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Effects of sulphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line

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1 The effects of various sulphonylureas and diazoxide on insulin secretion and the activity of various channels have been studied using tissue culture and patch-clamp methods in an insulin-secreting cell line derived from a rat islet cell tumour.

2 Tolbutamide, glibenclamide and HB699 increased the rate of insulin release by 2–5 fold. The concentrations of tolbutamide and glibenclamide giving half-maximum effects on insulin secretion were approximately 40 μM and 0.2 μM, respectively.

3 Diazoxide (0.6–1.0 mM) *per se*, had either no effect or produced a small increase in insulin secretion, whereas when secretion was maximally stimulated by the combination of glucose (3 mM) and leucine (20 mM), it produced inhibition. Tolbutamide-induced release was also inhibited by diazoxide.

4 Tolbutamide, glibenclamide, HB699 and HB985 reduced the open-state probability of the ATP-K^+ channel in a dose-dependent manner. Tolbutamide and glibenclamide were shown to be effective regardless of which side of the membrane they were applied.

5 In whole cell recording, in which the total ATP-sensitive K^+ conductance of the cell could be measured, dose-inhibition curves for tolbutamide and glibenclamide were constructed, resulting in K_i values of 17 μM and 27 nM, respectively. The value of K_i for tolbutamide was unchanged when ATP (0.1 mM) was present in the electrode.

6 Diazoxide (0.6 mM) activated the ATP-K^+ channels only when they had first been inhibited by intracellular ATP (0.1 mM) or bath applied tolbutamide (3–30 μM). The inhibition produced by glibenclamide could not be reversed by diazoxide.

7 Neither tolbutamide (1.0 mM) nor glibenclamide (10 μM) altered the open-state probability of the Ca^{2+}-activated K^+ channel or the Ca^{2+}-activated non-selective cation channel which are present in this cell line.

8 It is concluded that the sulphonylureas and related hypoglycaemic drugs and diazoxide regulate insulin secretion by direct effects on the ATP-K^+ channel or a protein closely associated with this channel.

Introduction

Although the sulphonylurea drugs have been in use for the treatment of diabetes for over 30 years, their mechanism of action remains unknown. Their ability to lower the plasma glucose concentration in diabetes appears to be due, at least in part, to their ability to stimulate insulin secretion (as reviewed by Lebovitz, 1985). Diazoxide, a benzothiadiazine, can induce hyperglycaemia and at least part of this effect appears due to a direct action on the B-cell inhibiting insulin secretion which has been stimulated by a variety of agents (Milner & Hales, 1969). It has recently been discovered that B-cells (Cook & Hales, 1984) and the insulin secreting cell line, CRI-G1, (Sturgess *et al.*, 1986a) contain a K^+ channel, the opening of which is inhibited by adenosine 5'-triphosphate (ATP) applied to the cytosolic membrane surface. This ATP-sensitive K^+ channel (ATP-K^+) may play a major role in mediating glucose-stimulated insulin secretion. Cell-attached recordings from B-cells have shown that the opening of this

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channel is reduced on exposure of the B-cell to high glucose concentrations (20 mM) and that glucose metabolism must occur (Ashcroft *et al.*, 1984). Therefore it is likely that the link between glucose metabolism and the initial depolarization of B-cells (which causes the opening of voltage-dependent calcium channels and hence insulin release) is the inhibition of this K^+ channel by raised intracellular ATP. The electrical effects of sulphonylureas on B-cells (Dean & Matthews, 1970) and their effects in stimulating insulin-secretion have been linked with a decrease in K^+ permeability of the plasma membrane (Henquin & Meissner, 1982). It was of interest, therefore, to determine whether sulphonylureas had any effect on the ATP-sensitive K^+ channel. We found in single channel studies that the sulphonylurea drugs, tolbutamide and glibenclamide, both reduced the open state probability of the ATP-sensitive K^+ channel and that their relative potency was of the same order as their pharmacological efficacy in man (Sturgess *et al.*, 1985; Ashford *et al.*, 1986). These findings have been confirmed and extended by Trube *et al.* (1986), who also showed that the drugs did not affect Ca^{2+}-dependent K^+ channels present in B-cells (Cook *et al.*, 1984; Findlay *et al.*, 1985) and the CRI-G1 cell line (Sturgess *et al.*, 1986a), and that the drug diazoxide could reverse inactivation of the ATP-sensitive K^+ channel. Subsequently we have discovered in the insulin-secreting cell line, CRI-G1, a second cation channel which can be inhibited by adenine nucleotides (Sturgess *et al.*, 1986b); therefore, this represents another possible target for the drugs.

In order to test further our suggestion that the ATP-sensitive K^+ channel is the sulphonylurea receptor in insulin-secreting tissues (Sturgess *et al.*, 1985), we have studied in parallel, on the CRI-G1 cell line, the effects of a range of sulphonylurea and related compounds (Figure 1) on insulin secretion and on the ATP-sensitive K^+ channel. The latter has been studied by patch clamp methods using the whole cell, cell-attached or isolated membrane patch configurations. We have also, using these techniques, examined the effects of diazoxide on this cell line. Finally, we have conducted experiments to determine whether the Ca^{2+}-activated, adenine nucleotide-sensitive non-selective cation ($Ca\text{-}NS^+$) channel in these cells (Sturgess *et al.*, 1986b) is sensitive to sulphonylureas.

Methods

Cell culture and secretion studies

Cells of the rat pancreatic islet cell line CRI-G1 were cultured and passaged at 3–4 day intervals as previously described (Carrington *et al.*, 1986).

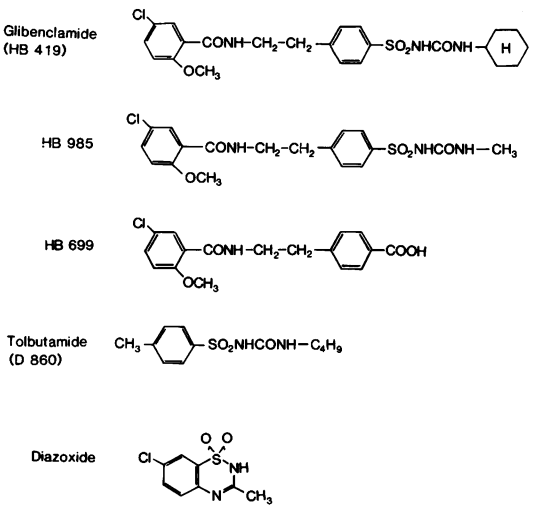

Figure 1 Structures of diazoxide and the various sulphonylureas used in this study.

Cells were plated at a density of 1.5×10^5 cells per dish in Dulbecco's Modified Eagle Medium plus foetal calf serum (5%, v/v), HEPES (10 mM), penicillin (50,000 units l^{-1}) and streptomycin (50 mg l^{-1}) and cultured for 48 h before their use in studies of insulin secretion. On the day of the experiment the medium was removed and the cells were incubated in air for 30 min at 37°C in 2 ml of sterile filtered buffered salt solution (BSS), composition (mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10 and BSA 1 g l^{-1}, pH 7.4. The 30 min incubation was then repeated in a fresh 2 ml of BSS. At the end of this period the medium was removed and a further 2 ml of BSS, containing the additions stated, was added to the cells. Media containing various stimuli of secretion and drugs were made up as follows: leucine (40 mM in BSS; 2 × working strength), glucose (30 mM in BSS; 10 × working strength), tolbutamide and HB699 (dissolved in minimum volume of 0.1 M NaOH or 0.1 M KOH, and diluted in BSS to 10 mM or 5 mM; 10 × working strength), dizoxide (dissolved in minimum volume of 0.1 M KOH and diluted in BSS to 4 mM; 4 × working strength) and glibenclamide (dissolved in methanol and diluted in BSS to 5 μM; 10 × working strength). Stock solutions were sterilised by filtration and where necessary the pH of the working solution was readjusted to 7.4.

Groups of three dishes were used for each incubation condition. The insulin content of the medium at zero time was determined by removing the medium from a group of three dishes immediately following its addition. For the other incubation conditions the cells were incubated at 37°C for 15 min

over which period it has been shown that insulin release is linear with time (data not shown). At the end of the incubation period, or at zero time, the medium was aspirated into 1.8 ml centrifuge tubes and centrifuged at 11,000 r.p.m. for 1 min in order to sediment any loose cells. The supernatants were removed and assayed for insulin (Hales & Randle, 1963) either immediately or after storage at −20°C. Rat insulin (Novo, Bagsvaerd, Denmark) was used as a standard. At the end of the experiment, the cells from three dishes chosen at random from those used for 15 min incubations were detached by treatment with trypsin/EDTA solution and counted in a haemocytometer. Insulin release was expressed as pmol per 10^6 cells $15\,min^{-1}$.

Solutions

Cells were used for patch clamp studies 2–6 days (inclusive) after plating; before use cells were washed thoroughly in solution A which consisted of (mM): NaCl 135.0, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0 and HEPES 10.0, pH 7.2 with NaOH.

For whole cell voltage clamp studies, the cells were bathed in solution A and the recording pipette contained (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 2.0, KEGTA 10.0, HEPES 10.0, pH 7.2 with KOH resulting in final free calcium and magnesium concentrations of 0.03 μM and 0.65 mM, respectively (solution B). In experiments on outside-out patches the bath contained either solution A or was replaced with a solution containing (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH (solution C). In some experiments on outside-out patches the recording pipette contained (mM): KCl 140.0, KEGTA 1.0, free $Ca^{2+} < 10^{-8}$ M, HEPES 10.0, pH 7.2 with KOH (solution D). Hence the effects of the various drugs could be tested on the ATP-K^+ channel under symmetrical or asymmetrical K^+ conditions. To activate the Ca-NS^+ channel, the electrode solution was replaced with one containing (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 1.0, HEPES 10.0, pH 7.2 with KOH (solution E). In inside-out patch experiments the pipette contained solution C and for cell-attached experiments contained (mM): KCl 140.0, $MgCl_2$ 5.0, $CaCl_2$ 5.0, HEPES 10.0, pH 7.2 with KOH (solution F). The bath medium was solution A for cell-attached recordings and on formation of an inside-out patch was replaced with a solution containing (mM): KCl 140.0, $MgCl_2$ 1.0, $CaCl_2$ 0.9, KEGTA 1.0, HEPES 10.0, pH 7.2 with KOH resulting in a final free calcium concentration of 1 μM (solution G).

Tolbutamide and diazoxide were added from 50 mM and 25 mM stock solutions prepared in 0.1 M KOH, and the numbered Hoechst compounds from 20 mM stocks in 0.1 M KOH. Glibenclamide was added from a 5 mM stock in methanol (appropriate control experiments using 0.02% methanol, equivalent to using 1 μM glibenclamide, indicated that it had no effect on the recordings). In some whole-cell and outside-out experiments, 0.1 mM K_2 ATP was included in the pipette (solution B). The $MgCl_2$ concentration was increased to 1.1 mM in order to compensate for Mg^{2+} chelation by ATP. This value was calculated by the programme described by Hesketh *et al.* (1983). Drugs were applied to cells or cell-free membrane patches by superfusing the whole bath using a continuous gravity feed system and continuous expiration by suction. The bath could be perfused at a rate of $1–2\,ml\,s^{-1}$ without loss of the seal resistance, hence complete exchange of solutions could be achieved in 5–10 s. ATP (Na^+ and K^+ salts, vanadium free) and leucine were obtained from Sigma (Poole, Dorset) and the hypoglycaemics, tolbutamide, glibenclamide, HB 699 and HB 985 were provided by Hoechst Aktiengesellshaft, Frankfurt, West Germany. Diazoxide was donated by Allen and Hanburys Ltd, Ware, U.K. All electrophysical experiments were performed at room temperature, 22–25°C.

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

Electrical recording and analysis

All four different recording modes of the patch-clamp technique were employed: the whole-cell, cell-attached, inside-out and outside-out patch configurations (Hamill *et al.*, 1981). Recording pipettes were pulled from borosilicate glass capillaries, and for isolated patch formation had resistances of between 8–12 MΩ when filled with electrolyte solution. For whole-cell recording, pipettes were used which had a resistance of between 3–5 MΩ. An EPC-7 (List Electronic) or a Dagan 8900 patch clamp amplifier was used to record current signals, and these were monitored during the experiment using an oscilloscope, and stored on magnetic tape (Racal 4DS). Current signals were subsequently replayed into a chart recorder (Gould 2200), which filtered the records at 0.14 kHz; these traces were used for illustrative purposes. Outward current, defined as current from the intracellular to the extracellular side of the membrane, is indicated as upward deflections in all traces. The potential across the membrane is described following the usual sign convention for membrane potential (i.e. inside negative). The degree of channel block (obtained from single channel recordings) was estimated by determining the open-state probability (P_{OPEN}) of the channel(s) in the patch before, during and after drug application.

The open-state probability was determined off-line using an analysis programme which incorporated a

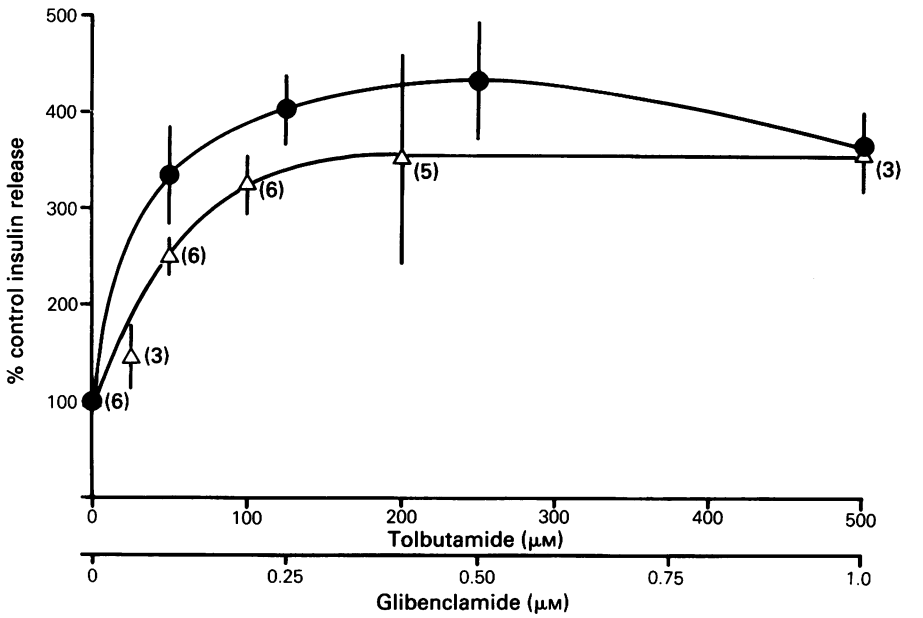

Figure 2 Dose-dependent stimulation of release of insulin from CRI-G1 cells by tolbutamide (△) and glibenclamide (●). Values are means of 3 samples for glibenclamide and (*n*) samples for tolbutamide; vertical lines indicate s.d. The control rates of insulin release were 5.2 ± 1.1 pmol per 10^6 cells $15\,min^{-1}$ and 0.76 ± 0.10 pmol per 10^6 cells $15\,min^{-1}$ for tolbutamide and glibenclamide, respectively. Significant differences ($P < 0.05$, unpaired *t* test) exist between control and test values for all points except the 25 μM tolbutamide point.

50% threshold crossing parameter to detect events (Dempster, 1988) and run either on a minicomputer (PDP 11/23) or an IBM AT microcomputer. Stretches of data of between 30 and 90 s duration were replayed at the recorded speed, filtered at 600–1000 Hz using an 8-pole Bessel filter, and digitized at a frequency of 3.3–5 kHz using a CED 502 (PDP 11/23) or Data Translation 2801 interface (IBM AT). The open-state probability of the channel was obtained by measuring the total time (by integration) that individual channels spent in the open state and expressing this time as a proportion of the total time of the recording, taking into consideration the number of channels observed in the patch. In order to assess the actions of the sulphonylureas when applied in the whole-cell experiments and to construct dose-inhibition curves for tolbutamide and glibenclamide, the following protocol was used. The cell was voltage-clamped at a holding potential of −70 mV and small voltage pulses (of alternate ± 10 mV, 200 ms in duration and at 2 s intervals) were applied (Trube *et al.*, 1986). Little or no ATP present in the recording pipette resulted in a progressive activation of the ATP-K^+ conductance due to dialysis of the cell interior. Dose-inhibition curves were obtained by measuring the amplitudes of the current responses (I) during exposure to the sulphonylurea and comparing them with those observed under control conditions (I_c). Values for the controls were obtained by calculating the mean amplitude before and after application of tolbutamide, enabling the slow process of 'run down' to be taken into account as the experiment progressed. The inhibition caused by glibenclamide proved to be virtually irreversible, therefore, the mean amplitude of the currents before drug application were taken as controls. The dose-inhibition curves were fitted by the following equation.

$$I/I_c = 1/(1 + (\alpha/c)^n)$$

where α = tolbutamide/glibenclamide concentration, c = half maximal inhibitory concentration and n = Hill coefficient.

Results

Insulin release studies

The sulphonylureas, tolbutamide and glibenclamide, were found to be potent stimuli for insulin release in this cell line, comparable in the response produced to a combination of leucine and glucose (Table 1 and Figure 2). The effects of these drugs were shown to be concentration-dependent (Figure 2), half-maximal release being elicited by approximately 40 μM tolbu-

Table 1 Effects of diazoxide on insulin release stimulated by leucine plus glucose and by tolbutamide on CRI G1 cells

Treatment	*Insulin release* (pmol per 10^6 cells $15\,min^{-1}$)
Control	0.45 ± 0.20
Diazoxide (1 mM)	0.70 ± 0.10
Leucine (20 mM) + glucose (3 mM)	3.85 ± 0.50
Leucine (20 mM) + glucose (3 mM) + diazoxide (1.0 mM)	0.90 ± 0.40
Tolbutamide (0.5 mM)	4.35 ± 0.15
Tolbutamide (0.5 mM) + diazoxide (1.0 mM)	3.00 ± 0.25

The results are expressed as mean ± s.e.mean of triplicate dishes of cells used in a single experiment. Each dish contained 6×10^5 cells. The insulin content of the medium at zero time was 0.95 ± 0.05 pmol per 10^6 cells.

tamide and 200 nM glibenclamide. Insulin release was also stimulated in these cells by HB 699 (0.1 mM producing an approximately two fold increase). HB 699 is a benzoic acid derivative of the non-sulphonylurea moiety of glibenclamide, which has been shown previously to possess hypoglycaemic properties (Geisen *et al.*, 1985) and cause insulin release *in vivo* (Ribes *et al.*, 1981) and *in vitro* (Glatt & Schatz, 1981).

In contrast, diazoxide (1 mM), a known hyperglycaemic agent which inhibits insulin release in man (Dollery *et al.*, 1962; Seltzer & Allen, 1965) and other animals (Tabachnick & Gulbenkian, 1968), had little effect on the basal insulin release from these cells but was an extremely effective inhibitor of maximally stimulated insulin release produced by leucine plus glucose (Table 1). However, this concentration of diazoxide did not completely inhibit insulin release stimulated by 0.5 mM tolbutamide (Table 1).

Membrane patch studies

Before single channel studies of B-cells, it was thought that the sulphonylureas stimulated insulin secretion by binding to the extracellular surface of the cell (Gylfe *et al.*, 1984). Initial patch-clamp studies supported this conclusion by demonstrating that both tolbutamide and glibenclamide inhibited ATP-K^+ channels when applied directly to outside-out membrane patches from this cell line (Sturgess *et al.*, 1985; Ashford *et al.*, 1986). It was found that both drugs produced a dose-dependent inhibition of the ATP-K^+ channel activity, that glibenclamide was the more potent and also showed a lack of reversibility on wash-out (data not shown). However, it has been found that tolbutamide is also able to block the ATP-K^+ channel when applied to the intracellular face of the membrane (Trube *et al.*, 1986). As can be seen in Figure 3(a,b), both tolbutamide and glibenclamide inhibit the ATP-K^+ channel in a dose-dependent manner when applied to inside-out membrane patches formed from this cell line. Furthermore, this figure shows that the relative potencies of tolbutamide and glibenclamide on inside-out patches are unchanged compared to outside-out patches (Sturgess *et al.*, 1985; Ashford *et al.*, 1986) and that the action of glibenclamide is not reversible on wash. A comparison of the degree of inhibition elicited by those sulphonylureas on the separate membrane faces (Table 2) suggests that these drugs may be more effective when applied to inside-out (i.e. intracellular face) than outside-out (extracellular face) patches, although a significant difference was obtained only for 1 mM tolbutamide.

As it has previously been argued that the highly potent sulphonylureas such as glibenclamide interact with two distinct sites in the plasma membrane of B-cells (one site recognising the sulphonylurea moiety, the other site a different chemical grouping (Brown & Foubister, 1984; Rufer & Losert, 1979), the action of HB699 on the ATP-K^+ channel was tested using outside-out membrane patches. This drug also produced a marked inhibition of the ATP-K^+ channel activity (Figure 4a), with a potency similar to that of glibenclamide (Table 2). There appeared to be little reversal on washout of this agent. In addition, the glibenclamide derivative HB985 (which has a methyl group in place of the cylcohexane ring, see Figure 1) was also active in inhibiting the ATP-K^+ channel (Figure 4b). The potency of HB985 was 100 fold less than that of glibenclamide (Table 2) and again no reversal of action occurred on washout of the drug.

In contrast to the hypoglycaemic agents, the hyperglycaemic drug, diazoxide, produced activation of the ATP-K^+ channel when applied to the extracellular surface of outside-out membrane patches (Figure 5a). However, this effect could only be observed if the channel activity had already been partially inhibited by the presence of 0.1 mM ATP in the electrode, no activation occurred otherwise (data not shown). Thus, diazoxide, like the sulphonylureas, probably produces its effects by way of a membrane site. This is also suggested by the data in Figure 5b, where spontaneous channel openings recorded from a cell-attached patch are shown under control conditions with only saline in the bath and then inhibited by the addition of glucose plus leucine to the bathing medium. These concentrations of glucose plus leucine also induced the appearance of action potentials under these recording conditions (data not shown). Application of 0.6 mM diazoxide in addition

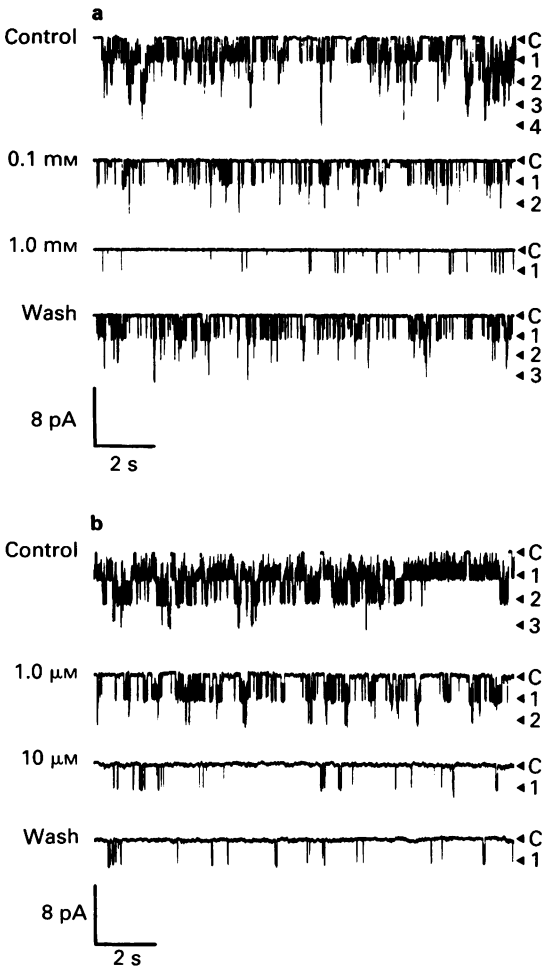

Figure 3 Single channel currents recorded from inside-out membrane patches, exposed to symmetrical 140 mM KCl, illustrating dose-dependent inhibition of the ATP-K^+ channel by (a) tolbutamide and (b) glibenclamide. Note the difference in concentrations of these drugs causing inhibition of this channel and that the action of tolbutamide is reversible unlike that of glibenclamide. The membrane potential was −50 mV for both (a) and (b) and openings (inward currents) are shown as downward deflections. The closed state (c) and the number of channels open at a given time are indicated to the right of this and all subsequent figures. Note that in these membrane patches the maximum number of channels open simultaneously is four and three, respectively. P_{OPEN} values are as follows: (a) control 0.146; 0.1 mM 0.024; 1.0 mM 0.010; wash 0.060; (b) control 0.470; 1 μM 0.127; 10 μM 0.020; wash 0.015.

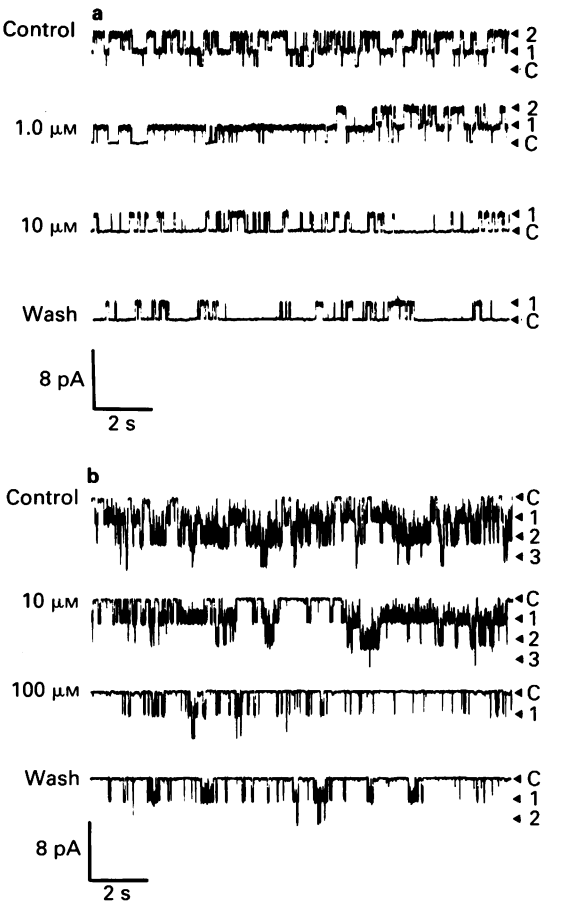

Figure 4 Dose-dependent inhibition of the ATP-K^+ channel by the glibenclamide analogues (a) HB 699 and (b) HB 985. Single channel currents recorded from outside-out membrane patches exposed to symmetrical 140 mM KCl, held at membrane potentials of (a) +50 mV and (b) −50 mV. Single channel openings are denoted by upward deflections (outward currents) in (a) and downward deflections (inward currents) in (b). Note that HB 699 is effective at a lower concentration than HB 985 and that no reversal of effect occurred on washout of HB 699, but occasionally slight reversal was observed with HB 985. P_{OPEN} values are as follows: (a) control 0.330; 1 μM 0.277; 10 μM 0.043; wash 0.035; (b) control 0.208; 10 μM 0.093; 100 μM 0.019; wash 0.051.

to the glucose plus leucine in the bathing medium (i.e. not in the electrode solution), partially reversed the inhibition of the ATP-K^+ channel induced by the glucose and leucine and also inhibited the generation of action potentials.

Another major class of K^+ selective channels present in B-cells (Cook *et al.*, 1984; Findlay *et al.*, 1985) and this cell line (Sturgess *et al.*, 1986a) is the Ca^{2+} activated K^+ channel. Neither tolbutamide nor glibenclamide had any effect on the activity of

Table 2 Inhibition of ATP-K^+ channel activity by tolbutamide, glibenclamide and analogues of glibenclamide on isolated membrane patches

[*Drug*]		*Patch configuration*	P_{OPEN} (% inhibition)	
Tolbutamide	100 μM	o/o	70.3 ± 11.8	(4)
		i/o	85.1 ± 3.1	(8)
Tolbutamide	1 mM	o/o	80.0 ± 5.1	(7)
		i/o	92.3 ± 1.5	(9)*
Glibenclamide	1 μM	o/o	70.3 ± 5.0	(9)
		i/o	79.1 ± 2.5	(8)
Glibenclamide	10 μM	o/o	81.5 ± 5.0	(8)
		i/o	93.0 ± 2.1	(6)
HB699	1 μM	o/o	38.9 ± 11.7	(5)
	10 μM	o/o	80.7 ± 4.6	(5)
HB985	10 μM	o/o	61.1 ± 6.8	(5)
	100 μM	o/o	85.9 ± 8.4	(3)

The results are expressed as % inhibition ± s.e.mean.
* $0.05 > P > 0.01$.

this channel when applied to inside-out or outside-out membrane patches. For example 10 μM glibenclamide had no effect on the open-state probability (control; 0.984; 10 μM glibenclamide; 0.986) of this channel recorded from an outside-out membrane patch, activated with 1 mM Ca^{2+} and at a membrane potential of +50 mV, agreeing with the observations of Trube *et al.* (1986). Recently it has been shown (Sturgess *et al.*, 1986b) that a calcium-activated, adenine nucleotide-sensitive, non-selective cation channel exists in these cells. It can be seen from Figure 6 that this channel is not sensitive to inhibition by the sulphonylureas. For example, regardless of the sidedness of the patch or the concentration of Ca^{2+} used to activate the channel (0.1 or 1.0 mM), tolbutamide (1 mM) produced no significant change ($n = 6$) in the P_{OPEN}, nor did 10 μM glibenclamide ($n = 5$).

Whole-cell studies

Detailed studies of the concentration-dependence and degree of reversibility of these drugs have proved impracticable on isolated membrane patches. The reasons for this are the wide variations in the channel activity between patches and, more importantly, the phenomenon of channel run-down. This process whereby the ATP-K^+ channel open-state probability decreases with time after patch excision (irrespective of sidedness of the patch) is well documented both in B-cells (Findlay *et al.*, 1985) and in cardiac (Trube & Hescheler, 1984) and skeletal (Spruce *et al.*, 1987) muscles. Thus as can be seen from Table 2, in order to obtain reliable data, relatively high concentrations of these drugs have to be tested.

An alternative method of assessing the concentration-dependence of these compounds is to use the whole-cell configuration (Trube *et al.*, 1986). By allowing dialysis of the cell interior with the pipette solution (and washout of ATP), the overall (cellular) potassium conductance observed, under voltage clamp, is due to the increased activation of the ATP-K^+ channel (Rorsman & Trube, 1985). This is illustrated in Figure 7a, where at the start of the trace (i.e. just after the formation of the whole-cell clamp) the cell has a relatively low conductance; this initially increases with time until a peak is reached and thereafter declines as run-down of channel activity occurs. Hence the run-down phenomenon is now continuous and can be quantified allowing activators and inactivators of the ATP-K^+ current to be tested at a wide range of concentrations. The total cell conductance after washout of intracellular ATP was 21.9 ± 1.4 nS ($n = 52$). In many experiments using this technique, 0.1 mM ATP was present in the patch pipette (this reduced the conductance to 12.2 ± 1.2 nS ($n = 10$)) in an attempt to slow the run-down process (Trube *et al.*, 1986) and so allow more concentrations of tolbutamide to be tested. The effects of tolbutamide, at various concentrations, on the ATP-K^+ channel current are illustrated in Figure 7b. It can be seen that it produces a dose-dependent inhibition of the current and that this is reversible on washing out the drug (i.e. the currents return to control values taking, by extrapolation, run-down into consideration). Glibenclamide also inhibited this K^+ current (Figure 7c) but with greater potency and irreversibly (even with 30 min continuous washing); thus, only one effective concentration of glibenclamide could be tested per cell. In addition, glibenclamide had a slower onset rate than

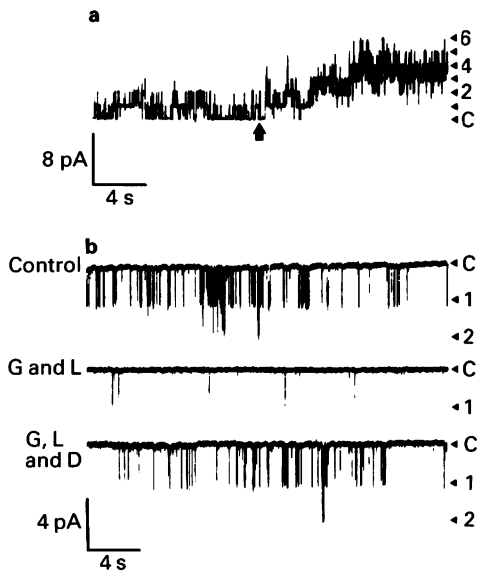

Figure 5 (a) Activation of ATP-K^+ channels by 0.6 mM diazoxide applied to an outside-out membrane patch (arrow), held at a membrane potential of zero mV. The extracellular membrane surface of the patch was exposed to solution A, whilst the intracellular surface was in contact with solution B plus 0.1 mM ATP. Channel openings are denoted by upward deflections (outward currents). (b) ATP-K^+ channel currents recorded from a cell-attached membrane patch at the cells' membrane potential. The cells were bathed in solution A, whilst the pipette contained solution F. Inward single channel currents are shown as downward deflections. Bath application of 3 mM glucose and 20 mM leucine (G and L) produced a substantial inhibition of the channel currents, this effect could be reversed by the presence of 0.6 mM diazoxide in addition to the glucose and leucine (G, L and D). P_{OPEN} values are as follows: control 0.0328; G and L 0.0007; G, L and D 0.0180.

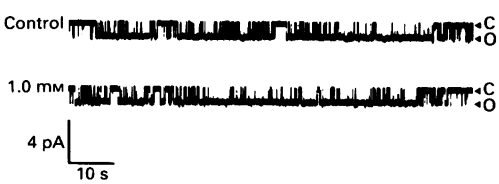

Figure 6 Lack of effect of 1 nM tolbutamide on the adenine nucleotide-sensitive Ca-NS^+ channel. Single channel current records from an outside-out membrane patch (inward currents) at a membrane potential of −50 mV. P_{OPEN} values are as follows: control 0.68; 1 mM tolbutamide 0.68. The pipette contained solution E (1 mM Ca^{2+} to activate the channel).

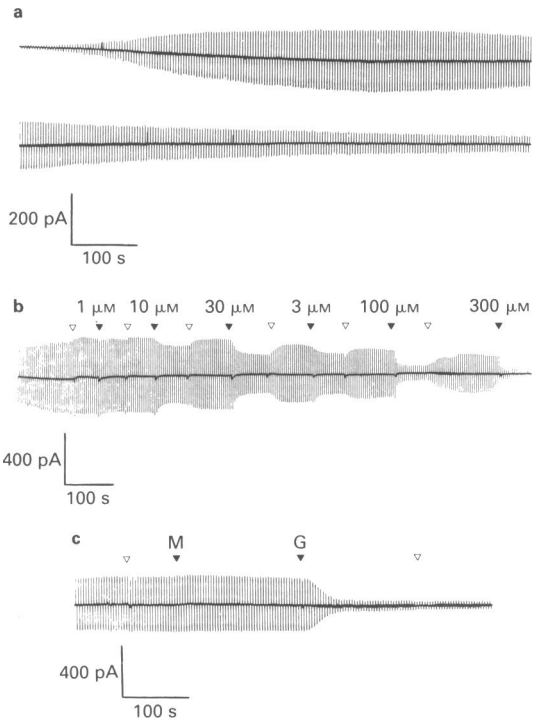

Figure 7 Recordings of whole-cell voltage-clamped K^+ currents from single CRI-G1 cells. The extracellular solution was A and the pipette contained solution B. The cell membrane was clamped at −70 mV and alternate voltage pulses of ±10 mV were applied. The current responses are denoted by the vertical lines. (a) Example of the whole-cell clamp (record starts within 1 min of attaining whole-cell clamp) showing the time course of activation of the ATP-sensitive K^+ conductance and its subsequent 'run-down'. (b) Effect of various concentrations of tolbutamide (▼) on the ATP-sensitive K^+ current. Note the reversible nature of the inhibition elicited by tolbutamide on wash (▽), taking the slow continuous run-down into account. The small downward deflections are superfusion artefacts. Before the addition of drugs, the cell was washed with solution A (▽) indicating that bath perfusion did not affect the size of the current pulses. (c) Effect of 100 nM glibenclamide (G▼) on the K^+ current. Note that the methanol control (M▼) had no effect (0.006%, used to dissolve glibenclamide), and that the action of glibenclamide was not reversible on washing (▽).

that of tolbutamide (compare Figures 7c and 9d for glibenclamide with 7b and 9c for tolbutamide). Dose-inhibition curves for both tolbutamide and glibenclamide were constructed and these are shown in Figure 8. Tolbutamide has an IC_{50} of 17 μM and a Hill coefficient of 1.0 in the absence of ATP in the pipette and there is no significant shift of the curve in

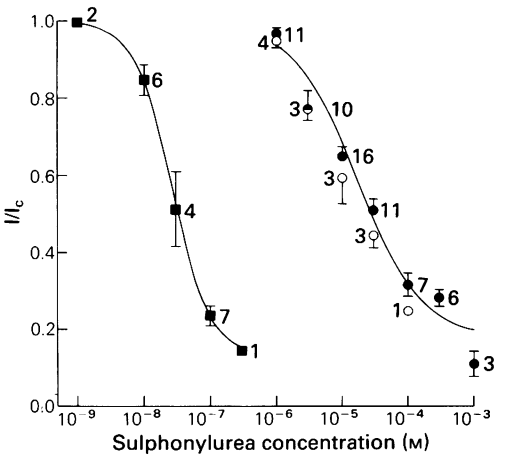

Figure 8 Concentration-inhibition curves for glibenclamide (■) and tolbutamide (●) upon ATP-K^+ currents recorded using the whole-cell configuration. The bath contained solution A whilst the electrode contained solution B. The cell membrane was clamped at −70 mV and alternate voltage pulses of ±10 mV were applied. Data are presented as fractions of control currents (I_c), taking run-down into consideration for the tolbutamide data, due to the reversible nature of its effect. The dose-dependence of tolbutamide was also tested on current partially inhibited by 0.1 mM ATP (○) present within the pipette (and therefore the cell). It can be seen that the presence of ATP had no effect on the tolbutamide inhibition. All points are the means of the indicated number of experiments; vertical lines show s.e.mean. The values of K_i (half-maximal inhibitory concentration) and the Hill coefficients were 27 nM and 17 μM and 1.5 and 1.0 for glibenclamide and tolbutamide, respectively. The curves were fitted according to the modified Marquardt non-linear least squares method (Harwell Library routine VB01A).

the presence of 0.1 mM ATP. These data, in agreement with Trube *et al.* (1986), indicate that ATP does not interfere with tolbutamide inhibition of the current. Glibenclamide produced a 50% inhibition of the K^+ current at a concentration of 27 nM and had a Hill coefficient of 1.5. Furthermore, using whole-cell recording under current-clamp, addition of tolbutamide (100–300 μM) or glibenclamide (100 nM) induced a depolarization of the cell from a value of between −60 and −70 mV to between −40 and −30 mV.

The action of diazoxide (0.6 mM) was also tested on the whole-cell ATP-K^+ current. In the absence of ATP in the pipette (and hence the cell) diazoxide had no effect on the current (Figure 9a), regardless of how much run-down had occurred. However, if ATP (0.1 mM) was present in the pipette resulting in a partial inhibition of the K^+ current, addition of diazoxide to the bath produced an increase in the current which was sustained and only slowly reversed on washout of the drug (Figure 9b). Diazoxide also reversed the inhibition induced by tolbutamide (Figure 9c) whether or not the K^+ current had previously been inhibited by ATP, but did not reverse the inhibition elicited by glibenclamide (Figure 9d).

Discussion

It is clear from these results that the cell line, CRI-G1, responds to the hypoglycaemic agents and diazoxide in terms of insulin release in a manner similar to freshly isolated B-cells. These data, in conjunction with our previous study (Sturgess *et al.*, 1985) showing that the sulphonylureas directly inhibited the ATP-K^+ channel, suggested that this cell-line would be a useful model for a more detailed study into the mechanisms of action of these drugs. Using the cell-attached membrane patch configuration, these drugs applied in the bathing solution have no direct access to the extracellular membrane within the patch electrode by virtue of the gigaohm seal (Sakmann & Neher, 1984) and so channel inhibition must occur via the drug partitioning into the rest of the extracellular membrane. In agreement with the study of Trube *et al.* (1986), tolbutamide and glibenclamide inhibited the ATP-K^+ channel whether applied to the intracellular or the extracellular membrane surface. However, from the data presented here, there is a possibility that both drugs are more effective when applied to the intracellular membrane face, i.e. the sulphonylurea receptor may be more easily accessed from the intracellular side.

The structural analogues of glibenclamide, HB699 and HB985, are both effective blockers of the ATP-K^+ channel in this cell line. The compound HB699, which is similar to the non-sulphonylurea moiety of glibenclamide, was shown to be slightly less effective than glibenclamide itself but much more potent than tolbutamide. Garrino *et al.* (1985) demonstrated that HB699 depolarized B-cells by decreasing K^+ permeability and induced insulin release. They found that HB699 was much less potent than glibenclamide and only slightly more potent than tolbutamide. These authors also showed that the effects of HB699 on $^{86}Rb^+$ efflux were reversible on washing unlike the effects, described here, on the ATP-K^+ channel. Our overall conclusion from the present studies is that HB699, and hence the non-sulphonylurea moiety, appears more potent than the sulphonylurea itself (i.e. tolbutamide) on the ATP-K^+ channel. It is possible that the highly active sulphonylureas are such potent secretagogues because they possess two active moieties in

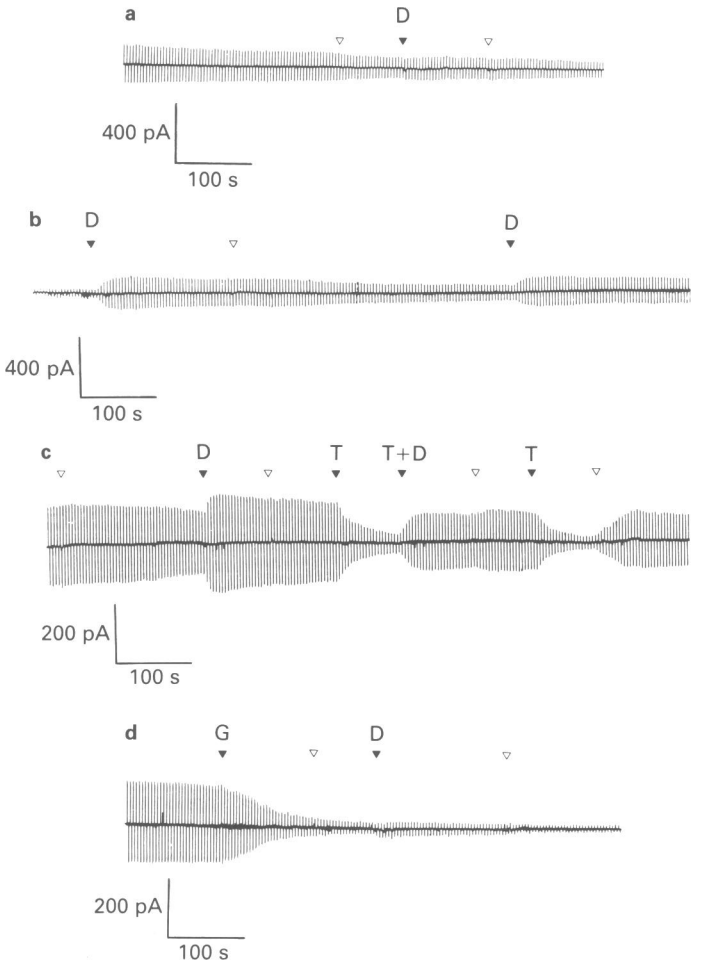

Figure 9 (a) Lack of effect of 0.6 mM diazoxide (D▼) on the ATP-sensitive K^+ current in the absence of any inhibition of the current other than run-down. Diazoxide tested 13 min after formation of whole-cell clamp. (b) In contrast to (a), with 0.1 mM ATP in the pipette producing a partial inhibition of the current, 0.6 mM diazoxide does induce an increase in the current which is not reversible on washout (▽) but appears to wane with time, such that re-application of the same concentration of diazoxide also induces an increase in current. (c) Currents enhanced by diazoxide (D▼) can be subsequently inhibited by 30 μM tolbutamide (T▼) and a further application of 30 μM tolbutamide plus 0.6 mM diazoxide will partially offset this inhibition (T + D). Note that the diazoxide effect is again not reversible on washout (▽) unlike that of tolbutamide. (d) Currents inhibited by 30 nM glibenclamide (G▼) are not reversible on washing (▽) and are not affected by 0.6 mM diazoxide (D▼).

one molecule, which can both interact with the 'receptor' (ATP-K^+ channels). Garrino *et al.* (1986), investigating the non-sulphonylurea moiety of gliquidone, came to a similar conclusion. However, the relatively low potency of HB985 is scarcely consistent with this notion, possessing as it does both moieties (the end cyclohexane ring of glibenclamide is replaced with a methyl group; see Figure 1), since it is less potent than HB699 which lacks the sulphonylurea moiety. Possibly the loss of the cyclohexane ring results in an unfavourable molecular configuration at the site for channel inhibition.

The half-maximal inhibitory concentration (K_i) of tolbutamide (17 μM) for inactivation of the ATP-K^+ channel in these cells is close to that obtained (7 μM) by Trube *et al.* (1986) for mouse pancreatic B-cells,

and, also in agreement with these authors, the dose-inhibition curve was unaffected by the presence of intracellular ATP. In addition, glibenclamide was found to have a K_i of 27 nM, i.e. to be approximately 500 fold more potent. This potency ratio was approximated in the insulin release studies where half-maximal release was elicited by 40 μM tolbutamide and 0.2 μM glibenclamide, i.e. a 200 fold difference in concentration. These values for relative potency are also close to that for hypoglycaemic potency *in vivo*.

The hyperglycaemic agent, diazoxide, activates the ATP-K^+ channel in these cells, in agreement with the data from mouse pancreatic B-cells (Trube *et al.*, 1986). It is clear from the data presented here that the activation of ATP-K^+ channels only occurs if channel activity is first depressed by 0.1 mM ATP applied internally or by bath applied tolbutamide (10–30 μM). In contrast diazoxide could not reverse the inhibition produced by glibenclamide on the channel. The lack of reversal of glibenclamide action by diazoxide may be correlated with the irreversible nature of the glibenclamide block. The ability of diazoxide to act in isolated membrane patches inhibited by ATP or tolbutamide is strongly suggestive of a membrane site of action for this drug.

The hypoglycaemic drugs and diazoxide are relatively selective for the ATP-K^+ channel, since they have no effect on the nucleotide-sensitive non-selective cation channel, or the calcium activated K^+ channel (see also Trube *et al.*, 1986), or on voltage-activated channels in B-cells (unpublished observations; Rorsman & Trube, 1985). It has recently been shown that tolbutamide can inhibit the activity of the ATP-K^+ channel present in cardiac tissue (Gee & Misler, 1987; Belles *et al.*, 1987) and that the sulphonylureas inhibit K^+ efflux in metabolically exhausted skeletal muscle, an effect attributed to inhibition of the ATP-K^+ channels (Castle & Haylett, 1987). The possibility is therefore strengthened, as we have suggested previously (Sturgess *et al.*, 1985), that there may be pharmacological effects of the sulphonylurea drugs on other tissues mediated by ATP-sensitive K^+ channels.

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