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Comparative Study of the Effects of Cromakalim (BRL 34915) and Diazoxide on Membrane Potential, $[Ca^{2+}]_i$ and ATP-Sensitive Potassium Currents in Insulin-Secreting Cells

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Summary. Patch-clamp and single cell $[Ca^{2+}]_i$ measurements have been used to investigate the effects of the potassium channel modulators cromakalim, diazoxide and tolbutamide on the insulin-secreting cell line RINm5F. In intact cells, with an average cellular transmembrane potential of -62 ± 2 mV ($n = 42$) and an average basal $[Ca^{2+}]_i$ of 102 ± 6 nM ($n = 37$), glucose (2.5–10 mM): (*i*) depolarized the membrane, through a decrease in the outward K_{ATP} current, (*ii*) evoked Ca^{2+} spike potentials, and (*iii*) caused a sharp rise in $[Ca^{2+}]_i$. In the continued presence of glucose both cromakalim (100–200 μM) and diazoxide (100 μM) repolarized the membrane, terminated Ca^{2+} spike potentials and attenuated the secretagogue-induced rise in $[Ca^{2+}]_i$. In whole cells (voltage-clamp records) and excised outside-out membrane patches, both cromakalim and diazoxide enhanced the current by opening ATP-sensitive K^+ channels. Diazoxide was consistently found to be more potent than cromakalim. Tolbutamide, a specific inhibitor of ATP-sensitive K^+ channels, reversed the effects of cromakalim on membrane potential and K_{ATP} currents.

Key Words patch-clamp · fura-2 · K_{ATP} channels · $[Ca^{2+}]_i$ · insulin-secreting cell · RINm5F cell · diazoxide · cromakalim (BRL 34915) · tolbutamide

Introduction

Control of insulin secretion from pancreatic β-cells of the islets of Langerhans is determined by the membrane potential (for reviews *see,* Petersen & Findlay, 1987; Ashcroft, 1988; Petersen, 1988). In intact resting cells, open ATP-sensitive potassium (K_{ATP}) channels (Cook & Hales, 1984) dominate the permeability of the membrane, and maintain a transmembrane potential close to the equilibrium potential for K^+ (Findlay, Dunne & Petersen, 1985; Rorsman & Trube, 1985; Dunne et al., 1986). Carbohydrate secretagogues close these channels, thereby causing a membrane depolarization (Ashcroft, Harrison & Ashcroft, 1984; Dunne et al., 1986) which is required for the opening of voltage-gated Ca^{2+} channels (Matthews & Sakamoto, 1975; Velasco, Petersen & Petersen, 1988) mediating the subsequent rise in the free intracellular calcium ion concentration ($[Ca^{2+}]_i$) (Wollheim & Biden, 1987). Since K_{ATP} channels play a fundamental role in governing the membrane potential and $[Ca^{2+}]_i$, pharmacological control of these channels may be of particular interest in the treatment of insulin-regulation disorders (Petersen & Dunne, 1989).

Sulphonylurea compounds, such as tolbutamide, glibenclamide and glipizide, have for a number of years been used to treat certain forms of Type II or non-insulin-dependent diabetes, due to their ability to enhance basal insulin levels (Henquin, 1980). Through the use of the patch-clamp technique it has been shown that each of these drugs will selectively close ATP-sensitive K^+ channels, thereby mimicking the effects of glucose (Trube, Rorsman & Ohno-Shosaku, 1986; Dunne, Ilott & Petersen, 1987; Ashcroft et al., 1987). Conversely, the structurally related diazoxide, used to treat certain forms of insulinomas (Altszuler, Hampshire & Morarv, 1977), has the opposite effect; inhibiting insulin release (Henquin et al., 1982) by repolarizing the membrane (Henquin & Meissner, 1982). Patch-clamp experiments have shown that this drug will specifically open K_{ATP} channels (Trube et al., 1986; Dunne et al., 1987), by a mechanism dependent on protein phosphorylation (Dunne, 1989).

Cromakalim (BRL 34915) is one member of a family of novel benzopyran derivatives (Hamilton, Weir & Weston, 1986), that has been found to have vasodilatory and antihypertensive effects on a number of smooth muscle preparations (for review *see,* Cook, 1988), and recently shown to open K^+ channels in arterial smooth muscle cells (Standen et al., 1989) and cardiac myocytes (Escande et al., 1988).

Since it has been reported from in vivo studies that cromakalim increases plasma glucose levels (Cook, Quast & Weir, 1988; Quast & Cook, 1989), the present study was undertaken to examine the effects of cromakalim on membrane potential, $[Ca^{2+}]_i$ and ATP-sensitive K^+ currents in the clonal insulin-secreting cell line RINm5F.

Materials and Methods

Cell Isolation and Maintenance

All experiments were carried out on the clonal insulin-secreting cell line RINm5F, maintained as previously described (Dunne et al., 1988*b*, 1989).

Media

The standard extracellular Na^+-rich solution used throughout these experiments, contained (in mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 $CaCl_2$ and 10 HEPES. The pH was set at 7.2 using NaOH. The standard intracellular K^+-rich solution contained (in mM): 140 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 HEPES, 1 EGTA and 1 ATP. No $CaCl_2$ was added and the pH set at 7.2 with KOH. Stock solutions of diazoxide (Glaxo Research, UK) and tolbutamide (Sigma, UK) were prepared in dimethylsulphoxide (DMSO), whereas stock solutions of cromakalim (BRL 34915) (±6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzopyran-3-ol) (Beecham Pharmaceuticals, UK) were prepared in 70% ethanol (vol/vol). The maximal concentrations of DMSO, 1%, and ethanol, 2%, used in the final solutions for experiments had no direct effect upon K^+ channels in 10 (Dunne et al., 1987) and five (7/7 applications) separate patches, respectively. The osmolarity of all solutions was 290 ± 5 mOsm/kg. All experiments were conducted at room temperature 22–25°C.

Patch-Clamp Experiments

Single-channel current recordings, from outside-out membrane patches and whole-cell current and voltage measurements (Hamill et al., 1981), were made with the K^+-rich solution in the pipette and the Na^+-rich solution in the bath. Patch-clamp pipettes (Type 101 PB, Ceebee Glass, Denmark) were found to have a final resistance of between 5 and 10 MΩ when filled. Exchange from control to test solutions was achieved manually under visual control (Dunne, Findlay & Petersen, 1988*a*). All current and voltage recordings were stored on FM tape (Racal 4DS recorder) for subsequent replay and analysis. Patch-clamp single-channel current recordings have been photographed directly from the oscilloscope screen, with upward deflections representing outward current flow (i.e., from the inside to the outside of the membrane patch). Whole-cell current (voltage-clamp) and voltage (current-clamp) records have been photographed directly from pen-recording traces, filtered at 20 Hz (low pass). Changes in potassium channel open-state probability have been expressed as a percentage of the precontrol level of activity (Dunne et al., 1989; Dunne, 1989). This particular method of quantification was preferred to that of expressing an absolute value of open probability, since the finite number of operational K^+ channels in a particular patch of membrane is often unknown (Dunne et al., 1989). Changes in whole-cell K_{ATP} currents, obtained using voltage pulses of 20 mV from a holding potential of −60 mV (100 msec duration, 1 pulse every 0.8 sec), have been expressed as a percentage of the precontrol value.

Measurements of Single Cell $[Ca^{2+}]_i$

RINm5F cells were loaded with fluorescence indicator fura-2 by a 30-min preincubation in the Na^+-rich solution with 3 μM fura-2-acetoxymethyl ester at room temperature. Changes in single cell $[Ca^{2+}]_i$ were assessed using dual-excitation microfluorimetry (Grynkiewicz, Poenie & Tsien, 1985), with a Spex (Glen spectra) DM 3000 CM system, as previously described (Yule & Gallacher, 1988). $[Ca^{2+}]_i$ was estimated from the ratio (R) of the fluorescence at 340 and 380 nm according to the formula (Schlegel et al., 1987):

$$[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$$

where K_d = 225 nM (Grynkiewicz et al., 1985), R_{max}, R_{min} and β are constants; 7.9 ± 0.4 (n = 6), 0.49 ± 0.08 (n = 6) and 3.9 ± 0.4 (n = 6), respectively. These constants were determined using the *in situ* calibration procedures described by Schlegel et al. (1987, 1988). All records have been corrected for autofluorescence at each wavelength (determined in unloaded cells) before the ratio was calculated.

Results

The Effects of Cromakalim (BRL 34915), Diazoxide and Tolbutamide on Membrane Potential and $[Ca^{2+}]_i$

The actions of cromakalim (BRL 34915), diazoxide and tolbutamide on the transmembrane potential of single RINm5F cells have been monitored using the whole-cell current-clamp variant of the patch-clamp technique. In total, 42 cells were investigated. The average spontaneous zero-current membrane potential, estimated within seconds of forming the whole cell was found to be −62 ± 2 mV (n = 42).

RINm5F cells have for a number of years been thought to be insensitive to changes in extracellular glucose, due to metabolic irregularities (Praz et al., 1983; Halban et al. 1983). In spite of this, however, Ribalet, Eddlestone and Ciani (1988) have recently shown that these cells will respond to glucose, by a mechanism involving the specific closure of ATP-sensitive K^+ channels. These data have been confirmed in our experiments. Glucose (2.5–10 mM) added to 28 separate whole cells (37 times) depolarized the membrane by on average 22 ± 2 mV (n = 37). This depolarization was associated with the generation of Ca^{2+} spike potentials (Fig. 1, upper panel) as originally described by Matthews and Sakamoto (1975). It is during the Ca^{2+} spike, that Ca^{2+} enters the cell and leads to an increase in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Fig.

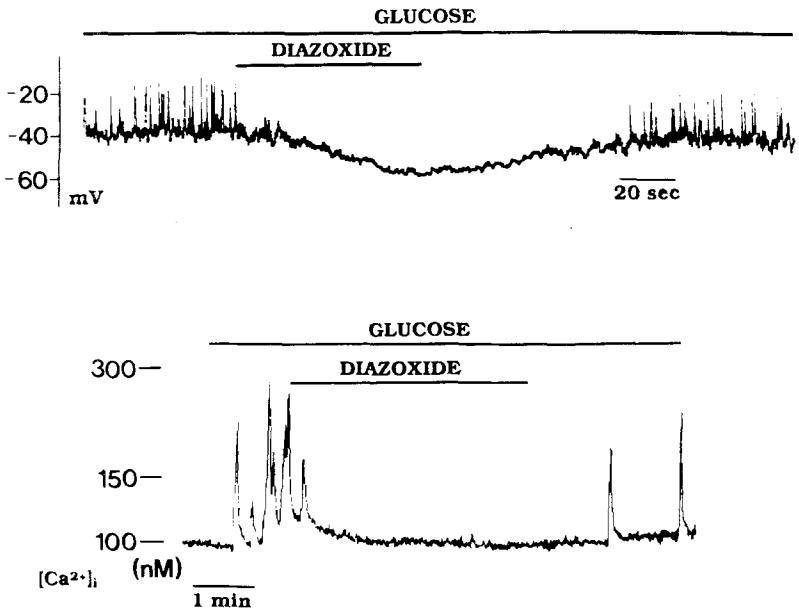

Fig. 1. The effect of diazoxide on the membrane potential, upper panel, and $[Ca^{2+}]_i$, lower panel, of glucose (2.5 mM)-stimulated RINm5F cells. The record shown in the upper panel was obtained using the patch-clamp whole cell current-clamp recording configuration, beginning 1200 sec after forming the whole cell and 35 sec after the addition of glucose to the bathing solution. Measurements of changes in $[Ca^{2+}]_i$, lower panel, have been made using dual-excitation microfluorimetry with fura-2. Diazoxide (100 μM) repolarizes the membrane in the presence of glucose, terminating Ca^{2+} spike potentials and lowering $[Ca^{2+}]_i$. Both records come from separated RINm5F cells

1, lower panel). Not all the cells, however, used in this study were able to generate Ca^{2+} spike potentials. In 33% of cells (14/42), stimulant-evoked depolarization was not associated with the presence of spike potentials, which most likely reflects differences in the division cycles of our RINm5F cell population (Rorsman, Arkhammar & Berggren, 1986). The average duration of Ca^{2+} spike potential firing in 25 out of 28 whole cells was found to be 302 ± 36 sec (n = 25), the other three cells having spike potentials that persisted for more than 15 min after initiating the experiment. The disappearance of Ca^{2+} spikes is probably due to the run-down of Ca channels, as has been described in other cell types (Byerly & Hagiwara, 1982; Fenwick, Marty & Neher, 1982; Katayama, Hofmann & Trautwein, 1985). Adding diazoxide (100 μM) to the cell in the continued presence of glucose, brings about a sharp repolarization of the membrane, which terminates the Ca^{2+} spike potentials (upper panel) and attenuates the glucose-induced rise in $[Ca^{2+}]_i$ (lower panel). When diazoxide was removed a renewed depolarization results, reintroducing Ca^{2+} spike potentials and increasing $[Ca^{2+}]_i$. This result was seen in 22 (29/30 applications) (upper panel) and six (lower panel) separate cells.

Cromakalim (BRL 34915) gave similar responses to those described for diazoxide (Fig. 2). In the record shown (upper panel), the cell had an initial membrane potential of −56 mV, and at the time the experiment commenced was found to be firing a number of spontaneous Ca^{2+} spike potentials. However, when glucose (2.5 mM) was added, a further depolarization resulted, which enhanced the frequency of spike-potential firing (upper panel). Cromakalim (100 μM) in the presence of glucose, repolarized the membrane, terminated Ca^{2+} spike potentials (upper panel) and lowered the glucose-induced rise in $[Ca^{2+}]_i$ (lower panel). At concentrations between 100 and 200 μM, cromakalim invariably reversed the effects of glucose upon membrane potential and $[Ca^{2+}]_i$ in 23 (26/29 applications) and 5 separate cells, respectively. Cromakalim in concentrations between 10 and 50 μM had weak and inconsistent effects; 3/6 applications to five separate cells caused a slight repolarization of the membrane, with 3/6 attempts having no effects. Figure 3 shows that the sulphonylurea tolbutamide (100 μM), a selective inhibitor of K_{ATP} channels, markedly repolarizes the membrane in the presence of cromakalim, a result typical of 5 separate whole cells (6/6 applications).

The Effects of Cromakalim (BRL 34915), Diazoxide and Tolbutamide on K_{ATP} Currents

The patch-clamp whole-cell voltage-clamp recording configuration has been used to investigate the effects of the potassium channel modulators on the whole cell K_{ATP} currents. Outward K^+ currents, elicited by 20 mV depolarizing pulses (100 msec duration, 1 pulse every 0.8 sec) from a holding potential of −60 mV, were reduced when glucose (2.5–10 mM) was added to the bathing solution, due to closure of ATP-sensitive potassium channels (Fig. 4). In the continued presence of glucose both

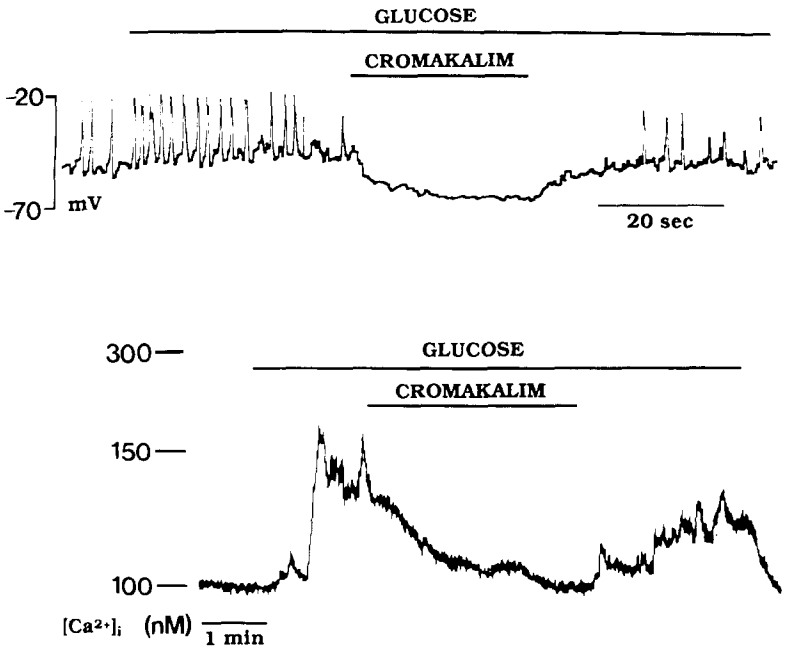

Fig. 2. The effect of cromakalim (100 μM) on the membrane potential, upper panel, and $[Ca^{2+}]_i$, lower panel, of glucose (2.5 mM)-stimulated RINm5F cells. The record shown in the upper panel was obtained using the whole cell current-clamp recording configuration, and begins 130 sec after forming the whole cell. Measurements of changes in $[Ca^{2+}]_i$, lower panel, have been made using dual-excitation microfluorimetry. Both records come from separate RINm5F cells

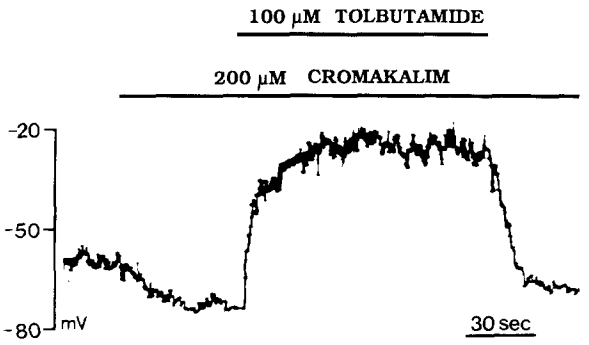

Fig. 3. The effect of tolbutamide (100 μM) on the cromakalim (200 μM)-induced repolarization of a single RINm5F cell. Cromakalim was added to the cell 20 sec after glucose had depolarized the membrane from −70 to −56 mV (*not shown*). The record begins 390 sec after forming the whole cell current-clamp recording configuration and 370 sec after the disappearance of Ca^{2+} spike potentials. Glucose was present for the duration of the experiment

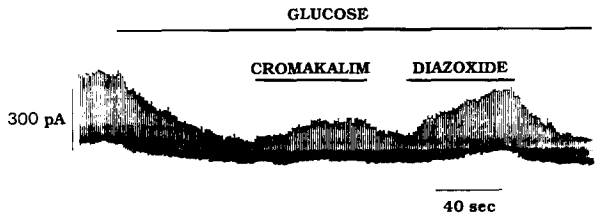

Fig. 4. A comparison between the actions of 200 μM cromakalim and 100 μM diazoxide on glucose (2.5 mM)-inhibited whole cell K_{ATP} currents in a single RINm5F cell. The record begins 600 sec after forming the whole cell recording configuration

cromakalim (100–200 μM) and diazoxide (100 μM) enhanced K_{ATP} currents, a result typical of 9 (11/11 applications) and 7 (8/8 applications) cells, respectively. A quantitative analysis of 12 of these cells has been presented in Fig. 5. Cromakalim-activated K_{ATP} currents were inhibited by tolbutamide (100 μM) in three cells (5/5 applications), as shown in Fig. 6. No significant effect of cromakalim was found between 10 and 50 μM (n = 4 separate cells, 6/6 applications).

The actions of cromakalim, diazoxide and tolbutamide on single K_{ATP} channel currents has been studied using the patch-clamp excised outside-out membrane patch recording configuration, with 1 mM ATP on the cytosolic side of the membrane. Figure 7 shows that, within the same patch of membrane, both cromakalim (100 μM) and diazoxide (100 μM) reversibly activate ATP-sensitive K^+ channels. Similar effects were seen in an additional 19 separate outside-out membrane patches, that were exposed to both cromakalim (100–200 μM) (23/23 times) (*see* Fig. 9*A*) and diazoxide (100 μM) (26/26 times) (*see* Fig. 9*C*).

Interactions between cromakalim and tolbutamide have been investigated. Figure 8 shows that in this particular patch of membrane openings of ATP-sensitive K^+ channels had ceased prior to the application of cromakalim, due to channel run-down (Findlay et al., 1985; Findlay & Dunne, 1986). Cromakalim (200 μM), added to the outside of the membrane, reversed the effects of run-down. In the continued presence of the drug, tolbutamide (100 μM) closed cromakalim-stimulated K^+ chan-

nels, a result seen in four separate patches (10/10 times). A quantitative analysis of the effects of tolbutamide on cromakalim-activated K^+ channels and cromakalim on tolbutamide-inhibited channels (seen 15/15 times in six separate patches) has been presented in Fig. 9*A* and *B*, respectively.

Discussion

Control of K_{ATP} channels in insulin-secreting cells through the use of pharmacological tools (Cook, 1988) is of considerable interest to the treatment of a number of insulin- and/or glucose-regulation disorders, since the gating of these channels governs the membrane potential of the cell. Tolbutamide mimics the effects of glucose on β-cells by closing K^+ channels, depolarizing the membrane, causing spike potentials and increasing $[Ca^{2+}]_i$. Diazoxide, on the other hand, has the opposite effect, lowering basal insulin levels by decreasing $[Ca^{2+}]_i$, through a repolarization of the membrane, brought about by the activation of ATP-sensitive K^+ channels (for reviews *see* Petersen, 1988; Dunne & Petersen, 1989). The actions of cromakalim on vascular smooth muscle cells also appear to be mediated by a direct effect on K^+ channels, since vasodilation has been shown to be associated with a hyperpolarization of the cell membrane, an enhanced ^{86}Rb or ^{42}K efflux (Hamilton et al., 1986; Shetty & Weiss, 1987) and the activation of ATP-sensitive potassium channels (Standen et al., 1989). Cromakalim also appears to activate whole-cell (Sanguinetti et al., 1988) and single K_{ATP} channel currents (Escande et al., 1988) in isolated cardiac muscle cells.

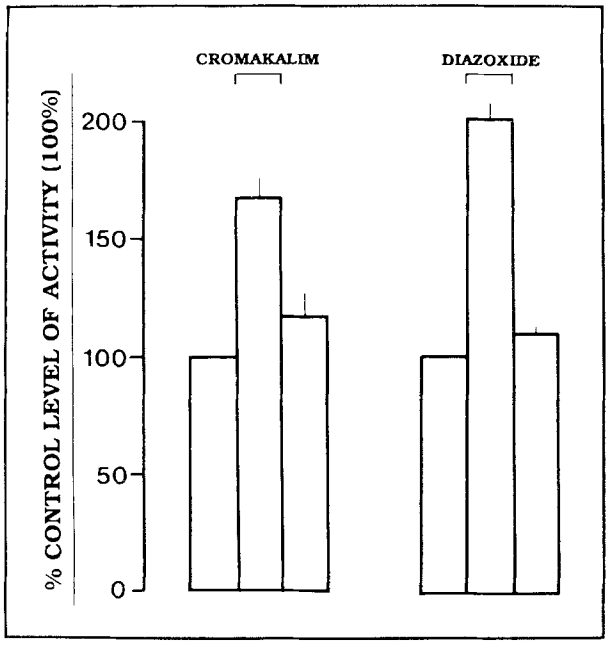

Fig. 5. Comparison between the effects on cromakalim (200 μM) and diazoxide (100 μM) on the average whole cell K_{ATP} current recorded in 12 separate RINm5F cells. Average values (mean ± SEM) have been expressed as a percentage of the control level of activity for eight and seven applications of cromakalim and diazoxide, respectively

In vivo cromakalim has been reported to enhance plasma glucose levels (Cook et al., 1988; Quast & Cook, 1989), effects that can now be explained by our experiments, since cromakalim activates K_{ATP} currents in excised outside-out membrane patches (Fig. 7) and in RINm5F whole cells (Fig. 4). In the presence of glucose open K_{ATP} channels would repolarize the membrane, terminate Ca^{2+} spike potentials and attenuate the glucose-induced rise in $[Ca^{2+}]_i$ (Fig. 2), the key intracellular regulator of insulin secretion (Wollheim & Biden, 1987). That the K^+ channels activated by cromakalim are indeed the ATP-sensitive channels, has been confirmed in experiments with tolbutamide, a potent inhibitor of these channels in insulin-secreting cells (Trube et al., 1986; Dunne et al., 1987). Cromakalim had no significant effect on tolbutamide-inhibited K^+ channels (Fig. 9), and the sulphonylurea (*i*) inhibited cromakalim-activated K^+ currents in whole cells (Fig. 6) and excised membrane patches (Figs. 8 and 9) and also (*ii*) reversed the effects of cromakalim on the membrane poten-

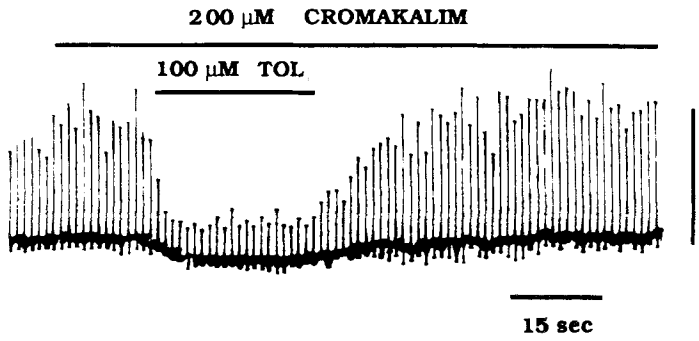

Fig. 6. Tolbutamide (100 μM)-inhibition of cromakalim (200 μM)-activated K_{ATP} currents in a single RINm5F whole cell voltage-clamp record. The experiment begins 700 sec after forming the whole cell and 100 sec after adding glucose (10 mM) to the bathing solution. Glucose was present for the duration of the experiment

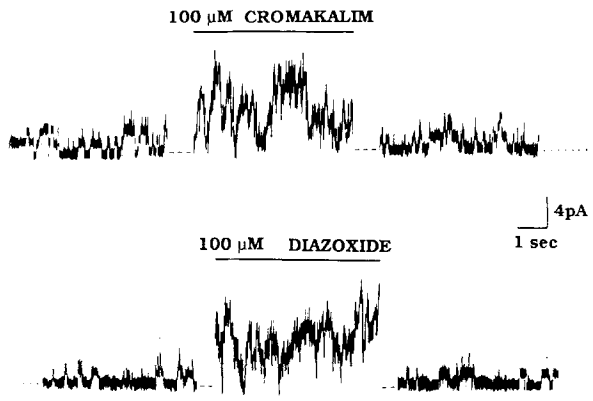

Fig. 7. Activation of ATP-sensitive K^+ channels by 100 μM cromakalim and 100 μM diazoxide. All single-channel current data comes from the same excised outside-out membrane patch, obtained with 1 mM ATP added on the cytosolic surface of the membrane. The delay between the end of the upper panel and the beginning of the lower panel is 60 sec

tial (Fig. 3). Interactions between diazoxide and tolbutamide have previously been described (Trube et al., 1986; Dunne et al., 1987).

Overall, the effects of cromakalim on K_{ATP} channels were significantly less marked than those of equimolar concentrations of diazoxide. Figure 9 shows that on average 200 μM cromakalim enhanced the K_{ATP} current recorded in outside-out membrane patches by 203 ± 10% (n = 8) of the control level of activity, whereas diazoxide, at half the concentration, increased the current by 341 ± 60% (n = 8). Similar effects were found in the measurements of whole-cell K_{ATP} currents (Figs. 4 and 5). Below 100 μM the effects of cromakalim on K_{ATP} currents, membrane potential and $[Ca^{2+}]_i$, were consistently found to be weak and insignificant, whereas diazoxide has been shown to be an effective opener of K_{ATP} channels at concentrations as low as 20 μM (Dunne et al., 1987; Schmid-Anto-

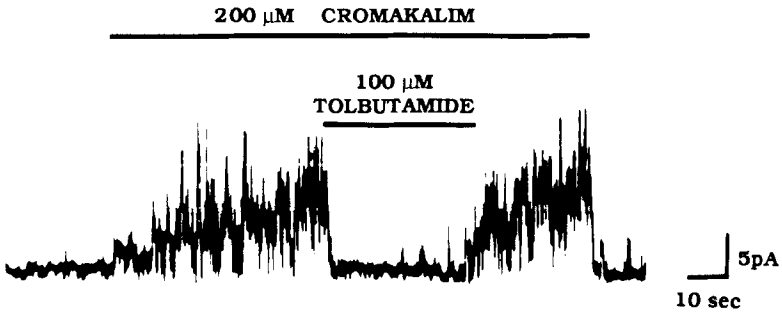

Fig. 8. Tolbutamide (100 μM)-inhibition of cromakalim (200 μM)-activated ATP-sensitive K^+ channels in a RINm5F outside-out membrane patch

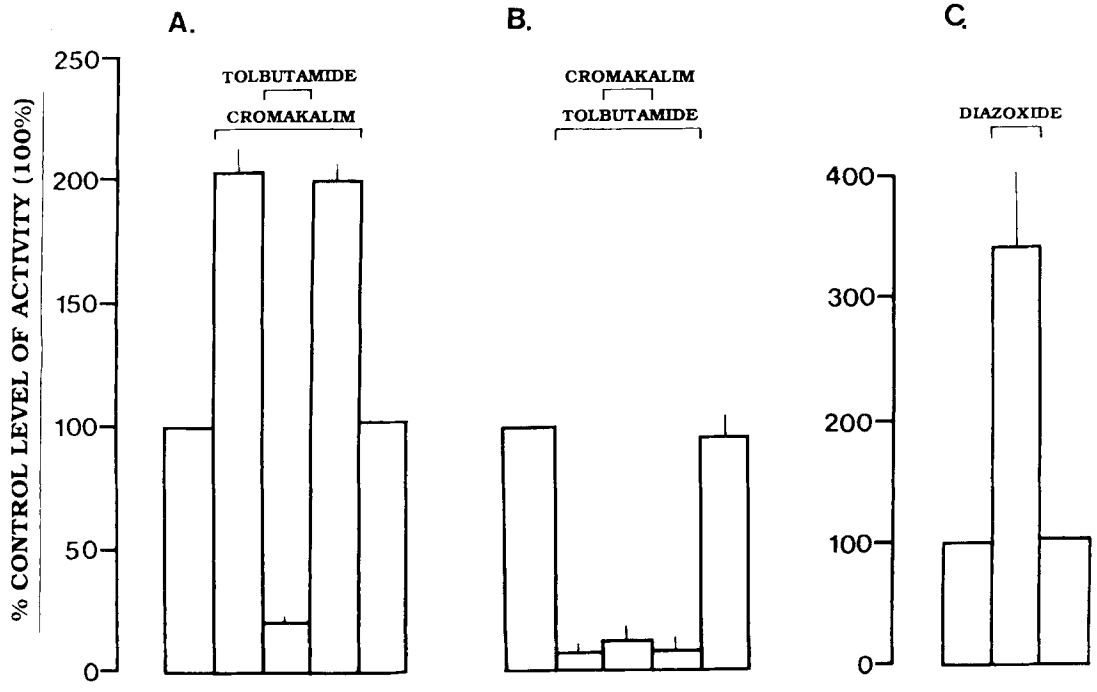

Fig. 9. Quantitative analysis of interactions between tolbutamide (100 μM) and cromakalim (200 μM), (*A*) and (*B*), and the effects of diazoxide (100 μM) on ATP-sensitive K^+ channels, (*C*). All data comes from RINm5F outside-out membrane patches. Average values (mean ± SEM) have been expressed as a percentage of the control level of activity for four (n = 10 applications), six (n = 15) and eight (n = 8) separate patches for (*A*), (*B*) and (*C*), respectively. Note the change in vertical scale used in (*C*).

marchi et al., 1987). Similarly, in cardiac myocytes cromakalim appears only to be an efficient activator of channels at concentrations around 300 μM (Escande et al., 1988).

The observations that the β-cell and cardiac K_{ATP} channel (Escande et al., 1988) have a low affinity for cromakalim, whereas the smooth muscle channel has a high affinity (Standen et al., 1989), may indicate a degree of structural diversity in ATP-sensitive K^+ channels in different tissues. Further evidence for this is provided by Faivre and Findlay (1989) who have recently shown that the sulphonamide diazoxide, a potent activator of both the β-cell (Dunne et al., 1987; Dunne, 1989) and smooth muscle K^+ channel (Standen et al., 1989), actually inhibits similar channels in ventricular muscle cells. Since, it is so far only in insulin-secreting cells that detailed investigations of the mechanism by which diazoxide (Dunne et al., 1987; Dunne, 1989) and cromakalim (Dunne, Aspinall & Petersen, 1990) opens channels have been carried out, the results of similar experiments with cardiac and smooth muscle cells should be interesting.

Regarding the physiological relevance of our findings, we can conclude that cromakalim is unlikely to be an effective substitute for diazoxide as a novel hyperglycemia-inducing compound, since the concentrations required by cromakalim to open K^+ channels are substantially higher than those required by diazoxide. Our findings do, however, have important implications for the possible use of cromakalim and cromakalim-related compounds as effective anti-hypertensive compounds. In vivo cromakalim has been shown to have significant effects upon K_{ATP} channels and both the electrical and mechanical activities of vascular smooth muscle cells at concentrations below 1 μM (Standen et al., 1989, and Hamilton et al., 1986, respectively). Within this particular dosage range cromakalim would have no effect upon the gating of K^+ channels in the insulin-secreting cells, and therefore no effect upon either plasma glucose or insulin levels, avoiding any possible hyperglycemic side effects.

In summary we have shown that cromakalim will open single- and whole-cell K_{ATP} currents in insulin-secreting cells. In the presence of glucose this results in membrane repolarization, the termination of Ca^{2+} spike potentials and the lowering of carbohydrate-inducing rises in $[Ca^{2+}]_i$.

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