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COMPARATIVE EFFECTS OF LN 5330 AND DIAZOXIDE ON INSULIN RELEASE AND $^{86}Rb^+$ PERMEABILITY IN PERIFUSED RAT ISLETS OF LANGERHANS

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Abstract—The benzothiadiazine derivative LN 5330 (chloro-7 trifluoromethyl-6 benzothiadiazine-1,2,4 dioxide-1,1) has been shown to inhibit insulin secretion and calcium uptake. The present study was carried out to investigate the effects of LN 5330 on insulin release and $^{86}Rb^+$ efflux from perifused rat pancreatic islets; a comparison was made with the structural analogue diazoxide. In the presence of 8.3 mM glucose, LN 5330 (100 μM) accelerated $^{86}Rb^+$ efflux while reducing insulin output from the islets. LN 5330 induced a dose-dependent acceleration of $^{86}Rb^+$ efflux and appeared to be a more potent activator of $^{86}Rb^+$ efflux than diazoxide. The stimulatory effect of LN 5330 on $^{86}Rb^+$ efflux persisted in the absence of extracellular calcium. In the absence of glucose, $^{86}Rb^+$ permeability also increased, LN 5330 being again significantly more efficient than diazoxide at an equimolar concentration. These data indicate that the benzothiadiazine derivative LN 5330 inhibits insulin secretion by increasing the potassium permeability of the plasma membrane. It is suggested that, like diazoxide, this drug could activate the ATP-sensitive K^+ channel.

Chloro-7 trifluoromethyl-6 benzothiadiazine-1,2,4 dioxide-1,1 (LN 5330) is a benzothiadiazine derivative. It is represented in Fig. 1 together with two potassium channel activators, the structural analogue diazoxide and the cyanoguanidine derivative pinacidil. Like diazoxide, LN 5330 inhibits glucose-induced insulin secretion from isolated perfused rat pancreas [1], and calcium uptake by isolated islets [2]. LN 5330, however, displays further properties which differentiate the two derivatives; unlike diazoxide, it stimulates glucagon secretion [3] and, in some instances, induces a stimulatory off-response in insulin output [1, 2].

As potassium permeability is involved critically in the regulation of B-cell membrane potential and hence the insulin secretion coupling [4, 5], the present work was carried out to investigate and compare the effects of LN 5330 and diazoxide on insulin release and $^{86}Rb^+$ permeability in perifused rat islets of Langerhans.

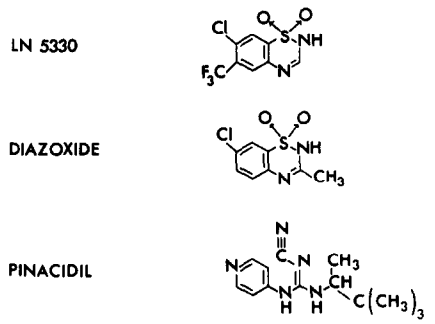

Fig. 1. Chemical structure of LN 5330 (chloro-7 trifluoromethyl-6 benzothiadiazine-1,2,4 dioxide-1,1) compared with those of diazoxide (chloro-7 methyl-3 benzothiadiazine-1,2,4 dioxide-1,1) and pinacidil (*N*″-cyano-*N*-4-pyridyl-*N*′-1,2,2-trimethylpropylguanidine).

MATERIALS AND METHODS

Experiments were performed on pancreatic islets from male Wistar albino rats fed *ad lib.* and weighing 120–180 g. Islets were isolated by collagenase digestion [6] according to a technique derived from that of Lacy and Kostianovsky [7]. They were incubated in 220 μL Krebs–Ringer bicarbonate buffer (pH 7.4) containing glucose 8.3 mM and supplemented with bovine serum albumin (Fraction V) 1 mg/mL and 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) 10 mM. In these conditions, under continuous gassing with O_2/CO_2 (95:5), islets were loaded for 90 min at 37° in the presence of $^{86}RbCl$ (37–185 MBq/mL; sp. act.: 37–296 MBq/mg rubidium). $^{86}Rb^+$ was used as the tracer for K^+ [8, 9].

At the end of the labelling period, the islets were washed twice with non-radioactive medium. Batches of 25 islets were perifused in parallel chambers using a system similar to that described by Henquin [10]. The volume of the chamber was 0.3 mL and the flow rate 1.4 mL/min. Switching the distribution valve of the perifusion system at min 40 started the medium change and this marked the beginning of the test period. No correction was made for the dead time

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(about 60 sec) between switching the valve and collection of the medium. The medium was a Krebs–Ringer bicarbonate buffer (ionic composition in mM: NaCl 118; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5) equilibrated with O_2/CO_2 (95:5) and supplemented with bovine serum albumin (Fraction V) 1 mg/mL. Ca^{2+}-free solutions were prepared by substituting $MgCl_2$ for $CaCl_2$ (the concentration of residual total calcium being less than 10 μM), and the medium was supplemented with 100 μM EGTA.

LN 5330 and diazoxide were used at concentrations ranging from 10 μM to 1 mM. The drugs were tested under three different sets of experimental conditions: in the presence of (1) 8.3 mM glucose and 2.5 mM calcium or (2) 8.3 mM glucose without extracellular calcium and (3) in the absence of glucose. For control experiments, the perifusion medium was derived alternately from two control solutions.

Insulin release and $^{86}Rb^+$ efflux were measured in effluent fractions collected over 2-min intervals from min 20 onwards, this time lag allowing a complete wash-out of the extracellular space [11].

$^{86}Rb^+$, in the effluent fraction and remaining in the islets at the end of the experiment, was counted by measuring the Cerenkov radiation, after addition to each sample of 3 mL of a 3 mM aqueous solution of the wavelength shifter 7-amino-1,3-naphtalenedisulfonic acid, potassium salt [12]. From the sum of the radioactivity remaining in the islets at the end of the experiment and the cumulated effluent radioactivity, the fractional efflux of $^{86}Rb^+$ was calculated for each collection interval and expressed as a percentage of the instantaneous islet content per min. For the concentration–response data, the peak $^{86}Rb^+$ efflux was the difference between the highest value recorded during stimulation and the basal value found for the same experiment between the 38th and the 40th min of perifusion, and was expressed as a percentage of this reference value.

Insulin was assayed by the radioimmunological method of Herbert *et al.* [13] using [^{125}I]insulin (C.I.S. International, Gif-Sur-Yvette, France); purified rat insulin as standard (Novo, Copenhagen, Denmark), the biological activity of which was 22.3 μUnits/ng; and anti-insulin serum (ICN Biochemicals, Miles, Puteaux, France). The sensitivity of the assay was 0.1 ng/mL. Insulin output per min was determined by multiplying the hormone concentration in the effluent by the flow rate. Results are expressed as a percentage of the reference value recorded between min 38 and 40.

Statistical analysis. Results are means ± SEM. Data were submitted to analysis of variance and multiple comparison tests [14].

Chemicals. LN 5330 was kindly supplied by Laroche-Navarron (Puteaux, France) and diazoxide by Schering (Bloomfield, NJ, U.S.A.). Both drugs were dissolved in equimolar NaOH (1 mol/L) and the volumes adjusted with NaCl (0.154 mol/L).

Collagenase was supplied by Serva (Heidelberg, F.R.G.); ^{86}RbCl by the Radiochemical Centre (Amersham, U.K.); HEPES by Gibco Europe (Paisley, U.K.); and bovine serum albumin (Fraction V) and ethyleneglycol-bis(β-aminoethyl-ether)*N,N,N′,N′*-tetraacetic acid (EGTA) by the Sigma Chemical Co. (St Louis, MO, U.S.A.).

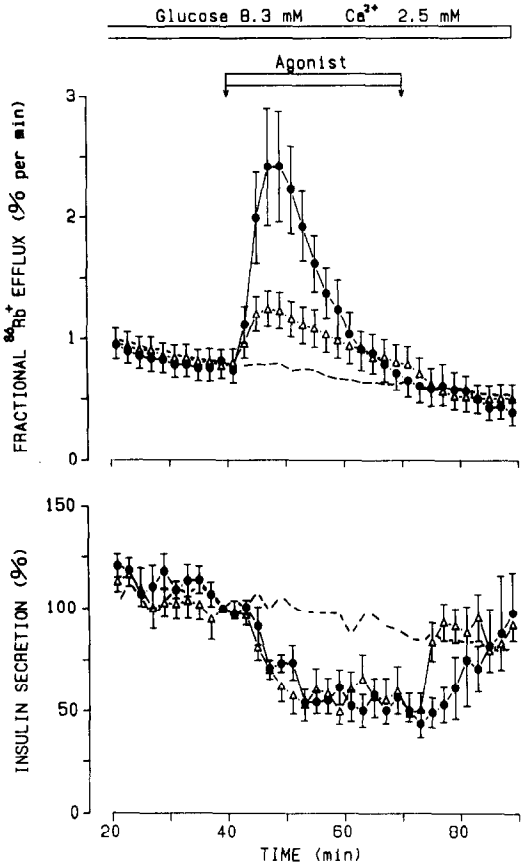

Fig. 2. Effects of LN 5330 (●) and diazoxide (△) on insulin release and $^{86}Rb^+$ efflux from rat pancreatic islets perifused in the presence of glucose 8.3 mM and Ca^{2+} 2.5 mM throughout. Broken lines show control experiments. Values are means ± SEM for six experiments.

RESULTS

Effects of LN 5330 and diazoxide in the presence of glucose (8.3 mM) and calcium (2.5 mM)

Effects on insulin release and $^{86}Rb^+$ efflux. The effects of 100 μM LN 5330 on the time course of $^{86}Rb^+$ outflow and insulin release were tested in comparison with diazoxide at an equimolar concentration (Fig. 2).

When the islets were perifused in the presence of 8.3 mM glucose, the basal rate of insulin recorded between min 38 and 40 averaged 31.8 ± 4.1 pg/min/islet (N = 18; reference value just before addition of the agonist). Addition of 100 μM LN 5330 caused a rapid and sustained inhibition of insulin release. The decrease in insulin output reached 50% and was significantly different from control values between min 46 and 78 ($P < 0.01$). The level of inhibition was the same as that caused by an equimolar concentration of diazoxide. However, although reversible for both drugs, the inhibitory effect of LN 5330

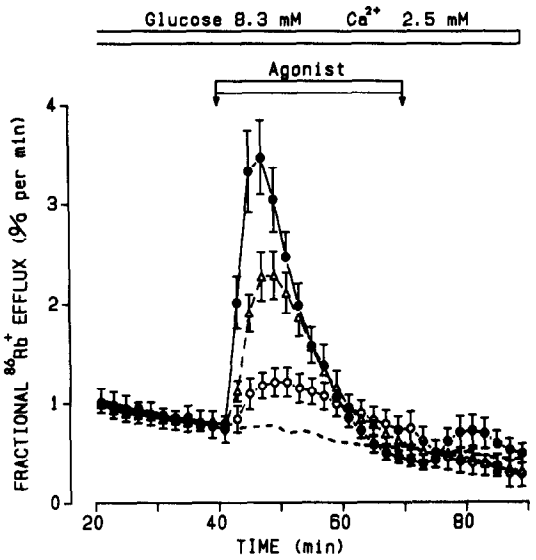

Fig. 3. Effects of increasing concentrations of LN 5330 [(○) 50 μM; (△) 100 μM; (●) 200 μM] on $^{86}Rb^+$ efflux from rat pancreatic islets perifused in the presence of glucose 8.3 mM and Ca^{2+} 2.5 mM throughout. Broken lines show control experiments. Values are means ± SEM for at least six experiments.

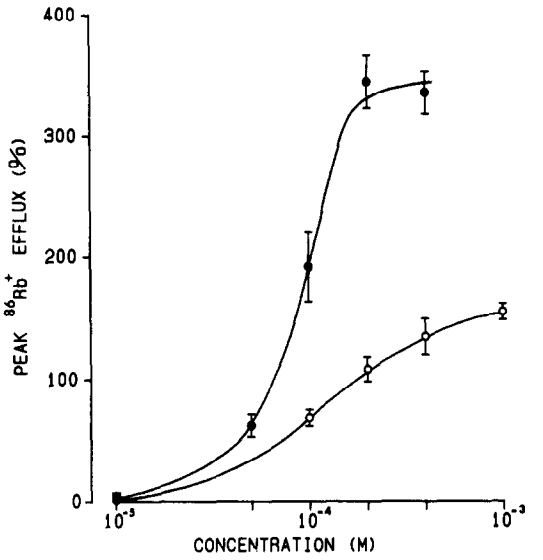

Fig. 4. Concentration–response curves for the stimulatory effect of LN 5330 (●) and diazoxide (○) on $^{86}Rb^+$ efflux from pancreatic islets perifused in the presence of glucose 8.3 mM and Ca^{2+} 2.5 mM throughout. Values are means ± SEM for at least five experiments.

lasted longer after withdrawal than the effect of diazoxide (significantly different between min 74 and 78, $P < 0.01$).

In the presence of 8.3 mM glucose, the fractional efflux rate of $^{86}Rb^+$ remained fairly stable. Addition of 100 μM LN 5330 caused a rapid and significant acceleration of $^{86}Rb^+$ efflux which was significantly more pronounced than that caused by an equimolar concentration of diazoxide ($P < 0.001$, between min 44 and 56), although the effect of the latter appeared to be more sustained (Fig. 2).

Concentration–response data for $^{86}Rb^+$ efflux. The effects of increasing concentrations of LN 5330 and diazoxide on $^{86}Rb^+$ efflux were tested. Figure 3 illustrates the concentration-dependent effect of LN 5330 (50–200 μM) on the kinetics of $^{86}Rb^+$ outflow from islets perifused throughout in the presence of 8.3 mM glucose. The acceleration of $^{86}Rb^+$ outflow caused by 50 μM LN 5330 was more sustained than the increase caused by higher concentrations and paralleled the effect of 100 μM diazoxide (Fig. 2). Figure 4 shows the concentration–response curves for the peak $^{86}Rb^+$ outflow caused by LN 5330 (10–400 μM) and diazoxide (10 μM–1 mM). Both benzothiadiazine analogues produced a dose-related stimulatory effect within a similar range of concentrations and LN 5330 proved to be a more effective activator of $^{86}Rb^+$ outflow than diazoxide. Indeed, the $^{86}Rb^+$ efflux during stimulation by 200 μM LN 5330 averaged 3.5 ± 0.4% per min (maximal effect), as compared to 2.2 ± 0.3% per min when islets were stimulated by 1 mM diazoxide ($P < 0.02$); basal values being 0.8 ± 0.1% per min and 0.9 ± 0.1% per min, respectively.

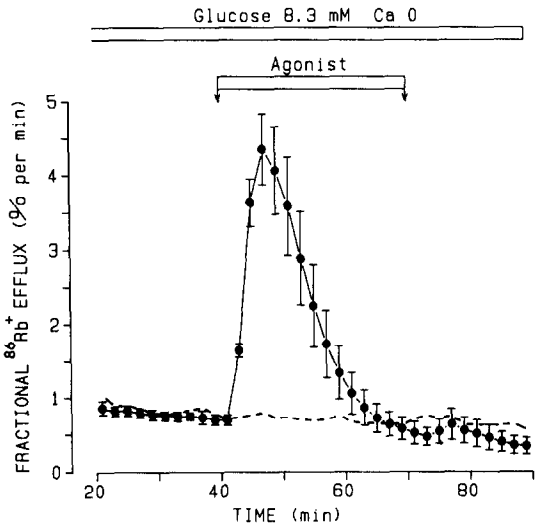

Fig. 5. Effect of LN 5330 (●) on $^{86}Rb^+$ efflux from rat islets perifused with a medium containing glucose 8.3 mM, in the absence of extracellular calcium. Broken lines show control experiments. Values are means ± SEM for three experiments.

Effects of LN 5330 and diazoxide in the absence of either calcium or glucose

Effects of LN 5330 in the absence of extracellular calcium (Fig. 5). In a Ca^{2+}-free medium supplemented with 0.1 mM EGTA, the rise in $^{86}Rb^+$ efflux produced by 100 μM LN 5330 persisted and was even significantly amplified. Indeed, $^{86}Rb^+$ outflow between min 46 and 48 averaged 4.4 ± 0.5%

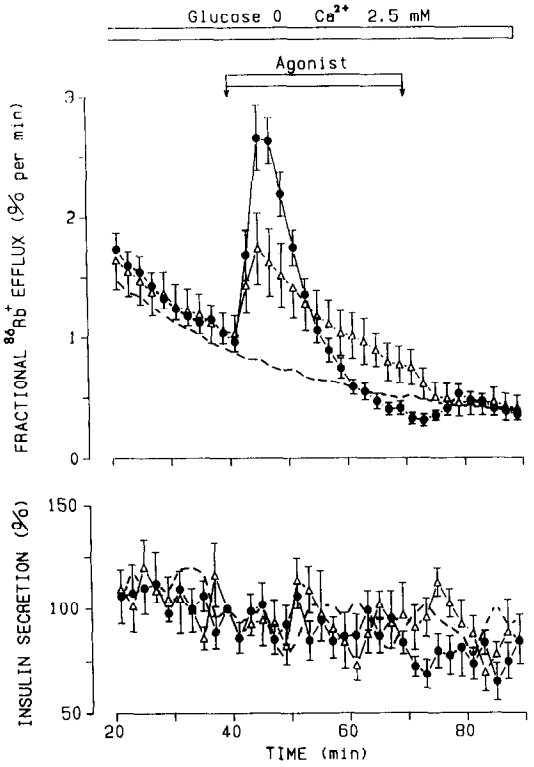

Fig. 6. Effects of LN 5330 (●) and diazoxide (△) on insulin release and $^{86}Rb^+$ efflux from rat islets perifused with a medium containing Ca^{2+} 2.5 mM but no glucose. Broken lines show control experiments. Values are means ± SEM for six experiments.

per min, as compared to 2.4 ± 0.5% per min in the presence of extracellular calcium ($P < 0.02$).

Effects of LN 5330 and diazoxide in the absence of glucose (Fig. 6). When the islets were perifused in the absence of glucose, the basal rate of insulin release between min 38 and 40 averaged 17.3 ± 2.3 pg/min/islet (N = 18). Insulin release was low, fairly stable and was not significantly affected by either drug.

Under these conditions, the fractional efflux rate of $^{86}Rb^+$ decreased slowly with time. Addition of 100 μM LN 5330 caused a prompt but transient acceleration of $^{86}Rb^+$ efflux. The maximal increase in $^{86}Rb^+$ outflow, between min 44 and 48, was significantly higher in the presence of LN 5330 than 100 μM diazoxide ($P < 0.001$). However, whereas the rate of $^{86}Rb^+$ efflux accelerated by diazoxide, although slowly declining, remained clearly higher than in the controls, the efflux rate accelerated by LN 5330 decreased rapidly to and below control values and became significantly lower, as compared with diazoxide between min 60 and 66 ($P < 0.01$).

DISCUSSION

The benzothiadiazine derivative LN 5330, structurally related to diazoxide, inhibits glucose-induced insulin secretion from perifused rat islets of Langerhans. This result is in accordance with previous data obtained in the isolated perfused rat pancreas [1, 3], as well as in incubated rat islets [2]. Under our experimental conditions, the inhibition of insulin output by LN 5330 was similar to that caused by an equimolar concentration of diazoxide, although LN 5330 was shown to be half as potent as diazoxide in isolated perfused rat pancreas [1]. Unlike previous observations in perfused pancreas [1] or incubated islets [2], there is no stimulatory effect after withdrawal of LN 5330 (50–400 μM, not shown). It has been shown that LN 5330, but not diazoxide, increases glucagon output from isolated perfused rat pancreas [3]. However, it is well known that glucagon stimulates insulin secretion [15]. A possible explanation for this discrepancy could rest on a difference in the degree of glucagon wash-out, depending on the experimental preparation.

It is now well established that diazoxide hyperpolarizes the B-cell membrane by increasing its permeability to potassium [16, 17]. The subsequent hyperpolarization inactivates voltage-dependent Ca channels, resulting in a reduction of Ca^{2+} influx and insulin release [18]. Furthermore, diazoxide has been shown to increase the opening probability of ATP-sensitive K channels of the B-cell membrane [19–22]. Recent data suggest that the diazoxide-induced activation of ATP–K^+ channel currents involves phosphorylation of the channel or some closely associated membrane protein [23].

LN 5330, while decreasing insulin output, accelerated $^{86}Rb^+$ efflux from the islets. This effect persisted in a Ca-free medium, indicating that it does not require the presence of extracellular calcium. In the absence of glucose, $^{86}Rb^+$ permeability was still increased by LN 5330, excluding the possibility that an impairment of glucose metabolism accounted for the effects observed with the drug. These results suggest that the benzothiadiazine derivative LN 5330 inhibits insulin release by a direct interaction with K^+ permeability of the B-cell membrane. Like diazoxide, this drug could interact with the ATP-sensitive K^+ channel. The increase in K^+ permeability may be expected to hyperpolarize the plasma membrane and consequently inactivate the voltage-dependent Ca^{2+} channels. Indeed, LN 5330 has been shown to inhibit calcium uptake by pancreatic islets [2].

As regards the concentration–response data, LN 5330 and diazoxide caused dose-dependent increases in the $^{86}Rb^+$ efflux from pancreatic islets perifused in the presence of glucose, within a similar range of concentrations. However, LN 5330 appeared clearly to be a more effective activator of the potassium channel than diazoxide. This observation is not in agreement with previous results obtained in isolated perfused rat pancreas, showing that LN 5330 was half as potent as diazoxide in inhibiting insulin release [1]. As discussed above, a possible explanation of this discrepancy could be the stimulatory effect of LN 5330 on glucagon secretion [3] which could in turn interfere with insulin release, altering the resulting potency of the drug.

Among the various potassium channel openers

investigated recently as being smooth muscle relaxants, the cyanoguanidine derivative pinacidil, which seems to have some structural analogy with benzothiadiazine derivatives LN 5330 and diazoxide (Fig. 1), has been shown to inhibit insulin release from pancreatic islets [24]. This drug has its effect by a similar cellular mechanism as does diazoxide, i.e. by opening ATP-sensitive potassium channels, whereas the lesser inhibitory effects of cromakalim and nicorandil on insulin release may involve actions other than on potassium channels in B-cells [25]. It has been shown that pinacidil is less potent than diazoxide at opening the potassium channels in B-cells at concentrations ranging from 10 μM to 1 mM [26]. Our results clearly show that LN 5330 is more potent than diazoxide at increasing $^{86}Rb^+$ permeability in a similar range of concentrations. Thus, this structural analogue of diazoxide could be a valuable tool for studying potassium channels in B-cells and determining further the structural requirements needed to mediate the action of potassium channel activators.

In conclusion, these experimental data indicate that the benzothiadiazine derivative LN 5330 inhibits insulin secretion by increasing the potassium permeability of the plasma membrane. The acceleration of the $^{86}Rb^+$ efflux is dose-dependent, LN 5330 being more potent than diazoxide and the effect is not subordinate to the presence of glucose or calcium in the medium. It is suggested that this drug, like diazoxide, could activate the ATP-sensitive K^+ channels of the B-cell.

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