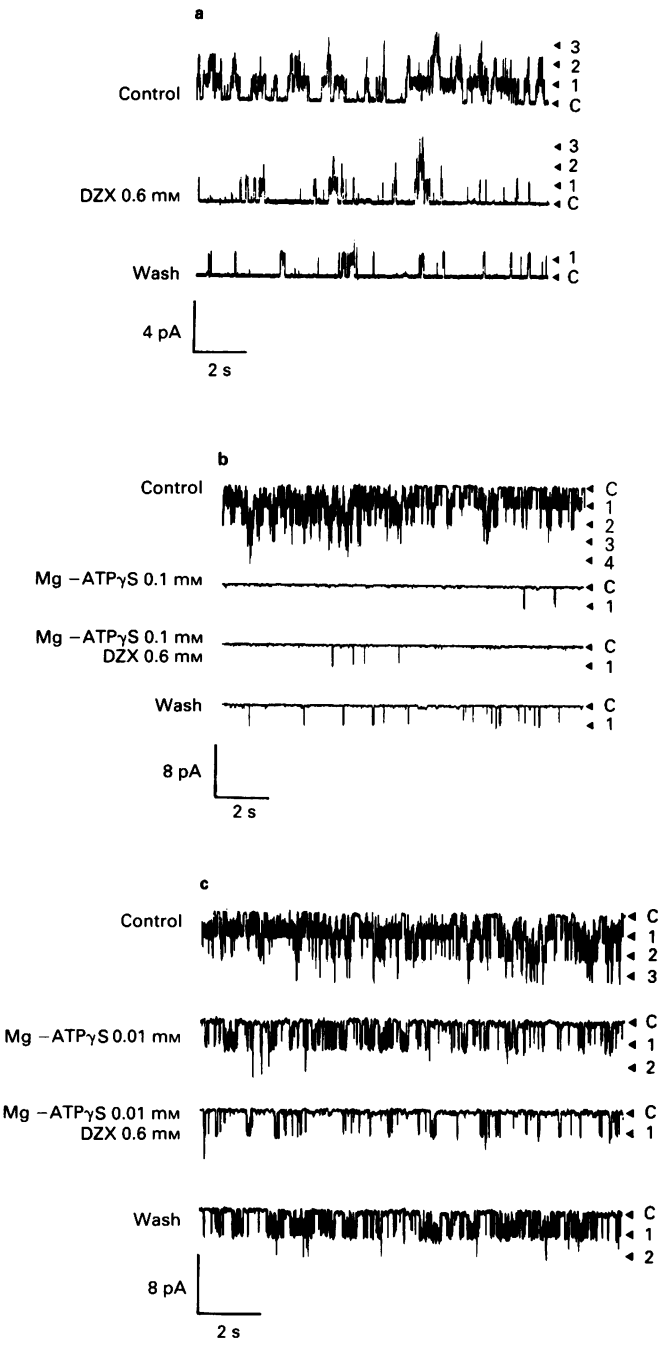

Figure 1 (a) Single channel currents recorded from an outside-out membrane patch exposed to a physiological cation gradient and voltage-clamped at a membrane potential of 0 mV. The bath contained solution A whilst the pipette contained solution B to which 0.1 mM Mg-AMP-PCP had been added (Control). Application of 0.6 mM diazoxide (DZX) to the extracellular surface of the patch inhibited K_{ATP} channel activity an effect not reversed upon wash. The $N_f.P_o$ values were as follows: control 0.947; diazoxide 0.080; wash 0.065. (b) Single channel currents recorded from an inside-out patch exposed to symmetrical 140 mM KCl, held at a membrane potential of − 50 mV. The extracellular surface was in contact with solution D whilst solution E bathed the intracellular surface. Application of 0.1 mM ATPγS virtually abolished K_{ATP} channel opening and concomitant addition of 0.6 mM diazoxide (DZX) had no effect. The inhibition by Mg-ATPγS was poorly reversed on wash. The values for $N_f.P_o$ were as follows: control 0.303; Mg-ATPγS 0.001; Mg-ATPγS and diazoxide 0.001; wash 0.008. (c) A similar experiment to (b) in which 0.01 mM ATPγS was used. At this concentration, where K_{ATP} channel currents were less inhibited, diazoxide (0.6 mM) was still ineffective at increasing channel activity. Only the inhibitory effect of diazoxide was apparent. Washing out of both the Mg-ATPγS and diazoxide resulted in some reversal. The values for $N_f.P_o$ were as follows: control 0.760; Mg-ATPγS 0.216; Mg-ATPγS and diazoxide 0.096; wash 0.154. For abbreviations, see text.

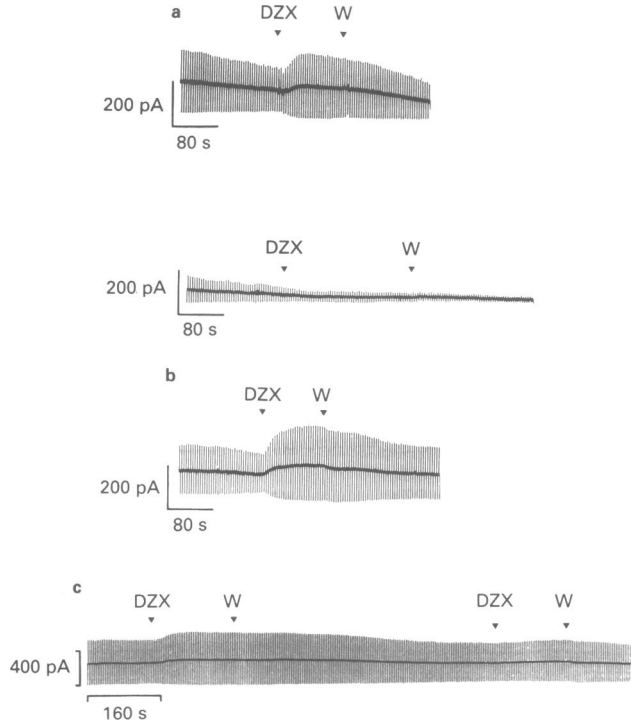

Figure 2 Recordings of whole-cell, voltage-clamped K_{ATP} currents. Cells were voltage clamped at a holding potential of − 70 mV and alternate ± 10 mV pulses of 200 ms duration applied every 2 s. Both records shown in (a) are taken from the same cell bathed in solution A, whilst the pipette contained solution B to which 0.1 mM Mg-AMP-PNP had been added. The upper trace shows the effect of 0.6 mM diazoxide (DZX) soon after the peak whole-cell current had been reached. Note the transient increase in the amplitude of the current pulses (denoted by the vertical lines) and the concomitant change in holding current (indicated by the horizontal line). Washing out of the diazoxide (W) made little difference to the time course for K_{ATP} current run-down. The lower trace is a continuation of the upper with a break of approximately 1 min. A second application of 0.6 mM diazoxide (DZX) was ineffective in eliciting activation of the K_{ATP} current. (b) The effect of diazoxide upon whole-cell K_{ATP} currents recorded under the conditions described above but with 0.1 mM Mg-ATPγS dialysing the cell interior. Diazoxide (0.3 mM) induced an increase in the amplitude of the currents in response to the voltage pulses with a concomitant increase in the holding current. This effect waned gradually with time of exposure and appeared relatively unaffected by the washout of the drug. A second application of the same concentration of diazoxide resulted in a reduced response which also gradually diminished with time. (c) The effect of diazoxide (0.6 mM) on cells dialysed with 0.1 mM Mg-ATP. Note the sustained activating effect of diazoxide under these conditions. For abbreviations, see text.

cell K_{ATP} current (Figure 2a and Table 1). In the presence of Mg-AMP-PCP in the pipette, the activation elicited by diazoxide was greater than in the presence of a similar concentration of Mg-AMP-PNP although a subsequent application of diazoxide, once the response had returned to control, resulted in no significant effect (Table 1). When Mg-ATPγS dialysed the cell interior (Figure 2b) the activation induced by diazoxide was similar to that observed when Mg-AMP-PNP was present intracellularly and a second application also had no significant effect (Table 1). In general it was noted that the diazoxide-induced activation of whole-cell K_{ATP} currents with Mg-ATP present intracellularly was generally longer-lived (Figure 2c) when compared with cells dialysed with the other, poorly hydrolysed, nucleotides. A quantitative assessment of the peak response obtained to diazoxide using various adenine nucleotides and phosphates is given in Table 1.

In order to test whether the diazoxide-induced activation depended upon the K_{ATP} channels being inhibited by the dialysing nucleotide, experiments were performed in which the cells were dialysed with ADP, AMP or adenosine and the effects of diazoxide examined. The results, shown in Table 1, indicate that the potency of diazoxide progressively decreases with a reduction in the length of the phosphate chain (i.e. ATP > ADP > AMP > adenosine). In the presence of adenosine tetraphosphate or sodium tripolyphosphate, diazoxide was poorly effective or ineffective in producing activation. A second application of diazoxide, irrespective of which nucleotide dialysed the cell interior, failed to produce a significant activation of the whole-cell K_{ATP} current (Table 1). This lack of effect may be due to the loss of an unknown cellular component during the course of whole-cell recording.

In cells dialysed with Mg-AMP-PCP, diazoxide induced a concentration-dependent enhancement of whole-cell K_{ATP} currents (Figures 3a,b) which generally declined in the continued presence of the drug with a time course unaffected by washout of the diazoxide. The rate of the current decline appeared to decrease with increasing concentration of diazoxide. The relationship between diazoxide-induced activation and the concentration of the dialysing nucleotide was also examined. Data obtained from cells dialysed with a range of Mg-AMP-PCP concentrations revealed that as the concentration of Mg-AMP-PCP was increased the amplitude of the whole-cell K_{ATP} current diminished (Figure 4a,b) indicating that this analogue is also a potent inhibitor of K_{ATP} channel activity. The half-maximal inhibitory concentration for this nucleotide is approximately 100 μM, a value similar to that obtained for Mg-ATP (Sturgess *et al.*, 1988). As the overall conductance of the cell diminished, application of diazoxide (0.3 mM) resulted in a progressively larger degree of activation relative to pre-drug levels (Figure 4c). Note that mixed results were obtained with 1 mM AMP-PCP dialysing the cell

Table 1 Whole-cell K_{ATP} current activation induced by diazoxide (0.6 mM) in the presence of various dialysing nucleotides and related compounds (0.1 mM)

Dialysing nucleotide or related molecule	*1st Application* (I_D/I_C)	*2nd Application* (I_D/I_C)
AMP-PCP	*1.85 ± 0.15 ($n = 4$)	1.11 ± 0.13 ($n = 4$)
ATPγS	*1.48 ± 0.17 ($n = 5$)	1.10 ± 0.07 ($n = 5$)
AMP-PNP	*1.45 ± 0.10 ($n = 5$)	*0.77 ± 0.06 ($n = 5$)
#ATP	*1.27 ± 0.05 ($n = 7$)	1.18 ± 0.10 ($n = 5$)
ADP	*1.26 ± 0.13 ($n = 5$)	1.06 ± 0.12 ($n = 2$)
AMP	*1.15 ± 0.04 ($n = 3$)	1.19 ± 0.12 ($n = 2$)
Adenosine	*1.08 ± 0.02 ($n = 3$)	ND
Adenosine tetraphosphate	*1.07 ± 0.02 ($n = 2$)	1.01 ± 0.03 ($n = 2$)
Sodium tripolyphosphate	1.04 ± 0.01 ($n = 3$)	ND
Various (saline control)	1.00 ± 0.02 ($n = 8$)	ND

For abbreviations, see text.
Cells were dialysed with solution B, consequently nucleotides are predominantly the Mg^{2+} complexed species.
*$P < 0.05$ relative to control value obtained on perfusion of the bath with normal saline containing no diazoxide. On some occasions the effects of a second application were not determined (ND).
#Taken from Kozlowski *et al.* (1989).

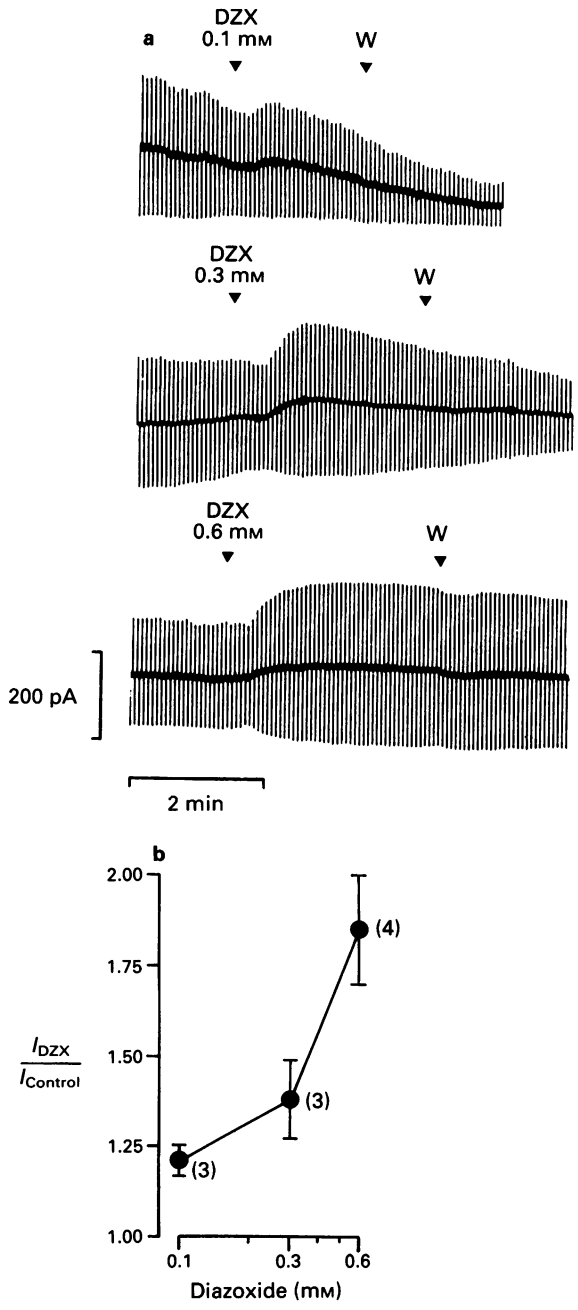

Figure 3 (a) Effects of different concentrations of diazoxide upon whole-cell K_{ATP} currents. In all three records (taken from different cells), solution A bathed the cell exterior, whilst solution B containing 0.1 mM Mg-AMP-PCP dialysed the interior of the cells. Application of diazoxide (DZX) induced a concentration-dependent increase in the whole-cell K_{ATP} current. Note the time course of the decline of whole-cell K_{ATP} currents activated by diazoxide varied between cells and was generally unaffected by washout of the drug (W). (b) Concentration-response relationship for activation of K_{ATP} currents (I) by diazoxide under the conditions described in (a). $I_{DZX}/I_{Control}$ is the ratio of the peak current obtained in the presence of diazoxide (I_{DZX}) and the current amplitude under control conditions ($I_{Control}$) immediately prior to application of the drug. Vertical lines represent the ± s.e.mean values with the number of determinations being indicated in parentheses adjacent to the corresponding data point. Note only one concentration of diazoxide was tested per cell. For abbreviations, see text.

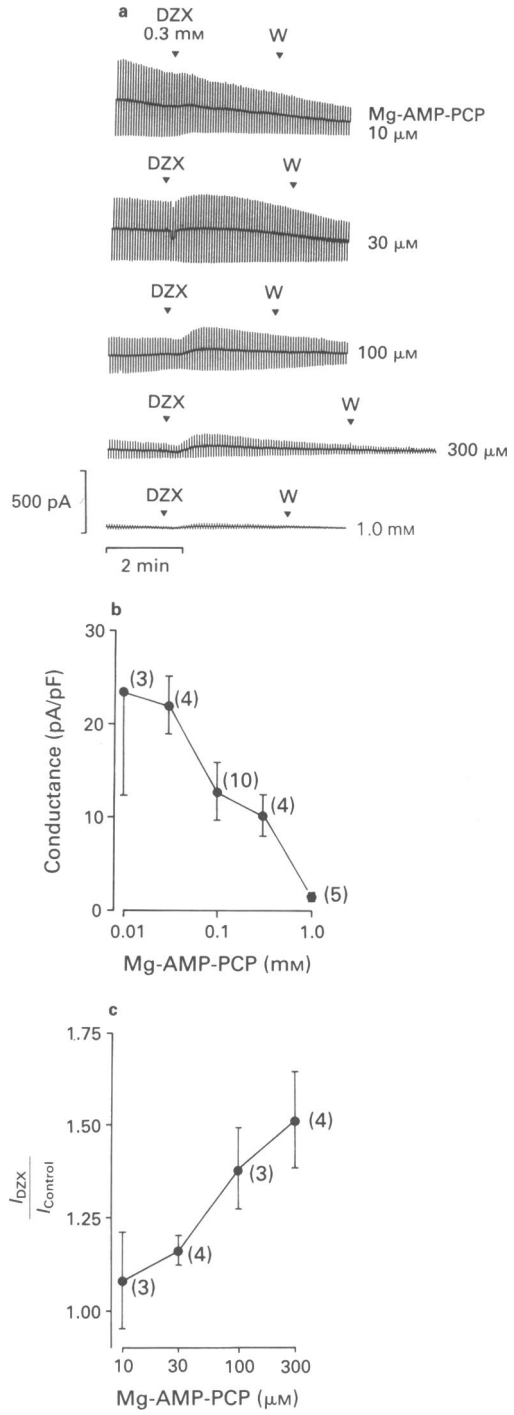

Figure 4 (a) Effect of 0.3 mM diazoxide (DZX) upon whole-cell K_{ATP} currents recorded from cells dialysed with various Mg-AMP-PCP concentrations. The five records shown were recorded from different cells. Each cell was bathed in solution A whilst the recording pipettes contained solution B to which different concentrations of Mg-AMP-PCP were added. The concentration of Mg-AMP-PCP dialysing the cell interior is indicated to the right of each current record. Note that the magnitude of the whole-cell K_{ATP} current became smaller as the concentration of Mg-AMP-PCP was increased, and that as the concentration of the nucleotide was increased the activation induced by diazoxide became larger relative to the amplitude of the current prior to application of the drug. (b) Relationship between the mean peak whole-cell K_{ATP} current (pA/pF) and the concentration of Mg-AMP-PCP dialysing the interior of the cells. The vertical lines indicate ± s.e.mean values whilst the number of determinations is shown adjacent to its corresponding data point. (c) Concentration-response relationship for 0.3 mM diazoxide applied to cells dialysed with a range of Mg-AMP-PCP concentrations under the recording conditions described in (a). Diazoxide has a progressively greater activating effect as the concentration of the nucleotide is increased. Data for 1 mM Mg-AMP-PCP have not been included (see text). For abbreviations, see text.

interior (increases in I_D/I_C of between 0.07 and 7.62 occurred). One explanation for this variability is that, because the whole-cell current was very small under these conditions, it was difficult to determine the current maximum and conse-

quently diazoxide may have been added after the peak and following substantial K_{ATP} current run-down in some experiments. It has been demonstrated that diazoxide does not reverse the rundown process (Sturgess *et al.*, 1989).

In order to determine whether the activation produced by diazoxide was Mg^{2+}-dependent, cells were dialysed with solution C which was nominally Mg^{2+}-free. Application of diazoxide to cells which were dialysed with nucleotides in Mg^{2+}-free conditions resulted in no activating action and a significant inhibition of the whole-cell K_{ATP} current by diazoxide was observed for all nucleotides tested (Table 2 and Figure 5). These inhibitory effects were quantified after 2.5 min exposure to diazoxide since a steady-state inhibition was not attained under these conditions (Kozlowski *et al.*, 1989). There was little variation in the amount of inhibition induced by diazoxide in the absence or in the presence of different dialysing nucleotides. Thus, activation of the whole-cell K_{ATP} current by diazoxide was dependent upon the presence of intracellular Mg^{2+} ions. Substitution of the Mg^{2+} present in solution B by Mn^{2+}, in the presence of 0.1 mM AMP-PCP, prevented K_{ATP} current activation by 0.3 mM diazoxide (I_D/I_C; 1.05 ± 0.05, $n = 6$). This effect was significantly ($P < 0.05$) smaller when compared to the increase observed with 0.1 mM Mg-AMP-PCP intracellularly (I_D/I_C; 1.38 ± 0.11, $n = 3$). These data strongly suggest that the diazoxide-induced activation process has a specific requirement for Mg^{2+} ions.

Base specificity

Application of diazoxide (0.6 mM) to cells dialysed with solution B containing 0.1 mM Mg-GTPγS (Figure 6a) resulted in an increase in the whole-cell K_{ATP} current (I_D/I_C; 1.43 ± 0.13, $n = 4$) similar to that induced in the presence of 0.1 mM Mg-ATPγS (see Table 1). In three of the four cells the effect of a subsequent application of diazoxide after washout was examined. No significant second response being observed (I_D/I_C; 1.16 ± 0.14, $n = 3$). The K_{ATP} current activation induced by diazoxide in the presence of GTPγS was also Mg^{2+}-dependent (Figure 6b). In the absence of Mg^{2+}, diazoxide inhibited the K_{ATP} current to 0.70 ± 0.08 ($n = 3$) of control, a value similar to that obtained in other Mg^{2+}-free conditions (Table 2). In contrast, cells dialysed with a pyrimidine base nucleotide, 0.1 mM Mg-CTP (Figure 6c), were relatively insensitive to 0.6 mM diazoxide (I_D/I_C; 1.11 ± 0.09, $n = 3$).

Protease modulation of diazoxide action

Trube *et al.* (1989) reported that intracellularly-applied trypsin produced an increase in the K_{ATP} current and reduced its sensitivity to nucleotides in pancreatic β-cells. Therefore the effects of trypsin were determined on the diazoxide-induced, Mg^{2+}-nucleotide dependent activation of K_{ATP} currents. In the presence of 0.1 mg ml^{-1} trypsin and 0.1 mM Mg-AMP-PCP, intracellularly, the whole-cell conductance was significantly ($P < 0.05$) higher than the conductance of cells dialysed with 0.1 mM Mg-AMP-PCP alone (Table 3) and was similar to that obtained for these cells in the absence of intracellular ATP under these recording conditions (Sturgess *et al.*, 1988). Furthermore in the presence of trypsin, diazoxide failed to activate the whole-cell K_{ATP} current (Figure 7) which remained at the increased level. Addition of 1 mM PMSF (a serine protease inhibitor which prevents the proteolytic action of trypsin) to the intracellular dialysing solution together with trypsin (0.1 mg ml^{-1}) and Mg-AMP-PCP (0.1 mM) restored the ability of diazoxide to induce activation of the whole-cell K_{ATP} current (Figure 7). In addition, the presence of PMSF opposed the stimulatory effect of trypsin on the whole-cell K_{ATP} conductance in the presence of 0.1 mM Mg-AMP-PCP (Table 3). These latter observations confirm that the effects of trypsin are the result of its proteolytic activity.

Table 2 Inhibitory action of diazoxide (0.6 mM) on whole-cell K_{ATP} currents recorded from cells dialysed with various nucleotides in the absence of Mg^{2+} ions (Solution C)

Nucleotide	I_D/I_C
AMP-PCP 0.1 mM	0.73 ± 0.07 ($n = 4$)
ATPγS 0.01 mM	0.79 ± 0.04 ($n = 3$)
AMP-PNP 0.01 mM	0.79 ± 0.02 ($n = 3$)
*ATP 0.1 mM	0.87 ± 0.03 ($n = 6$)
*ATP 0.01 mM	0.84 ± 0.03 ($n = 4$)
AMP 0.1 mM	0.81 ± 0.03 ($n = 3$)
*No nucleotide (solution C)	0.75 ± 0.05 ($n = 7$)

For abbreviations, see text.
*Taken from Kozlowski *et al.* (1989).

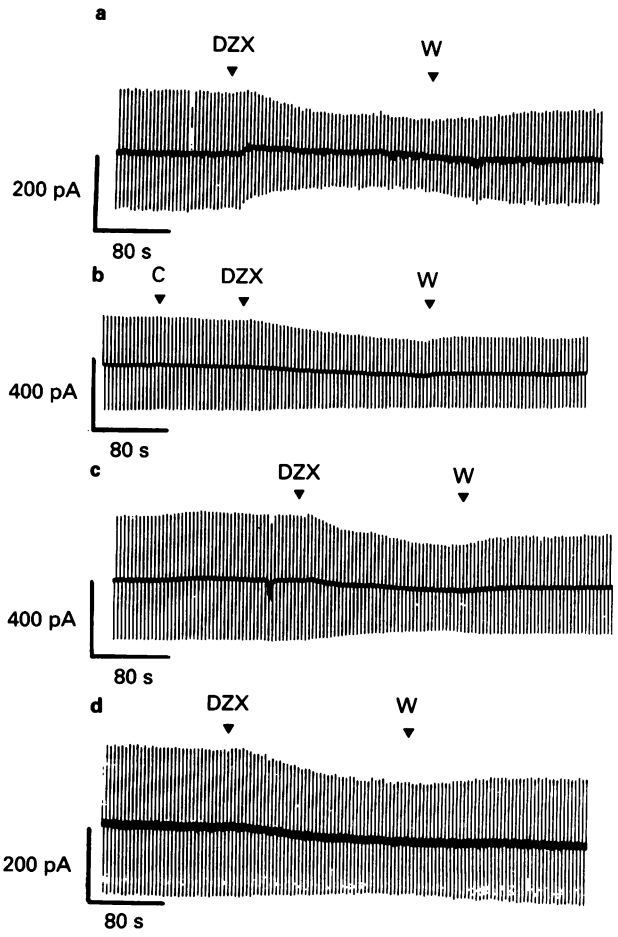

Figure 5 Recordings of whole-cell voltage clamped K_{ATP} currents. Records shown in (a), (b), (c) and (d) are taken from different cells. In all four cases cells were bathed in solution A whilst the pipette contained solution C nominally free of Mg^{2+} ions (< 5 mM) to which nucleotides were added. The records shown were obtained from cells dialysed with (a) 0.1 mM AMP-PCP, (b) 0.01 mM AMP-PNP, (c) 0.01 mM ATPγS and (d) 0.1 mM AMP. In the presence of all the nucleotides application of 0.6 mM diazoxide (DZX) induced an inhibition of the whole-cell K_{ATP} current. Note, in (b) a control perfusion of the bath with bathing solution containing no diazoxide (indicated by the letter C on the current trace) did not alter the magnitude of the current. For abbreviations, see text.

Effects of CRI-G1 cell lysate

In all the aforementioned experiments, diazoxide was added after the peak whole-cell K_{ATP} current had been reached, with the result that the maximal activation did not exceed 100%

of the control levels. However, when diazoxide was added soon after formation of the whole-cell configuration (i.e. before there is a substantial loss, due to dialysis, of intracellular contents as shown by the increase in overall cell conductance (see Kozlowski *et al.*, 1989) a very large activation (>350%, $n = 3$) was observed (Figure 8a). This difference may well be indicative of a process which depends upon a substance lost from the cell during dialysis. To examine this possibility, cells were dialysed with an electrode containing solution B, exogenous ATP and diluted cell lysate prepared according to the protocol described in the Methods. Application of diazoxide to cells dialysed with the lysate solution increased the whole-cell K_{ATP} current (Figure 8b) with I_D/I_C being increased to 1.67 ± 0.34 ($n = 3$) and 1.54 ± 0.15 ($n = 9$) by 0.6 and 0.3 mM diazoxide, respectively. The ATP content of the diluted lysate solution, measured by a luciferase assay, was between 0.4 and 14.8 μM. In control experiments, diazoxide (0.3 mM) was without effect in cells dialysed with 5 μM ATP alone (Figure 8c), with I_D/I_C remaining at approximately control levels (0.98 ± 0.05; $n = 3$). Furthermore, application of diazoxide (0.6 mM) elicited activation (I_D/I_C; 1.66 ± 0.55, $n = 3$) in the presence of lysate but with ATP omitted. Hence, under these whole-cell recording conditions, activation is not solely due to the presence of a nominal concentration of Mg-ATP but also dependent largely upon the presence of some intracellular regulatory factor.

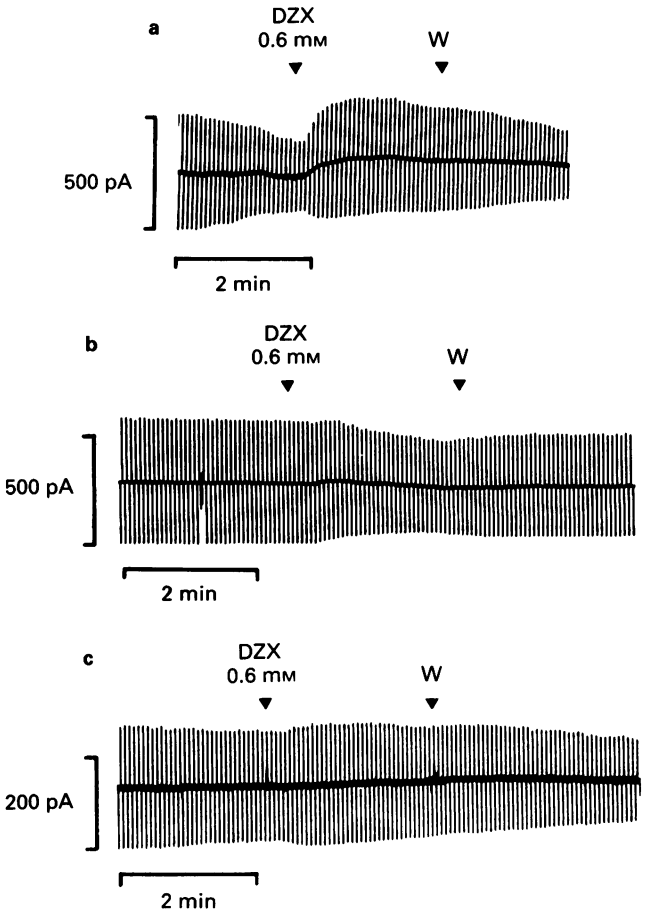

Figure 6 Whole-cell K_{ATP} currents recorded from three different cells with purine and pyrimidine bases. The cells were bathed in solution A. (a) Currents recorded from a cell with solution B containing 0.1 mM Mg-GTPγS dialysing the cell interior. Application of 0.6 mM diazoxide (DZX) induced a marked activation of the K_{ATP} current which waned gradually with time and was unaffected by washout (W) of the drug. (b) Currents recorded from a cell with solution C containing 0.1 mM GTPγS in the absence of Mg^{2+} ions dialysing the cell interior. Application of 0.6 mM diazoxide resulted in an inhibition of whole-cell K_{ATP} currents which was poorly reversed upon washout of the drug (W). (c) Currents recorded from a cell with solution B containing the pyrimidine base Mg-CTP (0.1 mM) as the dialysing solution. Application of 0.6 mM diazoxide resulted in a slight activation of the currents with washout of the drug (W) having little effect. For abbreviations, see text.

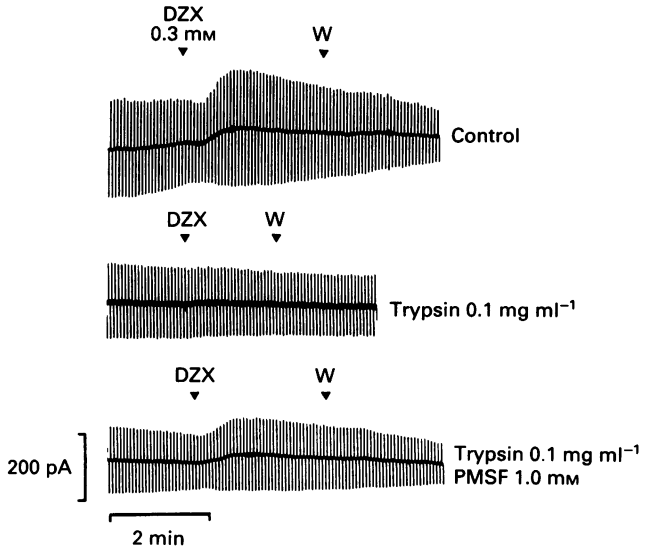

Figure 7 Effects of trypsin upon diazoxide induced activation of whole-cell K_{ATP} currents. Each trace shown was recorded from a different cell. Cells were bathed in solution A with solution B, which also contained 0.1 mM AMP-PCP, as the dialysis solution. Application of 0.3 mM diazoxide (DZX) to the cell under these conditions resulted in a marked activation of the currents (top trace). When 0.1 mg ml^{-1} trypsin was added to the dialysing solution, in the continued presence of AMP-PCP (0.1 mM), diazoxide was unable to elicit an activation (centre trace). In contrast, cells with both 0.1 mg ml^{-1} trypsin and 1.0 mM PMSF in the presence of 0.1 mM Mg-AMP-PCP were responsive to 0.3 mM diazoxide which elicited an activation of the currents (bottom trace). Washout of diazoxide is indicated by (W) on each current record. For abbreviations, see text.

Discussion

Previous studies on isolated patches and whole-cell configurations of insulin-secreting cells have shown that K_{ATP} channel

Table 3 The lack of effect of diazoxide (0.6 mM) on K_{ATP} currents recorded in the presence of intracellular trypsin

Pipette solution	*Whole-cell conductance* (pA/pF)	*Effect of DZX* (I_D/I_C)
Mg-AMP-PCP 0.1 mM	12.7 ± 3.1 ($n = 10$)	1.38 ± 0.11 ($n = 3$)
Mg-AMP-PCP 0.1 mM	27.9 ± 6.6 ($n = 8$)	0.98 ± 0.04 ($n = 9$)
trypsin 0.1 mg ml^{-1}		
Mg-AMP-PCP 0.1 mM	15.4 ± 6.1 ($n = 4$)	1.30 ± 0.12 ($n = 4$)
trypsin 0.1 mg ml^{-1}		
PMSF 1.0 mM		

For abbreviations, see text.
For all experiments the cells were bathed in solution A whilst the cell interior was dialysed with solution B.

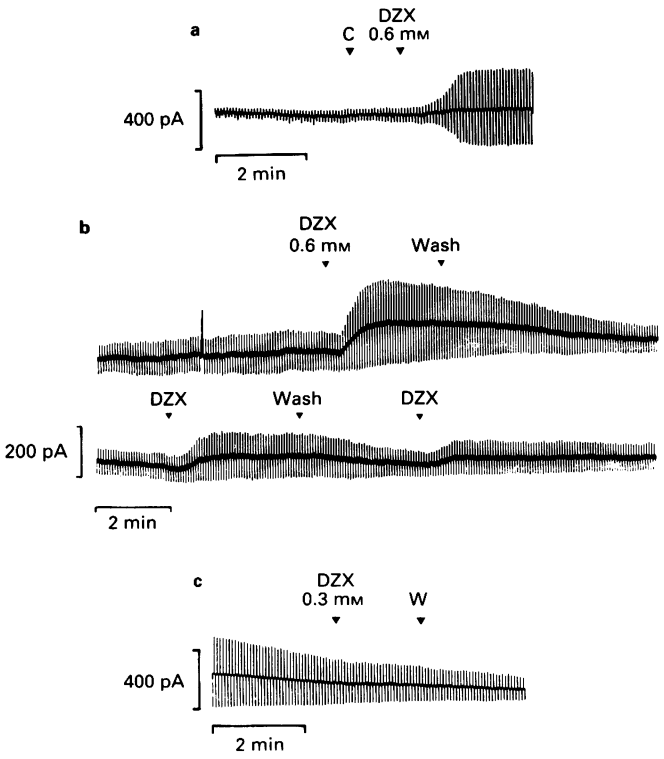

Figure 8 (a) Effect of diazoxide on whole-cell K_{ATP} currents soon after formation of whole-cell mode. The cell was bathed in solution A throughout the experiment, whilst solution C containing 0.1 mM AMP in the absence of Mg^{2+} ions dialysed the cell interior (conditions under which diazoxide does not normally activate K_{ATP} channels; cf. Table 2). Application of 0.6 mM diazoxide (DZX) to the cell soon after achieving a stable voltage-clamp but before the peak whole-cell K_{ATP} current was reached resulted in a large activation of the current. Note, a control perfusion of the bath with bathing solution containing no drug (indicated by the letter C on the current trace) did not alter the magnitude of the current at this stage of the experiment. (b) Effect of diazoxide on whole-cell K_{ATP} currents recorded from a cell bathed in solution A and with solution B, to which the lysate had been added, dialysing the cell interior. Note the large and transient nature of the activation induced by 0.6 mM diazoxide. (c) Effect of diazoxide on whole-cell K_{ATP} currents from a cell bathed in solution A and with solution B containing 5 mM ATP as the dialysis medium. Note that under these conditions, 0.3 mM diazoxide (DZX) is ineffective in eliciting K_{ATP} channel activation.

activation by diazoxide requires the presence of intracellular ATP (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988; Kozlowski *et al.*, 1989). In cell-free patches isolated from insulin-secreting cells, this activation is dependent upon the presence of intracellular Mg-ATP suggesting that phosphorylation is involved (Kozlowski *et al.*, 1989; Dunne, 1989). The findings of the current study are consistent with such a mechanism. Thus, in cell-free patches, diazoxide was unable to activate K_{ATP} channels which were inhibited by the non-hydrolysable ATP analogue, Mg-AMP-PCP. Indeed, only the underlying inhibitory action of the drug was observed (see also Kozlowski *et al.*, 1989). Although Mg-ATPγS has been reported to substitute for Mg-ATP in many processes requiring phosphorylation (Yount, 1975), diazoxide did not activate K_{ATP} channels in cell-free patches in the presence of this nucleotide. Hence the enzyme(s) utilizing ATP, or the protein(s) accepting the phosphate(s) may be specific for ATP. These results parallel the findings of Ohno-Shosaku *et al.* (1987) that in inside-out patches isolated from mouse pancreatic β-cells, K_{ATP} channel refreshment (a transient increase in channel activity relative to control following washout of Mg-ATP) is only mediated by Mg-ATP, whereas Mg-AMP-PNP, Mg-AMP-PCP and Mg-ATPγS are ineffective. The lack of effect observed in the presence of Mg-ATPγS despite the effectiveness of Mg-ATP might be due to differences in the rates of thiophosphorylation, phosphorylation and dephosphorylation (Palvino *et al.*, 1985), suggesting that continuous hydrolysis of ATP is necessary for channel activation.

If phosphorylation is involved in K_{ATP} channel activation then either the channel (or a regulatory protein) has first to be phosphorylated to allow diazoxide to elicit its effect, or diazoxide itself must stimulate a kinase which subsequently mediates phosphorylation. Both protein kinase A and protein kinase C have been reported to increase K_{ATP} channel activity (De Weille *et al.*, 1989; Ribalet *et al.*, 1989), although an interaction of diazoxide with protein kinase C is perhaps unlikely since activation can occur in the absence of intracellular Ca^{2+} (Kozlowski *et al.*, 1989).

The whole-cell, voltage-clamp data clearly indicate that there is also a second mechanism for the activating action of diazoxide on K_{ATP} currents in this cell-line, which is also dependent upon the presence of a purine Mg-nucleotide phosphate in the dialysing solution. However, the activation elicited under these conditions is not phosphorylation-driven as it occurs in cells dialysed with AMP and ADP and the non-hydrolysable ATP analogues Mg-AMP-PNP and Mg-AMP-PCP. This activation is also Mg^{2+}-dependent as in the absence of Mg^{2+} ions, or in the presence of Mn^{2+} ions, a nucleotide-independent inhibitory effect of diazoxide is observed similar to that reported previously in the presence of Mg-free ATP (Kozlowski *et al.*, 1989). These results, using whole-cell and cell-free patch K_{ATP} current recording, imply that diazoxide activation of K_{ATP} channels may be mediated by two distinct processes. In isolated patches, activation by diazoxide appears to depend on phosphorylation whilst under whole-cell recording conditions it depends on the presence of a purine nucleotide and Mg^{2+} ions. It is therefore possible that, for the second mechanism, the Mg^{2+} complexed species of the nucleotide binds to a modulatory site, loosely associated with the channel, which is lost or altered upon patch excision. In support of this hypothesis is the observation that a relatively long-lived activation of the whole-cell K_{ATP} current occurs in cells dialysed with Mg-ATP when compared to cells dialysed with non-hydrolysable ATP analogues, implicating phosphorylation in the sustained effect. Furthermore, in agreement with the single channel data, the activating action of diazoxide in cells dialysed with Mg-ATPγS is similar in duration to that in cells dialysed with Mg-AMP-PCP and Mg-AMP-PNP, which suggests that ATPγS does not substitute for ATP in maintaining a longer-lived activation.

The ability of diazoxide to activate K_{ATP} channels in cells dialysed with AMP or ADP in the presence of Mg^{2+} ions indicates that activation is not due to reversal of K_{ATP} current inhibition since these nucleotides, particularly at the low concentration used in this study (0.1 mM), are poor K_{ATP} channel inhibitors in this cell-line (Sturgess, 1988) and other insulin-secreting cell types (Cook & Hales, 1984; Misler *et al.*, 1986; Ashcroft, 1988). Furthermore, a small but significant activation occurs in the presence of adenosine which is totally ineffective at inhibiting K_{ATP} channel activity in this cell-line (Sturgess, 1988) and RINm5F cells (Ribalet & Ciani, 1987). The small increase in K_{ATP} current observed in the presence of adenosine tetraphosphate also indicates that the triphosphate configuration is optimal. In addition, the relative lack of activation with sodium tripolyphosphate or CTP in the dialysing solution indicates that a purine base in conjunction with a phosphate is also necessary for diazoxide-induced activation. Further experiments using Mg-AMP-PCP, as the most potent nucleotide for diazoxide-induced activation of whole-cell K_{ATP} current, have demonstrated that this activation is diazoxide-concentration dependent (for a constant nucleotide concentration intracellularly) and nucleotide-concentration dependent (at a constant diazoxide concentration).

In the presence of trypsin and Mg-AMP-PCP intracellularly, the whole-cell conductance was increased to a level similar to that observed in the absence of nucleotides or divalent cations (Sturgess *et al.*, 1988; Kozlowski & Ashford, 1990); an effect prevented by the addition of PMSF to the dialysing solution. This observation suggests that proteolysis of some intracellular protein, susceptible to the proteolytic action of trypsin, is responsible for the increase in conductance. Trypsin was also found to prevent the activating action of diazoxide on whole-cell K_{ATP} current, an effect again inhibited by PMSF. In view of these data it is tempting to speculate that trypsin may functionally uncouple the putative nucleotide binding site from its effector, thereby preventing an activating action of diazoxide. A similar hypothesis has been proposed to explain the altered sensitivity of K_{ATP} channels to ATP in the presence of intracellularly applied trypsin in patches isolated from mouse pancreatic β-cells (Trube *et al.*, 1989). Alternatively, it is possible that in the presence of trypsin, the K_{ATP} channels are maximally activated and are therefore not susceptible to further activation by diazoxide.

It was noted that a second application of diazoxide to cells dialysed with a range of nucleotides in the presence of Mg^{2+} ions resulted in a reduced response to the drug, an effect which may be due to the loss or inactivation, through dialysis of the cell contents, of a regulatory protein. To test this hypothesis, cells were dialysed with an electrode solution containing cell extract and the effects of diazoxide examined. The results obtained show that diazoxide is indeed able to activate K_{ATP} currents recorded from whole-cell dialysed with a lysate containing a low concentration (0.4 to 15 μM) of Mg-ATP. At Mg-ATP concentrations of this order of magnitude, diazoxide does not induce K_{ATP} channel activation, suggesting the lysate contains a cellular component lost or altered during cell dialysis which is required for activation. This possibility is supported by the observation that large activating effects of diazoxide are observed soon after formation (i.e., before substantial dialysis can occur) of the whole-cell configuration. It is unlikely that this increased effectiveness of diazoxide is due to the high intracellular ATP content of the cells *per se*, soon after achieving the whole-cell configuration (approximately 5 mM; Ashcroft *et al.*, 1987), since diazoxide is ineffective under conditions of high intracellular ATP in whole-cell recordings (Trube *et al.*, 1986; Sturgess, 1988).

Thus, in conclusion, we suggest that diazoxide can activate K_{ATP} channels in CRI-G1 insulin-secreting cells by two separate pathways. In cell-free membrane patches we propose that diazoxide induces K_{ATP} channel activation by a mechanism involving phosphorylation of the channel or some associated protein by Mg-ATP (see Kozlowski *et al.*, 1989). In intact cells an alternative activatory pathway may exist in which an intracellular regulatory protein, which binds purine Mg^{2+} nucleotides, interacts with the channel and allows diazoxide to induce activation. It is not, at present, clear which of these mechanisms dominates in intact cells where Mg-ATP is the major intracellular purine nucleotide present. It is also not clear as to how diazoxide mediates its inhibitory effects on K_{ATP} channels. However, this secondary inhibitory action is clearly nucleotide-independent and apparent under conditions which do not favour activation (see also Kozlowski *et al.*, 1989). Perhaps under these conditions diazoxide mimics the action of the sulphonylureas. However, in view of the intracellular milieu required for this inhibitory effect it is unlikely to occur in intact pancreatic β-cells. With regard to the potassium channel activating action of diazoxide, it will be interesting to determine whether the dual mechanism proposed above is of general significance and underlies some of the activatory actions of other potassium channel openers in other tissues (Quast & Cook, 1989; Edwards & Weston, 1990).

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