Block of ATP-regulated potassium channels by phentolamine and other α -adrenoceptor antagonists

M.J. Dunne

Department of Biomedical Science, University of Sheffield, Sheffield S10 2TN

1 The patch clamp technique has been used to characterize the effects of phentolamine, an unselective blocker of α_1 - and α_2 -adrenoceptors, on the electrical activity of isolated RINm5F insulin-secreting cells and the gating of ATP-regulated potassium (K^+_{ATP}) channels.

2 Current-clamp experiments carried out by use of both conventional whole-cell recordings and nystatin-perforated cells, have demonstrated that phentolamine (5–20 μ M) in the complete absence of α -adrenoceptor agonists, caused a sharp depolarization of the cell membrane from approximately –66 mV to –42 mV. This depolarization was associated with the generation of calcium action potential-like spikes. In the continued presence of phentolamine, diazoxide (100 μ M) reversed these effects by causing a hyperpolarization of the cell, thereby preventing Ca^{2+} spikes.

3 Unitary current events from K^+_{ATP} channels were recorded from both outside-out membrane patches and saponin permeabilized or open-cells. When added to either the inside or the outside of the plasma membrane, phentolamine (0.1–100 μ M) blocked openings from these channels. The effects of phentolamine were rapid, sustained and fully reversible. Phentolamine was apparently a more effective blocker of channels from the inside than the outside of the membrane.

4 The K_1 value, corresponding to 50% inhibition of channels was estimated to be approximately 0.7 μ M when phentolamine was added to the inside of the membrane and the Hill coefficient approximately 1.

5 Yohimbine (1–10 μ M) and the chemically 2-substituted imidazoline α -adrenoceptor antagonists, antazoline (25 μ M) and tolazoline (25 μ M) were also found to block K^+_{ATP} channels in isolated patches of membrane.

6 In conclusion the present study demonstrates that phentolamine and other imidazoline adrenoceptor antagonists have effects upon ATP-sensitive K^+ channels that are not associated with stimulation of the adrenoceptor.

Keywords: Patch-clamp; K^+_{ATP} channels; adrenoceptor; RINm5F; insulin secreting cell; yohimbine; imidazoline

Introduction

Phentolamine, which blocks both α_1 - and α_2 -adrenoceptors, has effects upon insulin-secreting cells that cannot only be attributable to antagonism of the adrenoceptor. In the absence of any exogenous agonist, phentolamine initiates insulin secretion both *in vivo* (Lundquist, 1972) and *in vitro* (Malaisse *et al.*, 1967; Efendic *et al.*, 1975; Smith & Furman, 1988; Chan *et al.*, 1988; Schulz & Hasselblatt, 1989; Garrino & Henquin, 1990). In addition, phentolamine has been found to alleviate the diazoxide-induced inhibition of insulin secretion and the enhanced rate of $^{86}Rb^+$ efflux from pre-loaded islets (Burr *et al.*, 1971; Henquin *et al.*, 1982), effects that can be explained by the recent observations by Plant & Henquin (1990) that phentolamine inhibits whole-cell K^+ currents in isolated cells. Closure of adenosine 5'-triphosphate (ATP)-sensitive potassium (K^+_{ATP}) channels, initiates a depolarization of the cell membrane, resulting in the opening of voltage-operated Ca-channels and a subsequent rise in the cytosolic Ca^{2+} concentration, the key internal regulator of insulin secretion (as reviewed by Petersen & Findlay, 1987; Ashcroft, 1988; Ashcroft & Ashcroft, 1990).

In the present study the actions of phentolamine have been investigated under current clamp conditions (whole-cell and nystatin perforated cells) and at the single channel current level. The results indicate that phentolamine has effects upon individual cells that are similar to those of glucose by causing a marked inhibition of K^+_{ATP} channels, a depolarization of the cell membrane and the initiation of Ca^{2+} spike potentials.

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.*, 1986).

Media

The standard extracellular Na^+ -rich solution used throughout these experiments, contained (mm): NaCl 140, KCl 4.7, $MgCl_2$ 1.3, $CaCl_2$ 2.5 and HEPES 10. The pH was adjusted to 7.2 with NaOH. The standard intracellular K^+ -rich solution contained (mm): KCl 140, NaCl 10, $MgCl_2$ 1.13, HEPES 10, EGTA 1 and ATP 1. No $CaCl_2$ was added and the pH was adjusted to 7.2 with KOH. Stock solutions of diazoxide (Glaxo Research Limited, UK), antazoline, tolazoline and nystatin (Sigma, UK) were prepared in dimethylsulphoxide (DMSO). The maximal concentrations of DMSO (1%) used in the final solutions for experiments had no direct effect upon K^+ channels (Dunne *et al.*, 1987). Phentolamine mesylate was obtained from Ciba-Geigy, UK. The osmolality of all solutions was 290 ± 5 mosmol kg^{-1} . All experiments were carried out at room temperature, 22–25°C.

Patch-clamp experiments

Patch-clamp recordings from excised outside-out membrane patches, nystatin perforated and whole cells (Hamill *et al.*, 1981) were made with the K^+ -rich solution in the pipette and the Na^+ -rich solution in the bath. For perforated patch experiments ATP was omitted from the K^+ -rich solution and nystatin (20 μ g ml^{-1}) added. Permeabilized or open-cell experiments (Dunne *et al.*, 1986), in which the plasma membrane was perforated by saponin (0.05%) (Sigma, UK) in contact with the membrane outside the area from which the single-channel current recording was made, were performed with the K^+ -rich solution (without ATP) in the bath and the Na^+ -rich solution (without $CaCl_2$) in the pipette. Patch-clamp pipettes (Type 101 PB, Ceebee Glass, Denmark) were found to have a final resistance of between 5 and 10 M Ω when filled.

Exchange of the bathing solution from control to test, was achieved manually under visual control by a series of outlet pipes (Dunne *et al.*, 1988).

All current and voltage recordings have been either photographed directly from the pen recording trace, filtered at 20 Hz (low pass) or digitized and then plotted by use of a CED software package (Cambridge, UK). Upward deflections represent outward current flow (i.e. from the inside to the outside of the membrane patch). Changes in potassium channel open-state probability (P) have been quantified from pre-recorded stretches of data lasting between 15 and 20 s, and are expressed as a fraction of the pre-control value (P_c) (Dunne, 1989). Analysis has been performed with a CED 1401 interface and a software package supplied by CED (Cambridge, UK).

Results

Nystatin-perforated and conventional whole-cells have been used to investigate the effects of phentolamine on the electrical activity of individual cells. Under current-clamp conditions the average resting membrane potential was found to be -67 ± 4 mV (mean $\pm$ s.e.mean, $n = 7$ separate cells). When challenged with phentolamine (added directly to the bath) over the range of 5–20 μ M, the cells underwent a rapid depolarization, which was maximal within 45 s of stimulation, found on average to be 24 ± 2 mV ($n = 7/7$ applications) and was associated with the persistent generation of Ca^{2+} action potential spikes. In the continued presence of phentolamine, 100 μ M diazoxide reversed the effects of the drug by causing a marked repolarization of the membrane which led to the termination of spike potentials ($n = 6$ separate cells) (Figure 1). The maximal effect of diazoxide was seen between 8 and 22 s following its addition to the bath.

Effects of phentolamine on unitary current events from ATP-sensitive potassium channels have been studied with both permeabilized or open-cells and excised outside-out patches. A typical open-cell experiment is shown in Figure 2. The record begins 25 s after 0.05% saponin was added to the bath solution. Dialysis of the cell leads to the wash-out of cytosolic ATP and the subsequent activation of K^+_{ATP} channels, as has been previously described (Dunne *et al.*, 1986). At the points indicated ATP (1 mM) and phentolamine (10 μ M) were individually added to the bathing solution, which, under these conditions, is now in contact with the inside of the plasma membrane. Both ATP and phentolamine inhibited the K^+_{ATP} channels, reducing the relative open-state probability

Figure 1 Effects of diazoxide on the phentolamine-induced electrical activity of an isolated insulin-secreting cell. The continuous voltage record, which was obtained with a nystatin-perforated cell (current-clamp mode), illustrates that in the continued presence of phentolamine (5 μ M), diazoxide (100 μ M) reverses the effects of the drug on the membrane potential.

Figure 2 Inhibition of ATP-sensitive potassium channels by phentolamine. The current record shows how both ATP (1 mM) and phentolamine (10 μ M) inhibit K^+_{ATP} channels in a RINm5F open-cell. The current trace starts 25 s after permeabilization of the cell was complete.

(P/P_c) from 1 to 0.16 and 0.31, respectively. The actions of both ATP and phentolamine were fully reversible upon removal of the compounds from the bath; $P/P_c = 1.17$ and 1.05, respectively. Over the range 0.1 to 100 μ M, phentolamine was added to a total of 10 separate open-cells. In one of these it was possible to complete the full range of phentolamine concentrations several times, and the concentration-response relationship for this patch has been shown in Figure 3. The K_i value of 50% channel inhibition was estimated to be approximately 0.7 μ M, and the data fitted by a Hill coefficient of 0.95, according to the equation:

$$\frac{P}{P_c} = \frac{1}{1 + ([x]/K_i)^h} \quad (1)$$

where K_i is the concentration of phentolamine that produces half-maximal inhibition of K^+_{ATP} channels ($P/P_c = 0.5$), $[x]$ is the concentration of the inhibitor and h is the slope parameter (Hill coefficient).

Inhibition of K^+_{ATP} channels by 0.1 μ M phentolamine was seen on a further 3/4 occasions in 4 separate cells; 1 μ M phentolamine on 6/6 occasions in 4 separate cells; 10 μ M phentolamine, $n = 5/6$ in 6 separate cells and 100 μ M closed channels in 4 separate cells, $n = 4/4$.

The effects of 5 μ M phentolamine on K^+_{ATP} channels when added to the outside of the membrane is shown in Figure 4. The record was obtained from an excised outside-out membrane patch, recorded with 1 mM ATP on the cytoplasmic face of the membrane. Phentolamine reversibly inhibited channels reducing P/P_c from 1 to 0.26 in the record shown, and to an average value of 0.45 ± 0.1 in 4 separate patches.

Channel blockade from either the inside or the outside of the membrane was rapid, readily reversible and was not

Figure 3 Concentration-dependent block of K^+_{ATP} channels by phentolamine. The concentration-response curve was obtained from a single open-cell exposed to phentolamine over the range 0.1 to 100 μ M. Each point represents the mean (s.e.mean shown by vertical bars) of between 4 and 6 additions of each of the concentrations of phentolamine to the patch. The curve is drawn to equation 1 (see Results section), with a Hill coefficient of 0.95 and a K_i value of 0.7 μ M.

Figure 4 Block of K^+_{ATP} channels by phentolamine applied to the outside of the membrane. The record was taken from an excised outside-out patch, recorded with 1 mM ATP on the cytoplasmic face of the membrane. The scale bar on the left-hand side of the current trace corresponds to the number of coincident K^+ channel openings.

associated with any significant reduction in the amplitude of the single channel current event.

Inhibition of ATP-sensitive potassium channels (open-cell records) was also brought about by the α -adrenoceptor antagonists antazoline (25 μ M) which reduced P/P_c from 1 to 0.28 ± 0.08 ($n = 4$), tolazoline (25 μ M) $P/P_c = 0.49 \pm 0.06$ ($n = 4$) see Figure 5, and by yohimbine (10 μ M) $P/P_c = 0.19 \pm 0.09$ ($n = 3$). The actions of antazoline, tolazoline and yohimbine were not associated with any marked decrease in the amplitude of the unitary current events.

Discussion

The present work illustrates that phentolamine is a highly effective blocker of ATP-sensitive potassium channels in insulin-secreting cells, which agrees with the data of Plant & Henquin (1990) who have recently shown that the α -adrenoceptor antagonist inhibits whole-cell K^+ currents and ^{86}Rb efflux from pre-loaded islets. Phentolamine-evoked closure of K^+_{ATP} channels explains how the drug is able to initiate changes in the cell electrical activity similar to those brought about by either glucose or glyceraldehyde in these

cells (Dunne, 1990) by promoting a depolarization of the membrane and the generation of Ca^{2+} spike potentials. Figure 1 shows that diazoxide — a specific activator of K^+_{ATP} channels in insulin-secreting cells (Trube *et al.*, 1986; Dunne *et al.*, 1987; Dunne, 1989), reverses the effects of phentolamine on the cell electrical activity and this fits well with the previous observations that phentolamine counteracts the actions of diazoxide on ^{86}Rb efflux and insulin-secretion (Burr *et al.*, 1971; Henquin *et al.*, 1982).

By use of a combination of excised outside-out patches and permeabilized cells, it has been demonstrated that phentolamine is an effective blocker of K^+_{ATP} channels when it is added directly to either the inside or the outside of the plasma membrane. The concentration-dependent block of K^+ channels in open-cells was half maximal at 0.7 μ M, fitted by a Hill coefficient of 0.95 and not associated with significant effects upon the amplitude of the single-channel current event. The efficacy of channel blockade was found to be more potent when phentolamine was applied internally, since 5 μ M phentolamine in outside-out patches reduced the relative open-state permeability of channels by approximately 55% vs 80% in open-cells. This finding may indicate that the binding site for phentolamine is located on the inner plane of the plasma membrane. Indeed if this is the case it may explain why using the whole-cell recording configuration, where it is harder to completely 'wash' the membrane, Plant & Henquin (1990) reported the effects of phentolamine to be poorly reversible. In my experiments the actions of phentolamine upon whole-cells and nystatin-perforated cells were readily reversible, but the relative concentrations of the drug were very much lower than those used by Plant & Henquin (1990).

The K_i value corresponding to 50% closure of K^+_{ATP} channels, was estimated to be approximately 0.7 μ M, which indicates that phentolamine is as effective a blocker of K^+_{ATP} channels as tolbutamide, $K_i = 3\text{--}18 \mu\text{M}$ (for review see Ashcroft & Ashcroft, 1990). The efficacy of channel inhibition was apparently not enhanced by the presence of cytosolic ATP, since in outside-out patches with 1 mM ATP in the pipette solution, phentolamine was less efficient at closing the channels than in open-cell experiments, which were carried out in the complete absence of ATP. One may speculate that, as with tolbutamide (Zunkler *et al.*, 1988), the potency of the drug may be further enhanced by the presence of internal ADP.

There can be little doubt that imidazoline-based adrenoceptor antagonists bind to sites other than the adrenoceptor (Michel & Insel, 1989). By analogy with the effects of the sulphonylureas on K^+_{ATP} channels in insulin-secreting cells (Ashcroft & Ashcroft, 1990), it is not yet clear from these preliminary studies, whether one of these sites, an 'imidazoline receptor', is part of the K^+_{ATP} channel or a separate protein. There is, however, firm evidence to suggest that the two are very closely associated, since all the imidazoline α -adrenoceptor antagonists so far tested, block K^+_{ATP} channels and initiate insulin secretion; phentolamine (as discussed), antazoline and tolazoline (Figure 5; Schultz & Hasselblatt, 1989) and efroxan (Chan & Morgan, 1990; Dunne *et al.*, 1991). This observation can also be extended to include the substituted imidazoline idazoxan, which at low concentrations has little effect upon insulin secretion in the absence of an adrenoceptor agonist (Ostenson *et al.*, 1988; Chan & Morgan, 1990) but at higher concentrations (>100 μ M) was found to reverse partially the diazoxide-induced inhibition of insulin secretion (Chan & Morgan, 1990) and block K^+_{ATP} channels (Dunne *et al.*, 1990). Yohimbine, on the other hand, is an α -adrenoceptor antagonist that does not contain an imidazoline structure but is also an efficient blocker of K^+_{ATP} channels (Plant & Henquin, 1990).

The pharmacological regulation of ATP-regulated potassium channels is complex (as reviewed by Petersen & Dunne, 1989; Quast & Cook, 1989; Ashcroft & Ashcroft, 1990). Channel blockers include the sulphonylureas, plant extracts and local anaesthetics. Since we can now add to this list certain α -adrenoceptor antagonists, there are two major

Figure 5 Blockade of K^+_{ATP} channels by tolazoline (a) and antazoline (b); both records come from separate open-cell patches. The scale bar on the left-hand side of each current trace corresponds to the number of coincident K^+ channel openings.

pharmacological implications of this study. Firstly, as pointed out by Plant & Henquin (1990), the results of many *in vivo* and *in vitro* studies with phentolamine and other α -adrenoceptor antagonists, may now need to be re-interpreted. Secondly, these data may provide us with a platform to develop an alternative strategy to the sulphonylurea-based drugs in the therapeutic treatment of diabetes mellitus. Such an approach would be feasible if it were possible to isolate the α -adrenoceptor blocking actions of compounds like phentolamine and efaroan from the effects upon K^+_{ATP} channels and

this may turn out to be feasible through the use possibly of pure enantiomeric forms of the drugs.

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