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Phentolamine and yohimbine inhibit ATP-sensitive K^+ channels in mouse pancreatic β-cells

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1 The effects of phentolamine and yohimbine on adenosine 5'-triphosphate (ATP)-sensitive K^+ channels were studied in normal mouse β-cells.

2 In the presence of 3 mM glucose, many ATP-sensitive K^+ channels are open in the β-cell membrane. Under these conditions, phentolamine inhibited ^{86}Rb efflux from the islets. This inhibition was faster with 100 than with 20 μM phentolamine but its steady-state magnitude was similar with both concentrations. Yohimbine (20–100 μM) also inhibited the efflux rate but was not as potent as phentolamine.

3 In the presence of 6 mM glucose, most ATP-sensitive K^+ channels are closed in the β-cell membrane. Their opening by 100 μM diazoxide caused a marked acceleration of ^{86}Rb efflux from the islets. This acceleration was almost entirely prevented by 20 μM phentolamine. It was barely affected by 20 μM yohimbine and reduced by 50% by 100 μM yohimbine.

4 ATP-sensitive K^+ currents were studied in single β-cells by the whole cell patch-clamp technique. Phentolamine (20–100 μM) caused a progressive but almost complete and irreversible inhibition of the current. The effects of yohimbine were faster but smaller; the inhibition was still incomplete with 100 μM yohimbine.

5 The increase in ATP-sensitive K^+ current produced by 100 μM diazoxide was prevented by 100 μM phentolamine but only partially attenuated by 100 μM yohimbine.

6 It is concluded that phentolamine inhibits ATP-sensitive K^+ channels in pancreatic β-cells. This novel effect of phentolamine resembles that of hypoglycaemic sulphonylureas. It may account for previously unexplained effects of the drug. These observations also call for reinterpretation of many studies in which phentolamine was used as an allegedly specific blocker of α-adrenoceptors.

Introduction

Phentolamine is a classical blocker of α-adrenoceptors. However, its use has sometimes led to the erroneous conclusion that these receptors are involved in certain physiological or pharmacological events.

Because activation of α_2-adrenoceptors inhibits insulin release (Nakaki *et al.*, 1980), and because phentolamine reverses the inhibition of insulin release provoked by diazoxide (Blackard & Aprill, 1967; Burr *et al.*, 1971; Porter *et al.*, 1978), it was first thought that the latter drug activates α-adrenoceptors in β-cells. Subsequent studies showed that, among α-adrenoceptor antagonists, phentolamine (α_1, α_2), but not yohimbine (α_2) or dihydroergotamine (α_1, α_2) could prevent the acceleration of ^{86}Rb efflux and the inhibition of insulin release caused by diazoxide in rat islets (Henquin *et al.*, 1982). It was thus concluded that the effect of phentolamine was not mediated by α-adrenoceptors, but resulted from its ability to counteract the increase in K^+ permeability caused by diazoxide (Henquin & Meissner, 1982). The mechanism of action could not be further analyzed because it was only discovered later that diazoxide opens ATP-sensitive K^+ channels in β-cells (Trube *et al.*, 1986; Dunne *et al.*, 1987; Sturgess *et al.*, 1988).

More recently it has been reported that phentolamine reverses the hyperpolarization and the relaxation of vascular and tracheal muscles caused by cromakalim (McPherson & Angus, 1989; Murray *et al.*, 1989). Since cromakalim opens ATP-sensitive K^+ channels in smooth muscle (Standen *et al.*, 1989), it is possible that phentolamine interferes with the action of cromakalim at the K^+ channel level.

In the present study we have investigated whether phentolamine has direct effects on ATP-sensitive K^+ channels in mouse pancreatic β-cells. The effects of phentolamine were also compared to those of yohimbine.

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Methods

All experiments were carried out on islets obtained by collagenase digestion of the pancreas of fed female NMRI mice. ^{86}Rb efflux experiments were performed at 37°C and patch-clamp experiments at room temperature (20–24°C).

For measurement of the efflux of ^{86}Rb (used as tracer for K), the islets were first loaded with the tracer for 90 min in a medium containing 15 mM glucose and supplemented with $^{86}RbCl$ (1.5 to 3 MBq ml^{-1}; sp.act. 7.4 to 18.5 TBq mol^{-1}). The concentration of Rb never exceeded 0.4 mM (Garrino & Henquin, 1988). The islets were then transferred to a dynamic system of perifusion, permitting ^{86}Rb efflux to be monitored (Henquin, 1978). The solutions used had the following ionic composition (in mM): NaCl 120, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 24. They were gassed with O_2/CO_2 (94:6) to maintain a pH of 7.4 and were supplemented with 1 mg ml^{-1} bovine serum albumin fraction V (Boehringer, Mannheim, Germany).

For patch-clamp measurements, islets were first dispersed into single cells which were then cultured for 1–2 days (Plant, 1988). Recordings were made in a bath solution containing (in mM): NaCl 135, KCl 5.6, $CaCl_2$ 2.6, $MgCl_2$ 1.2, HEPES 10, and titrated to pH 7.4 with NaOH. Pipettes were filled with a solution containing (in mM): KCl 135, $MgCl_2$ 4, $CaCl_2$ 2, EGTA 10, Na_2ATP 0.65, Na_2GTP 0.1, HEPES 20, titrated to pH 7.15 with KOH. This solution has a low free Ca^{2+} concentration (50 nM) and a lower than physiological concentration of ATP. The technique of whole-cell recording and the method used to isolate ATP-sensitive K^+ currents from other membrane currents have been described previously (Garrino

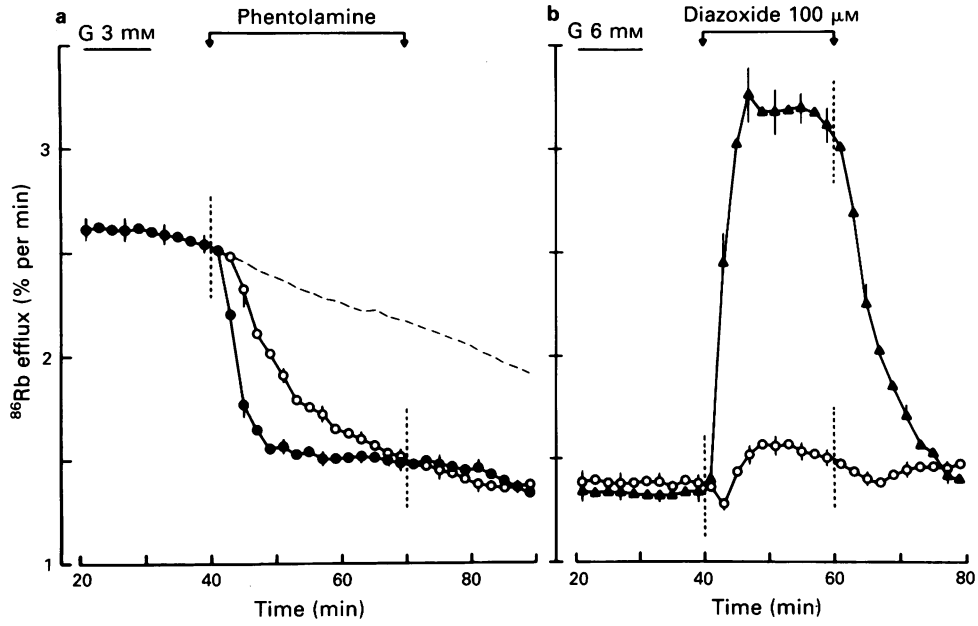

Figure 1 Effects of phentolamine on ^{86}Rb efflux from mouse islets. (a) Phentolamine was added at the concentration of 20 μM (○) or 100 μM (●) to a medium containing 3 mM glucose (G). Controls without phentolamine are shown by the broken line. (b) Diazoxide (100 μM) was added to a medium containing 6 mM glucose alone (▲) or with 20 μM (○) phentolamine. Values are means for 5 experiments with s.e.mean shown by vertical lines.

et al., 1989). Briefly, currents were measured at 15 s intervals at the holding potential (−70 mV), and in response to 100 ms voltage steps to −60 and −80 mV separated by 100 ms. At these potentials, which are more negative than the thresholds

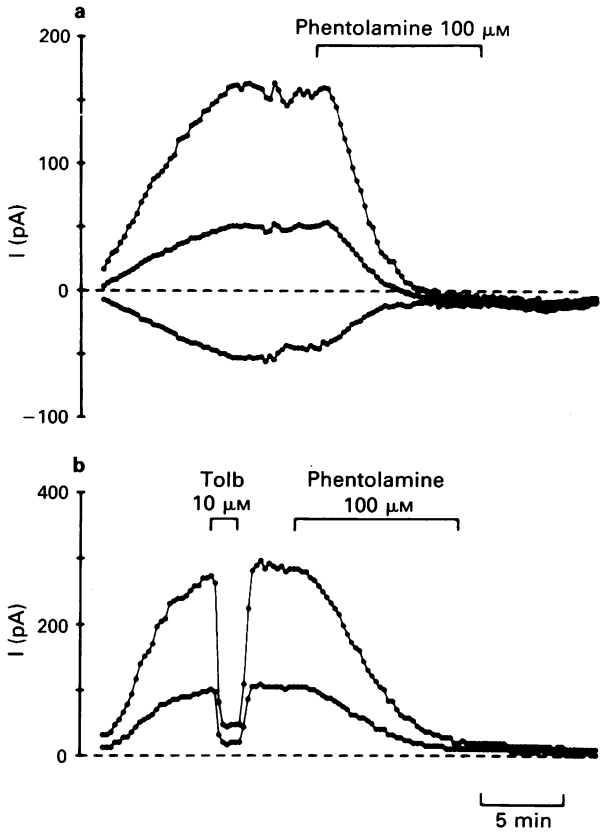

Figure 2 Inhibition of ATP-sensitive K^+ currents by 100 μM phentolamine in mouse β-cells. (a) The upper, middle and lower traces show currents recorded at −60, −70 and −80 mV respectively. (b) Comparison of the inhibition produced by 10 μM tolbutamide (Tolb) and 100 μM phentolamine. Currents were recorded at −60 mV (upper trace) and −70 mV (lower trace). The values shown correspond to currents measured at 15 s intervals.

for the activation of voltage-dependent Ca^{2+} and K^+ channels, and with the intracellular medium used, the current which develops with time is almost entirely a current through ATP-sensitive K^+ channels. This is indicated by the near complete block of the current by 100 μM tolbutamide, a specific blocker of ATP-sensitive K^+ channels (Trube *et al.*, 1986).

To study the voltage-dependent K^+ currents in the absence of ATP-sensitive K^+ currents, 100 μM tolbutamide was added to the bathing solution. The solutions were otherwise the same as those used for the measurement of ATP-sensitive K^+ currents. Voltage-dependent K^+ currents activate at potentials above −40 mV (Rorsman & Trube, 1986). To monitor the effect of phentolamine, cells were held at a potential of −70 mV and hyperpolarized for 50 ms to −100 mV, to measure the leakage current. This pulse was followed after 100 ms by a 150 ms step to 0 mV which activates voltage-sensitive K^+ currents. The Ca^{2+}- and voltage-activated K^+ current, which is also present in these cells, is not activated when cells are dialysed with pipette solutions containing the high concentration of calcium buffer (10 mM EGTA, 2 mM Ca) which was used here (Satin *et al.*, 1989). Recordings were started 30 s after rupturing the membrane patch and entering the whole-cell configuration.

Phentolamine mesylate was obtained from Ciba Geigy (Basel, Switzerland), yohimbine hydrochloride was from Aldrich Chemie (Steinheim, F.R.G.), tolbutamide from Hoechst A.G. (Frankfurt, F.R.G.) and diazoxide was provided by Schering Corp. (Bloomfield, NJ, U.S.A.). Stock solutions of diazoxide (40 mM) were prepared in 0.1 M NaOH.

Results

Effects of phentolamine on ^{86}Rb efflux

In the presence of 3 mM glucose, many ATP-sensitive K^+ channels are open in the β-cell membrane (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988). Under these conditions, the rate of ^{86}Rb efflux is high and decreases slightly with time (Figure 1a). Addition of phentolamine to the medium inhibited ^{86}Rb efflux. The rate of this inhibition was higher with 100 than with 20 μM phentolamine, but its steady-state magnitude was similar with both concentrations of the drug.

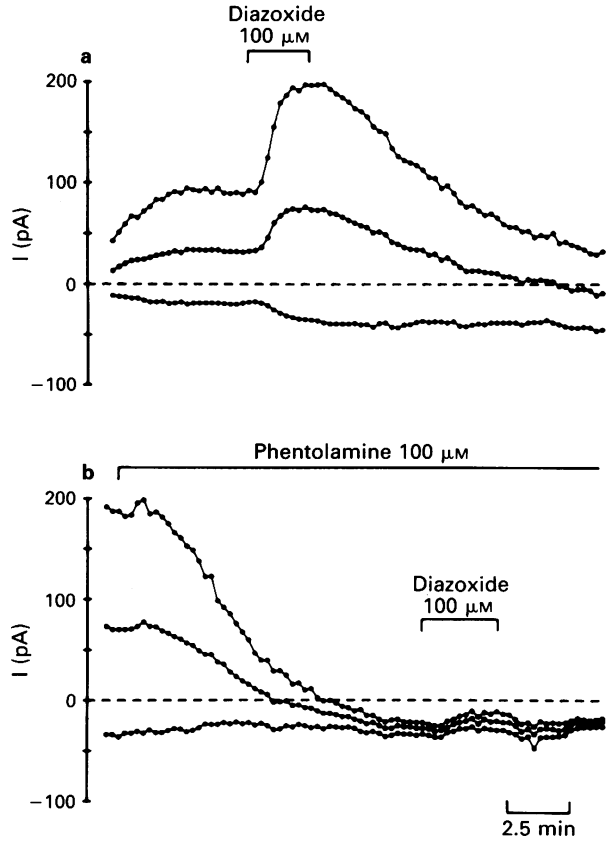

Figure 3 Effects of 100 μM diazoxide on ATP-sensitive K^+ currents in mouse β-cells, (a) under control conditions and (b) in the presence of 100 μM phentolamine. In both parts of the figure the upper, middle and lower traces are the currents recorded at intervals of 15 s at −60, −70 and −80 mV respectively.

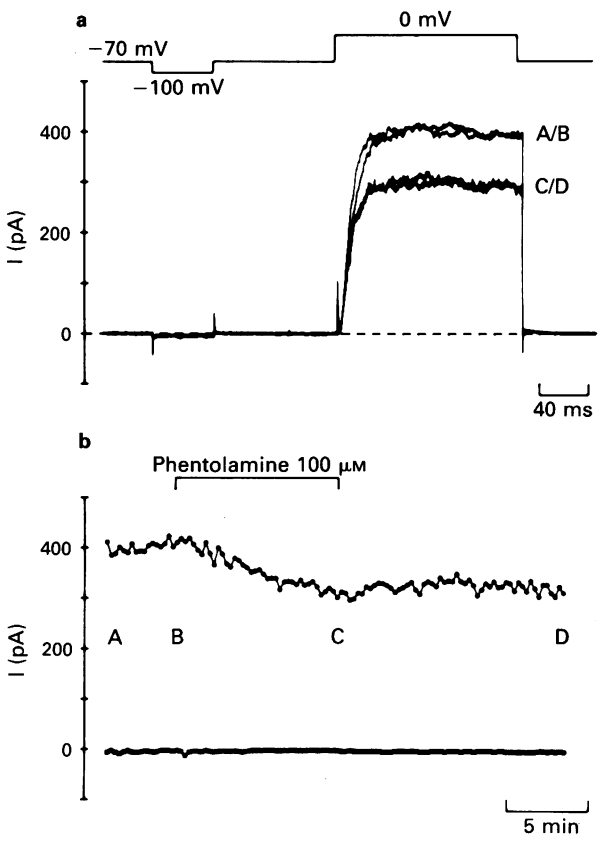

Figure 4 Effects of phentolamine on voltage-dependent K^+ currents in a mouse β-cell. (a) Currents recorded in response to the illustrated protocol at the beginning of the experiment (A), just before adding phentolamine (B), after 10 min of phentolamine application (C), and after washing for 14 min with a phentolamine-free solution (D). (b) Time-course of the effect of phentolamine: the times at which the currents shown in the upper part were recorded are indicated by capital letters. The lower trace near the 0 value shows that the leakage current measured during the hyperpolarizing pulse to −100 mV was not influenced by phentolamine.

In the presence of 6 mM glucose, most ATP-sensitive K^+ channels are closed in the β-cell membrane (Rorsman & Trube, 1985; Misler *et al.*, 1986; Ashcroft *et al.*, 1988), and the rate of ^{86}Rb efflux is lower and more stable than in 3 mM glucose (Figure 1b). Addition of 100 μM diazoxide markedly accelerated ^{86}Rb efflux. This acceleration was almost totally prevented by 20 μM phentolamine. This confirms our previous observation made in rat islets (Henquin *et al.*, 1982).

Effects of phentolamine on ATP-sensitive K^+ currents

At physiological ATP concentrations, only a small fraction of ATP-sensitive K^+ channels are active and whole cell currents are very small (Rorsman & Trube, 1985). As shown previously (Trube *et al.*, 1986; Garrino *et al.*, 1989), ATP-sensitive K^+ currents increase following breakage of the patch membrane and dialysis of the cell interior with a pipette solution containing less than the physiological concentration of ATP (Figure 2). In these experiments, with 0.65 mM ATP in the pipette solution, currents were not as large as those seen with lower ATP concentrations but were more stable, i.e. showed less run-down, allowing more accurate quantification of the changes produced by test agents.

After the currents had attained a steady level, addition of 100 μM phentolamine to the bathing solution resulted in a slow but very marked decrease in the ATP-sensitive K^+ current (Figure 2a). Currents were decreased by 94.3 ± 0.8% (mean ± s.e.mean, $n = 10$) compared to the current recorded before the addition of the drug. Figure 2b shows that 10 μM tolbutamide rapidly and reversibly blocked ATP-sensitive K^+ channels. This effect was much faster than that of phentolamine although the maximum block (88.3 ± 1.1%, $n = 6$) produced by this low concentration of the sulphonylurea was less than that produced by 100 μM phentolamine. Effects of similar magnitude, though slower than those observed with 100 μM, were observed with 20 and 50 μM phentolamine (results not shown). The effects of phentolamine were irreversible.

Since phentolamine inhibits the increase in ^{86}Rb efflux induced by diazoxide (Figure 1b), we tested the action of diazoxide on ATP-sensitive K^+ currents in the presence of phentolamine. Diazoxide increases ATP-sensitive K^+ currents in β-cells, the magnitude of its effect being dependent on the intracellular ATP concentration (Zünkler *et al.*, 1988). The effects of diazoxide under the conditions used in these experiments are shown in Figure 3a. On addition of diazoxide, the amplitude of the current change in response to a potential step from −70 to −60 mV increased by 65.9 ± 9.2 pA (10.5 ± 1.4 pA/pF; $n = 7$). Relative to the initial control current (100%), this corresponds to an increase to 268 ± 50%. Diazoxide had much less effect in the presence of 100 μM phentolamine (Figure 3b). It increased the evoked current by only 3.2 ± 0.4 pA (0.5 ± 0.1 pA/pF; $n = 5$), that is from 5.7 ± 0.8% to 9.1 ± 1.1% of the initial control current.

Effects of phentolamine on voltage-sensitive K^+ currents

To determine whether phentolamine specifically affects ATP-sensitive K^+ channels, we tested its effects on voltage-sensitive K^+ channels which are also present in the β-cell membrane. A 10 min application of 100 μM phentolamine decreased the voltage-sensitive K^+ current at 0 mV by 30.2 ± 3.3% ($n = 4$) compared to the control value. Neither the time-course of the voltage-sensitive current, nor the leakage current were affected by the treatment with phentolamine (Figure 4). As with the

ATP-sensitive K^+ current, the effects of phentolamine were irreversible.

Effects of yohimbine on ^{86}Rb efflux

In the presence of 3 mM glucose, yohimbine marginally decreased ^{86}Rb efflux from islet cells at the concentration of 20 μM, but caused a clear and reversible inhibition at 100 μM (Figure 5a). In the steady state this inhibition was less than that produced by 20 or 100 μM phentolamine. The acceleration of ^{86}Rb efflux brought about by 100 μM diazoxide in the presence of 6 mM glucose was only slightly attenuated by 20 μM yohimbine and reduced by half by 100 μM yohimbine (Figure 5b).

Effects of yohimbine on ATP-sensitive K^+ currents

Addition of 100 μM yohimbine to the medium resulted in an inhibition of the ATP-sensitive K^+ current which was more rapid than that observed with phentolamine. The inhibition was incomplete compared to the inhibition by 100 μM tolbutamide (Figure 6) and was, at least partially, reversible. In the steady-state, currents were decreased by 77.8 ± 1.3% (n = 5). Small effects of yohimbine were already detectable at a concentration of 10 μM.

On addition of 100 μM diazoxide to a medium containing 100 μM yohimbine, the amplitude of the current change in response to potential steps from −70 to −60 mV increased by 73.0 ± 12.2 pA (11.9 ± 2.2 pA/pF, n = 5). Relative to the initial control current (100%), this corresponds to an increase from 22.2 ± 1.3% in yohimbine alone to 97.9 ± 12.0% in yohimbine plus diazoxide.

Discussion

The present study conclusively shows that phentolamine inhibits ATP-sensitive K^+ channels in pancreatic β-cells. This conclusion is based on three sets of observations. Firstly, phentolamine decreased ^{86}Rb efflux from islets perifused with a low concentration of glucose. The only K^+ channels that are known to be open under these conditions are the ATP-sensitive K^+ channels (Rorsman & Trube, 1985; Ashcroft *et al.*, 1988; Misler *et al.*, 1986; Petersen & Findlay, 1987). More-

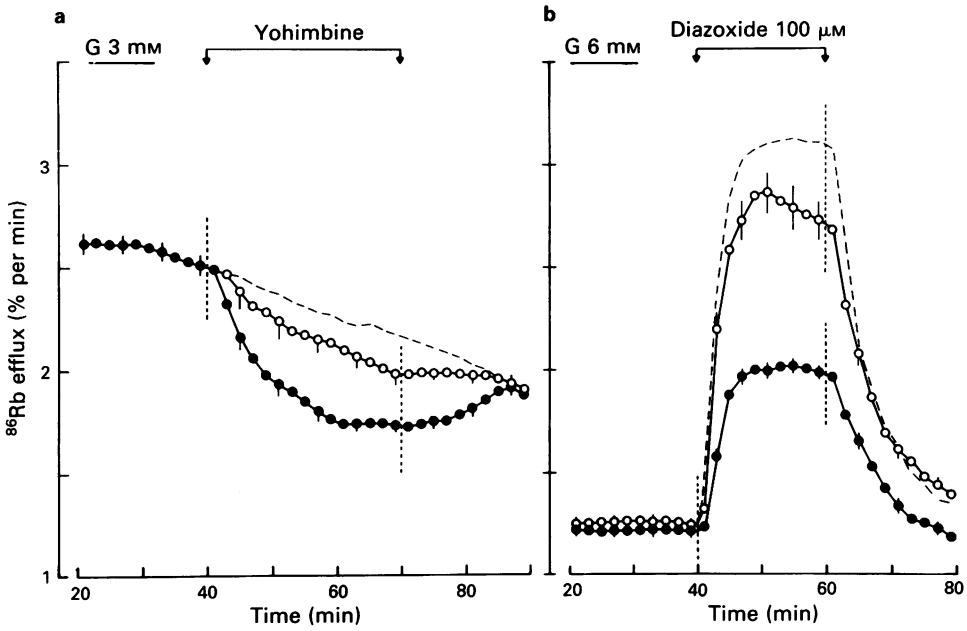

Figure 5 Effects of yohimbine on ^{86}Rb efflux from mouse islets. (a) Yohimbine was added at the concentration of 20 μM (○) or 100 μM (●) to a medium containing 3 mM glucose (G). (b) Diazoxide (100 μM) was added to a medium containing 6 mM glucose and 20 μM (○) or 100 μM (●) yohimbine. Controls without yohimbine are shown by the broken lines. Values are means for 4 experiments with s.e.mean shown by vertical lines.

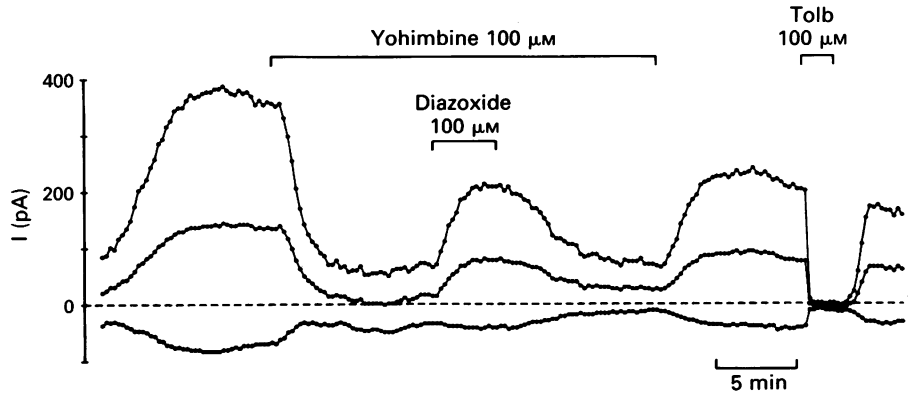

Figure 6 Inhibition of ATP-sensitive K^+ currents by 100 μM yohimbine in a mouse β-cell. Diazoxide (100 μM) was tested in the presence of yohimbine. Following washout of yohimbine, 100 μM tolbutamide (Tolb) was added to the bathing solution. The upper, middle and lower traces show currents recorded at −60, −70 and −80 mV respectively. The values shown correspond to currents measured at 15 s intervals.

over, the only drugs that are known to inhibit ^{86}Rb efflux in the presence of a low concentration of glucose are those which block ATP-sensitive K^+ channels. The best known of them are the hypoglycaemic sulphonylureas (Henquin & Meissner, 1982; Trube *et al.*, 1986). Secondly, ATP-sensitive K^+ currents measured in the whole cell configuration as described by Trube *et al.* (1986) were totally suppressed by phentolamine as they were by tolbutamide. Thirdly, phentolamine prevented the acceleration of ^{86}Rb efflux and the increase in ATP-sensitive K^+ currents otherwise produced by diazoxide, which is currently considered to be a selective activator of ATP-sensitive K^+ channels in β-cells (Trube *et al.*, 1986).

The present observations give direct support to previous presumptions that certain effects of phentolamine might be unrelated to blockade of α_2-adrenoceptors. An inhibition of ATP-sensitive K^+ channels may account for the ability of phentolamine to increase insulin release in the absence of an α-adrenoceptor agonist (Efendic *et al.*, 1975; Smith & Furman, 1988; Schulz & Hasselblatt, 1988; Garrino & Henquin, 1990) and to counteract the inhibition of release caused by diazoxide (Henquin *et al.*, 1982). On the other hand, it may now seem surprising that yohimbine does not share the properties of phentolamine on insulin release since it also inhibits ATP-sensitive K^+ channels. This is probably explained by the fact that yohimbine was generally tested at concentrations of 1–30 μM (Henquin *et al.*, 1982; Hillaire-Buys *et al.*, 1985; Schulz & Hasselblatt, 1988; Garrino & Henquin, 1990) and that the inhibition of ATP-sensitive K^+ channels is still incomplete at 100 μM.

A recent study has ascribed the insulin-releasing effect of phentolamine to its imidazoline structure rather than to its α-adrenoceptor blocking property (Schulz & Hasselblatt, 1989). This conclusion was based, firstly, on the evidence that the concentrations of phentolamine required to increase insulin release were higher than those required to counteract the inhibition by clonidine, and, secondly, on the insulin-releasing effects of imidazoline derivatives that do not block α-adrenoceptors. It is now well established that α-adrenoceptor agonists and antagonists which possess an imidazoline structure bind with high affinity to sites other than the α-adrenoceptor itself (Michel & Insel, 1989). The nature of these imidazoline binding sites is still unknown. The present study might suggest that ATP-sensitive K^+ channels are one of these sites, were not yohimbine, which is not an imidazoline, also able to inhibit these channels. One could even argue that the mere fact that both phentolamine and yohimbine inhibit K^+ channels indicates that this inhibition is related to blockade of the α-adrenoceptors. This problem is not easy to solve because all attempts to demonstrate directly that ATP-sensitive K^+ channels are opened by α_2-adrenoceptor agonists have been unsuccessful. Nevertheless, one observation does not support the above hypothesis: the concentrations of blockers that affect K^+ channels are much higher than those required to block the effects of α-agonists.

The mechanisms by which phentolamine blocks ATP-sensitive K^+ channels are not established, and their investigation was beyond the scope of this study. Some features of this inhibition deserve comment, however. The rate of inhibition by phentolamine was much lower than that by tolbutamide, and was dose-dependent, whereas the steady-state effect was not dose-dependent. These characteristics and the poor reversibility of the inhibition suggest that phentolamine has no direct or easy access to the blocking site of the channel. It is unlikely, however, that the effect is non-specific and the mere consequence of the accumulation of phentolamine in the membrane since voltage-dependent K^+ channels were only marginally affected. The inhibition of ATP-sensitive K^+ channels by yohimbine differed from that by phentolamine by its greater rapidity, reversibility and dose-dependency. Distinct mechanisms are thus likely to be involved.

The present findings have two important pathophysiological implications. Firstly, they call for reinterpretation of numerous human and animal studies in which phentolamine was used as an allegedly specific blocker of α-adrenoceptors. In particular, the role of the α-adrenergic control of insulin release in the absence of stress conditions may have been overestimated (Cerasi *et al.*, 1969; Buse *et al.*, 1970; Misbin *et al.*, 1970; Lundquist, 1972; Robertson & Porte, 1973). Secondly, our findings cast doubts on the hypothesis that an excessive α-adrenergic tone contributes to the impaired release of insulin in non-insulin-dependent diabetic patients (Robertson *et al.*, 1976). A cornerstone of this hypothesis was indeed the observation that phentolamine increased plasma insulin levels in these patients. A clinical study using idazoxan instead of phentolamine had already questioned this concept (Ostenson *et al.*, 1988).

In conclusion, both phentolamine and yohimbine are able to inhibit ATP-sensitive K^+ currents in pancreatic β-cells. Our findings suggest that care must be taken when interpreting results obtained with high concentrations of these blockers of α-adrenoceptors.

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