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A PYRIDOTHIADIAZINE (BPDZ 44) AS A NEW AND POTENT ACTIVATOR OF ATP-SENSITIVE K^+ CHANNELS

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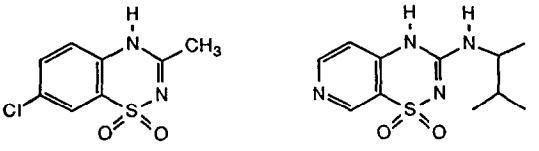
Abstract—The present study was undertaken to characterize the effects of [3-(1′,2′-dimethylpropyl)amino-4*H*-pyrido[4,3-*e*][1,2,4]thiadiazine 1,1-dioxide] (BPDZ 44), a new pyridothiadiazine derivative, on ionic and secretory events in rat pancreatic islets. The drug increased the rate of ^{86}Rb outflow regardless of the extracellular glucose concentration. The effects of BPDZ 44 on ^{86}Rb outflow persisted in the absence of extracellular Ca^{2+} but were abolished by glibenclamide. BPDZ 44 markedly decreased ^{45}Ca outflow and insulin output from islets perifused in the presence of 16.7 mM glucose and extracellular Ca^{2+}. The drug did not affect the increase in ^{45}Ca outflow mediated by K^+ depolarization. Lastly, in single B-cells, BPDZ 44 inhibited the glucose but not the KCl-induced rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). These data suggest that BPDZ 44 inhibits the insulin releasing process by activating ATP-sensitive K^+ channels. This K^+ channel activation will lead to a decrease in Ca^{2+} influx and reduction in $[Ca^{2+}]_i$.

Key words: Pyridothiadiazine; K^+_{ATP} channels; insulin release

Excitable cells are equipped with different types of Ca^{2+} and K^+ channels which play a pivotal role in signal transduction. The development of pharmacological compounds affecting the activity of these ionic channels is a valuable method for discovering new therapeutic agents. Indeed, drugs modulating the passive movements of Ca^{2+} have been successfully used for many years in the treatment of numerous cardiovascular disorders such as arrythmias, angina and hypertension [1]. By contrast, little attention has been paid to the therapeutic potential of pharmacological modulation of K^+ channels. In the last few years, however, some interest has been focused on these ionic channels when it was realized that smooth muscle relaxants such as diazoxide, minoxidil sulfate, cromakalim and pinacidil exerted their hypotensive effects by activating K^+ channels [2]. Diazoxide and pinacidil have also been shown to exert an inhibitory effect on the insulin-secreting process through activation of ATP-sensitive K^+ channels [3]. Although the K^+ channel openers presently available display several potential clinical applications, the diversity of K^+ channels offers the possibility to develop new agents selective for specific K^+ channel types.

Recently, we have synthetized pyridothiadiazine derivatives which are structural analogues of diazoxide [4]. Among these new compounds, BPDZ 44‡ (Fig. 1) was shown to be more potent than diazoxide in inhibiting the glucose-induced insulin secretion from incubated pancreatic islets [4].



DIAZOXIDE BPDZ 44

Fig. 1. Chemical structures of diazoxide and BPDZ 44.

The main objective of the present study was to determine whether the inhibitory effect of BPDZ 44 on the insulin releasing process was related to changes in transmembrane ionic movements and cytosolic Ca^{2+} concentrations.

MATERIALS AND METHODS

All experiments were performed with pancreatic islets removed from fed albino rats.

Measurements of insulin release from incubated islets. Groups of 10 islets, each derived from the same batch of islets, were preincubated for 30 min at 37° in 1 mL of a physiological salt medium (in mM: NaCl 115, KCl 5, $CaCl_2$ 2.56, $MgCl_2$ 1, $NaHCO_3$ 24) supplemented with 2.8 mM glucose, 0.5% (w/v) dialysed albumin and equilibrated against a mixture of O_2 (95%) and CO_2 (5%).

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‡ Abbreviations: BPDZ 44, [3-(1′,2′-dimethylpropyl)amino-4*H*-pyrido[4,3-*e*][1,2,4]thiadiazine 1,1-dioxide]; FOR, fractional outflow rate; $[Ca^{2+}]_i$, intracellular calcium concentration.

The islets were then incubated at 37° for 90 min in 1 mL of the same medium containing 16.7 mM glucose and, in addition, either diazoxide or BPDZ 44. The release of insulin was measured radioimmunologically using rat insulin as a standard [5].

Measurements of ^{86}Rb, ^{45}Ca *outflow and insulin release from perifused islets.* The media used for incubating, washing and perifusing the islets consisted of a physiological salt medium supplemented with 0.5% (w/v) dialysed albumin and gassed with O_2 (95%)/CO_2 (5%).

The methods used to measure ^{86}Rb efflux, ^{45}Ca efflux and insulin release from perifused islets have been described in prior publications [6–8]. Briefly, groups of 100 islets were incubated for 60 min in a medium containing 16.7 mM glucose and either ^{86}Rb (0.15–0.25 mM; 50 μCi/mL) or ^{45}Ca (0.02–0.04 mM; 100 μCi/mL). After incubation, the islets were washed three times and then placed in a perifusion chamber. The perifusate was delivered at a constant rate (1.0 mL/min). From the 31st to the 90th min, the effluent was continuously collected over successive periods of 1 min each. An aliquot of the effluent (0.6 mL) was used for scintillation counting while the remainder was stored at −20° for insulin radioimmunoassay [5]. At the end of the perifusion, the radioactive content of the islets was also determined. The outflow of ^{86}Rb or ^{45}Ca (cpm/min) was expressed as a fractional outflow rate (% of instantaneous islet content/min; FOR). The validity of ^{86}Rb as a tracer for the study of K^+ handling in the islets has been previously assessed [9].

Measurements of Fura-2 fluorescence from single islet cells. The methods used to isolate single rat pancreatic islet cells and used for the measurement of cytoplasmic free Ca^{2+} concentration with Fura-2-AM (Molecular Probes, Eugene, OR, U.S.A.) have been described previously [10]. Briefly, the cells were placed on glass coverslips and maintained in tissue culture during 72 hr before use. The cells were then incubated with Fura-2 (final concentration: 4 μM) for 1 hr and, after washing, the coverslips with the cells were mounted as the bottom of an open chamber placed on the stage of the microscope. Fura-2 fluorescence of single loaded cells was measured using dual-excitation microfluorimetry with a Spex photometric system (Optilas, Alphen aan den Rijn, Holland). The excitation wavelengths (340 nm and 380 nm) were alternated at the frequency of 1 Hz, the length of time for data collection at each wavelength being 0.05 sec. The emission wavelength was 510 nm. $[Ca^{2+}]_i$ was calculated from the ratios of the 340 nm and 380 nm signals after background substraction using the equation:

$$[Ca^{2+}]_i = K_d \cdot \frac{(R - R_{min})}{(R_{max} - R)} \cdot \frac{Sf2}{Sb2}$$

where K_d is the dissociation constant for the Fura-2–Ca^{2+} complex (224 nM at 37°). R_{max}, R_{min} and Sf2/Sb2 were determined in separate experiments by recording Fura-2 fluorescence in the absence of extracellular Ca^{2+} or in the presence of a saturating Ca^{2+} concentration.

The physiological salt medium used to perfuse the cells contained, in addition, HEPES-NaOH 20 mM, glucose 2.8 mM and was gassed with O_2 (95%)/CO_2 (5%). The open chamber (1 mL) was thermostated at 37° and perfused at a rate of 2 mL/min.

Drugs. Some media contained no $CaCl_2$ and were enriched with 0.5 mM EGTA (Sigma Chemical Co, St Louis, MO, U.S.A.). The different media also contained, as required, glucose (Merck, Darmstadt, F.R.G.), albumin (fraction V, Sigma), BPDZ 44 (synthetized at the Department of Medicinal Chemistry, University of Liège, Belgium), diazoxide (Essex Labo, Brussels, Belgium) and glibenclamide (Upjohn, Kalamazoo, MI, U.S.A.). BPDZ 44, diazoxide and glibenclamide were dissolved in dimethylsulfoxide which was added to both control and test media at final concentrations not exceeding 0.1% (v/v). When high concentrations of K^+ were used, the concentration of NaCl was lowered accordingly to keep osmolarity constant.

Calculations. All results are expressed as the mean (± SEM) together with the number of individual experiments. The magnitude of the increase in ^{86}Rb and ^{45}Ca outflow was estimated in each individual experiment from the integrated outflow of ^{86}Rb or ^{45}Ca observed during stimulation (45th to the 68th min) after correction for basal value (40th to the 44th min). Peak ^{45}Ca outflow was estimated from the difference in ^{45}Ca outflow between the highest value recorded during stimulation and the mean basal value found within the same experiment between the 40th to 44th min of perifusion. The inhibitory effect of BPDZ 44 on insulin release from islets perifused in the presence of 16.7 mM glucose was taken as the difference between the mean value for insulin output recorded in each individual experiment between the 40–44th and 60–68th min of perifusion. The statistical significance of differences between mean data was assessed by using Student's *t*-test.

RESULTS

Effect of BPDZ 44 on the glucose-induced insulin release from incubated islets

The addition of micromolar concentrations of BPDZ 44 to islets incubated in the presence of 16.7 mM glucose provoked a dose-dependent decrease in insulin release (Fig. 2). Indeed, after the addition of 1, 10, 50, 100 and 500 μM BPDZ 44 to the incubation medium, the insulin release represented 66.3 ± 11.0% (N = 8), 13.3 ± 1.6% (N = 8), 9.3 ± 0.6% (N = 8), 8.8 ± 0.9% (N = 8), and 7.9 ± 0.7% (N = 8) of the control value, respectively ($P < 0.05$ in each case).

The presence of diazoxide in the incubation medium also reduced the glucose-induced insulin release but the inhibitory effect of diazoxide was less pronounced than that of BPDZ 44. Thus, in the presence of 10 and 50 μM diazoxide, the release of insulin averaged 75.7 ± 3.4% (N = 8) and 29.2 ± 1.6% (N = 7) of that recorded in the presence of glucose (16.7 mM) but absence of drug ($P < 0.001$ in each case).

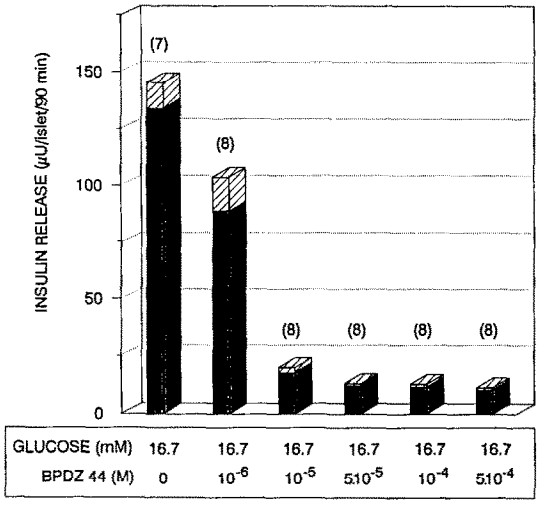

Fig. 2. Effect of increasing concentrations of BPDZ 44 on insulin release from islets incubated in the presence of 16.7 mM glucose. The upper edges of the open bars represent the means whilst the crosshatching at the top of the bars corresponds to the SEM. Figures in parentheses are numbers of individual experiments.

Effects of BPDZ 44 on ^{86}Rb and ^{45}Ca outflow from islets perifused in the absence or presence of non-insulinotropic glucose concentrations

In the absence of glucose in the perifusate, the addition of BPDZ 44 (50 μM) transiently increased (17 min) the rate of ^{86}Rb outflow (data not shown). The drug (50 μM) also induced a marked but sustained rise in ^{86}Rb outflow from islets perifused in the presence of 2.8 mM glucose and presence or absence of extracellular Ca^{2+} (data not shown).

Figure 3 illustrates the effect of BPDZ 44 (50 μM) on ^{86}Rb FOR from islets exposed to 5.6 mM glucose. In the presence or absence of extracellular Ca^{2+}, BPDZ 44 again provoked a rapid and sustained increase in the rate of ^{86}Rb outflow. The capacity of BPDZ 44 (50 μM) to stimulate ^{86}Rb FOR was identical whether the perifusing medium contained or was deprived of extracellular Ca^{2+} (Fig. 3). Thus, the increment in ^{86}Rb outflow evoked by BPDZ 44 averaged 1.89 ± 0.12%/min (N = 4) in the presence and 1.86 ± 0.38%/min (N = 4) in the absence of extracellular Ca^{2+}, respectively (P > 0.9).

The presence of the hypoglycemic sulfonylurea glibenclamide (10 μM) in the perifusate completely abolished the stimulatory effect of BPDZ 44 (50 μM) on ^{86}Rb outflow from islets perifused throughout in the presence of 5.6 mM glucose and extracellular Ca^{2+} (Fig. 3).

In the last series of experiments, we examined the effects of BPDZ 44 on ^{45}Ca FOR. The drug (50 μM) did not modify the ^{45}Ca outflow rate from islets perifused in the presence of extracellular Ca^{2+} and in the absence or presence of 5.6 mM glucose throughout (N = 6 in each case, data not shown).

Effects of BPDZ 44 on ^{86}Rb, ^{45}Ca outflow and insulin release from islets perifused in the presence of an insulinotropic glucose concentration

In the presence of 16.7 mM glucose and

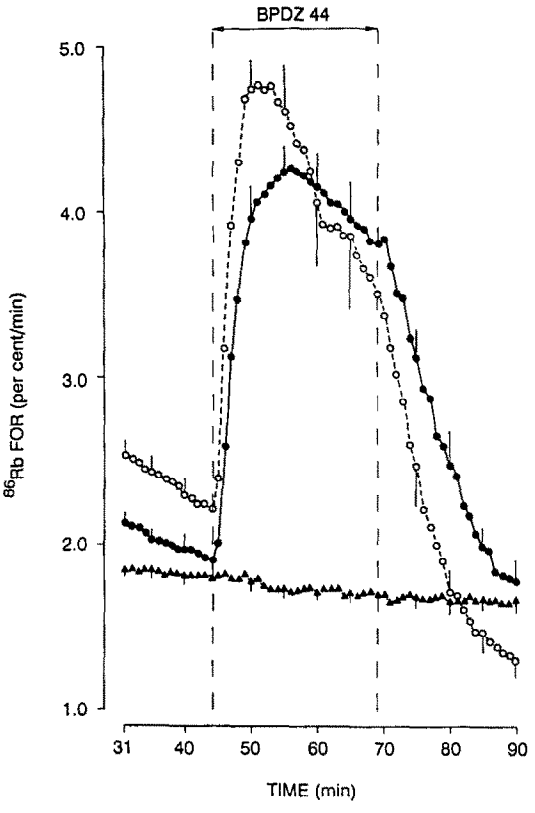

Fig. 3. Effect of BPDZ 44 (50 μM) on ^{86}Rb outflow from islets perifused throughout in the presence of 5.6 mM glucose. Basal media contained Ca^{2+} (●; 2.56 mM), Ca^{2+} and glibenclamide (▲; 10 μM) or were deprived of Ca^{2+} and enriched with EGTA (0; 0.5 mM). Mean values (±SEM) refer to four individual experiments.

extracellular Ca^{2+}, the addition of BPDZ 44 (50 μM) elicited a rapid and sustained increase in ^{86}Rb outflow (Fig. 4, upper panel). This stimulatory effect persisted in the absence of extracellular Ca^{2+} but was completely abolished when the perifusate was enriched with glibenclamide (10 μM, data not shown).

Incidentally, the capacity of BPDZ 44 to stimulate ^{86}Rb FOR was less marked in islets exposed to 16.7 mM (0.91 ± 0.14%/min; N = 13) than in islets exposed to 5.6 mM glucose (1.43 ± 0.19; N = 8) (P < 0.05).

When BPDZ 44 (50 μM) was administered to islets perifused in the presence of 16.7 mM glucose and Ca^{2+}, it provoked an immediate and sustained inhibition of ^{45}Ca FOR (Fig. 4, middle panel). The removal of the drug from the perifusing medium was followed by a delayed (10 min later), slow and modest re-ascension in ^{45}Ca outflow.

Lastly, BPDZ 44 (50 μM) did not affect ^{45}Ca FOR from islets perifused throughout with a medium containing 16.7 mM glucose but deprived of extracellular Ca^{2+} (data not shown).

In the continuous presence of 16.7 mM glucose and extracellular Ca^{2+}, the insulin output is marked and well sustained (Fig. 4, lower panel). Under the

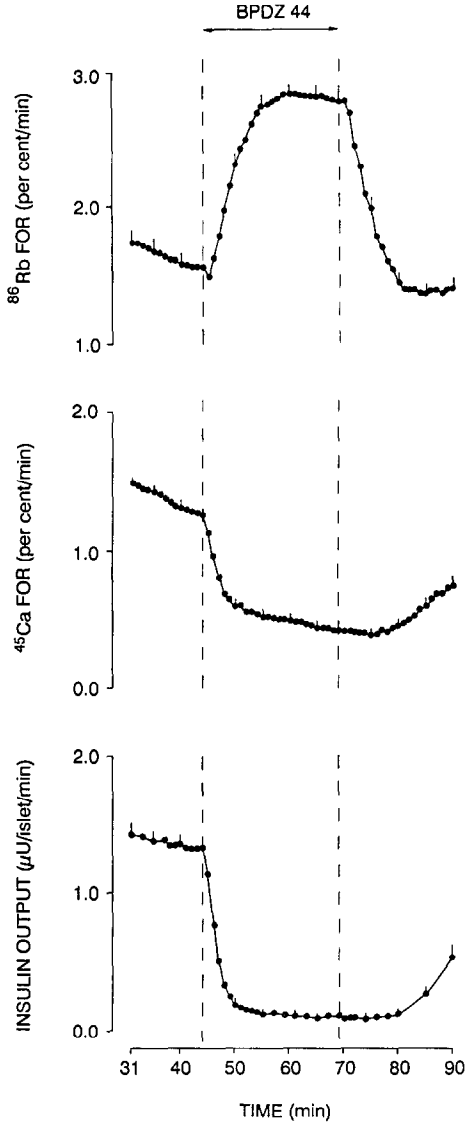

Fig. 4. Effect of BPDZ 44 (50 μM) on ^{86}Rb outflow (upper panel), ^{45}Ca outflow (middle panel) and insulin release (lower panel) from islets perifused throughout in the presence of 16.7 mM glucose. Basal media contained Ca^{2+} (2.56 mM). Mean values (±SEM) refer to four to nine individual experiments.

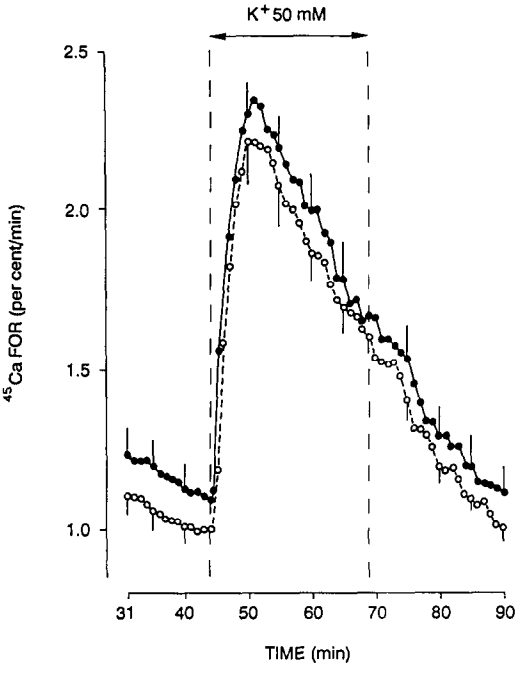

Fig. 5. Effect of a rise in the extracellular concentration of K^+ from 5 to 50 mM on ^{45}Ca outflow from islets perifused in the absence (0) and presence of BPDZ 44 (●; 50 μM). Mean values (±SEM) refer to four individual experiments.

latter experimental conditions, exposure to BPDZ 44 (50 μM) inhibited insulin release. The drug provoked a sharp, monophasic and sustained reduction of the glucose-induced insulin release. In the presence of BPDZ 44 (60–68th min), the release of insulin represented 9.3 ± 0.7% (N = 7, P < 0.001) of that recorded before the administration of the drug (40–44th min).

The inhibitory effect of BPDZ 44 on the insulin releasing process persisted after the removal of the drug from the medium. A slight increase in insulin output was only noticed 10 min after the withdrawal of the drug. This slowly reversible effect of BPDZ 44 was reminiscent of that seen on ^{45}Ca outflow (Fig. 4, compare middle and lower panel).

Effect of BPDZ 44 on KCl*-induced increase in* ^{45}Ca *outflow*

Raising the extracellular concentration of K^+ from 5 to 50 mM provoked a rapid and marked increase in ^{45}Ca outflow from islets perifused in the absence of glucose and presence of extracellular Ca^{2+} (Fig. 5). When the same experiment was repeated in the presence of BPDZ 44 (50 μM), the basal rate of ^{45}Ca outflow was unaffected. The basal ^{45}Ca outflow averaged 1.00 ± 0.05%/min (N = 4) in the absence and 1.10 ± 0.09%/min (N = 4) in the presence of BPDZ 44 throughout (P > 0.05). Moreover, BPDZ 44 (50 μM) also failed to modify the cationic response to K^+. Thus, the integrated outflow of ^{45}Ca measured during exposure to 50 mM K^+ averaged 0.90 ± 0.13%/min (N = 4) and 0.88 ± 0.10%/min (N = 4) in the absence and presence of BPDZ 44, respectively (P > 0.5). The peak ^{45}Ca outflow observed during stimulation averaged 1.24 ± 0.20%/min (N = 4) and 1.25 ± 0.12%/min (N = 4) in the absence and presence of BPDZ 44, respectively (P > 0.5).

Effect of BPDZ 44 on the glucose and KCl*-induced changes in cytosolic free* Ca^{2+} *concentration*

A rise in the glucose concentration from 2.8 to 16.7 mM provoked a marked increase in $[Ca^{2+}]_i$ (Fig. 6). The subsequent addition of BDPZ 44 (50 μM)

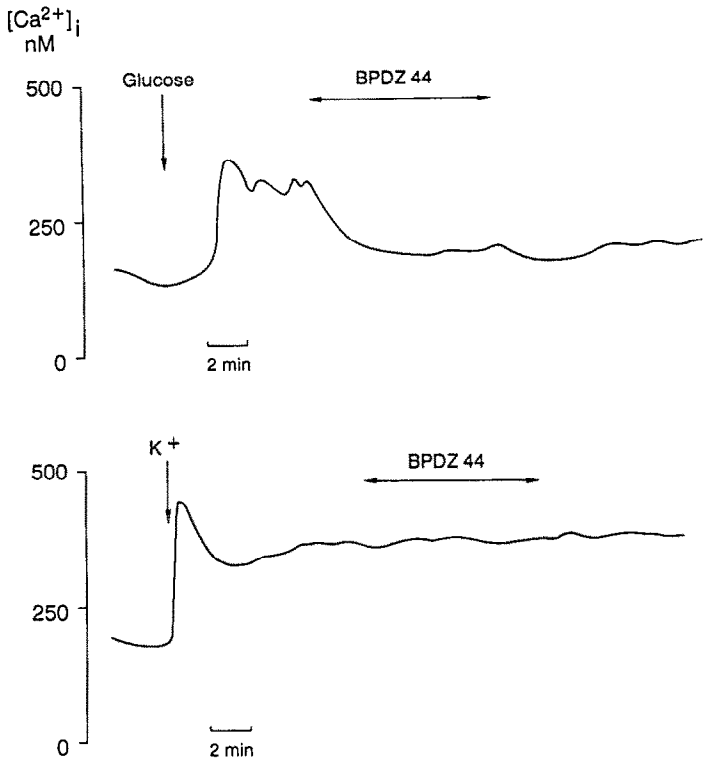

Fig. 6. Effect of BPDZ 44 (50 μM) on glucose (16.7 mM; upper panel) and KCl (50 mM; lower panel)-induced increase in $[Ca^{2+}]_i$ in single pancreatic B cell. The experiments were carried out in the presence of glucose (2.8 mM) and Ca^{2+} (2.56 mM) in the basal medium.

dramatically reduced the glucose-induced rise in cytosolic free Ca^{2+} concentration (Fig. 6). The inhibitory effect of BPDZ 44 was immediate, sustained but not rapidly reversible. By contrast, BPDZ 44 (50 μM) did not affect the drastic increase in $[Ca^{2+}]_i$ evoked by the addition of KCl (Fig. 6).

DISCUSSION

The present data indicate that BPDZ 44 is a potent inhibitor of the glucose-induced insulin release. The results also confirm that the capacity of the pyridothiadiazine derivative to inhibit the insulin releasing process is more marked than that of diazoxide [4].

BPDZ 44 was also shown to provoke a rapid, pronounced and sustained increase in ^{86}Rb outflow from prelabelled and perifused pancreatic islets. Although the measurement of ^{86}Rb FOR underestimates the real changes in K^+ fluxes [9], our findings suggest that the drug increases K^+ permeability of the pancreatic B-cell.

The K^+ channel activation mediated by BPDZ 44 may be expected to hyperpolarize the B-cell membrane. This will in turn inhibit the activity of the voltage-dependent Ca^{2+} channels, decrease Ca^{2+} entry, reduce $[Ca^{2+}]_i$ and ultimately inhibit the secretory process.

This proposed mechanism of action is attested by several experimental evidences. First, BPDZ 44 provoked a marked reduction in ^{45}Ca outflow from islets perifused in the presence of 16.7 mM glucose and extracellular Ca^{2+}. In islets exposed throughout to Ca^{2+} and insulinotropic concentrations of glucose, ^{45}Ca FOR is known to reflect a sustained stimulation of isotopic exchange between influent ^{40}Ca and effluent ^{45}Ca [6]. Thus, the inhibitory effect of BPDZ 44 on ^{45}Ca FOR can be viewed as the result of a reduction of Ca^{2+} entry into the islet cells. In agreement with such a proposal, the decrease in ^{45}Ca outflow mediated by BPDZ 44 did not occur when the islets were exposed to non-insulinotropic glucose concentrations or were perifused in the absence of extracellular Ca^{2+}. Second, measurements of Fura-2 fluorescence from single islet cells clearly revealed the capacity of BPDZ 44 to counteract the glucose-induced increase in cytosolic free Ca^{2+} concentration. Lastly, BPDZ 44 was unable to affect the increase in ^{45}Ca outflow and cytosolic free Ca^{2+} concentration provoked by a rise in the extracellular K^+ concentration. These cationic responses to K^+ depolarization, which can be inhibited by Ca^{2+} entry blockers [8], are known to be unaffected by K^+ channel openers [3, 7, 11]. The lack of effects of BPDZ 44 on the KCl responses indicates that the drug does not directly interfere with the voltage-sensitive Ca^{2+} channels and further supports the hypothesis that the primary effect of the pyridothiadiazine derivative is to raise the K^+ permeability of the pancreatic B-cell.

The enhancing effect of BPDZ 44 on ^{86}Rb outflow is reminiscent of that evoked by diazoxide and pinacidil [3, 7, 11–13]. These "K^+ channel openers" have been reported to specifically activate the ATP-sensitive K^+ channels equipping the B-cell plasma membrane. Although electrophysiological studies are needed to ascertain the exact identity of K^+ channels modulated by BPDZ 44, our physiological and pharmacological approach suggests that the pyridothiadiazine derivative might be a potent activator of K_{ATP} channels. Indeed, the drug increased ^{86}Rb outflow whatever the concentration of extracellular glucose [11]. This rise, however, was more marked in the presence of the sugar. On the other hand, the stimulatory effect of BPDZ 44 on ^{86}Rb FOR was completely abolished by glibenclamide, a hypoglycemic sulfonylurea known to close ATP-sensitive K^+ channels [14, 15]. Lastly, the BPDZ 44-induced rise in ^{86}Rb outflow persisted in the absence of extracellular Ca^{2+}. The latter observation can be taken as indirect evidence that the drug mainly interferes with a Ca^{2+}-insensitive mechanism of ^{86}Rb extrusion. All these findings, as well as the close similarities between the effects of BPDZ 44, pinacidil and diazoxide on ionic and secretory events in pancreatic B-cells [3, 7, 13], provide indirect support for the view that BPDZ 44 interferes with ATP-modulated K^+ channels.

In conclusion, the present data indicate that the pyridothiadiazine derivative BPDZ 44 markedly inhibits the insulin releasing process. This inhibitory effect appears to be mediated by the activation of ATP-sensitive K^+ channels leading to a decrease in Ca^{2+} influx and subsequent reduction in cytosolic free Ca^{2+} concentration.

Provided that the capacity of BPDZ 44 to inhibit insulin release and to stimulate ^{86}Rb outflow tightly correlates with its ability to bind to the K_{ATP} channel protein, it is tempting to propose the use of pyridothiadiazine derivatives as potent pharmacological tools for the characterization of K_{ATP} channels.

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