British Journal of Pharmacology (1996) 117, 1749–1755

Block of pancreatic ATP-sensitive K^+ channels and insulinotrophic action by the antiarrhythmic agent, cibenzoline

Ayako Ishida-Takahashi, [1]Minoru Horie, *Yoshiyuki Tsuura, *Hitoshi Ishida, Tomohiko Ai & Shigetake Sasayama

Departments of Internal Medicine and *Metabolism and Clinical Nutrition, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan

1 We investigated the effect of cibenzoline (a class Ia antiarrhythmic drug) on basal insulin secretory activity of rat pancreatic islets and ATP-sensitive K^+ channels (K_{ATP}) in single pancreatic β cells of the same species, using radioimmunoassay and patch clamp techniques.

2 Micromolar cibenzoline had a dose-dependent insulinotrophic action with an EC_{50} of 94.2±46.4 μM. The compound inhibited the activity of the K_{ATP} channel recorded from a single β-cell in a concentration-dependent manner. The IC_{50} was 0.4 μM in the inside-out mode and 5.2 μM in the cell-attached mode, at pH 7.4.

3 In the cell-attached mode, alkalinization of extracellular solution increased the inhibitory action of cibenzoline and the IC_{50} was reduced from 26.8 μM at pH 6.2 to 0.9 μM at pH 8.4. On the other hand, the action of cibenzoline in the excised inside-out mode was acute in onset with a small IC_{50}, indicating that the drug attains its binding site from the cytoplasmic side of the cell membrane.

4 In the inside-out mode, micromolar ADP reactivated the cibenzoline-blocked K_{ATP} channels in a manner similar to that by which ADP restored ATP-dependent block of the channel.

5 The binding of [^{3}H]-glibenclamide to pancreatic islets was inhibited by glibenclamide but not by cibenzoline. In contrast, the [^{3}H]-cibenzoline binding was displaced by unlabelled cibenzoline but not by glibenclamide. It is concluded that cibenzoline blocks pancreatic K_{ATP} channels via a binding site distinct from the sulphonylurea receptor.

Keywords: Antiarrhythmic agent; cibenzoline; ATP-sensitive K^+ channel; drug-induced hypoglycaemia; pancreatic β cells

Introduction

Cibenzoline, a class Ia drug and an imidazoline derivative [4,5-dihydro-2{2.2-diphenyl-cyclopropyl}1H-imidazole butanedivate 1:1}], has been used for the treatment of both atrial and ventricular tachyarrhythmia since the late 1980s (Touboul *et al.*, 1986). In isolated tissues, cibenzoline exhibits class I, and to a lesser extent, class III and IV antiarrhythmic activity. In patients with arrhythmias cibenzoline produces dose-proportional increases in QRS, QTc, AH, and HV intervals and in the ventricular effective refractory period (Harron *et al.*, 1992).

The compound also induces a sporadic hypoglycaemia (Hilleman *et al.*, 1987; Jeandel *et al.*, 1988; Lefort *et al.*, 1988; Garshot *et al.*, 1988; Houdent *et al.*, 1991). Although elevated blood levels of immunoreactive insulin (IRI) were observed in some cases (Lefort *et al.*, 1988; Garchot *et al.*, 1988; Houdent *et al.*, 1991), the mechanism underlying this drug-induced hypoglycaemia remains unknown. In an earlier paper (Horie *et al.*, 1992), we showed that antiarrhythmics with Vaughan Williams class Ia action (Bigger & Hoffmann, 1978), including cibenzoline, reversibly block ATP-sensitive K (K_{ATP}) channels in excised membranes from both rat heart and pancreatic β cells. More recently, Kakei *et al.* (1993) reported that a micromolar range of the compound inhibited whole-cell K conductance measured from single rat β cells (IC_{50} = 1.5 μM).

K_{ATP} channels in pancreatic β cells play a crucial role in regulating insulin secretion. Closure of them depolarizes β cells and promotes Ca^{2+} influx through the voltage-dependent Ca^{2+} channels, thereby triggering hormone secretion (Cook & Hales, 1984; Ashcroft *et al.*, 1984; Rorsman & Trube, 1985; Misler *et al.*, 1986; for review see Ashcroft, 1988). Sulphonylureas, potent insulin secretagogues, were found to act through a block of pancreatic and cardiac K_{ATP} channels via an intramembrane pathway (Zünkler *et al.*, 1989; Findlay, 1992). Similarly, cibenzoline also may affect pancreatic β cell K_{ATP} channels and cause hypoglycaemia via insulin oversecretion.

The cibenzoline half-block concentrations (IC_{50}) for β cell K_{ATP} channels were 1.5–2.5 μM (Horie *et al.*, 1992; Kakei *et al.*, 1993), which are very close to the therapeutic concentrations of the drug (~1 μM: Massarella *et al.*, 1986). Supposing these relative values also to be the case in humans, it would be expected that most patients receiving the drug would experience hypoglycaemia. Clinical hypoglycaemia associated with cibenzoline is, however, quite infrequent. The present study was designed, therefore, to test whether a cibenzoline block of the K_{ATP} channel is in fact associated with insulin release from rat pancreatic islets and if so, what concentration of the drug is required. We also examined how the compound interacted with the channel by using [1] radioimmunological measurement of insulin secretory capacity and [2] cell-attached and cell-free patch-clamp techniques along with [3] receptor binding assay.

Methods

Cell preparation

Islets of Langerhans were isolated from pancreas of male Wistar rats by collagenase digestion as previously described (Sutton *et al.*, 1986). Batches of isolated islets were provided for measurements of insulin secretion and for sulphonylurea displacement studies. For electrophysiological studies, cultured islet cells were prepared as previously reported (Tsuura *et al.*, 1992). Briefly, the islets were incubated for 30 min at 37°C in Ca^{2+}-free Krebs-Ringer bicarbonate (KRB) buffer medium. The composition of KRB medium was (in mM): NaCl 129.4,

[1]Author for correspondence at: Department of 3rd Internal Medicine, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan.

KCl 5.2, $CaCl_2$ 2.7, $MgSO_4$ 1.3, $NaHCO_3$ 24.8, glucose 3.3 and HEPES 10 (pH 7.4). Before cell preparation the KRB medium was supplemented with 16.7 mM glucose, 0.2% bovine serum albumin (BSA) and 3 mM ethyleneglycol *bis*(β-aminoethylether)-N, N, N′, N′-tetraacetic acid (EGTA). Islet cells were then mechanically dispersed in Ca^{2+}-free KRB medium by gently pipetting the isolated islets 5 to 10 times with 1000-μl micropipettes. Single cells were then transferred into RPMI 1640 medium (GIBCO; glucose concentration 11.1 mM) supplemented with 10% foetal calf serum, 100 μu ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin and cultured for 2–4 days on cover glasses (15 mm × 4 mm) at 37°C in a humidified incubator gassed with air and CO_2 (5%).

Measurement of insulin secretion

The insulin secretory capacity was measured by the batch incubation method (Tsuji *et al.*, 1988). Briefly, isolated islets were preincubated at 37°C for 30 min in a standard external medium supplemented with 5.5 mM glucose and 0.2% BSA. The composition of the standard extracellular medium was (in mM): NaCl 135, KCl 5, $CaCl_2$ 5, $MgSO_4$ 2 and HEPES-NaOH 5 (pH 7.4). Each batch of about 10 islets was then incubated for 30 min at 37°C in 0.7 ml medium containing various concentrations of cibenzoline or glibenclamide. Five batches were used for each test solution. After each incubation, 0.1 ml of the medium was withdrawn, diluted to one-tenth and stored at −20°C for later insulin assay. Immunoreactive insulin (IRI) was measured by radioimmunoassay with the polyethylene glycol method, with rat insulin (Novo Nordisk, Bagsvaerd, Denmark) used as standard.

Electrophysiology

A cover glass with cultured islet cells was transferred to a test chamber placed on the stage of an inverted microscope (Nikon, TMD, Tokyo). The chamber (volume 0.5 ml) was continuously perfused with the standard extracellular medium (the composition as indicated above). Single K_{ATP} channel activities were recorded in either cell-attached or inside-out mode of patch-clamp technique (Hamill *et al.*, 1981) at room temperature (22–24°C). Pipettes were made from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a vertical two-step puller (Narishige PP83, Tokyo) and had a resistance of 2–3 MΩ when filled with a standard pipette solution containing (in mM): KCl 150, $CaCl_2$ 2, and HEPES-KOH 5 (pH 7.4). Shanks of the pipettes were coated with Sylpot (Dow Corning Japan Ltd., Yokohama, Japan) and the tips were fire-polished immediately before use.

For the cell-attached mode, the cells were bathed in either standard extracellular medium or high K^+ extracellular medium containing (in mM): KCl 145, $MgSO_4$ 2, EGTA 0.5, and HEPES 5 (pH 7.4 by KOH) virtually to eliminate the transmembrane potential. To obtain a pH of 6.2, PIPES was used, for pH 8.4, TAPS was used. In the inside-out mode, the cytoplasmic side of cell-free patches was bathed with a standard intracellular solution containing (in mM): KCl 145, NaCl 10, $CaCl_2$ 0.1, $MgSO_4$ 2, EGTA 1, K_2ATP 0.001, and 5 HEPES (pCa 7.7, pH 7.2 with KOH).

All the data were fed directly to a chart recorder (Nihonkoden RJG-4122, Tokyo, Japan) and stored on a video tape via a pulse code modulation converting system (Sony PCM-501, Tokyo, Japan) for later off-line analyses by a computer (NEC 98XL, Tokyo, Japan). Data acquisition was performed at a sampling frequency of 1 kHz through a Bessel type filter (0.5 kHz, 24 dB $octave^{-1}$; NF electronic instruments, FV-624). The mean patch current (I) was measured as the average difference between baseline currents (no channel open) and open channel currents. The unitary amplitude of open channel currents (i) was estimated by analysis of the amplitude distribution using P-Clamp softwares (AXON Instruments, Inc. Foster City, CA, U.S.A.). The mean number of open channels in the presence of varying concentrations of cibenzoline (NPo) and the absence of the agent ($\overline{NP_o}$) were derived from the product of the number of available channels in the patch (N) and the probability of their being open (P_o) NP_o and $\overline{NP_o}$ were estimated from the mean patch currents ($I = NP_o i$) as I/i. Relative channel activities ($NP_o/\overline{NP_o}$) were fitted to the Hill equation with the Marquardt-Levenberg algorithm:

$$NPo\ /\overline{NPo} = (1 + ([\text{cibenzoline}]/IC_{50})^{n_H})^{-1} \quad (1)$$

where IC_{50} indicates the half maximal concentration for inhibition of the drug and n_H the Hill coefficient. The effect of each concentration of the compound was examined separately and the washout effect was then confirmed by recovery of channel activity up to >80% of the NP_o value before exposure to the compounds. However, at higher concentrations of the drug, the washout was not consistently seen, and the NP_o before exposure was employed as $\overline{NP_o}$ in such cases. For open and closed time histogram construction, half amplitude criteria were used to determine the open state.

Displacement of radioactive cibenzoline and glibenclamide

The displacing effect of cibenzoline on the sulphonylurea receptor binding in rat pancreatic β-cells was assessed as has been described previously (Schmid-Antomarchi *et al.*, 1987). Dispersed pancreatic islet cells were washed two times with 10 ml of KRB buffer supplemented with 0.2% BSA at 37°C. After resuspension in KRB buffer, they were incubated for 2 h at 23°C with either 2.0 nM [^{3}H]-glibenclamide (50.9 Ci mM^{-1}, NET-1024, New England Nuclear, Boston) or [^{3}H]-cibenzoline (8.0 Ci mM^{-1}, a generous gift from Fujisawa Pharmaceutical Co., Osaka, Japan) in the presence of various concentrations of non-radioactive cibenzoline or glibenclamide. Binding was terminated by rapid filtration through Whatman GF/C filters followed by washing five times with 5 ml of ice-cold distilled water. Radioactivity on filters was counted in 10 ml of an aqueous scintillation cocktail (Aquasol R-2, New England Nuclear). Results were expressed as the percentage of radioactivity of bound [^{3}H]-glibenclamide or [^{3}H]-cibenzoline which remained after adding non-radioactive compound.

Numerical data are expressed as mean ± s.e.mean. Statistical differences were evaluated by Student's paired and unpaired t test and considered significant at $P < 0.05$.

Drugs

Cibenzoline (a kind gift from Fujisawa Pharmaceutical Co., Osaka, Japan) was prepared as 50 mM stock solution and glibenclamide (Hoeschst Japan, Tokyo, Japan) was prepared as 100 μM stock solution by dissolving in dimethyl sulphoxide (DMSO, Nacalai tesque, Kyoto, Japan). Each stock solution was added to the bath solution immediately before use to the final concentration given in the text. Final DMSO concentrations were lower than 0.1%, which alone had no action on the insulin secretory capacity or single K_{ATP} channel activities.

Results

Cibenzoline increases insulin secretion from rat pancreatic islets

Figure 1 shows representative data of the effects of cibenzoline on the insulin secretory capacity observed in a single experiment. In the continued presence of 5.5 mM glucose, cibenzoline at concentrations over 3 μM significantly potentiated the insulin secretion. The insulinotrophic effect of cibenzoline was concentration-dependent. The smooth curve in the graph is the best fit to the Hill equation (see legend) with the half effective cibenzoline concentration (EC_{50}) of 67.6 μM and the Hill coefficient of 0.7. In a total of three experiments, mean ci-

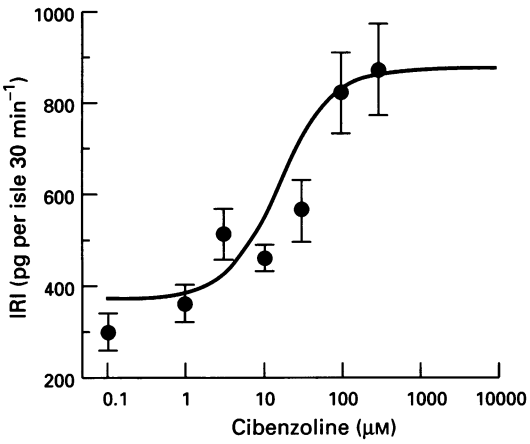

Figure 1 Cibenzoline-induced insulin secretion: IRI increases are plotted on a semilog scale as a function of cibenzoline concentration.

benzoline EC_{50} was 94.2 ± 46.4 μM and Hill coefficient was 1.2 ± 0.6. These results are in good agreement with recent receptor studies by Bertrand *et al.* (1992).

In another experiment using the same protocol, the effects of glibenclamide, a K_{ATP} channel blocking sulphonylurea, were examined and compared with those of cibenzoline. Insulin secretion was augmented from 909 ± 103 to 2278 ± 277 and 3257 ± 143 pg per islet 30 min^{-1} by glibenclamide at 100 nM and 1 μM respectively. In the same preparation, cibenzoline increased insulin levels to 1052 ± 181 and 2094 ± 277 pg per islet 30 min^{-1} at 10 μM and 100 μM. Thus, like sulphonylureas, cibenzoline inhibition of pancreatic K_{ATP} channels might stimulate insulin secretion.

Identification of pancreatic K_{ATP} channels

In earlier papers (Hone *et al.*, 1992; Kakei *et al.*, 1993), cibenzoline at <3 μM has been found to inhibit K_{ATP} channel activities recorded in excised inside-out patches when applied to the cytosolic side of membrane. The half blocking cibenzoline concentrations (IC_{50}) so far reported are, however, >10 fold lower than the EC_{50} value for its insulin-secreting action (94.2 μM). In order to understand the quantitative gap between the insulinotrophic effect and the channel blockade of cibenzoline, its action on K_{ATP} channels was studied in both on-cell and cell-free patches.

The experimental data in Figure 2 show pancreatic β cell K_{ATP} channel activities. The two records in the left panels represent channel activities most commonly found in on-cell (a) and excised inside-out (b) patches from our preparations. Spontaneous openings of K_{ATP} channels were observed in the presence of 5.5 mM glucose in the medium bathing the cell examined. Increase of the glucose concentration to 11 mM gradually closed the channel activity. Subsequent restoration of a glucose-free medium gradually restored the channel activity (Figure 2a), suggesting that the channel opening is dependent on extracellular glucose.

Upon excision of the cell-attached patch in the standard internal solution containing 1 μM ATP, the K_{ATP} channel activity usually increased suddenly. After the channel activity attained a steady state in the presence of 1 μM ATP, raising the ATP concentration to 1000 μM inhibited the channel activity in a reversible manner (Figure 2b). In both recording modes, the same channel activity was suppressed by glibenclamide (10 nM; not shown in figure).

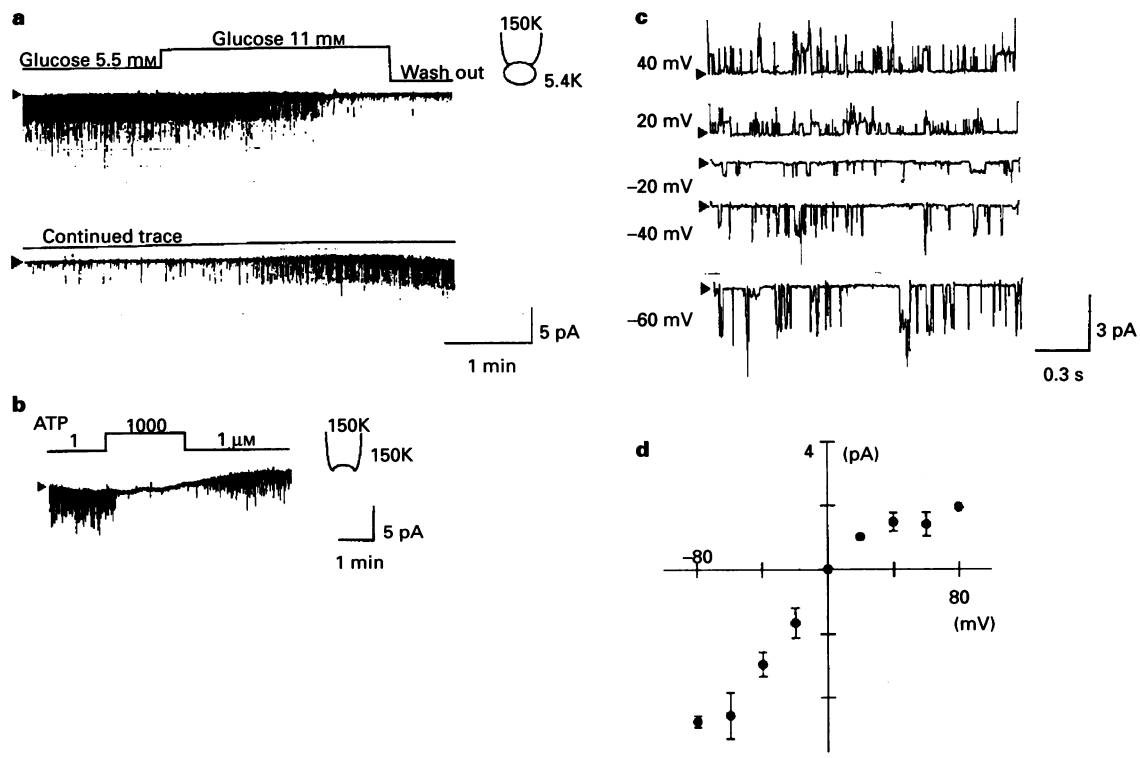

Figure 2 Identification of a single K_{ATP} channel of rat pancreatic β cells: slow chart record of single channel currents at 0 mV in the cell-attached configuration in the presence of 5.5 or 11.0 mM glucose as indicated by horizontal bars above the chart. (b) Single channel record at −40 mV inside-out mode in the presence of 1 or 1000 μM ATP as indicated above the chart record. (c) Fast chart record of single-channel currents at various membrane potentials. (d) Single channel current-potential relations recorded with pipette solutions containing 150 mM K.

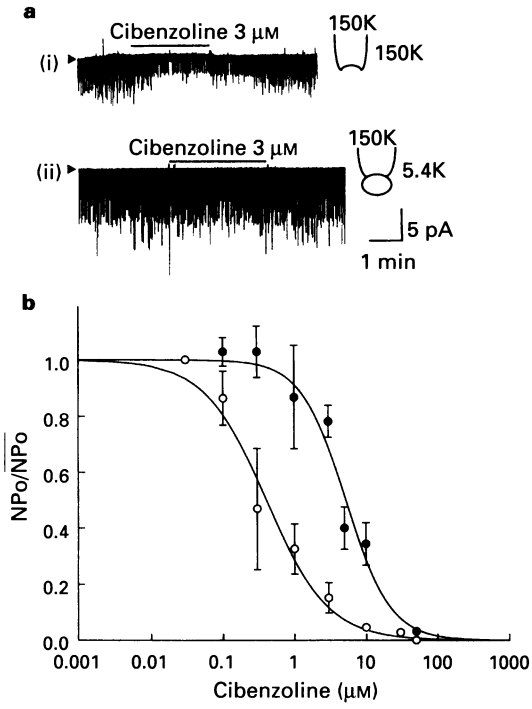

Figure 3 Cibenzoline block of K_{ATP} channels measured in inside-out and cell-attached mode: (a) (i) Slow chart record of single channel currents at −40 mV in inside-out mode in the absence or presence of 3 μM cibenzoline as indicated above chart record. (ii) Slow chart record of single-channel currents at 0 mV in cell-attached mode in absence or presence of 3 μM cibenzoline. (b) Concentration-inhibition relations of cibenzoline effect on K_{ATP} channels. Relative channel activities in the inside-out (○) ($n=45$) and the cell-attached (●) mode ($n=37$) are plotted against various concentrations of cibenzoline. Both experiments were carried out without glucose.

In Figure 2c and d, single-channel properties are presented: expanded current tracings at various membrane potentials (c) and unitary current-voltage relations obtained from 5 different patches (d). With 150 mM K-pipette solution, unitary current amplitude reversed at ~0 mV and showed a weak inward rectification. Since the recording was made from on-cell patches, the rectification must result from a naturally-occurring block by intracellular free Mg ions (Findlay, 1987). Single-channel conductance was calculated by a least-square fit to the negative limb of the relation and was 62.1 ± 0.3 pS. Taken together, the channels so far recorded are compatible with rat pancreatic K_{ATP} channels reported previously (Cook & Hales, 1984; Ashcroft *et al.*, 1984; Misler *et al.*, 1986; Tsuura *et al.*, 1992).

Cibenzoline inhibition of pancreatic K_{ATP} channels depends on the recording mode

Figure 3a illustrates typical results of the action of cibenzoline on K_{ATP} channel activities in excised inside-out (i) and on-cell (ii) patches as indicated in the insets. In the latter case, the compound was applied to the bathing medium (outside the pipette). In the inside-out patch, 3 μM cibenzoline reversibly suppressed the channel activity to 0.416, while the same concentration of the compound was without effect in the cell-attached mode. Higher concentrations of cibenzoline were required to obtain the same size of channel blockade under these recording conditions (for example 50 μM as in Figure 4a(ii)).

The blocking actions of cibenzoline were extensively tested at various concentrations in both recording modes. In Figure 3b, relative channel activities ($NP_0/\overline{NP_0}$) measured in either condition are plotted as functions of drug concentrations. These concentration-inhibition relationships are best fitted by

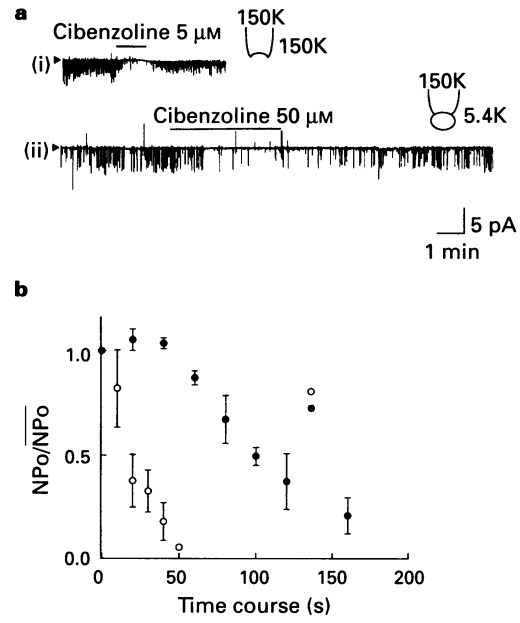

Figure 4 Time required to obtain the same blocking effect is shorter in the inside-out mode than in the cell-attached mode. (a) Slow chart record of single channel currents in inside-out (i) and cell-attached (ii) mode in absence or presence of 50 μM cibenzoline. (b) Mean patch currents were calculated every 10 s after exposure to cibenzoline and were normalized by that measured in the absence of cibenzoline (immediately before the drug application). Relative channel open probabilities in inside-out (○) and cell-attached (●) mode so obtained are plotted against the time (in s).

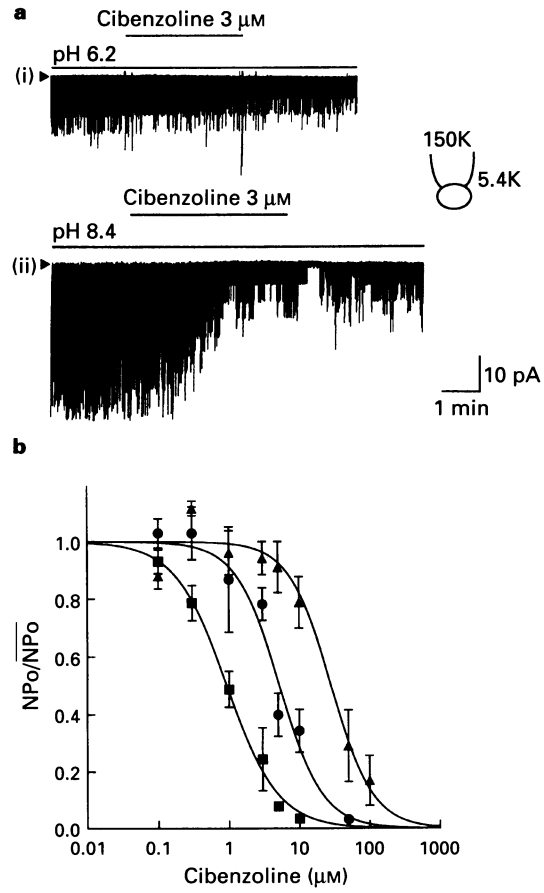

Figure 5 K_{ATP} channel blockade is changed by extracellular pH: (a) Slow chart record of single-channel currents at 0 mV in cell-attached configuration at extracellular pH (pH_o) of 6.2 (i) and of 8.4 (ii). Concentration-inhibition relationships of cibenzoline effect on K_{ATP} channel activities at varying pH_o of 6.2 (▲) ($n=21$), 7.4 (●) and of 8.4 (■) ($n=29$).

the Hill equation (*Eqn. 1* in Methods) with IC_{50} of 5.2 ± 0.7 μM in the cell-attached mode (filled circles) and 0.40 ± 0.06 μM in the inside-out mode (open circles). Therefore, ~13 fold higher concentrations of cibenzoline were needed to produce a similar size of channel blockade, partially explaining the discrepancy between the different EC_{50} levels obtained from insulin-measurement and electrophysiological experiments. Thus, difference in experimental set-up may result in discrepancy between the EC_{50} for insulin secretion and the IC_{50} for channel blockade by cibenzoline.

Different kinetics of channel blockade between cell-attached and cell-free patches

In the cell-attached mode, the time course for drug action and its washout was usually longer, and the washout was occasionally incomplete at higher concentrations. In contrast, in the excised inside-out patches, cibenzoline blocked the channel activity in a readily reversible manner. Figure 4a represents two chart records showing the time course of channel blockade by cibenzoline in inside-out ((i) 5 μM) and cell-attached ((ii) 50 μM) modes. According to concentration-inhibition relations in Figure 3 the compound at 5 and 50 μM could be expected to have similar potency in the two different modes and to produce >90% suppression of the channel activity. In Figure 4b, relative channel activities of cibenzoline are plotted as a function of time (in s). The time required to halve the control level of channel openings after exposure to cibenzoline was 109.2 ± 10.5 s in cell-attached (filled circles: $n=6$) and 24.7 ± 6.0 s in inside-out (open circles: $n=4$) mode. In the cell-attached condition, there was a time delay before the start of channel suppression. Thus, cibenzoline induced a similar size of channel block at a ~4-times faster rate when applied from the cytosolic side of cell-free patches.

Extracellular pH-dependence of cibenzoline block in the cell-attached mode

Since extracellular cibenzoline has higher IC_{50} and takes longer to cause the blockade, we considered it likely that the compound blocks the K_{ATP} channel by an interaction with the channel pore from the cytoplasmic side of the cell membrane. This notion was tested by examining the dependence of channel block on extracellular pH (pH_o) in the cell attached mode. Since the pK_a value for cibenzoline is reported to be 10.6, a ten fold increase in pH_o (alkalinization) from the physiological range of pH would produce 10 fold higher concentrations of the uncharged form of the compound, which can more easily permeate the cell membrane than the charged form.

As represented in Figure 5a, cibenzoline was without effect at pH_o of 6.2 but entirely blocked the channel at pH_o of 8.4. The inhibitory action appeared to be so potent that the washout effect was not observed in this experiment. In bathing solutions with pH_o of 6.2, 7.4 and 8.4, channel blockade by cibenzoline was assessed as relative channel opening according to the method employed in Figure 3. These values are plotted as a function of the concentrations in Figure 5b. The smooth curves in the graph were best fits to the eqn. 1 (in Methods), yielding IC_{50} of 0.94 ± 0.07 μM (filled squares; $n=1.2 \pm 0.1$) at 8.4 pH_o and 26.84 ± 4.5 μM (filled triangles; $n=1.3 \pm 0.2$) at 6.2 pH_o, respectively. Data plots for 7.4 pH_o and its fitted curve are the same as in Figure 3b (filled circles). It is, therefore, clear that channel blockade by external cibenzoline strongly depends on the pH of the extracellular medium. The higher the concentration of membrane-permeable cibenzoline (unchanged form), the more potent became its block.

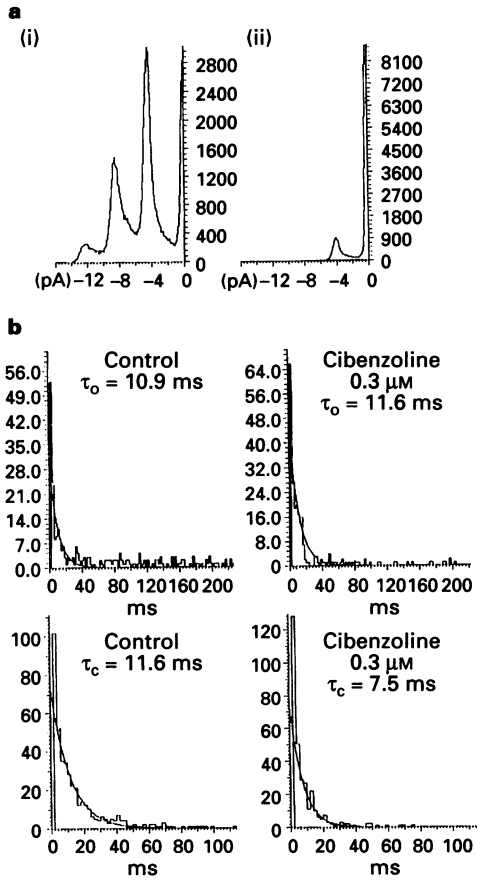

Figure 6 Lack of effect of cibenzoline on unitary amplitude or intraburst fast kinetics of K_{ATP} channels: (a) Cibenzoline did not affect unitary amplitude of K_{ATP} channels in absence (i) or presence (ii) of cibenzoline. (b) Open time (upper) and closed time (bottom) histograms in control (left) and 0.3 μM cibenzoline (right) at −40 mV holding potential.

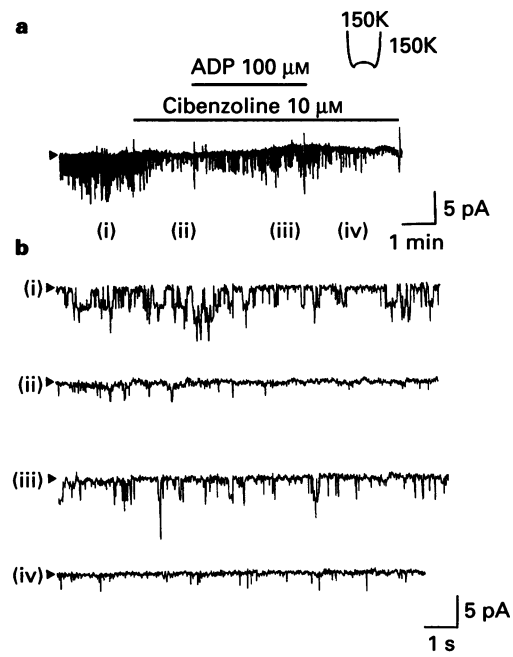

Figure 7 Intracellular ADP reactivated K_{ATP} channels which had been suppressed by cibenzoline. (a) Slow chart record of single channel currents in the absence (i) or presence of 10 μM cibenzoline (ii), (ii), (iv) and in the absence (i), (ii), (iv) or presence of 100 μM ADP (iii). Each is a fast chart record.

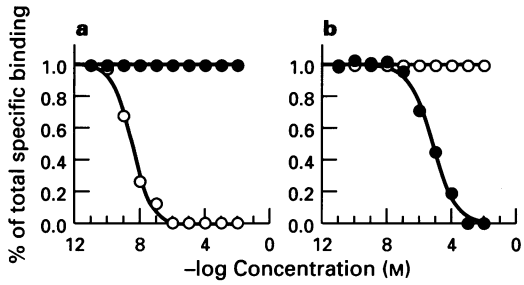

Figure 8 Inhibition of [^{3}H]-glibenclamide (a) and [^{3}H]-cibenzoline (b), binding to pancreatic islet cells by increasing concentrations of non-radioactive cibenzoline (●) and glibenclamide (○). Similar results indicating failure of cibenzoline to displace [^{3}H]-glibenclamide binding and of glibenclamide to displace [^{3}H]-cibenzoline binding were also found in three separate experiments.

Cibenzoline closes the channel by promoting interburst interval

Figure 6a illustrates two amplitude histograms constructed from single-channel recordings from the excised inside-out patch at −60 mV in the absence (a) or presence (b) of 3 μM cibenzoline. The Gaussian fit to the amplitude histogram gave the unitary amplitude (i) for the first step of channel opening of 4.332 pA in control and 4.357 pA in cibenzoline. In a total of 6 experiments, i values were not significantly altered by the compound (0.3–3 μM).

In the same recording condition, where only a single channel opening was seen throughout the experiment, analyses for burst distribution showed no remarkable change in dwell time in the absence (Figure 6b, upper left panel; $\tau_o = 10.9$ ms) or presence (Figure 6b, upper right panel; $\tau_o = 11.6$ ms) of 0.3 μM cibenzoline. Close time distributions could be fitted by the process expressed by double exponentials, and the values for τ_c of rapid component were not significantly changed by cibenzoline (3.7 ms in control and 2.7 ms in 0.3 μM cibenzoline). In contrast, the τ_c of the slow component doubled in cibenzoline, indicating that the compound increased the interburst duration, thereby reducing the open probability.

ADP attenuates the K_{ATP} channel blockade by ATP and cibenzoline

Pancreatic K_{ATP} channel activities are known to be modulated by intracellular ligands: the ATP-dependent closure of the channel was restored by micromolar ADP applied to the inner side of excised inside-out patches (Dunne & Petersen, 1986; Kakei *et al.*, 1986; Findlay, 1987). Figure 7 shows that 100 μM ADP restored the channel activity that had been suppressed by 10 μM cibenzoline. ADP-dependent recovery of the channel activity was consistently seen in the total of 4 experiments during the cibenzoline (10 μM) block. In contrast, the same concentration of ADP was without effect on the glibenclamide-induced block (data not shown in figure).

Effects of cibenzoline and glibenclamide on the [^{3}H]-cibenzoline and [^{3}H]-glibenclamide binding to rat pancreatic islet cells

Figure 8a shows displacement of [^{3}H]-glibenclamide bound to pancreatic islet cells by cibenzoline and glibenclamide. The [^{3}H]-glibenclamide binding was inhibited by increasing concentrations of non-radioactive glibenclamide with IC_{50} of 2.9 nM. However, non-radioactive cibenzoline failed to displace [^{3}H]-glibenclamide even at 1 mM. On the other hand, the [^{3}H]-cibenzoline binding was inhibited by cibenzoline in a dose-dependent manner with IC_{50} of 6.7 μM while glibenclamide was without effect even at 100 μM. Thus, the binding sites of these two compounds appear to differ.

Discussion

Mechanism of the insulinotrophic action of cibenzoline

The present study shows that cibenzoline, a newly-synthesized class Ia antiarrhythmic agent, binds and blocks rat pancreatic K_{ATP} channels from the cytoplasmic side of the cell membrane, thereby inducing insulin release. Its mode of binding to the channel differs from the mode of sulphonylureas, which is thought to be an intra-membrane path (Zünkler *et al.*, 1989; Findlay, 1992). From the extracellular side, cibenzoline blockade requires several minutes to reach its maximal effect. Due to the long time course of block and its acceleration by the intracellular application of the drug, it is likely that the compound blocks the K_{ATP} channel by an interaction with the pore from the cytoplasmic side of the channel.

The steep pH_o-dependence of cibenzoline blockade in the cell-attached mode also supports this conclusion: alkalinization of extracellular medium results in a stronger blockade of the channel activity. Since the pK_a value for cibenzoline is 10.6, a ten fold change of alkalinization (at pH < 10.6) should produce 10 fold higher concentration of the unchanged form of the drug, which can more easily permeate the cell membrane than the charged form. Thus, the drug does not reach the binding site through the conduction pathway from the external surface, but rather through the membrane phase. The membrane route might involve the unchanged form of the compound. In receptor-binding experiments (Figure 8), glibenclamide and cibenzoline bindings did not affect each other, indicating the presence of different receptors for these two compounds.

The site of action of the imidazolines, which is known as the imidazoline-guanidine receptor site (IGRS) or the non-adrenergic idazoxan binding site (NAIBS), is different from variants of the α-adrenoceptor and recognizes agents with either guanidine or imidazoline moieties (Edwards & Weston, 1993). Various compounds which possess either an imidazoline (antazoline, cirazoline, clonidine, and phentolamine) or a guanidine (guanabenz) moiety within their structure were reported to inhibit K_{ATP} generated levcromakalim (Ibbotson *et al.*, 1993). Cibenzoline is a 2-substituted imidazoline derivative, and its action may be mediated via the NAIBS. More recently, like cibenzoline, the anorectic compound ciclazindol, which has an imidazoline moiety within its structure, was reported to inhibit K_{ATP} channels (Noqck *et al.*, 1992) and not to displace [^{3}H]-glibenclamide (Lee *et al.*, 1995). The agent may have direct modulatory action on K_{ATP} channels in central nervous centre controlling appetite.

Clinical significance of cibenzoline blockade of K_{ATP} channels

Blockade of pancreatic K_{ATP} channels by cibenzoline may be relevant to drug-induced hypoglycaemia. However, a significant hypoglycaemic attack has been rare among the arrhythmia patients treated with cibenzoline (Hilleman *et al.*, 1987; Jeandel *et al.*, 1988; Lefort *et al.*, 1988; Garchot *et al.*, 1988; Houdent *et al.*, 1991), presumably because the plasma drug level seldom exceeds 10 μM, the concentration that produces ~50% block of rat pancreatic β cell K_{ATP} channels (Figure 5). However, it is important to monitor carefully blood sugar and IRI levels in patients receiving cibenzoline, especially in those with renal dysfunction and diabetes mellitus, especially because cibenzoline binding sites are different from those of the sulphonylurea receptor. Therefore, cibenzoline should be used most carefully in patients with diabetes mellitus with nephropathy who are receiving sulphonylureas.

We thank Dr S. Hayashi for comments on the manuscript. This work was supported by grants-in-aid for Scientific Research from the Japanese Ministry of Education, Science and Culture.

References

ASHCROFT, F.M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. *Annu. Rev. Neurosci.*, **11**, 97–118.

ASHCROFT, F.M., HARRISON, D.E. & ASHCROFT, S.J.H. (1984). Glucose induces closure of single potassium channels in isolated rat pancreatic β-cells. *Nature*, **312**, 446–448.

BERTRAND, G., GROSS, R., PETIT, P., LOUBATIÈRES-MARIANI, M.M. & RIBES, G. (1992). Evidence for a direct stimulatory effect of cibenzoline on insulin secretion in rats. *Eur. J. Pharmacol.*, **214**, 159–163.

BIGGER, J.T. JR. & HOFFMANN, B.F. (1985). Antiarrhythmic drugs. In *The Pharmacological Basis of Therapeutics*. ed. Gilman, A.G., Goodman, L.S., Rall, T.W. & Murad, F. pp. 748–783. New York, U.S.A.: MacMillan.

COOK, D.L. & HALES, N. (1984). Intracellular ATP directly blocks K^+ channels in pancreatic B-cells. *Nature*, **311**, 271–273.

DUNNE, M.J. & PETERSEN, O.H. (1986). Intracellular ADP activates K^+ channels that are inhibited by ATP in an insulin-secreting cell lines. *FEBS Lett.*, **208**, 59–62.

EDWARDS, G. & WESTON, A.H. (1993). The pharmacology of ATP-sensitive potassium channels. *Annu. Rev. Pharmacol. Toxicol.*, **33**, 597–637.

FINDLAY, I. (1987). The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. *J. Physiol.*, **391**, 611–631.

FINDLAY, I. (1992). Inhibition of ATP-sensitive K^+ channels in cardiac muscle by the sulphonylurea drug glibenclamide. *J. Pharmacol. Exp. Ther.*, **261**, 540–545.

GARCHOT, B.A., BEZIER, M., CHERRIER, J.F. & DAUBEZE, J. (1988). Cibenzoline and nypoglycaemia. *Lancet*, **8605**, 280.

HARRON, D.W.G., BROGDEN, R.N., FAULDS, D. & FITTON, A. (1992). Cibenzoline A review of its pharmacological properties and therapeutic potential in arrhythmias. *Drugs*, **43**, 734–759.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HILLEMAN, D.E., MOHIUDDIN, S.M., AHMED, I.S. & DAHL, J.M. (1987). Cibenzoline-induced hypoglycemia. *Drug. Intell. Clin. Pharm.*, **21**, 38–40.

HORIE, M., HAYASHI, S., YUZUKI, Y. & SASAYAMA, S. (1992). Comparative studies of ATP sensitive potassium channels in heart and pancreatic β cells using Vaughan-Williams class Ia antiarrhythmics. *Cardiovasc. Res.*, **26**, 1087–1094.

HOUDENT, C., NOBLET, C., VANDOREN, C., LEVESQUE, H., MORIN, C., MOORE, N., COURTOIS, H. & WOLF, L.M. (1991). Hypoglycémie induite par la cibenzoline chez le sujet âgé. *Rev. Med. Interne.*, **12**, 143–145.

IBBOTSON, T., EDWARDS, G. & WESTON, A.H. (1993). Antagonism of levcromakalim by imidazoline- and guanidine-derivatives in rat portal vein: involvement of the delayed rectifier. *Br. J. Pharmacol.*, **110**, 1556–1564.

JEANDEL, C., PREISS, M.A., PIERSON, H., PENIN, F., CUNY, G., BANNWARTH, B. & NETTER, P. (1988). Hypoglycaemia induced by cibenzoline. *Lancet*, **8596**, 1232–1233.

KAKEI, M., KELLY, R.P., ASHCROFT, S.J.H. & ASHCROFT, F.M. (1986). The ATP-sensitivity of K^+ channels in rat pancreatic B-cells is modulated by ADP. *FEBS Lett.*, **208**, 63–66.

KAKEI, M., NAKAZAKI, M., KAMISAKI, T., NAGAYAMA, I., FUKAMACHI, Y. & TANAKA, H. (1993). Inhibition of the ATP-sensitive potassium channel by class I antiarrhythmic agent, cibenzoline, in rat pancreatic β-cells. *Br. J. Pharmacol.*, **109**, 1226–1231.

LEE, K., KHAN, R.N., ROWE, I.C.M., HALL, A.C. & ASHFORD, M.L.J. (1995). Inhibition of K_{ATP} channel currents by ciclazindol in the CRI-G1 insulin secreting cell line. *Br. J. Pharmacol.*, Proc. Suppl. **92**, (in press).

LEFORT, G., HAISSAGUERRE, M., FLORO, J., BEAUFFIGEAU, P., WARIN, J.F. & LATAPIE, J.L. (1988). Hypoglycémies au cours de surdosages par un nouvel antiarythmique: la cibenzoline. *Presse Méd.*, **17**, 687–691.

MASSARELLA, J.W., KHOO, K., SZUNA, A.J., SANDOR, D.A., MORGANROTH, J. & AOGAICHI, K. (1986). Pharmacokinetics of cibenzoline after single and repetitive dosing in healthy volunteers. *J. Clin. Pharmacol.*, **26**, 125–130.

MISLER, S., FALKE, L.C., GILLIS, K. & MCDANIEL, M.L. (1986). Metabolite-regulated potassium channel in rat pancreatic B cells. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7119–7123.

NOACK, T., EDWARDS, G., DEITMER, P., GREENGRASS, P., MORITA, T., ANDERSSON, P-O., CRIDDLE, D., WYLLIE, M.G. & WESTON, A.H. (1992). The involvement of potassium channels in the action of ciclazindol in rat portal vein. *Br. J. Pharmacol.*, **106**, 17–24.

RORSMAN, P. & TRUBE, G. (1985). Glucose dependent K^+-channels in pancreatic β-cells are regulated by intracellular ATP. *Pflügers Arch.*, **405**, 305–309.

SCHMID-ANTOMARCHI, H., WEILLE, J.D., FOSSET, M. & LAZDUNSKI, M. (1987). The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K^+ channel in insulin-secreting cells. *J. Biol. Chem.*, **262**, 15840–15844.

SUTTON, R., PETERS, M., MCSHANE, P., GRAY, D.W.R. & MORRIS, P.J. (1986). Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation*, **42**, 689–691.

TOUBOUL, P., ATALLAH, G., KIRKORIAN, G., DE ZULOAGA, C., DUFOUR, A., AYMARD, M.F., LAVAUD, P. & MOLEUR, P. (1986). Electrophysiologic effects of cibenzoline in humans related to dose and plasma concentration. *Am. Heart J.*, **112**, 333–339.

TSUJI, K., TAMINATO, T., USAMI, M., ISHIDA, H., KITANO, N., FUKUMOTO, H., KOH, G., KUROSE, T., YAMADA, Y., YANO, H., SEINO, Y. & IMURA, H. (1988). Characteristic features of insulin secretion in the streptozotocin-induced NIDDM rat model. *Metabolism*, **37**, 1040–1044.

TSUURA, Y., ISHIDA, H., OKAMOTO, Y., TSUJI, K., KUROSE, T., HORIE, M., IMURA, H., OKADA, Y. & SEINO, Y. (1992). Impaired glucose sensitivity of ATP-sensitive K^+ channels in pancreatic β-cells in streptozotocin-induced NIDDM rats. *Diabetes*, **41**, 861–865.

ZUNKLER, B.J., TRUBE, G. & PANTEN, U. (1989). How do sulfonylureas approach their receptor in the B-cell plasma membrane? *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 328–332.

(Received July 24, 1995
Revised November 15, 1995
Accepted December 8, 1995)