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Amantadine and sparteine inhibit ATP-regulated K-currents in the insulin-secreting β-cell line, HIT-T15

[1]Frances M. Ashcroft, *Amanda J. Kerr, John S. Gibson & Beatrice A. Williams

University Laboratory of Physiology, Parks Road, Oxford OX1 3PT and *Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford OX3 9DU

1 The effects of pharmacological agents that potentiate insulin release were studied on ATP-regulated K-currents (K-ATP currents) in the insulin-secreting β-cell line HIT-T15 by use of patch-clamp methods.

2 The tricyclic drug, 1-adamantanamine (amantadine), reversibly inhibited both whole-cell currents (with a K_i of 120 μM) and single channel currents in inside-out patches. This effect was principally due to an increase in a long closed state which reduced the channel open probability. The related compound, 1-adamantanol, in which the amino group is substituted by a hydroxyl one, did not inhibit K-ATP currents substantially.

3 The alkaloid, sparteine, reversibly inhibited both whole-cell K-ATP currents (K_i = 171 μM) and single channel currents in inside-out patches.

4 The results suggest that sparteine and amantadine can block the K-ATP channel from either side of the membrane and support the idea that at least part of the stimulatory effect of these agents on insulin secretion results from inhibition of this channel.

Keywords: K-channel; ATP-regulated K-channel; sparteine; amantadine; amantanol; insulin secreting β-cell line (HIT-T15)

Introduction

Glucose metabolism by the pancreatic β-cell causes a slow depolarization of the β-cell membrane which triggers a characteristic pattern of electrical activity and thereby initiates insulin secretion (for reviews see Henquin & Meissner, 1984; Ashcroft & Rorsman, 1989). This slow depolarization is mediated by a decrease in membrane K^+ permeability which results from the inhibition of ATP-regulated K-channels (K-ATP channels) (Ashcroft *et al.*, 1984; Rorsman & Trube, 1985; Misler *et al.*, 1986). It is believed that an increase in the cytosolic ATP/ADP ratio links glucose metabolism to channel inhibition (Cook & Hales, 1984; Ashcroft & Rorsman, 1989). K-ATP channels are also inhibited by sulphonylureas, such as tolbutamide, which are used clinically in the treatment of non-insulin-dependent diabetes mellitus (Sturgess *et al.*, 1985; Trube *et al.*, 1986).

Recently, several other moieties, unrelated to the sulphonylureas, have been found to stimulate insulin release from isolated islets in the absence of glucose, whilst concomitantly reducing resting membrane K^+ permeability (Henquin, 1990). Among these are the tricyclic compound, 1-adamantanamine (amantadine) (Garrino & Henquin, 1987) and the alkaloid sparteine (Paolisso *et al.*, 1985). It seems probable that the effect of these compounds on insulin release is also mediated via inhibition of the K-ATP channel, since this channel contributes most of the resting K-permeability of the β-cell (Ashcroft *et al.*, 1988). Amantadine is of special interest since substitution of its amine group alters its efficacy as an insulin secretagogue; thus, carboxyl substitution (1-adamantanecarboxylic acid) reduces its potency, whilst hydroxyl substitution (1-adamantanol) renders it ineffective (Garrino & Henquin, 1987). Clinically, amantadine is used in parkinsonism and as a viral prophylactic (Parkes, 1974) although its mode of action is unknown. Sparteine has been used as an anti-arrhythmic or oxytocic agent (Goodman & Gilman, 1965).

In the present paper, we use the patch-clamp technique to investigate directly the effect of amantadine, amantanol and sparteine on K-ATP channels in the insulin-secreting cell-line HIT-T15. The results demonstrate that amantadine and sparteine reversibly inhibit the K-ATP channel when applied from either side of the cell membrane, whilst amantanol has little inhibitory effect.

[1] Author for correspondence.

Methods

Preparation

Experiments were carried out on the hamster clonal β-cell line HIT-T15 which secretes insulin in response to glucose (Ashcroft *et al.*, 1986). Cells were cultured as described previously (Ashcroft *et al.*, 1986), and used for up to 4 days after splitting and replating. All experiments were carried out on single cells.

Data recording and analysis

We used the whole-cell and inside-out configurations of the patch-clamp technique (Hamill *et al.*, 1981). Pipettes were pulled from borosilicate glass (Boralex, Rochester Scientific, U.S.A.), coated with Sylgard (Dow Corning, Seneffe, Belgium) close to their tips and fire-polished immediately before use. They had resistances between 2 and 5 MΩ when filled with intracellular solution. Currents were recorded with a List EPC 5 or EPC 7 patch-clamp amplifier (List Electronic, Darmstadt, F.R.G.) and stored on FM or video tape for later analysis.

We used a pulse protocol similar to that described by Trube *et al.* (1986) to monitor the effects of drugs on the whole-cell K-ATP current: the membrane potential was held at −70 mV and 10 ms pulses of +10 mV or −10 mV were applied alternately every 100 ms. For single channel recordings, the membrane was held at −70 mV: at this potential, K-ATP currents were inward under the ionic conditions of our experiments. The reference potential for all measurements was the zero current potential of the pipette before establishment of the seal.

Whole-cell current amplitudes were measured when the response to several successive current pulses was stable, after redisplay on a digital oscilloscope. They are expressed as the fraction I/I_c where I_c is the control current amplitude immediately before drug application and I is the current amplitude measured in the presence of the drug. The dose-response curves were fitted to the Hill equation using a least squares method.

$$\frac{I}{I_c} = \frac{1}{1 + ([X]/K_i)^h} \quad \text{eqn. 1}$$

where [X] is the concentration of inhibitor, h is the slope parameter (Hill coefficient) and K_i is the concentration that gives half-maximal inhibition ($I/I_c = 0.5$).

For analysis of channel kinetics and open probability, single channel currents were filtered (−3 db down at 2 kHz; 8-pole Bessel filter), amplified (×10) and digitized at 5 kHz using an Axolab interface and the programme PCLAMP (Axon Instruments). They were subsequently analysed using a Dell 320LX computer with in-house software. The open probability was determined from data segments of 100–200 s duration by measuring the total time spent at each unitary current level and expressing this as a fraction of the total time.

Records shown in this paper have been replotted with a Gould 3200 chart recorder which filtered the data at 15 Hz (Gould, Cleveland, U.S.A.).

Solutions

During whole-cell experiments the bath contained extracellular solution (solution A, mM): NaCl 135, KCl 5, $CaCl_2$ 5, $MgSO_4$ 2, HEPES 5 (titrated to pH 7.4 with NaOH). The pipette was filled with an intracellular solution (solution B) containing (mM): KCl 107, $MgSO_4$ 2, $CaCl_2$ 1, EGTA 11 (0.06 μM free Ca^{2+}), HEPES 11 (pH 7.2 with KOH; additional K^+ about 30 mM). Na_2ATP, 0.3 mM, was also included to prevent run-down of K-ATP currents; this concentration of ATP blocks K-ATP currents only slightly in whole-cell recordings (Trube *et al.*, 1986).

In the case of inside-out patch recordings, the pipette was filled with a high K^+ extracellular solution in which KCl replaced NaCl (solution C). K-ATP channel activity declines with time after patch excision in inside-out patches (Niki *et al.*, 1989). We attempted to minimize this rundown in two ways. In early experiments, the bath contained intracellular solution (B) to which 0.1 mM ATP, 0.1 mM ADP and 2 mM $MgCl_2$ was added (cf. Ashcroft & Kakei, 1989; Niki *et al.*, 1989). More recently, it has been reported that Mg-free solutions abolish rundown of K-ATP channel activity in CRI-G1 β-cells (Kozlowski & Ashford, 1990). In later experiments, therefore, the bath contained (solution D, mM): KCl 140, $CaCl_2$ 4.6, EDTA 10 (about 30 nM free Ca^{2+} and 1 nM free Mg^{2+}), HEPES 10 (pH 7.2 with KOH).

1-Adamantanamine (amantadine) hydrochloride (the generous gift of Merz (F.R.G.)) and sparteine (Sigma) were dissolved in water; 1-adamantanol (amantanol, Sigma) was dissolved in dimethylsulphoxide (DMSO, final concentration <1%).

The bath solution could be exchanged within 30 s. Experiments were carried out at room temperature (22°C).

Statistics

All data in the text and figures are given as the mean of n observations ± s.e. of the mean (s.e.mean).

Results

Control conditions

Following establishment of the whole-cell configuration, there was a progressive increase in the input conductance of the HIT-T15 cell, from around 0.5 nS initially to a mean value of 7.5 ± 0.6 nS ($n = 27$) after about 5 min. This increase of conductance represents the activation of K-ATP channels caused by wash-out of ATP from the cell as it can be largely prevented by inclusion of 3 mM ATP in the pipette and it is inhibited by tolbutamide (Niki *et al.*, 1989). The effect of drugs on the whole-cell K-ATP current was tested only after the input conductance was stable.

Inside-out patches showed a variable degree of channel activity with between 2 and 40 channels open simultaneously. These were identified as K-ATP channels by the following criteria (Niki *et al.*, 1989): (i) the mean single channel conductance was 62 ± 2 pS ($n = 12$); (ii) channel activity was voltage-insensitive and (iii) was inhibited by ATP (1 mM) and by tolbutamide (100 μM). In most patches, the effect of ATP on channel activity was tested before the application of any drug.

Effect of amantadine

The inhibitory effect of amantadine on the whole-cell K-ATP current is shown in Figure 1a(i). Drug concentrations greater than 10 μM rapidly and reversibly inhibited whole-cell K-ATP currents. Figure 1b shows the relationship between the K-ATP current and amantadine concentration; the solid line is fitted to the Hill equation (eqn. 1), with a K_i of 120 μM and a Hill coefficient of 0.92.

The whole-cell K-ATP current is the product of the single channel current (i), the number of functional K-ATP channels in the cell membrane (N) and the channel open probability (P). To determine which of these parameters is affected by amantadine we tested the effect of the drug on single K-ATP channel currents in inside-out patches.

Addition of 0.5 ($n = 3$; Figure 2) or 1 mM ($n = 15$) amantadine to the intracellular membrane surface reversibly reduced K-ATP channel activity in all cases. In some patches a small

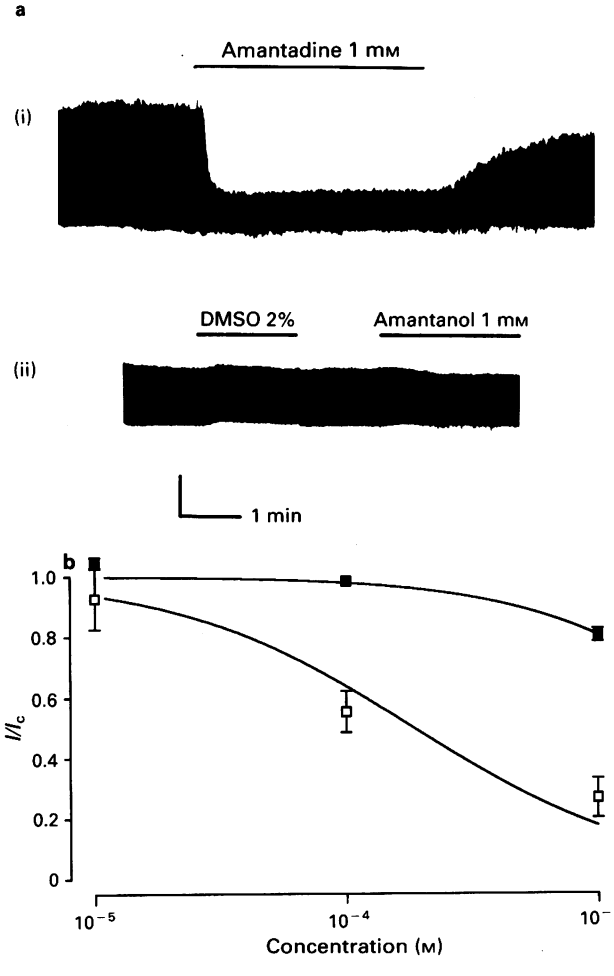

Figure 1 The effect of external application of amantadine and amantanol on the whole-cell K-ATP current. (a) (i)–(ii): Currents recorded in response to ±10 mV pulses from a holding potential of −70 mV. The time scale results in the current transients appearing as a continuous trace. Amantadine (1 mM, (i)), DMSO (2%, (ii)) and 1% DMSO + 1 mM amantanol (ii) were applied during the period denoted by the bars. Data were obtained from two different cells. The scale bar indicates 40 pA in (i) and 100 pA in (ii). (b) Dose-inhibition curve for amantadine (□) and amantanol (■). Values are given as a fraction of the control current (I/I_c). The points represent the mean of 3 or 4 cells and the vertical lines ±s.e.mean. The curve through the amantadine data (□) is fitted to eqn. 1 of the text with $K_i = 120$ μM and h = 0.92; that through the amantanol data (■) is fitted to eqn. 1 using K_i = 5 mM and h = 1.03.

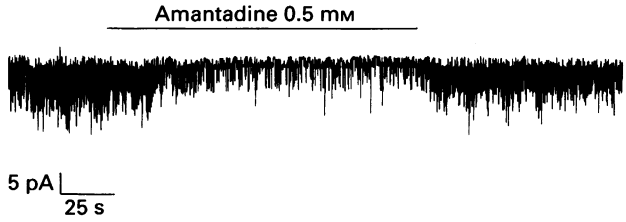

Figure 2 The effect of internal application of amantadine. Single channel currents recorded from an inside-out patch at a membrane potential of −70 mV. Amantadine (0.5 mM) was applied during the period indicated by the bar.

conductance channel was also present. This channel is probably the same as that reported in rat β-cells (Findlay *et al.*, 1985; Ashcroft *et al.*, 1988): it was not substantially affected by amantadine (Figure 2).

Figure 3 shows single channel currents and amplitude histograms obtained before and after addition of 1 mM amantadine to the intracellular solution. It is clear the drug does not alter the single channel current amplitude; nor does it affect the single channel conductance (data not shown). As we were unable to obtain a patch containing only a single active channel, we used binomial analysis to determine the effect of amantadine on N and P. An example of this analysis is shown in Figure 4, where the probability that a given number of channels are open (P_n) is plotted against the actual number of channels open (n). The binomial theorem provided a good fit to the data obtained in control solution, assuming that 3 channels are active with an individual open probability of

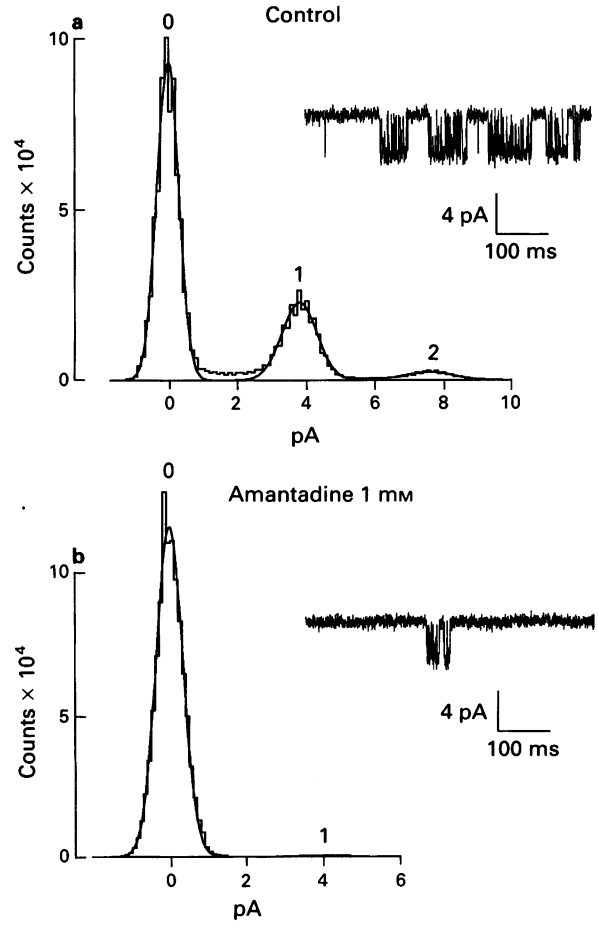

Figure 3 Amplitude histograms and single channel currents (inset) before (a) and after (b) the addition of 1 mM amantadine. Membrane potential, −70 mV. The histograms have been fit with a Gaussian function and the numbers above each peak indicate the number of channels open.

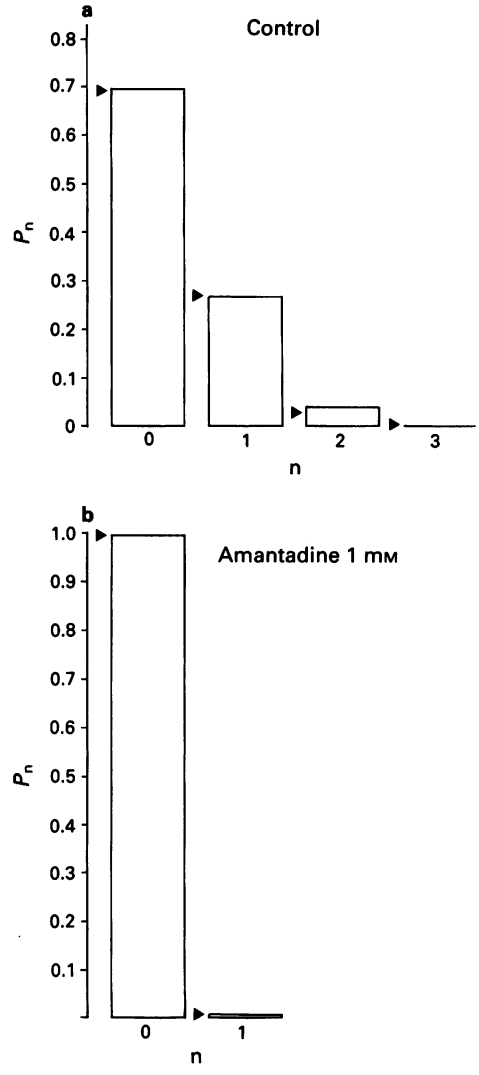

Figure 4 Binomial analysis of single channel data before (a) and after (b) addition of 1 mM amantadine to the intracellular solution. Membrane potential, −70 mV. Ordinate scale: probability that a given number of channels are open (P_n). Abscissa scale: number of channels open (n). The columns show the experimentally measured probabilities and the arrows the probabilities predicted by the binomial theorem.

0.115. In the presence of 1 mM amantadine, the data could only be fitted by assuming that a single channel was active with a markedly decreased open probability of 0.005. This suggests that the drug not only produces a decrease in the channel open probability but also causes some channels to enter a very long-lived closed state (i.e. a reduction in N). Because of the large decrease in channel activity, however, it is not possible to exclude the possibility that N is unaffected and the open probability of each channel is substantially reduced by an equal amount.

Mean values of P were 0.098 ± 0.031 in control solution and 0.005 ± 0.003 ($n = 3$) after addition of 1 mM amantadine (measured in the same patch). At lower concentration (0.5 mM), the drug also reduced the channel open probability, from 0.19 ± 0.02 (control) to 0.041 ± 0.008 ($n = 3$). Thus the open probability is decreased to approximately 21% and 5% of its control value by 0.5 mM and 1 mM amantadine respectively. This is somewhat more than is found for the whole-cell currents (Figure 1b). Whereas 1 mM amantadine substantially reduced the number of active channels in all three patches, 0.5 mM only did so in one patch.

The effect of 0.5 mM amantadine on the channel kinetics is examined in Figure 5. K-ATP channel openings occur in bursts, separated by longer closed periods (see inset Figure 3).

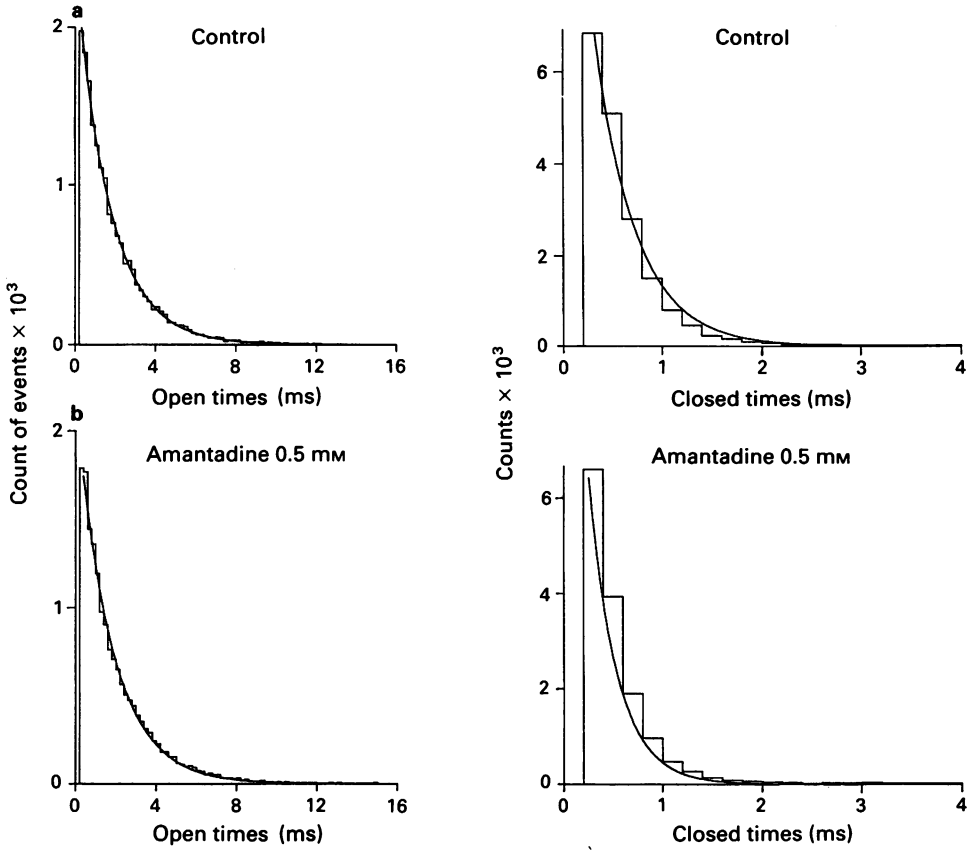

Figure 5 Distribution of open times (left) and closed times (right) during the burst before (a) and after (b) addition of 0.5 mM amantadine. Membrane potential, −70 mV. Both are fitted to a single exponential function $f(t) = A.\exp(-t/\tau_0)$ using a least squares algorithm. (a) Control: open times: A = 2440; τ_0 = 1.73 ms; closed times: A = 14711; τ_0 = 0.414 ms. Bin size displayed 0.2 ms. (b) Amantadine; open times: A = 2178; τ_0 = 1.68 ms; closed times: A = 16089; τ_0 = 0.28 ms. Bin size displayed 0.2 ms.

Our analysis is confined to the open and closed times within a burst of openings during which only a single channel was active: as we were unable to obtain any single channel patches, we do not know whether successive bursts of openings are due to the activity of the same or a different channel and a quantitative analysis of the interburst times is therefore not useful. In the absence of the drug, open times could be fitted by a single exponential with a time constant of 1.8 ± 0.1 ms (n = 5). Closed times were best described by the sum of two exponentials; the fast component, which corresponds to the intraburst closed times, comprised more than 90% of closings and had a mean lifetime of 0.37 ± 0.02 ms. These values are similar to those reported previously for HIT cells (Niki *et al.*, 1989) and mouse β-cells (Rorsman & Trube, 1985). Amantadine (0.5 mM) had little effect on either the open time or the closed time within the burst. Mean values obtained for open and closed times in the presence of the drug were 1.4 ± 0.1 ms (n = 3) and 0.39 ± 0.02 ms (n = 3), respectively. It is very clear from the records (Figures 2, 3), however, that amantadine dramatically reduces the burst duration and prolongs the interburst interval so that the channel enters a very long closed state (or states). The increase in this long closed state is responsible for the decrease in the channel open probability produced by the drug. Unfortunately, it was not possible to obtain an accurate estimate of the lifetime of the long closed state because of the very long recording periods that are necessary and the presence of channel rundown. Furthermore, we were unable to obtain a single channel patch.

The rate of onset of amantadine block of the whole-cell currents (48 ± 7 s; n = 4) was slower than that found for inside-out patches (13 ± 4 s; n = 6). The recovery of whole-cell K-ATP currents following removal of the drug was even slower (215 ± 31 s, n = 3, compared with 22 ± 6 s, n = 5, in inside-out patches). Thus we suggest that the site of action of amantadine is more accessible from the intracellular side of the membrane.

Effects of amantanol

In contrast to amantadine, application of amantanol to the bath had little effect on whole-cell K-ATP currents (Figure 1a(ii)). Even at the concentration of 1 mM, inhibition was only 20% (Figure 1b). Likewise, the activity of single K-ATP channels in inside-out patches was unaffected by 1 mM amantanol (n = 6; Figure 6). Application of DMSO at twice the maximal concentration used in experiments with amantanol was without effect (Figure 1a(ii)).

Effects of sparteine

The inhibitory effect of sparteine on whole-cell K-ATP currents is shown in Figure 7a. Currents were inhibited reversibly

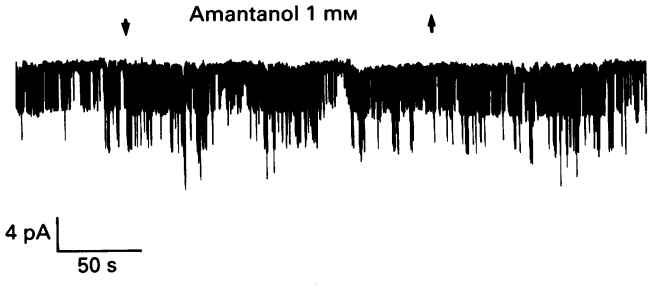

Figure 6 The effect of internal application of amantanol. Single channel currents recorded from an inside-out patch at a membrane potential of −70 mV. Amantanol (1 mM) was applied during the period indicated by the arrows.

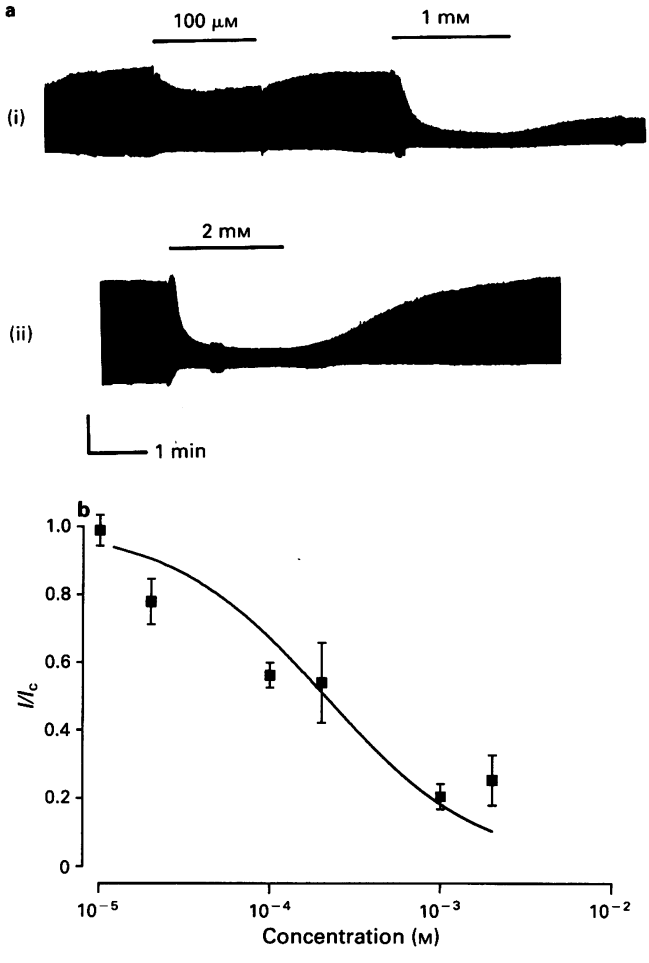

Figure 7 The effect of external application of sparteine on whole-cell currents. (a) (i)–(ii) Whole-cell currents recorded in response to +10 mV pulses from a holding potential of −70 mV in two different cells. Sparteine, at the concentrations indicated was applied during the period denoted by the bars. The scale bar indicates 100 pA. (b) Dose-inhibition curve for sparteine. Values are given as a fraction of the control current (I/I_c). The points represent the mean of 3 or 4 cells and the vertical lines ± s.e.mean. The curve is fitted to eqn. 1 of the text with K_i = 171 μM and h = 0.96.

at concentrations of sparteine between 20 μM and 2 μM in all cells tested (n = 16). Figure 7b shows the dose-response curve for sparteine inhibition; the solid line is fitted to the Hill equation (eqn. 1), with a K_i of 171 μM and a Hill coefficient of 0.96. It is apparent that sparteine is far less potent than the related alkaloid quinine, which produces almost complete inhibition of K-ATP currents at a concentration of 100 μM (Bokvist *et al.*, 1990).

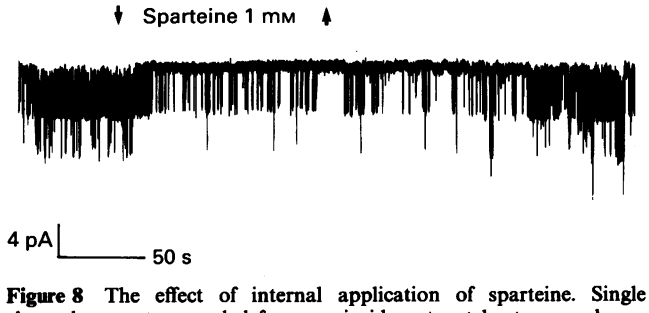

Figure 8 The effect of internal application of sparteine. Single channel currents recorded from an inside-out patch at a membrane potential of −70 mV. Sparteine (1 mM) was applied during the period indicated by the arrows.

Sparteine was also tested on single K-ATP channel currents recorded in inside-out patches. Bath concentrations of 1 and 2 mM reversibly reduced channel activity in all cases (n = 12) with no alteration of single channel current amplitude (Figure 8). Similar results have been obtained with inside-out patches from human β-cells (F.M. Ashcroft & M. Kakei, unpublished observation). The single channel current amplitude is also unaffected by quinine (Bokvist *et al.*, 1990).

Discussion

Our results show that amantadine and sparteine reversibly inhibit the K-ATP channel in HIT-T15 cells. Both these drugs have been shown to reduce the resting membrane K-permeability of β-cells and thereby elicit insulin release in the presence of non-stimulatory levels of glucose (Paolisso *et al.*, 1985; Garrino & Henquin, 1987). It seems probable that these effects of sparteine and amantadine result from their ability to inhibit K-ATP channels, since K-ATP channel activity principally accounts for the resting K-permeability of the β-cell (Ashcroft *et al.*, 1988). This hypothesis is supported by the observation that sparteine antagonizes the increase in ^{86}Rb efflux induced by diazoxide (Paolisso *et al.*, 1985), an activator of K-ATP channels (Trube *et al.*, 1986). Although a quantitative comparison is difficult, owing to the difference in experimental protocols, the concentrations reported for stimulation of insulin secretion by amantadine and sparteine (Paolisso *et al.*, 1985; Garrino & Henquin, 1987) are compatible with the K_i's we obtained for K-ATP channel inhibition.

Although our results suggest that the primary mechanism by which sparteine and amantadine increase insulin release is via inhibition of the K-ATP channel, additional effects of these drugs cannot yet be excluded. Sparteine, in particular, has been described as poorly reversible and as having only a transient effect on insulin secretion at high concentrations (2 mM, Paolisso *et al.*, 1985), effects which are less reconcilable with an action solely on the K-ATP channel. Whilst it is possible that these observations result from cellular accumulation of sparteine, the drug has been reported to affect other channels (e.g. Ohta & Narahashi, 1973; Senges & Ehe, 1973; Schauf *et al.*, 1976); furthermore, the related alkaloid quinine is known to affect β-cell metabolism (Henquin *et al.*, 1975).

The Hill coefficients obtained for inhibition by both amantadine and sparteine were close to unity, consistent with the idea that these drugs interact with their target in a one-to-one stoichiometry. Their site of action cannot be determined from the data presented here, but it is clear that both drugs can act from either side of the membrane since they are effective in inside-out patches as well as whole-cell recordings. As both amantadine and sparteine are lipid soluble to some extent, it is possible that they may reach their target site by partitioning into the lipid phase of the membrane. A similar suggestion has been made to account for the ability of the sulphonylurea tolbutamide to block the channel from both the inside and outside of the membrane (Trube *et al.*, 1986). The block by amantadine also resembles that produced by tolbutamide in that the single channel current amplitude is unchanged, the open and closed times within a burst of openings are unaffected and the principal effect is a decrease in the burst duration and an increase in the interburst interval (Gillis *et al.*, 1989).

The lack of an inhibitory effect of amantanol, compared to amantadine, is of considerable interest. Our experiments on inside-out patches allow us to discount the possibility that this difference results from the lower lipid permeability of amantanol preventing access to the inner face of the membrane (Garrino & Henquin, 1987). We still cannot exclude, however, the idea that this lower hydrophobicity may render a site within the membrane inaccessible. A more intriguing possibility is that the target site of amantadine is sufficiently specific that substitution of its amine group by an hydroxyl one renders it impotent. Our results do not allow us to determine

whether this site is on the channel protein itself or on a regulatory protein that modulates channel activity. The difference in the efficacy of amantadine and amantanol may be of use in elucidating the structure and control mechanisms of the K-ATP channel.

Amantadine is currently used in the treatment of Parkinson's disease, particularly to improve akinesia and rigidity. It is believed to augment the presynaptic release of dopamine although the mechanism by which it does so is unclear. Recent reports of K-ATP channels in substantia nigra pars compacta neurones (Röper *et al.*, 1990; Amoroso *et al.*, 1990), however, raise the intriguing possibility that the therapeutic effect of amantadine may be a consequence of inhibition of K-ATP channels in nigrostriatal neurones.

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