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The effects of cromakalim on ATP-sensitive potassium channels in insulin-secreting cells

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1 The single-channel current recording technique has been used to investigate the effects of cromakalim, diazoxide and ATP, separately and combined, on the opening of ATP-sensitive potassium channels in the insulin-secreting cell-line RINm5F. The actions of these drugs have been studied using the permeabilized open-cell variation of the patch-clamp technique.

2 In the absence of internal ATP, cromakalim (80–200 μM) was unable to open ATP-sensitive K^+ channels but when ATP was present both cromakalim and diazoxide caused channel openings.

3 Interactions between ATP and cromakalim seemed competitive. Concentrations of cromakalim in the range 80–200 μM readily activated channels inhibited by 0.1 mM ATP, but had no effects when the concentration of ATP was increased to 0.5–2 mM. Only when the concentration of cromakalim was increased to 400–800 μM could opening of 0.5–2 mM ATP-inhibited channels be regularly observed. In the continued presence of cromakalim (400–800 μM), an increase in the internal concentration of ATP from either 0.25 to 0.5 mM or 1 to 2 mM, inhibited cromakalim-activated K^+ channels.

4 Activation of ATP-inhibited K^+ channels was abolished by replacing ATP with ATPγS and cromakalim had no effects on ATPγS-inhibited channels. This suggests that cromakalim may open K_{ATP} channels in insulin-secreting cells by a mechanism which involves protein phosphorylation.

Introduction

Potassium selective ion channels whose gating is regulated by changes in the internal concentration of adenosine-5'-triphosphate (ATP) (K_{ATP} channels) have been described in the membranes of cardiac muscle (Noma, 1983; Kakei & Noma, 1984), skeletal muscle (Spruce *et al.*, 1985; 1987), smooth muscle (Davies *et al.*, 1989), hypothalamic neurones (Ashford *et al.*, 1989) and pancreatic β-cells (Cook & Hales, 1984; Findlay *et al.*, 1985; Rorsman & Trube, 1985). In intact β-cells, where openings can be recorded under resting conditions, K_{ATP} channels govern the regulation of insulin secretion. When challenged with carbohydrate secretagogues, metabolism of the sugars results in the specific closure of K_{ATP} channels, initiating a depolarisation of the membrane (Ashcroft *et al.*, 1984; Rorsman & Trube, 1985; Dunne *et al.*, 1986). This depolarisation is required for the opening of voltage-gated Ca^{2+} channels (Velasco *et al.*, 1988; Velasco & Petersen, 1989), which mediates an increase in the free intracellular calcium ion concentration—a key internal regulator of insulin secretion (Wollheim & Biden, 1987). It is not surprising, therefore, that pharmacological agents that influence the gating of K_{ATP} channels (Cook, 1988) in insulin-secreting cells have had a significant influence on the treatment of insulin- and/or glucose-regulation disorders (Petersen, 1988; Petersen & Dunne, 1989).

It has previously been shown that compounds such as the sulphonylurea drugs (tolbutamide, glibenclamide, glipizide, etc), used to treat certain forms of Type II or non-insulin dependent diabetes, mimic the effects of glucose on insulin-secreting cells by specifically closing K_{ATP} channels (Sturgess *et al.*, 1985; Trube *et al.*, 1986; Dunne *et al.*, 1987). Conversely, the sulphonamide diazoxide has been used to treat insulinomas of the pancreas (Altszuler *et al.*, 1977) since it inhibits insulin release (Henquin *et al.*, 1982), through a repolarisation of the cell membrane (Henquin & Meissner, 1982) brought about by the specific activation of K_{ATP} channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989).

Cromakalim, a putative antihypertensive agent, has been shown to relax smooth muscle cells (Hamilton *et al.*, 1986). Since this relaxation is associated with hyperpolarisation of the membrane and an enhanced ^{86}Rb and ^{42}K efflux, it has been suggested that cromakalim acts by specifically opening K^+ channels (Hamilton *et al.*, 1986; Shetty & Weiss, 1987). In cardiac smooth muscle cells the actions of cromakalim are antagonised by glibenclamide (Quast & Cook, 1988; Winquist *et al.*, 1989), indicating that the site of action of the drug may be the ATP-sensitive K^+ channels, recently confirmed in experiments carried out by Escande *et al.* (1988) and Sanguinetti *et al.* (1988). Results presented in this study suggest that cromakalim opens ATP-sensitive K^+ channels in insulin-secreting cells. This may explain why, *in vivo*, cromakalim increases plasma glucose levels (Cook *et al.*, 1988).

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.*, 1988b; 1989a).

Patch-clamp experiments

Single-channel current recordings (Hamill *et al.*, 1981) were obtained using the open- or permeabilized cell variant of the patch-clamp technique (Maruyama & Petersen, 1984; Dunne *et al.*, 1986). The generation of the open-cell recording configuration and the arrangement of the superfusion system used to exchange control with test solutions has been previously described (Dunne *et al.*, 1986; 1987; 1988a). Patch-clamp pipettes (Type 101 PB, Ceebee Glass, Denmark) had a final resistance of between 5 and 10 MΩ when filled with the Na^+-rich solution. The pipette voltage in all cases was held at 0 mV throughout the experiment. All current records were stored on FM tape (Racal 4DS recorder) for subsequent replay and analysis. Changes in potassium channel open-state probability have been estimated from continuous stretches of current records lasting between 15 and 30 s and expressed as a percentage of the pre-control level of activity (100%) (Dunne *et al.*, 1989a; Dunne, 1989). This method of quantification was preferred to that of expressing a finite value of open probability, since within a particular patch of membrane the definitive

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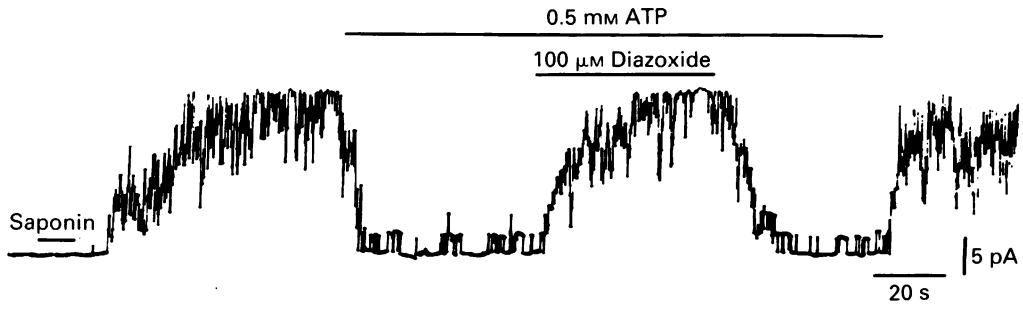


Figure 1 Diazoxide (100 μM)-activation of 0.5 mM ATP inhibited K^+ channels in a RINm5F open-cell. The record begins 30 s after a tight seal had been formed between the outer surface of an intact cell and tip of a patch-clamp pipette. Saponin (0.05%) was added to the bath solution at the point indicated. Potassium channels activated by permeabilization of the cell were inhibited by ATP and reactivated in the presence of the nucleotide by diazoxide. Channel openings are represented by upward deflections.

number of operational K^+ channels is often unknown (Dunne *et al.*, 1989a). Patch-clamp single-channel current recordings have either been photographed from the oscilloscope screen, filtered at 500 Hz (low pass) or from pen-recording trace, filtered at 20 Hz (low pass). Upward deflections in the recordings represent outward current flow, i.e. from the inside to the outside of the membrane patch.

Media

The standard extracellular Na^+-rich solution used throughout these experiments, contained (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.13, glucose 2.5, HEPES 10, and EGTA 1. No Ca^{2+} was included and the pH was adjusted to 7.2 with NaOH. This solution was used initially in the bath, following the removal of tissue culture media. Before experimentation the Na^+-rich solution was replaced by a standard K^+-rich medium containing (mM): KCl 140, NaCl 10, $MgCl_2$ 1.13, HEPES 10 and EGTA 1. No Ca^{2+} was included and the pH was adjusted to 7.2 with KOH. Stock solutions of cromakalim ((±)-6-cyano-3,4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzopyran-3-ol) (Beecham Pharmaceuticals, U.K.) were prepared in 70% ethanol (vol/vol) and stock solutions of diazoxide (Glaxo Research Limited, U.K.) prepared in dimethylsulphoxide (DMSO). The maximal concentrations of ethanol (2%) and DMSO (1%) used in the final solutions for experiments had no effects on the gating of K_{ATP} channels in 5 (11/12 applications) and 10 (Dunne *et al.*, 1987) separate open-cells, respectively. The osmolality of all solutions was 290 ± 5 mosm kg^{-1}. All experiments were carried out at room temperature (22–25°C).

Results

Opening of ATP-inhibited potassium channels by cromakalim and diazoxide

As described in a previous paper from our laboratory (Dunne *et al.*, 1986), permeabilization of an intact cell by saponin (0.05%), which was in contact with the plasma membrane outside the area from which the recording was being made, resulted in a massive increase in the K^+ current across the isolated patch of membrane, presumably due to the wash-out of cytosolic ATP (Figure 1). ATP (0.5 mM) added to the bath solution, which was now in contact with the inside of the

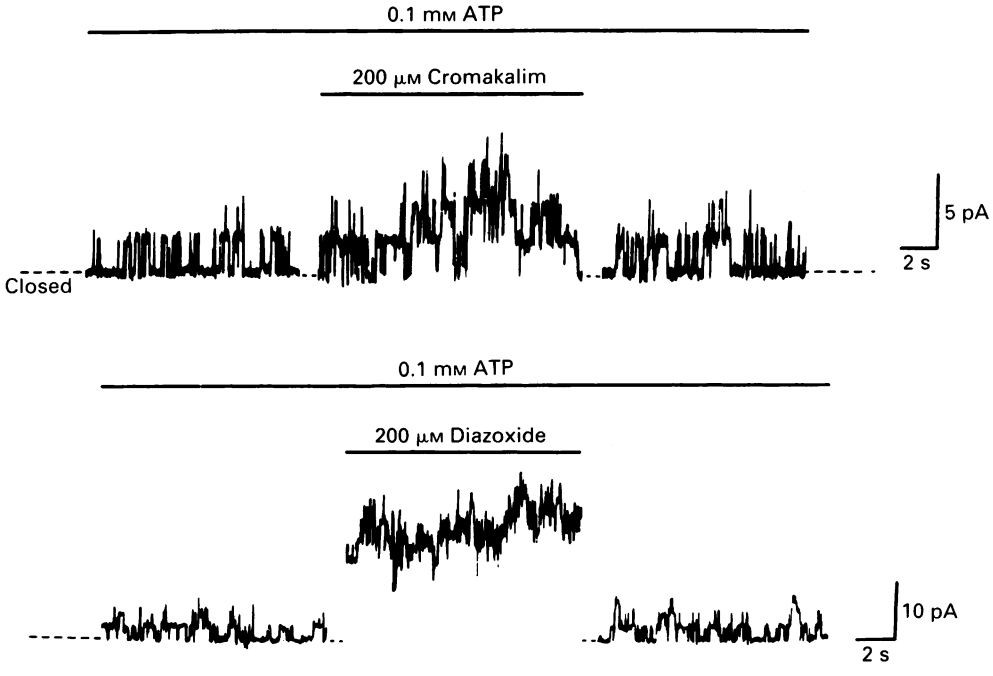


Figure 2 Cromakalim (200 μM) and diazoxide (200 μM) induced-opening of 0.1 mM ATP-inhibited K^+ channels. All current traces come from the same open-cell recording, that was exposed to 0.1 mM ATP 40 s before the first current trace. The interval between the upper and lower panel is 20 s. Note how in the lower panel the vertical scale has been compressed in order to accommodate the activation of channels brought about by diazoxide. The zero current value recorded when all the channels were closed has been indicated in this and subsequent figures by a dashed line.

membrane, inhibited this current. In the continued presence of ATP, diazoxide (100 μM) reversed the effects of the nucleotide by reactivating ATP-inhibited K^+ channels. When diazoxide was removed the K^+ current was suppressed, until finally removal of ATP increased the current to the pre-stimulus level of activity.

Figure 2 shows a typical result from a series of experiments in which comparisons were made between the effects of diazoxide and cromakalim on the gating of K_{ATP} channels, using the open-cell recording configuration. All the single-channel current data come from the same isolated patch of membrane, which had been exposed to 0.1 mM ATP before the beginning of the first current trace. The upper panel shows that cromakalim (200 μM) opened ATP-inhibited K^+ channels, whereas the lower panel shows the larger effect of diazoxide (200 μM). The interval between the end of the upper panel and the beginning of the lower was 20 s. At equimolar concentrations diazoxide was consistently found to be a stronger activator of K^+ channels than cromakalim (note how the vertical scale in the lower panel has been compressed). The experiment shown in Figure 2 was carried out in 3 additional open-cells. The effects of cromakalim were mixed; 6 out of 10 applications opened channels, with 4 attempts having no effects, diazoxide on the other hand activated K^+ channels on all 14 occasions it was added (14/14). A quantitative analysis of the actions of cromakalim (200 μM) and diazoxide (200 μM) on K^+ channels inhibited by 0.1 mM ATP is shown in Figure 3.

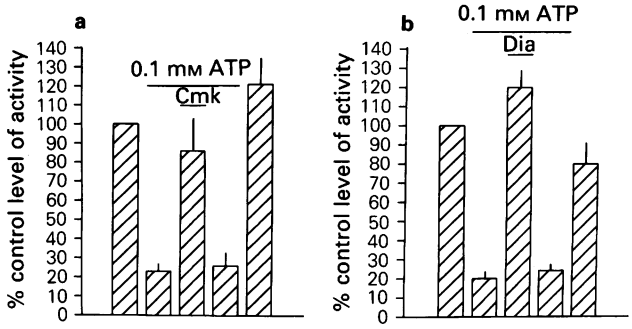
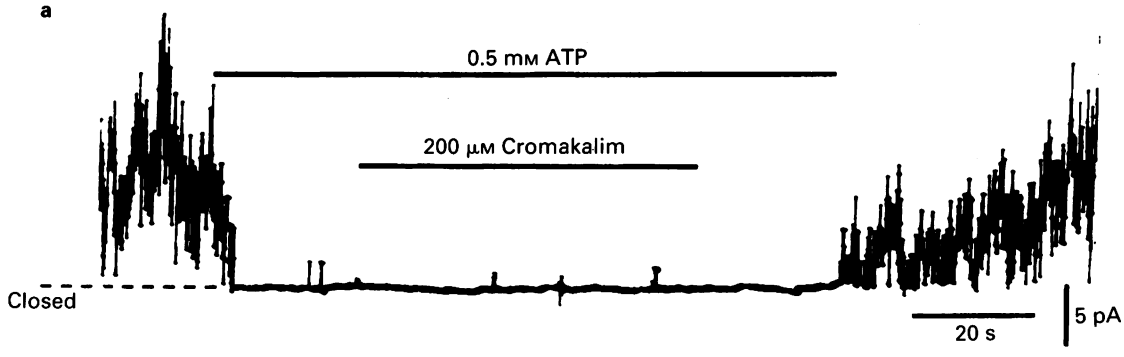


Figure 3 Quantitiative analysis of the effects of cromakalim (Cmk, 200 μM) and diazoxide (Dia, 200 μM) on the current recorded from 0.1 mM ATP-inhibited K^+ channels. Changes in open-state probability have been assessed from 4 separate RINm5F open-cells (including that shown in Figure 2) and expressed as an average value of the precontrol level of activity (100%); vertical bars represent s.e.mean.

In the presence of 0.5 mM ATP, cromakalim, at concentrations between 80 and 200 μM, had no significant effect on the channels (Figure 4a). In 5 separate open-cells, 80 μM cromaka-



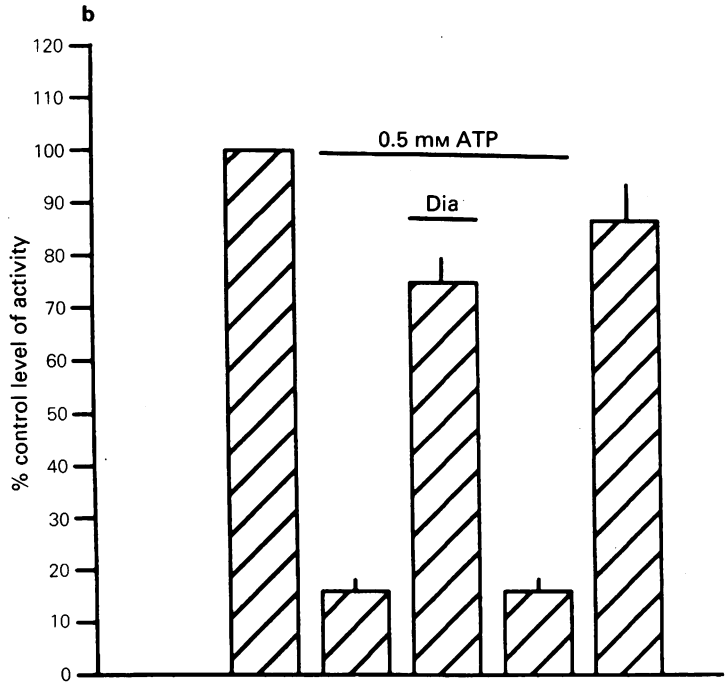


Figure 4 The continuous RINm5F open-cell current recording in (a) shows that cromakalim (200 μM) had no effect on 0.5 mM ATP-inhibited K^+ channels. (b) Shows a quantitative analysis of 5 separate RINm5F open-cells in which 100 μM diazoxide (Dia) was found to activate K^+ channels inhibited by 0.5 mM ATP. All data have been expressed as an average of the pre-control level of activity (100%); vertical bars show s.e.mean.

lim activated channels on 1 out of 8 attempts, with 13/15 applications of 200 μM cromakalim having no effects. In contrast, diazoxide (100 μM) opened K^+ channels inhibited by 0.5 mM ATP on 24/24 applications to 5 separate open-cells (Figures 1 and 4b). At higher concentrations cromakalim did open K_{ATP} channels. In five open-cells that were exposed to 400 and 800 μM concentrations of cromakalim, 400 μM had effects on 6/9 attempts, whereas 800 μM activated channels on 17/19 occasions. One typical experiment is shown in Figure 5a. Following permeabilization of this particular cell, 0.5 mM ATP almost completely inhibited the K^+ current. The addition of 400 μM cromakalim in the continued presence of ATP produced a sharp increase in the number of channel openings, which was enhanced even further by 800 μM cromakalim. When the concentration of cromakalim was lowered, channel openings were reduced, and decreased even further upon the complete removal of the drug from the inside of the membrane. Effects similar to those shown in Figure 5a were seen on all the 6 occasions when this protocol was carried out in 6 separate open-cells (Figure 5b). Finally, the actions of diazoxide and cromakalim on K^+ channels inhibited by 1 mM ATP have been investigated. Seven out of 9 applications of cromakalim (400 to 800 μM) opened K^+ channels ($n = 4$ open-cells), whereas no effects of the drug were seen below 400 μM ($n = 9$ open-cells). One hundred μM diazoxide activated 1 mM ATP-inhibited K^+ channels on each of the 54 occasions it was added to 18 separate open-cells.

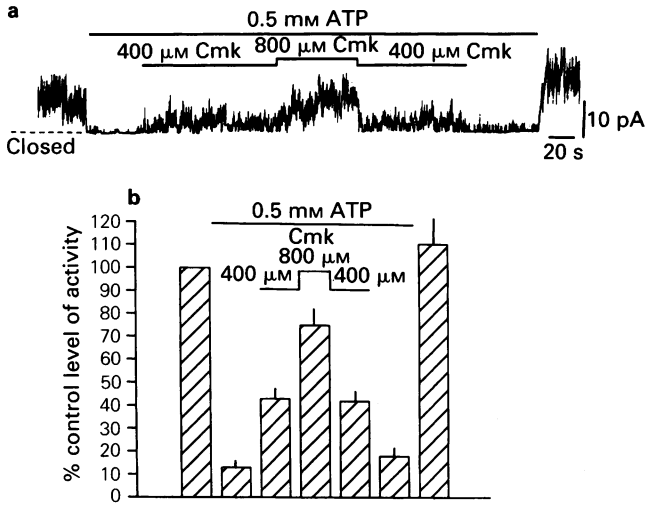


Figure 5 (a) The concentration-dependent activation of 0.5 mM ATP-inhibited K^+ channels by 400 and 800 μM cromakalim (Cmk) in a single RINm5F open-cell, whereas (b) shows the overall effects of 400 and 800 μM cromakalim on ATP inhibited channels. The data in (b) have been gathered from 4 separate open-cells (including the one presented in (a)). All values have been expressed as averages of the pre-control level of activity (100%); vertical bars show s.e.mean.

Interactions between cromakalim (BRL 34915), diazoxide and ATP

Interactions between cromakalim and ATP were complex. Although 200 μM cromakalim caused no consistent activation of K^+ channels inhibited by either 0.5 mM, 1 mM or 2 mM ATP, the drug was able to open K^+ channels inhibited by 4 mM ATP and 1 mM ADP. Figure 6a shows a typical experiment in which K_{ATP} currents suppressed by 4 mM ATP/1 mM ADP, were activated, firstly by 200 μM cromakalim then by 100 μM diazoxide. A quantitative analysis of this and 3 other experiments where the protocol was carried out 4/4 times is shown in Figure 6b.

In the complete absence of internal ATP, cromakalim (200–800 μM) had mixed effects. In 16 separate open-cells, 16/20 applications of cromakalim to the inside of the membrane had no effects upon the gating of K^+ channels, 4/20 attempts resulted in channel inhibition. In order to open ATP-sensitive K^+ channels it would appear that, like diazoxide (Dunne *et al.*, 1987), cromakalim requires the presence of ATP on the cystolic face of the membrane. This requirement for ATP is, however, complicated by the fact that ATP will itself compete with cromakalim for the gating of channels. In Figure 7 it is seen that K^+ channels inhibited by 1 mM ATP were reactivated by 400 μM cromakalim. However, when the concentration of ATP was increased to 2 mM, the actions of cromakalim were abolished. Similar effects were seen in 3 other open-cells,

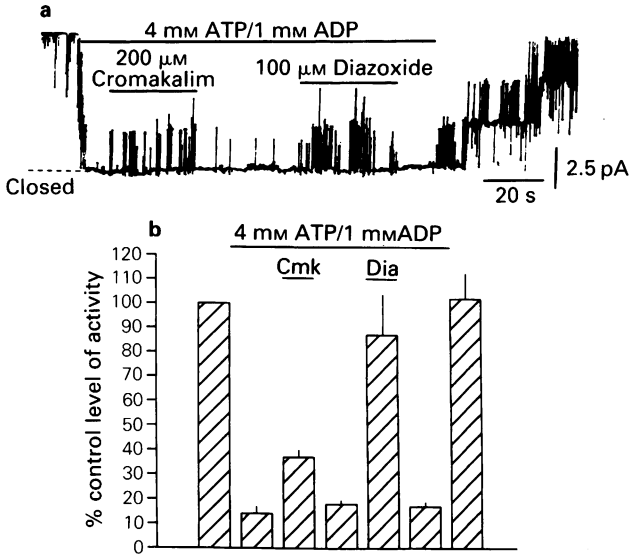


Figure 6 (a) Cromakalim (200 μM)- and diazoxide (100 μM)-activation of 4 mM ATP/1 mM ADP-inhibited K^+ channels in a single RINm5F open-cell. (b) Quantitative analysis of the effects of cromakalim (Cmk) and diazoxide (Dia) on ATP/ADP-inhibited K^+ channels. All data have been expressed as the average of the pre-control level of activity (100% from 4 separate cells (including the one presented in (a)). Vertical bars show s.e.mean.

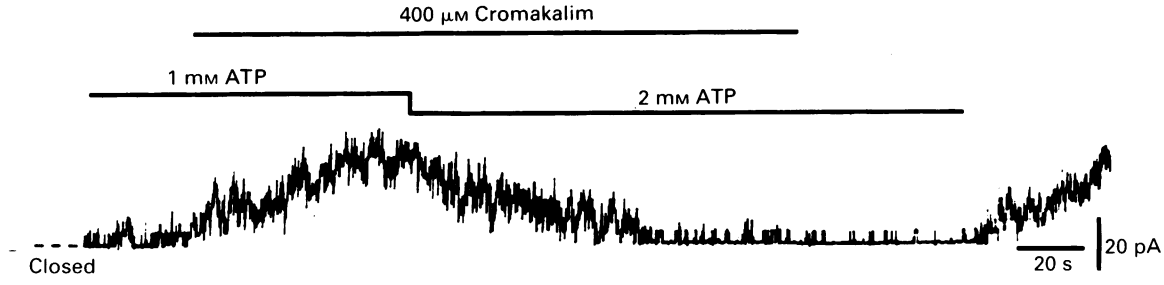


Figure 7 Interactions between ATP and cromakalim. In the continuous open-cell current recording shown, 1 mM ATP-inhibited K^+ channels were reactivated in the presence of ATP by cromakalim (400 μM), but inhibited again as the concentration of ATP was increased to 2 mM

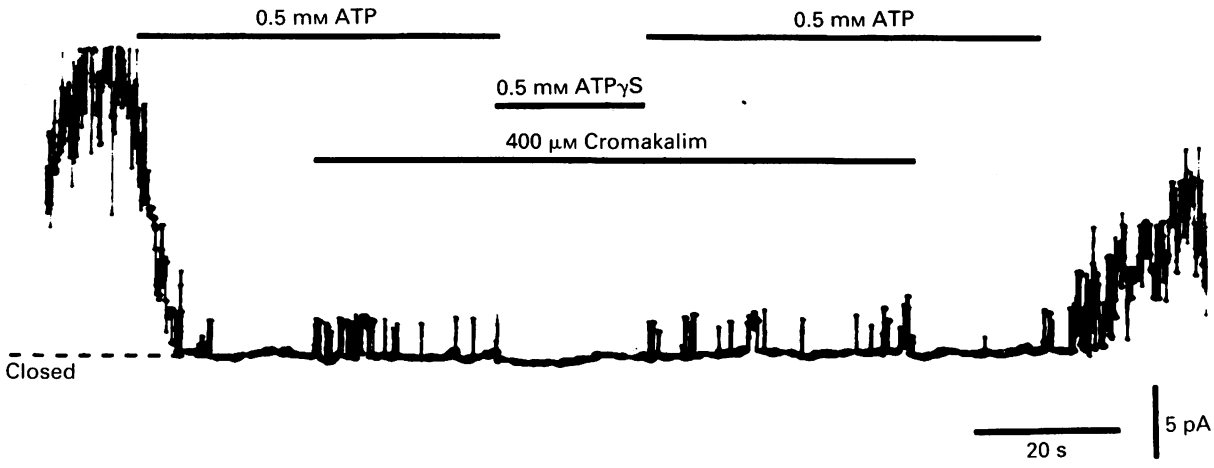


Figure 8 The ability of cromakalim to open K^+ channels inhibited by ATP (0.5 mM) but not those inhibited by ATPγS (0.5 mM). In the continuous open-cell current recording shown, K^+ channels inhibited by 0.5 mM ATP were reactivated by 400 μM cromakalim. When ATP was replaced by ATPγS in the continued presence of cromakalim, the effects of cromakalim were lost. Finally when ATP was readmitted a renewed activation of K^+ channels occurred.

in which interactions between 400 μM cromakalim and 0.25 mM/0.5 mM ATP and 1 mM/2 mM ATP were studied (7/7 times).

The continuous current record shown in Figure 8 represents a typical experiment in which we investigated the possibility that the opening of K^+ channels by cromakalim, requires protein phosphorylation. In the record shown, K^+ channels were inhibited by 0.5 mM ATP and reactivated by 400 μM cromakalim. When ATP was removed in the continued presence of cromakalim and replaced by 0.5 mM ATPγS, the cromakalim response was inhibited. The removal of ATPγS and readmission of ATP brought about a renewed activation of K^+ channels. Similar effects were seen in 3 other open-cell recordings on 4/4 occasions. In an additional 5 patches cromakalim (400–800 μM) had no effect on K^+ channels inhibited by 0.5 mM ATPγS (n = 7/7 attempts).

Discussion

In this study we have used the patch-clamp technique to investigate the mechanism by which cromakalim activates ATP-inhibited K^+ channels in the glucose-sensitive clonal insulin-secreting cell line, RINm5F (Ribalet *et al.*, 1988; Dunne *et al.*, 1989b). The experiments described have been carried out using the open-cell variation of the patch-clamp technique, since this particular recording configuration not only allows us consistent access to the inside of the plasma membrane but also delays the rate at which K_{ATP} channels run-down (Findlay & Dunne, 1986; Dunne *et al.*, 1986; Ohno-Shosaku *et al.*, 1987). Our data suggest: (i) that cromakalim activates ATP-inhibited K^+ channels from the inside of the plasma membrane, (ii) that the effects of the drug are concentration-dependent, (iii) that competitive interactions between ATP and cromakalim exist and (iv) that the mechanism by which cromakalim opens channels may be dependent on protein phosphorylation, of either the K_{ATP} channel or a related control unit.

Since the effects of diazoxide on ATP-sensitive K^+ channels in insulin-secreting cells have been extensively characterised (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989), it is interesting to compare the actions of cromakalim with those of the sulphonamide. There are a number of similarities: (i) both drugs require internal ATP to open channels regularly, (ii) ATP nevertheless competes with the two compounds for the gating of K_{ATP} channels (Figure 7) and (iii) both cromakalim and diazoxide open channels by a mechanism involving hydrolysis of ATP (Figure 8). With specific reference to this last point, it could be argued that since cromakalim/ATP interactions are complex, ATPγS may be a more potent inhibitor of the channels and therefore compete with the drug for the control of channel openings. However, since previous experiments from our laboratory have shown that both ATP and its various analogues have very similar efficacies of channel inhibition (Dunne *et al.*, 1988b; Dunne, 1989), this possibility can be dismissed.

Overall the effects of cromakalim on K^+ channels are far less impressive than those of diazoxide. In the presence of 0.1 mM ATP, diazoxide was found to be some 70% more effective at opening channels than the equivalent concentration of cromakalim (Figures 2 and 3), whereas in the presence of 0.5 mM ATP the degree of channel activation brought about by 100 μM diazoxide could only be matched by 800 μM cromakalim (compare Figures 4b and 5b). Below 200 μM the effects of cromakalim were consistently found to be weak, whereas diazoxide, on the other hand, has been shown to be an effective activator of K^+ channels at concentrations of 20 μM and less (Dunne *et al.*, 1987; Schmid-Antomarchi *et al.*, 1987). In the study carried out by Escande *et al.* (1988) on the cardiac ATP-sensitive K^+ channel, cromakalim was also apparently more effective at a concentration of 300 μM.

It is interesting to note that, even though 200 μM cromakalim had little effect on K^+ channels inhibited by 0.1 to 1 mM ATP, the drug readily opened channels in the presence of 4 mM ATP and 1 mM ADP (Figure 6). K_{ATP} channels in these cells are acutely sensitive to changes in the internal ratio of ATP/ADP (Dunne & Petersen, 1986; Kakei *et al.*, 1986; Dunne *et al.*, 1988b), which has led to the proposal that changes in ATP:ADP may couple carbohydrate metabolism to K^+ channel closure (Petersen, 1988; Petersen & Dunne, 1989). Since the particular concentrations of ATP and ADP used in these experiments are similar to those estimated, by a variety of techniques, to be present in the insulin-secreting cells (Dunne *et al.*, 1988b; Wollheim *et al.*, 1988), these findings indicate that under quasi-physiological conditions cromakalim may open ATP-sensitive K^+ channels in these cells.

With regard to the pharmacological significance of our results two major conclusions can be drawn. Firstly, since the effective concentrations of cromakalim are relatively high in comparison to those of diazoxide, our results suggest that cromakalim is unlikely to replace the sulphonamide as a novel hyperglycaemia-inducing agent. Secondly, our data have implications for the possible use of cromakalim as an antihypertensive agent. Since it has been demonstrated that cro-

makalim has significant effects *in vitro* on both the mechanical and electrical properties of smooth muscle cells at concentrations of 10 μM and less (Hamilton *et al.*, 1986; Allen *et al.*, 1986; Weir & Weston, 1986), there is (in this dose range) unlikely to be much effect on the opening of the β-cell ATP-sensitive K^+ channel, and therefore little risk of hyperglycaemia as a side effect of the drug.

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