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Effects of pinacidil, RP 49356 and nicorandil on ATP-sensitive potassium channels in insulin-secreting cells

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1 The whole-cell patch-clamp technique has been used to investigate the effects of pinacidil, RP 49356 and nicorandil on membrane potential and adenosine 5'-triphosphate (ATP)-sensitive K^+ channel currents in the insulin-secreting cell line RINm5F. Interactions between pinacidil, RP 49356, nicorandil, diazoxide and ATP have been studied in excised outside-out membrane patches and open-cells.

2 In RINm5F whole-cells (current-clamp mode) continually exposed to glucose, pinacidil, RP 49356 and nicorandil, at concentrations greater than 100 μM, consistently reversed the effects of the sugar by repolarizing the membrane and terminating voltage-gated Ca^{2+} spike-potentials.

3 The actions of pinacidil, RP 49356 and nicorandil on membrane potential may be explained by their effects on the opening of ATP-sensitive K^+ channels, since all three compounds activated channels in whole-cells (voltage-clamp mode), excised outside-out membrane patches and open-cells, at concentrations greater than 100 μM. Below 100 μM the actions of pinacidil, RP 49356 and nicorandil were weak and inconsistent.

4 The mechanism of channel activation appears to depend on the presence of cytosolic ATP, since in its absence, pinacidil, RP 49356 and nicorandil (> 100 μM) had either no effects or inhibited K^+ channels.

5 Pinacidil, nicorandil and RP 49356 (200–500 μM) also appeared to open K^+ channels inhibited by quasi-physiological concentrations of ATP (4 mM) and ADP (1 mM). However, in comparison to diazoxide their effects were weak.

6 Since pinacidil, RP 49356 and nicorandil have been shown to have effects on smooth muscle preparations at concentrations of 30 μM and below, the data presented in this study may have important implications for any prospective therapeutic application of the drugs, since at the concentrations required to relax smooth muscle cells, hyperglycaemia will be avoided.

Introduction

The pharmacological control of adenosine 5'-triphosphate (ATP)-sensitive potassium (K_{ATP}) channels in insulin-secreting cells, first identified by Cook & Hales (1984), has important implications for the therapeutic treatment of insulin regulation disorders (Cook, 1988), since these channels govern the transmembrane potential of pancreatic β-cells (Petersen & Findlay, 1987; Petersen & Dunne, 1989). Glucose metabolism closes K^+ channels found to be open in intact cells, thus initiating a membrane depolarization (Ashcroft *et al.*, 1984; Dunne *et al.*, 1986) which is functionally required for the activation of voltage-gated L-type calcium channels (Velasco *et al.*, 1988; Velasco & Petersen, 1989), responsible for Ca^{2+} spike potentials (Matthews & Sakamoto, 1985) and an increase in the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Wollheim & Biden, 1987; Dunne *et al.*, 1990b). Tolbutamide mimics the effects of glucose on these cells since it is a selective inhibitor of K_{ATP} channels (Sturgess *et al.*, 1985; Dunne *et al.*, 1987). The sulphonamide diazoxide, on the other hand, reverses the effects of the sugar, causing hyperglycaemia (Henquin *et al.*, 1982), through the activation of channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989).

The actions of the putative antihypertensive compounds nicorandil and pinacidil are also thought to involve novel modulatory effects on K^+ channels (Cook, 1988; Quast & Cook, 1989), and the vasorelaxant RP 49356 (Mondot *et al.*, 1988) has been shown to have a direct effect in opening K_{ATP} channels in cardiac myocytes (Escande *et al.*, 1988; Thuringer & Escande, 1989). Due to the current interest in the pharmacological regulation of K^+ channels and the fact that pinacidil has recently been shown to inhibit glucose-induced insulin release from pancreatic β-cells, by a mechanism involving an increase in the rate of ^{86}Rb efflux (Lebrun *et al.*, 1988), my aim was to investigate the effects of pinacidil, RP 49356 and nicorandil on ATP-sensitive K^+ channels in insulin-secreting cells using the patch-clamp technique.

Methods

Cell isolation and maintenance

All experiments were carried out on the clonal insulinoma cell line RINm5F, maintained as previously described (Wollheim *et al.*, 1988; Dunne *et al.*, 1989).

Patch-clamp experiments and analysis

Eletrophysiological investigations of insulin-secreting cells were carried out using the patch-clamp technique (Hamill *et al.*, 1981). Single-channel current recordings from excised outside-out membrane patches, as well as whole-cell current- and voltage-clamp experiments, were made with K^+-rich solution in the pipette and Na^+-rich solution in the bath. Permeabilized or open-cell experiments (Dunne *et al.*, 1986), in which the plasma membrane is perforated by saponin (0.05%) in contact with the plasma membrane outside the area from which the single-channel current recording is made, were carried out with Na^+-rich solution in the pipette and K^+-rich solution in the bath. Patch-clamp pipettes (Type 101PB, Ceebee Glass, Denmark) had a final resistance of between 5 and 10 MΩ when filled. Exchange from control to test solution was achieved manually under visual control through a series of outlet pipes (Dunne et *al.*, 1987; 1988a,b).

All whole-cell and single-channel current data were prefiltered at 3 kHz (low pass) (Kemo, UK) and stored on FM tape (Racal 4DS recorder) for subsequent replay and analysis. Changes in potassium channel open-state probability were quantified from pre-recorded stretches of data lasting between 15 and 20 s and are expressed as a fraction of the pre-control level of activity (100%), as previously described (Dunne *et al.*, 1989; Dunne, 1989). This particular method of quantification was preferred to that of expressing a finite value of open-probability, since the definitive number of operational K_{ATP} channels in a particular patch of membrane is often unknown.

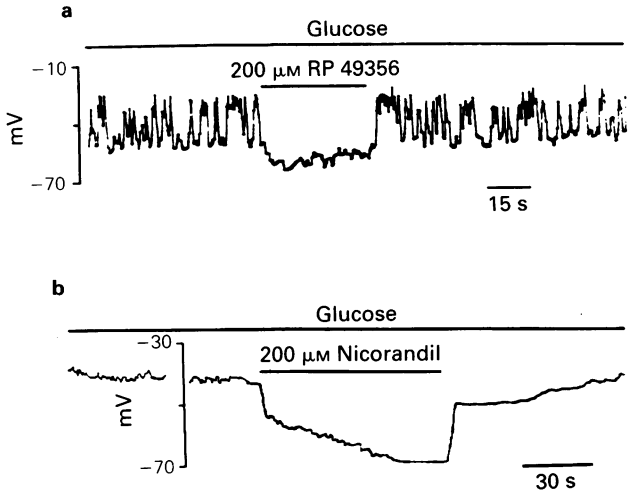


Figure 1 RP 49356 (a) and nicorandil (b) modify the glucose (5 mM)-induced electrical activity of RINm5F cells. Both records come from separate RINm5F whole-cells (currents-clamp mode). The experiment shown in (a) began 600 s after formation of the whole-cell, whereas the one shown in (b) began 480 s after initiation of the intracellular recording and 450 s after the run-down of Ca^{2+} spike-potentials. Both records started 40 s after the addition of glucose to the bathing solution.

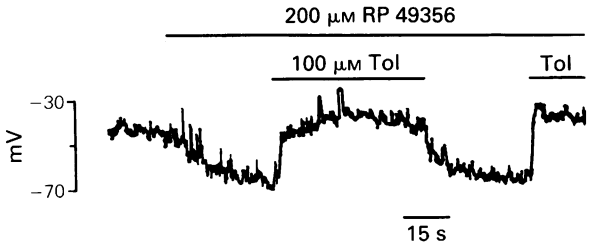


Figure 2 Tolbutamide (Tol) reversed the effects of RP 49356 on the glucose (5 mM)-induced electrical activity of a single RINm5F cell. The voltage recording began 400 s after initiation of the intracellular recording, 200 s after the run-down of Ca^{2+} spike-potentials and 50 s after the addition of glucose to the bathing solution.

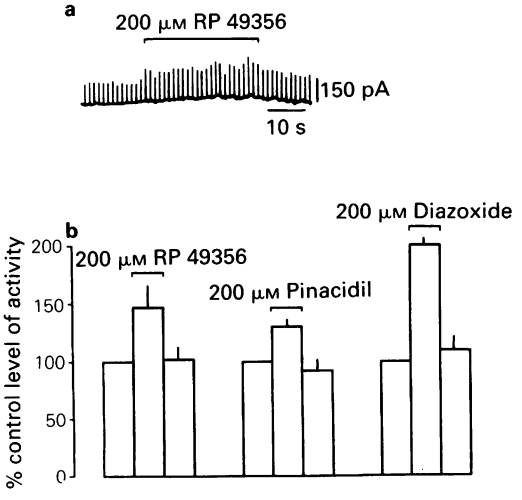


Figure 3 (a) RP 49356 (200 μM) activated K_{ATP} currents in a RINm5F whole-cell (voltage-clamp mode). The record began 700 s after initiation of the intracellular recording and 200 s after the addition of glucose (5 mM) to the bath, which reduced the K_{ATP} current to 41% of the control level of activity (not shown). (b) Shows a quantitative analysis of the effects of 200 μM concentrations of RP 49356, pinacidil and diazoxide on whole-cell K_{ATP} currents. All data have been expressed as a percentage of the pre-test level of activity (100%) (i.e. in the presence of 5 mM glucose), from 4, 4 and 7 separate cells, and includes the data presented in (a). In (b) each column represents the mean and vertical bars show s.e.mean.

Changes in whole-cell K_{ATP} currents, generated with depolarizing voltage pulses of 10/20 mV from a holding potential of −60 mV (100 ms duration, 1 pulse every 0.8 s) are also expressed as a percentage of the pre-test value (100%). All records presented were directly photographed from either the oscilloscope screen or from the pen-recording trace (Devices, UK). In all single-channel current records shown, upward deflections 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 and HEPES 10. When the solution was used in the bath (whole-cell and excised outside-out membrane patches), 2 mM $CaCl_2$ was added, whereas when it was used to fill the patch-clamp pipette (open-cell experiments) $CaCl_2$ was omitted and 0.5 mM EGTA added. The pH of all Na^+-rich solutions was adjusted to 7.2 with NaOH. The standard intracellular K^+-rich solution contained (mM): KCl 140, NaCl 10, $MgCl_2$ 1.13, EGTA 0.5 and HEPES 10. ATP 1 mM was added to this solution when it was used to fill patch pipettes (whole-cell and outside-out membrane patch experiments). The pH of all K^+-rich solutions was adjusted to 7.2 with KOH. Stock solutions of RP 49356 (N-methyl (pyridyl-3)-2 tetrahydrothiopyranne carbothioamide-2-t-oxide-1-r), nicroandil (Rhone-Poulenc Ltd, UK), and pinacidil ((±)-2-cyano-1-(4-pyridyl)-3-(1,2,2-trimethyl-propyl) guanidine monohydrate) (Leo Laboratories, UK) were prepared in 70% ethanol (vol/vol), whereas stock solution of tolbutamide (Sigma, UK) and diazoxide (Glaxo Research Ltd., UK) were prepared in dimethylsulphoxide (DMSO). The maximal concentrations of DMSO (1%) and ethanol (2%) used in these experiments had no effects on K^+ channels (Dunne *et al.*, 1987; Dunne *et al.*, 1990a). The osmolality of all solutions was 290 ± 5 mosm kg^{-1}. All experiments were carried out at room temperature (22–25°C).

Results

The effects of pinacidil, RP 49356 and nicorandil on membrane potential

The actions of pinacidil, RP 49356 and nicorandil on the transmembrane potential of RINm5F cells were investigated using the whole-cell current-clamp variation of the patch-clamp technique. In total 42 cells were used in this study. On average the spontaneous membrane potential, estimated within seconds of beginning the intracellular recording, was found to be −62 ± 2 mV (n = 42) (mean ± s.e.mean). Twenty eight of these cells were exposed to glucose (5–10 mM) added directly to the bath solution, 37 times. Glucose initiated (i) a depolarization of the membrane, found to be on average 22 ± 2 mV (n = 37) and (ii) caused the generation of spike-potentials. The effects of 200 μM RP 49356 on the glucose-induced electrical activity of a single RINm5F whole-cell is shown in Figure 1a. In the continued presence of the sugar, RP 49356 repolarized the membrane which led to the termination of spike-potentials. Of the 28 cells exposed to glucose in this particular series of experiments, only 3 were able to sustain Ca^{2+}-spikes for longer than 15 min. In the remaining 25 cells, the average duration of spike-potentials was found to be 302 ± 36 s. One such experiment is illustrated in Figure 1b, which also shows that 200 μM nicorandil reversed the effects of glucose by repolarizing the membrane.

Similar effects to those seen in Figure 1 were found for pinacidil, RP 49356 and nicorandil, at concentrations between 200 and 500 μM, in 4 (4/4 applications), 8 (9/10 applications) and 4 (4/4 applications) separate cells, respectively. At concentrations below 100 μM the effects of pinacidil, RP 49356 and nicorandil were weak and inconsistent (n = 7 separate cells).

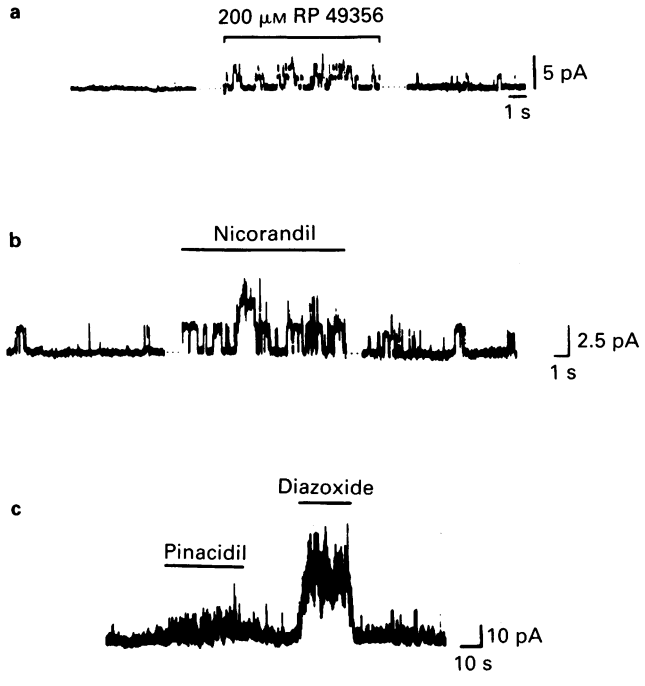


Figure 4 RP 49356 (200 μM) (a), nicorandil (200 μM) (b), pinacidil (200 μM) (c) and diazoxide (200 μM) (c) open ATP-sensitive K^+ channels in excised outside-out membrane patches. All 3 current traces came from separate cells.

Since the effects of glucose on insulin-secreting cells are initiated by changes in the gating of K_{ATP} channels, interactions between pinacidil, RP 49356, nicorandil and tolbutamide were investigated. Figure 2 shows that in the continued presence of glucose, 200 μM RP 49356 repolarized the membrane. When tolbutamide (100 μM) was then added in the presence of both glucose and RP 49356, a sharp, sustained depolarization of the cell was initiated. Removal of tolbutamide repolarized the membrane, which was followed by a renewed depolarization upon readmission of the sulphonylurea to the bath. This result was confirmed in a further 3 separate cells (4/5 attempts), and also seen for pinacidil (200 μM) ($n = 4$ cells) and nicorandil (200 μM) ($n = 3$ cells).

Pinacidil, RP 49356 and nicorandil enhance K_{ATP} currents

The effects of pinacidil, RP 49356 and nicorandil on whole-cell K_{ATP} currents were studied under voltage-clamp conditions. Outward K_{ATP} currents, elicited by 10 to 20 mV depolarizing voltage pulses from a holding potential of −60 mV (100 ms duration, 1 pulse every 0.8 s), were inhibited by glucose (5–10 mM) to, on average, 35 ± 9% of the control level of activity (not shown) due to the closure of K_{ATP} channels (Dunne *et al.*, 1990c) and enhanced by pinacidil (200 μM), RP 49356 (200 μM) (Figure 3a), nicorandil (200 μM) ($n = 3$ separate cells) and diazoxide (200 μM) in the continued presence of the sugar. A quantitative analysis of the effects of pinacidil ($n = 4$ cells), RP 49356 ($n = 4$ cells) and diazoxide ($n = 7$ cells) is shown in Figure 3b.

Single K_{ATP} currents from excised outside-out membrane patches, with 1 mM ATP added to the cytosolic face of the membrane, were also found to be activated by RP 49356 (200–

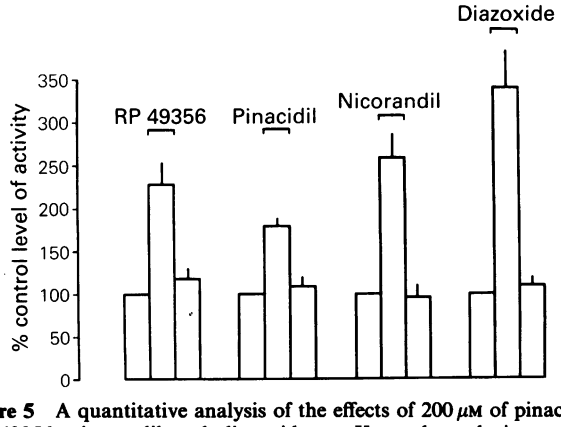
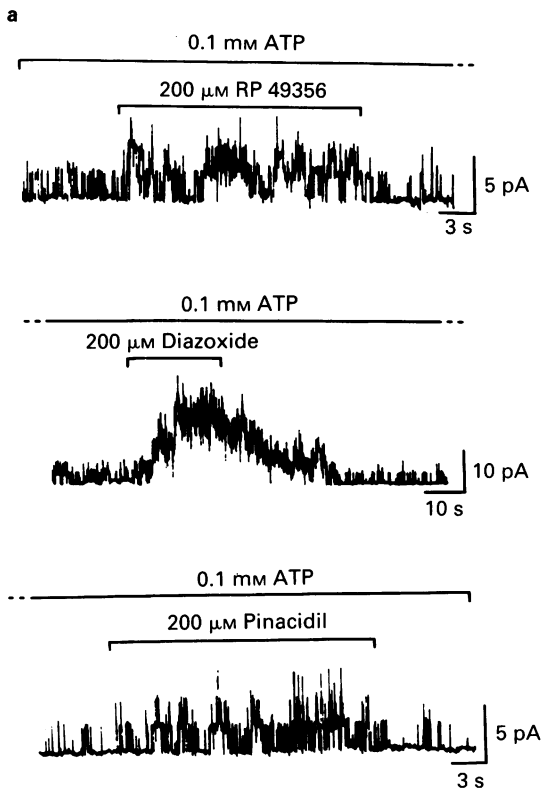


Figure 5 A quantitative analysis of the effects of 200 μM of pinacidil, RP 49356, nicorandil and diazoxide on K_{ATP} channels in excised outside-out membrane patch experiments. Average current values for the effects of RP 49356, pinacidil, nicorandil and diazoxide have been expressed as a percentage of the control level of activity (100%) from 10, 9, 5 and 8 separate cells, respectively, and the data shown in Figure 4 have been included. Each column represents the mean and vertical bars show s.e.mean.



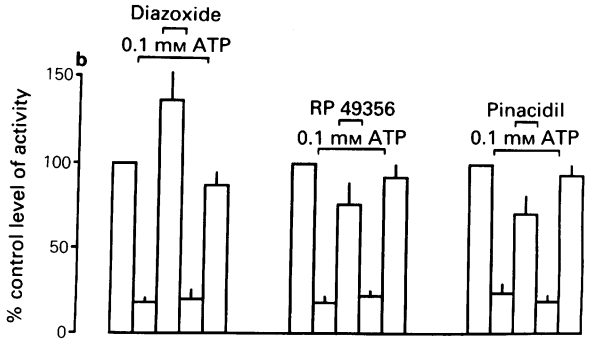


Figure 6 RP 49356, diazoxide and pinacidil open K^+ channels inhibited by 0.1 mM ATP. The data shown (a) are from the same RINm5F open-cell. The intervals between the end of the first record and the beginning of the second and the end of the second and the start of the third are 10 and 30 s, respectively. (b) Shows a quantitative analysis of the effects of 200 μM of diazoxide, RP 49356 and pinacidil on K_{ATP} channels inhibited by ATP (0.1 mM), and includes the experiments shown in (a). Average current values for the effects of diazoxide ($n = 5$ open-cells), RP 49356 ($n = 4$) and pinacidil ($n = 5$) have been expressed as a percentage of the control level of activity (100%). Vertical bars show s.e.mean.

500 μM) (Figure 4a), nicorandil (200–500 μM) (Figure 4b) and pinacidil (200–500 μM) (Figure 4c). A quantitative analysis of the effects of these compounds along with diazoxide (100 μM) is shown in Figure 5.

Opening of ATP-sensitive K^+ channels by pinacidil, RP 49356 and nicorandil requires cytosolic ATP

The open-cell recording configuration was used to study the mechanism by which pinacidil, RP 49356 and nicorandil open ATP-sensitive K^+ channels. Figure 6a shows 3 continuous current recordings taken from the same RINm5F open-cell. Following cellular perforation with 0.05% saponin (not shown) the activity of K^+ channels was reduced by adding 0.1 mM ATP to the inside of the plasma membrane. In the continued presence of ATP, 200 μM concentrations of RP 49356, diazoxide and pinacidil were all found to activate channels reversibly (note that the vertical scale in the centre panel has been compressed to accommodate the larger effect of diazoxide). A quantitative analysis of this and an additional 4 separate open-cell experiments, where direct comparisons were made between pinacidil (4/4 applications), RP 49356 (6/6 applications) and diazoxide (16/16 applications), has been presented in Figure 6b. Nicorandil (200 μM) was also found to open K^+ channels inhibited by 0.1 mM ATP in 3 separate cells (3/3).

When added to the inside of the membrane in the absence of ATP, the effects of pinacidil, RP 49356 and nicorandil were mixed. Out of 8 applications of pinacidil to 7 separate cells; 1 attempt activated channels, 1 addition had no effects whereas 6 applications were found to reduce the K_{ATP} current (Figure 7a). Similar inhibitory effects were found for both RP 49356 and nicorandil on 5/7 and 4/8 occasions in 6 and 4 separate cells, respectively. Inhibition was apparently not associated with any significant change in the frequency of channel opening, but was characterised by a decrease in the single-channel current amplitude (Figure 7).

Interactions between the putative K^+ channel modulators and ATP and ADP have also been studied. Figure 8a shows a typical experiment in which direct comparisons were made between the activator effects of 500 μM RP 49356, 500 μM pinacidil and 100 μM diazoxide on K^+ channels inhibited by 4 mM ATP and 1 mM ADP, whereas Figure 8b shows the quantitative analysis of this and an additional 6 separate open-cell experiments. Nicorandil was also found to open channels inhibited by ATP and ADP in 3 separate cells (n = 3/3 applications).

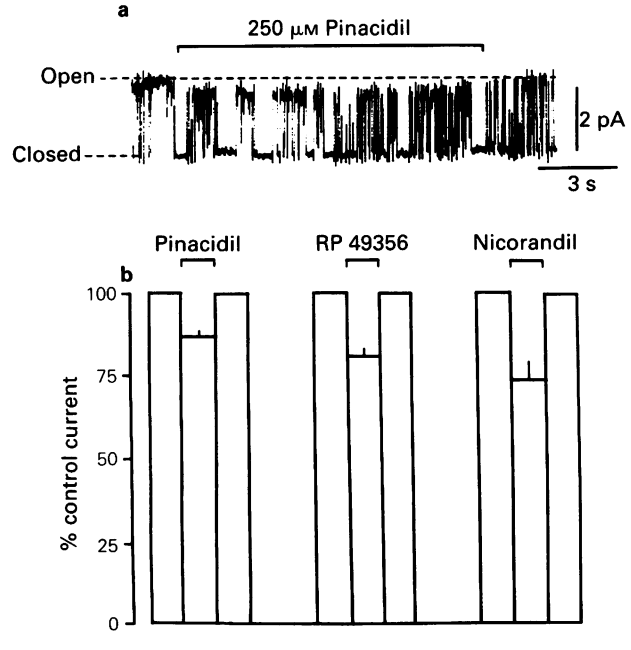


Figure 7 (a) Inhibition of K_{ATP} channels by pinacidil when added to the cytosolic face of the membrane in the absence of ATP. (b) A quantitative analysis of the effects of pinacidil, RP 49356 and nicorandil on K_{ATP} channels in the absence of internal ATP. Average data have been expressed as a percentage of the pre-control current value (100%), from 4, 4 and 4 separate open-cells, respectively, and include the data shown in (a). Vertical bars show s.e.mean.

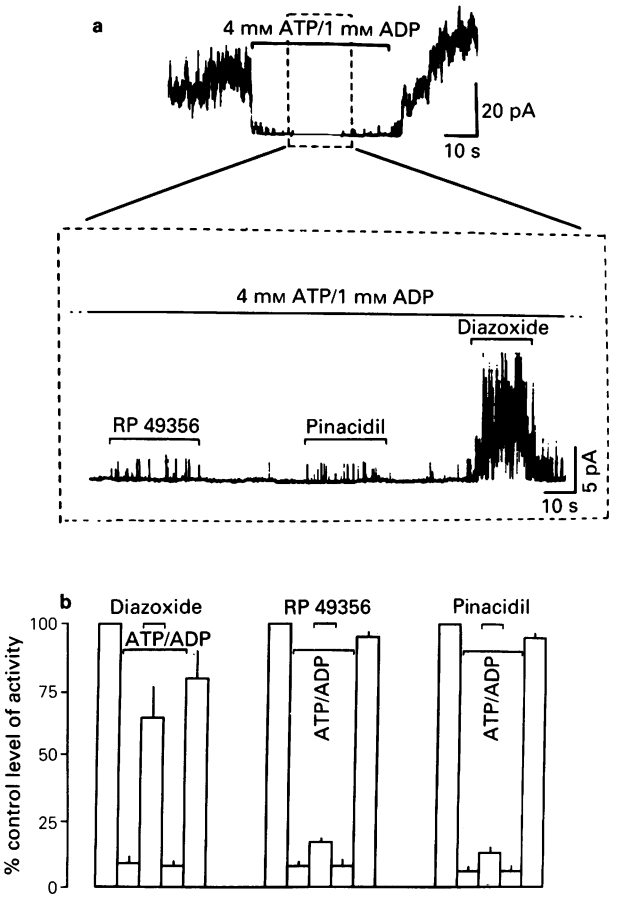


Figure 8 Pinacidil, RP 49356 and diazoxide open K^+ channels inhibited by 4 mM ATP and 1 mM ADP. (a) A typical continuous current trace, recorded from a RINm5F open-cell, whereas (b) illustrates the quantitative analysis of the effects of 500 μM concentrations of RP 49356 and pinacidil and 100 μM diazoxide on K_{ATP} channels inhibited by ATP (4 mM) and ADP (1 mM), and includes the experiments shown in (a). Average current values for the effects of diazoxide (n = 6 open-cells), RP 49356 (n = 4) and pinacidil (n = 4) have been expressed as a percentage of the control level of activity (100%). Vertical bars show s.e.mean.

Discussion

The patch-clamp technique has been used in the present study to investigate the effects of novel K^+ channel modulator compounds on ATP-sensitive K^+ channels in insulin-secreting cells. The effects of glucose on RINm5F cells (Ribalet *et al.*, 1988; Dunne *et al.*, 1990b) were found to be abolished by pinacidil, RP 49356 and nicorandil (Figure 1). All three compounds were found to enhance the flow of current through ATP-sensitive K^+ channels in whole-cells (Figure 3), excised outside-out membrane patches (Figures 4 and 5) and open-cells (Figures 6 and 8). Since carbohydrate-metabolism and tolbutamide-evoked inhibition of K_{ATP} channels in these cells results in the initiation of a depolarization of the membrane, the effects of pinacidil, RP 49356 and nicorandil on these channels may explain why, in whole-cells, each of the drugs repolarized the membrane and abolished spike-potentials. In total, 89% of the cells used in these experiments were unable

to generate Ca^{2+}-spikes for longer than 15 min. The disappearance of spike-potentials is probably related to the run-down of Ca^{2+} channels in the whole-cell, as has been found for a number of other cell types (Byerly & Hagiwara, 1982; Fenwick *et al.*, 1982; Katayama *et al.*, 1985).

The mechanism of K^+ channel activation was studied in permeabilized open-cells and outside-out membrane patches. These data suggest: (i) that internal ATP is required in order to observe channel activation regularly and (ii) that pinacidil, RP 49356 and nicorandil will open channels inhibited by ATP and ADP at concentrations present in intact cells (Wollheim *et al.*, 1988; Dunne *et al.*, 1988b). Qualitatively, the effects of the novel K^+ channel modulators are very similar to those of diazoxide, a hyperglycemia-inducing sulphonamide that has been used clinically to treat certain forms of insulinomas of the pancreas (Altsuzler *et al.*, 1977), due to its ability to open K_{ATP} channels (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989). However, in comparison to the effects of diazoxide, the actions of pinacidil, RP 49356 and nicorandil were weak (Figures 3, 4, 5, 6 and 8), suggesting that they are unlikely to replace the sulphonamide as effective hyperglycemia-inducing agents.

The experiment shown in Figure 7 is particularly interesting, since not only does it suggest thst ATP is obligatory for the actions of pinacidil, RP 49356 and nicorandil, but the data may indicate that each of the compounds will inhibit channels in the absence of ATP. The inhibition of K^+ channels was not characterized by any significant change in open-state probability but rather by a decrease in the single-channel current amplitude. Interactions between ATP and diazoxide have been investigated in some detail. Dunne *et al.* (1987) first concluded that internal ATP was required for diazoxide to open channels and that competitive interactions between ATP (ATP^{4-}) and diazoxide were apparent. More recently, both Dunne (1989) and Kozlowski *et al.* (1989) showed that the actions of diazoxide are dependent upon protein phosphorylation of either the channel or a related control unit. These conclusions were reached by the observations that (i) in the presence of non-hydrolyzable forms of ATP ($ATP\gamma S$, AMP-PNP and AMP-PCP) the activator effects of diazoxide were lost (Dunne, 1989; Kozlowski *et al.*, 1989) and (ii) in the complete absence of internal Mg-ATP, diazoxide actually closed channels (Kozlowski *et al.*, 1989). The activation of K^+ channels in Mg^{2+}-free solutions shown by Dunne *et al.* (1987) probably results from the presence of 3 μM Mg^{2+} in the test solution, which presumably provides enough Mg-ATP complex to augment the effects of the drug. In insulin-secreting cells cromakalim, which has little influence on channels below 100 μM (Kozlowski *et al.*, 1989, Dunne *et al.*, 1990a,b), is an effective modulator of channel gating at higher concentrations (Dunne *et al.*, 1990a,b). Since, like diazoxide, the actions of cromakalim are also dependent on protein phosphorylation (Dunne *et al.*, 1990a), it is interesting to speculate that a similar mechanism of channel activation may underlie the effects of pinacidil, RP 49356 and nicorandil.

In conclusion, the data presented in this study suggest that pinacidil, RP 49356 and nicorandil open ATP-sensitive K^+ channels in insulin-secreting cells, both from the inside and the outside of the plasma membrane. In the presence of glucose, K^+ channel activation results in a repolarization of the membrane and the termination of Ca^{2+} spike-potentials. This would then lead to an attenuation of the secretagogue-induced rise in $[Ca^{2+}]_i$, the key internal regulator of insulin secretion (Wollheim & Biden, 1987) and an inhibition of insulin release, as has been found for pinacidil (Lebrun *et al.*, 1988). However, since: (i) in a number of preparations, vaso-relaxant effects of nicorandil and pinacidil have been regularly observed at concentrations below 10 μM (Bray *et al.*, 1987; Cook *et al.*, 1988; Hermsmeyer, 1988; Videbaek *et al.*, 1988; Wilson *et al.*, 1988) and (ii) RP 49356 has been shown to open K_{ATP} channels in cardiac myocytes at concentrations around 30 μM (Thuringer & Escande, 1989), at these particular doses, the compounds will have little effect on β-cell electrical activity. This could be important for any possible therapeutic application of pinacidil, RP 49356 and nicorandil as effective antihypertensive drugs, since at the concentrations required to relax smooth muscle cells, hyperglycemia would be avoided.

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