Effect of Linogliride on Hormone Release From Perfused Rat Pancreas

Fuel Dependence and Desensitization by Tolbutamide

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We examined the effect of the hypoglycemic drug linogliride on hormone release from the in vitro perfused rat pancreas. Linogliride stimulated insulin release in the absence of glucose either in the presence or absence of a physiological mixture of amino acids. In addition, linogliride inhibited amino acid–induced glucagon release. Half-maximal effects of linogliride on insulin and glucagon release were achieved at concentrations as low as 26 and 3 μM, respectively. The effects of linogliride on hormone release largely resembled those of tolbutamide. In the absence of amino acids, the stimulation of insulin release by linogliride or tolbutamide was transient. When the pancreas had been preperfused for 20 min with tolbutamide, linogliride no longer had an effect on hormone release. Likewise, tolbutamide remained without effect in pancreases preperfused with linogliride. These data suggest that linogliride and tolbutamide may have a similar mechanism of action.
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Linogliride and its analogue pirogliride are substituted guanidines, which lower blood glucose levels (1–4). Pharmacological and biochemical studies indicate that a major mode of hypoglycemic action is through the stimulation of insulin secretion (1–4). We were interested in defining the mechanism of action of linogliride. We also wanted to determine the effect of linogliride on glucagon and somatostatin release. In addition, we wanted to determine whether the effects of linogliride depend on exogenous fuel. Previous studies with isolated islets indicated that both linogliride and its congener pirogliride require the presence of glucose to be effective. We decided to address these issues with studies of hormone release from the in vitro perfused rat pancreas. Contrary to results published on isolated islets (2), we found that linogliride also stimulated insulin release in the absence of exogenous fuel. The linogliride-induced absolute increase in insulin release was particularly large in the presence of a physiological mixture of amino acids. Linogliride also stimulated somatostatin release and potently inhibited amino acid–induced glucagon release.

In the absence of amino acids, the stimulation of insulin release by either linogliride or tolbutamide was transient. Surprisingly, in the continued presence of tolbutamide, linogliride no longer had an effect on hormone release. Likewise, tolbutamide remained without effect in pancreases that were continuously perfused with linogliride.

Our data suggest that linogliride, like glucose, stimulates insulin and somatostatin release and inhibits glucagon release. We hypothesize that linogliride, like tolbutamide, affects ATP-sensitive K^+ channels or a regulatory protein closely associated with this channel. An electrophysiological test of this hypothesis is described by Ronner et al. (this issue, p. 885).

RESEARCH DESIGN AND METHODS

Fed male Sprague-Dawley rats (250–350 g body wt) were obtained from Hilltop (Scottsdale, PA). Before surgery, the rats were anesthetized with pentobarbital sodium (30 mg/kg body wt i.p.). After removal of the pancreas, the anesthetized rats were killed by exsanguination.

The in vitro perfusion of the isolated rat pancreas (without stomach and spleen but with a segment of duodenum attached) was carried out at 37°C with a flow rate of 4 ml/min. The perfusion medium consisted of 137 mM NaCl, 2.7 mM KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.7 mM sodium phosphate, 23 mM sodium bicarbonate, and dextran (4% wt/vol, 40,000 M_r; Sigma, St. Louis, MO); the medium was gassed with a mixture of 5% (vol/vol) CO_2 in pure O_2. In some ex-

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periments, the perfusion medium contained a physiological mixture of amino acids at the following concentrations (total concentration 4000 μM): 437 μM alanine, 187 μM arginine, 40 μM aspartic acid, 93 μM citrulline, 121 μM glutamic acid, 500 μM glutamine (fresh), 298 μM glycine, 78 μM histidine, 94 μM isoleucine, 162 μM leucine, 370 μM lysine, 48 μM methionine, 69 μM ornithine, 82 μM phenylalanine, 349 μM proline, 568 μM serine, 270 μM threonine, 74 μM tryptophan, and 201 μM valine. Linogliride fumarate (McNeil, Spring House, PA) and tolbutamide sodium (Upjohn, Kalamazoo, MI) were dissolved directly in the perfusion medium on the day of the experiment. Samples of the perfusate were collected for 1 min into tubes containing 100 μl of a solution of Trasylol (1000 KIU/ml) and bovine serum albumin (BSA; 2.5 mg/ml; Sigma).

We performed assays for insulin, glucagon, and somatostatin. The hormone content of the samples was determined by radioimmunoassay (RIA) with single-antibody charcoal separation techniques. An antibody against insulin was obtained from ICN (Costa Mesa, CA), monoiodinated receptor-grade pork insulin was from Du Pont-NEN (Wilmington, DE), and rat insulin was a gift from Lilly (Indianapolis, IN). Glucagon was assayed with the following components: antiserum 04A, obtained from the Diabetes Research Fund of the University of Texas (Dallas, TX); iodinated glucagon from Cambridge Medical Diagnostics (Billerica, MA); and purified beef/pork glucagon from Lilly. Somatostatin was assayed with an antiserum from INCStar (Stillwater, MN), [^{125}I-Tyr^{1}]-somatostatin-14 from Du Pont-NEN, and somatostatin-14 standard from Calbiochem (San Diego, CA). The midpoints for the RIAs were at ~4, 0.2, and 0.15 ng/ml for insulin, glucagon, and somatostatin, respectively.

For statistical methods and curve-fitting procedures, data are shown as means ± SE. In figures where the error bar is not visible, the error was smaller than the symbol. The significance of differences in mean hormone release between individual time points was calculated with Student's two-tailed *t* test for paired or unpaired samples as indicated. The secretory profiles were integrated between both 5 and 19 and 21 and 40 min. The value of the first integral was considered representative of baseline hormone release. For the construction of concentration-response curves, baseline release linearly extrapolated to 20 min was subtracted from the second integral. SEs of the integrated secretory response were calculated from means ± SE at each time point in a set of perfusions. Curves obeying the formula

$$H = a + \{b/[1 + (K_{0.5}/d)^n]\} \quad (1)$$

were fitted to the concentration-response data with a weighted nonlinear regression with the Marquardt-Levenberg algorithm provided with the Sigmaplot version 4.0 sci-

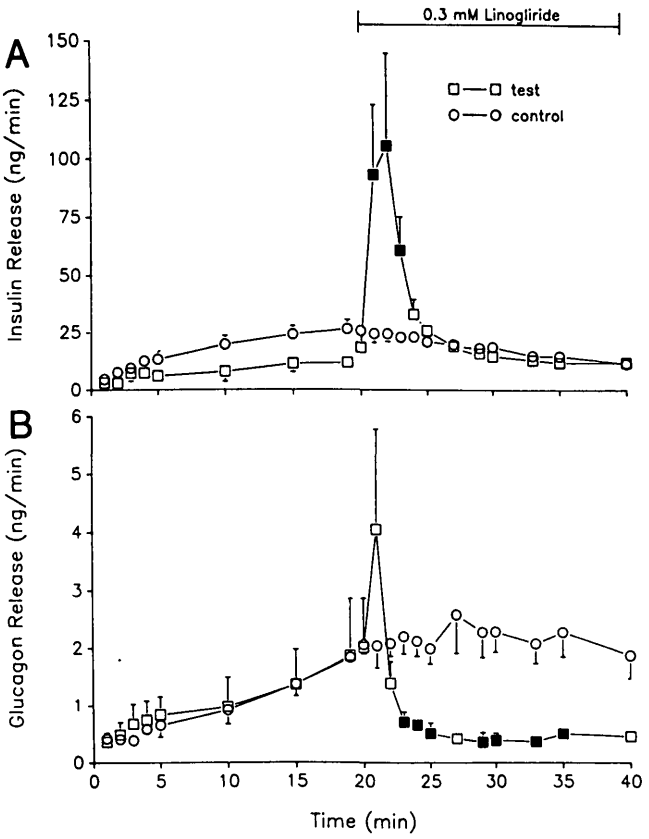

FIG. 1. Effect of linogliride on insulin (*A*) and glucagon (*B*) release from rat pancreas perfused in absence of exogenous fuel. Linogliride (300 μM) was present in perfusion solution from 20 to 40 min. Values are means ± SE of hormone release observed in 4 control perfusions and 2 perfusions with 300 μM linogliride. ■, Significant difference from release observed in control at same time point ($P \leq 0.05$ by Student's unpaired *t* test).

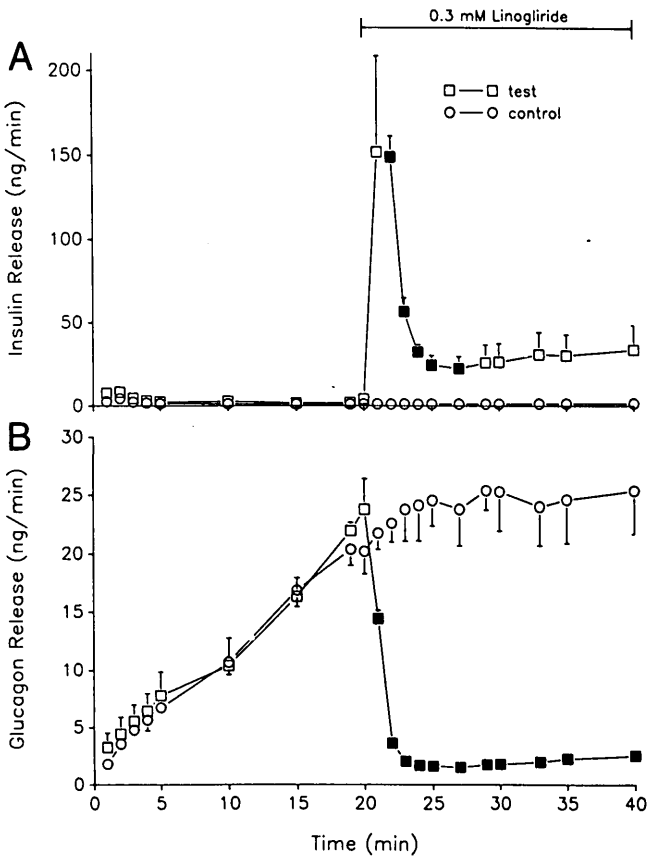

FIG. 2. Effect of linogliride on insulin (*A*) and glucagon (*B*) release in presence of 4 mM physiological mixture of amino acids. Same type of experiment as in Fig. 1. Values are means ± SE of hormone release observed in 3 perfusions. ■, Significant difference from mean at same time point in absence of linogliride ($P \leq 0.05$ by Student's unpaired *t* test).

entific plotting program (Jandel Scientific, Corte Madera, CA). In Eq. 1, H is the observed corrected hormone release, a is the estimated hormone release in the absence of linogliride, b is the maximal inhibition or stimulation of hormone release by linogliride, $K_{0.5}$ is a measure of the apparent affinity of linogliride, d is the test concentration of linogliride, and n resembles Hill's cooperativity coefficient. For the fitting process, the means were assigned weights of 1/SE.

BSA (Sigma) was RIA grade. Trasylol was obtained from Miles (New Haven, CT) as a sterile solution of 10,000 KIU/ml in isotonic NaCl. All other reagents used in the perfusion media were obtained from Sigma.

RESULTS

We studied the effect of linogliride on insulin release. Linogliride (300 μM) stimulated insulin release in the absence of fuels (Fig. 1), in the presence of a physiological mixture of amino acids (4 mM; Fig. 2), in the presence of 5 mM glucose (Fig. 3*A*), and in the presence of both 5 mM glucose and 4 mM amino acids (Fig. 3*B*). In the absence of amino acids (Figs. 1*A* and 3*A*), the stimulatory effect of linogliride was markedly short lived; however, in the presence of amino acids (Figs. 2*A* and 3*B*), the stimulatory effect of linogliride lasted >20 min.

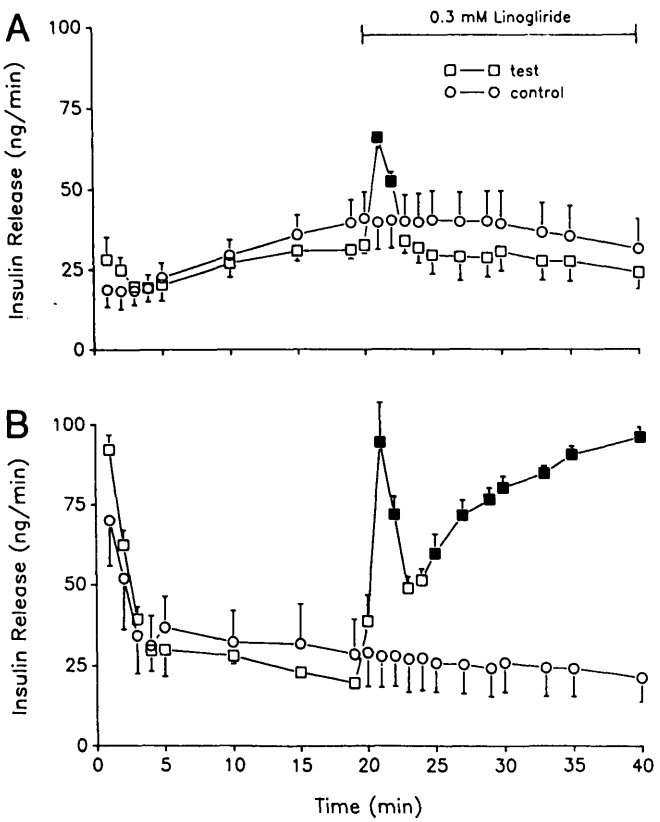

FIG. 3. Effect of linogliride on insulin release in presence of 5 mM glucose and combination of 4 mM amino acids and 5 mM glucose. Same type of experiment as in Fig. 1. Values are means ± SE of hormone release. *A*: perfusion with 5 mM glucose (n = 5 for control and n = 3 for test). There were no statistically significant differences between test and control. ■, Significant difference from 19-min time point within test perfusions ($P < 0.05$ with Student's t test for paired data). *B*: perfusion with combination of 4 mM amino acids and 5 mM glucose (n = 3 for both control and test). ■, Significant difference from release at same time point in control ($P \leq 0.05$ by Student's unpaired t test).

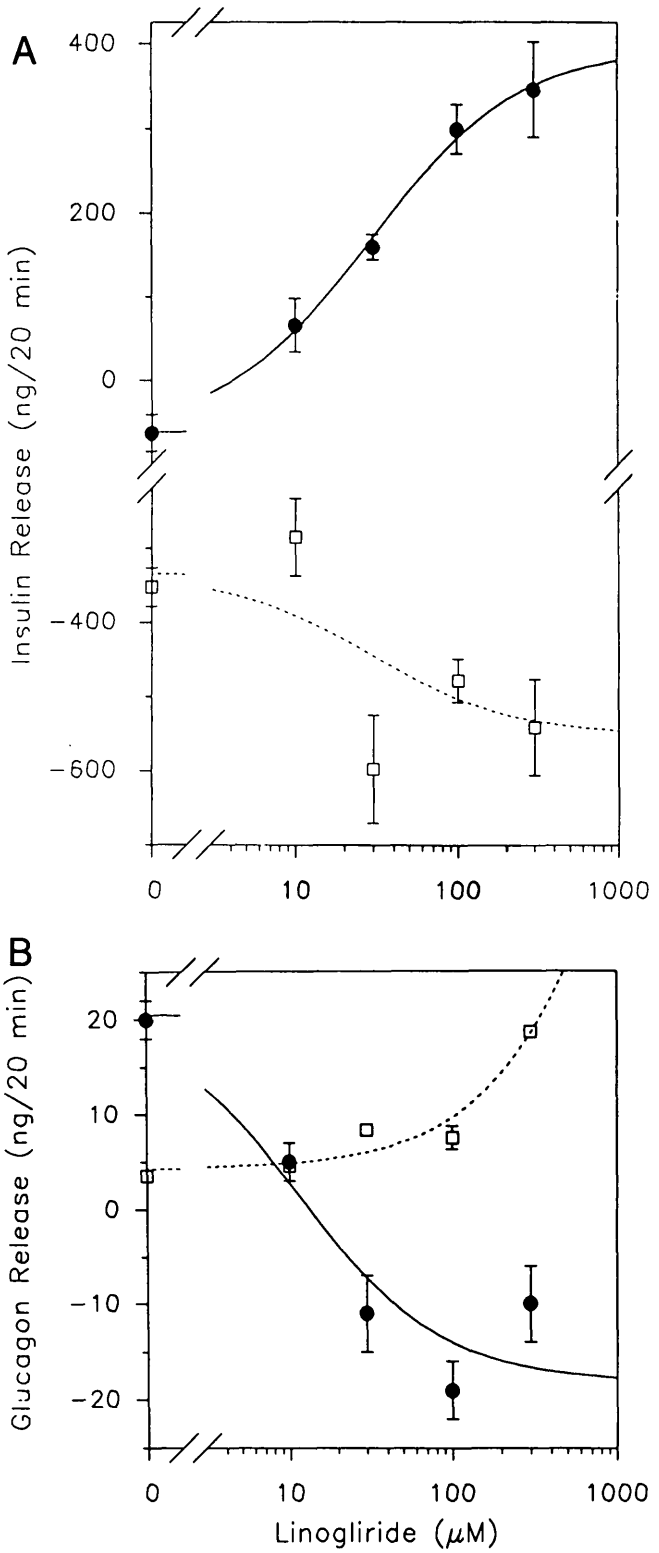

FIG. 4. Concentration-response curve for effect of linogliride on release of insulin (*A*) and glucagon (*B*) in absence of exogenous fuel. Hormone release was integrated between 21 and 40 min, then corrected for baseline release based on measurements in 5- to 19-min period. ●, Data from type of experiment illustrated in Fig. 1; hormone release was fitted to Eq. 1 (see METHODS). For insulin release, best fit was obtained with $K_{0.5}$ = 32 μM, n = 0.93 (*solid line*). For glucagon release, best fit was obtained with $K_{0.5}$ = 11 μM, n = 2.47 (*solid line*). □, Effect of linogliride in presence of tolbutamide; data derived from type of experiment illustrated in Fig. 8. Hormone release was fitted to Eq. 1 with n set to 1. Best fit was obtained with $K_{0.5}$ = 29 μM for insulin release and $K_{0.5}$ = 1.4 mM for glucagon release (*dashed lines*).

Perfusion experiments similar to those in Figs. 1–3 were also performed with lower concentrations of linogliride (i.e., 10, 30, and 100 μM). The results are summarized in the concentration-response curves in Figs. 4–7. A summary of the mathematical analysis of these data is provided in Table 1. For perfusions carried out in the presence of 5 mM glucose alone, only drug-induced peak hormone release (but not integrated hormone release) provided a meaningful concentration-response curve (Fig. 6). In all experiments, $K_{0.5}$ values did not change much if n was not allowed to float but instead set to 1 (Table 1). In addition, $K_{0.5}$ values were generally markedly higher for peak than for integrated hormone release.

We also studied the effect of linogliride on glucagon release. In the absence of any fuel, linogliride had a dual effect on glucagon release: initially, it tended to stimulate glucagon release slightly; later, it inhibited glucagon release (Fig. 1*B*). Concentration-response data of hormone release during the entire test phase are shown in Fig. 4*B*. In the presence of 5 mM glucose, linogliride had a very minor effect on glucagon release, and a meaningful concentration-response curve could not be derived. In the presence of a physiological mixture of amino acids, linogliride potently inhibited glucagon release (Figs. 2*B* and 5*B*). On the other hand, in the presence of both 4 mM amino acids and 5 mM glucose, glucagon release was <3 ng/min; it was depressed further by linogliride to as little as 0.5 ng/ml, but no clear-cut concentration-response curve could be derived from our data.

Linogliride stimulated peak somatostatin release during the test phase by as much as 0.3 ng/20 min (integrated release in response to 300 μM linogliride: 1.1 ± 0.2 ng/20

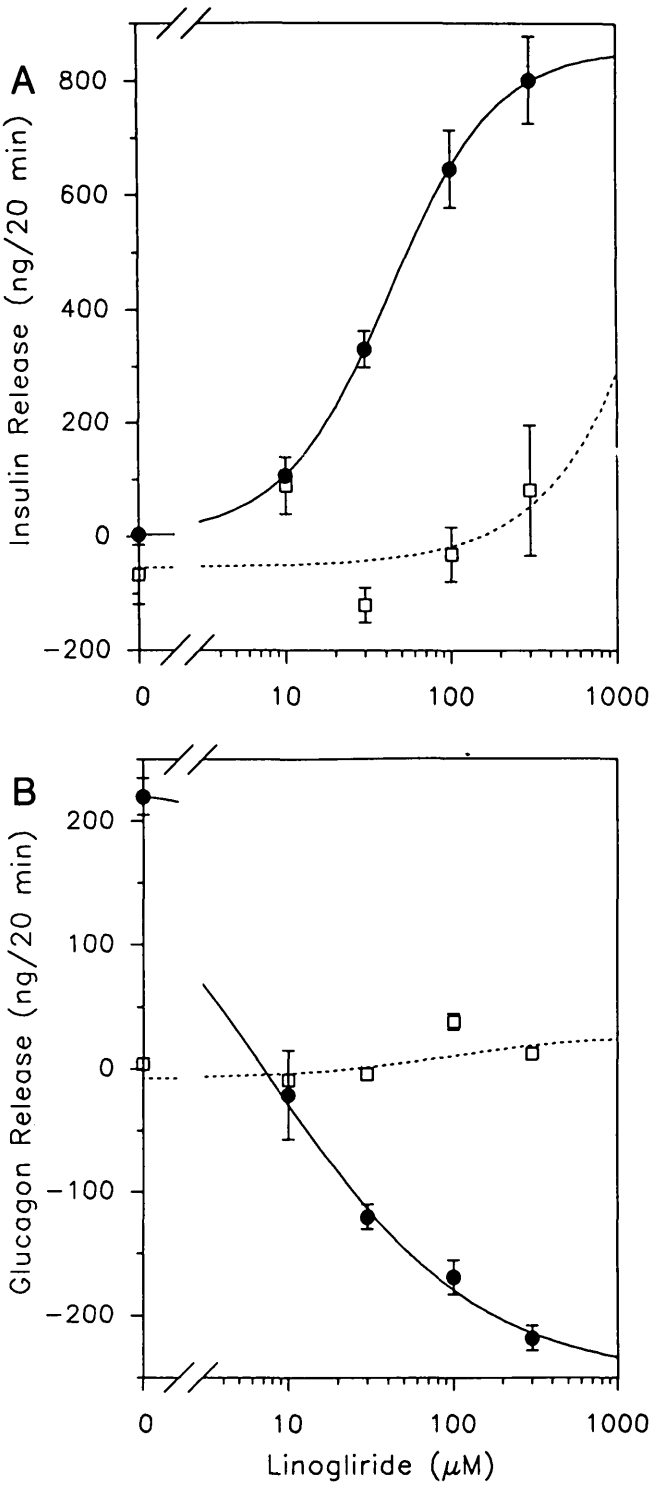

FIG. 5. Concentration-response curve for linogliride-induced insulin (*A*) and glucagon (*B*) release in presence of amino acids (4 mM). Data were derived from perfusions similar to those in Fig. 2 with same type of analysis as in Fig. 4. ●, Effect of linogliride alone. Best fit to Eq. 1 was obtained with $K_{0.5}$ = 43 μM and n = 1.35 for insulin release and with $K_{0.5}$ = 8.2 μM and n = 0.71 for glucagon release (*solid lines*). □, Effect of linogliride in presence of tolbutamide. For n = 1, best fit was obtained with $K_{0.5}$ = 3 mM for insulin release and $K_{0.5}$ = 87 μM for glucagon release (*dashed lines*).

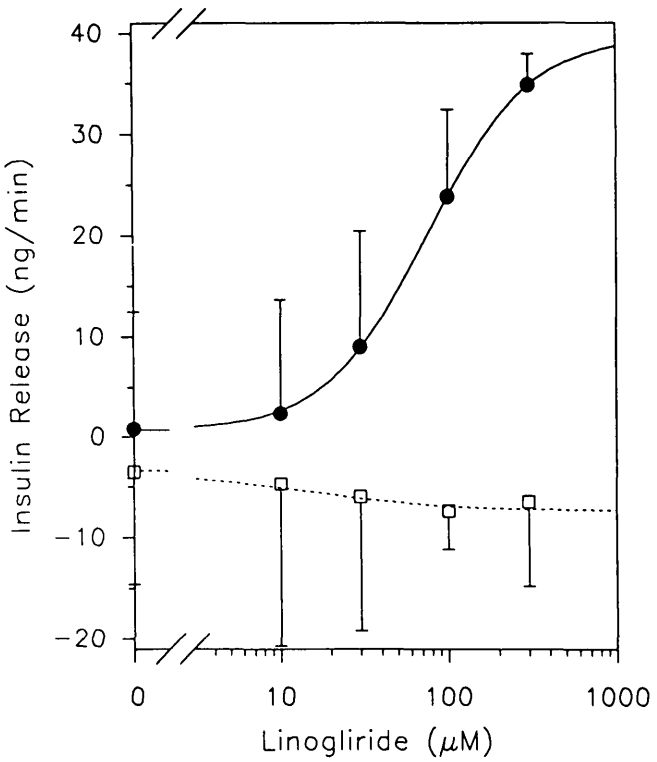

FIG. 6. Concentration-response curve for linogliride-induced peak insulin release in presence of 5 mM glucose. ●, Data derived from perfusions similar to those in Fig. 3*A*. Peak hormone release (observed at 25, 24, 22, 22, and 21 min for 0, 10, 30, 100, and 300 μM linogliride, respectively) was corrected for release at 19 min. Equation 1 (see METHODS) was best fitted with $K_{0.5}$ = 76 μM, and n = 1.42 (*solid line*). □, Effect of linogliride in presence of tolbutamide. Best fit with n = 1 was obtained for $K_{0.5}$ = 14 μM.

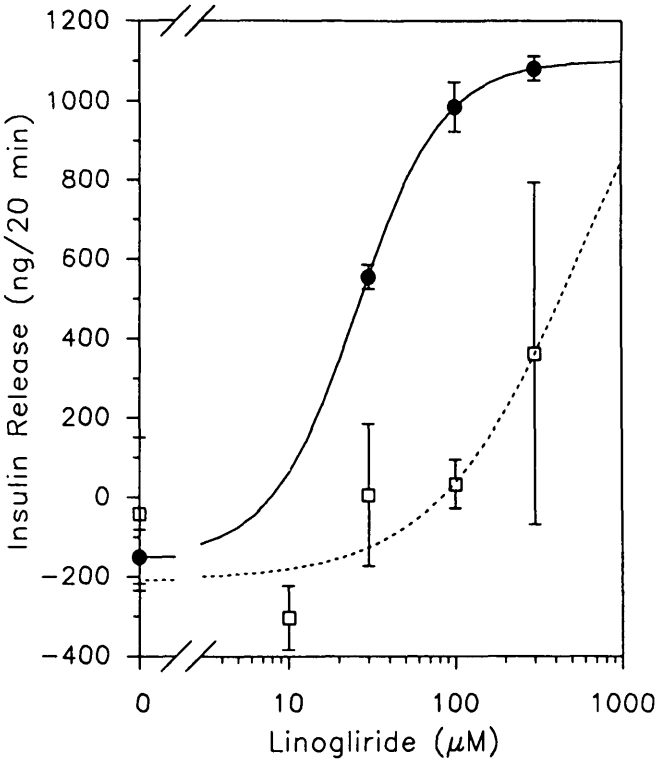

FIG. 7. Concentration-response curve for linogliride-induced insulin release in presence of amino acids (4 mM) and glucose (5 mM). ●, Data derived from perfusions similar to those shown in Fig. 3*B*. Hormone release was integrated between 21 and 40 min, then corrected for baseline release based on measurements in 5- to 19-min period. Best fit to Eq. 1 (see METHODS) was obtained with $K_{0.5}$ = 26 μM and n = 1.68 (*solid line*). □, Effect of linogliride in presence of 100 μM tolbutamide. Best fit was obtained for $K_{0.5}$ = 0.6 mM if n = 1 (*dashed line*).

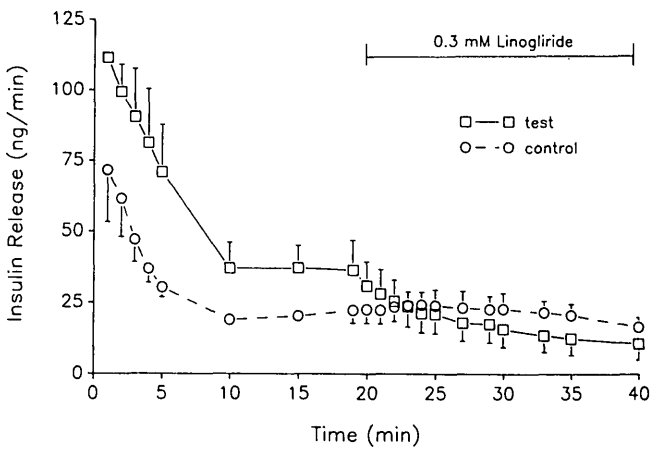

FIG. 8. Effect of linogliride on insulin release in absence of fuel in pancreases preexposed to tolbutamide. Pancreases were perfused as described in METHODS, but perfusion medium contained 100 μM tolbutamide from 0 to 40 min. Linogliride (300 μM) was present from 20 to 40 min. Values are means ± SE of hormone release observed in 3 perfusions each. There was no statistically significant difference between test and control data when compared at same time point; likewise, no data points during test phase differed significantly from hormone release from test pancreases at 19-min time point ($P > 0.05$ by Student's *t* test).

min without fuel, 1.4 ± 0.4 ng/20 min with 5 mM glucose, 1.9 ± 0.2 ng/20 min in the presence of both 4 mM amino acids and 5 mM glucose). However, these data did not allow us to obtain an estimate of the affinity constant $K_{0.5}$ for the effects of linogliride on somatostatin release.

To compare the mechanisms of action of tolbutamide and linogliride, pancreases were perfused with tolbutamide throughout the experiment and then stimulated with linogliride once the effects of tolbutamide on hormone release had worn off. Exposure to 100 μM tolbutamide desensitized the pancreas to linogliride in that linogliride no longer stimulated insulin release (Fig. 8). Data from similar perfusions with both tolbutamide and linogliride were again used to construct concentration-response curves. For the different fuels, these data are shown in Figs. 4–7 (□). Because the hormone release data showed considerable scatter, n was always set to 1 in Eq. 1. We also performed the converse experiments; i.e., we perfused pancreases in the continuous presence of linogliride (300 μM) and then stimulated them with tolbuta-

TABLE 1
Fuel dependence of effect of linogliride on pancreatic hormone release

	Peak hormone release			Integrated hormone release		
Perfusate fuel	$K_{0.5}$ (μM)	n	Maximal secretory effect (ng/min)	$K_{0.5}$ (μM)	n	Maximal secretory effect (ng/20 min)
Insulin release						
None	216 (186)	0.96	176	32 (30)	0.93	470
Amino acids (4 mM)	170 (183)	1.13	300	43 (77)	1.35	853
Glucose (5 mM)	76 (108)	1.42	39			
Amino acids (4 mM) + glucose (5 mM)	33 (40)	1.75	81	26 (26)	1.68	1252
Glucagon release						
None	(16)*		−2*	11 (12)	2.47	−34
	(8)†		−2†			
Amino acids (4 mM)	(10)*		−26*	8 (9)	0.71	−473
	(3)†		−24†			

Pancreases were perfused in the presence of the fuel indicated. Table provides summary of potency of linogliride taken from concentration-response curves in Figs. 4–7. Peak hormone release was measured against release observed at the 19-min time point within same set of perfusions. Integrated hormone release refers to baseline-corrected release during 20-min exposure to linogliride. $K_{0.5}$, half-maximally effective concentration of linogliride; values in parentheses are for fittings obtained with the slope factor n in Eq. 1 set to 1 (see METHODS). Maximal secretion refers to the maximal effect of linogliride as derived from fitted curves (i.e., *b* in Eq. 1); −, inhibitory effect.
*Measured at the 30-min time point.
†Measured at the 40-min time point.

mide (100 μM). Although tolbutamide still transiently, although not statistically significantly, stimulated insulin release when linogliride was used at the low concentration of 30 μM, tolbutamide had no effect at 100–300 μM linogliride (data not shown). Likewise, tolbutamide failed to have an effect on glucagon and somatostatin release in linogliride-preperfused pancreases.

We compared the secretory effects of linogliride with those of tolbutamide. To determine the effect of tolbutamide, insulin release was integrated during the first 20 min of perfusion (thereby using the 19-min value also for the 20-min time point); the net effect was taken as the difference between hormone release in the presence and absence of 100 μM tolbutamide (see Figs. 1–3 and 8 for representative examples; $n \geq 14$ in each perfusion series). Hormone release induced by linogliride was calculated from data similar to those in Figs. 1–3 and as follows: the release observed between the 20- and 40-min time points was corrected for baseline hormone release between the 5- and 19-min time points, thereby correcting for the trend in release seen between these two integrals in control perfusions without linogliride. Tolbutamide (100 μM) and linogliride (300 μM, $n = 3$, except in the absence of fuel [$n = 2$]) had qualitatively essentially the same effects on hormone release, except that linogliride did not stimulate insulin release in the presence of 5 mM glucose (Fig. 9).

DISCUSSION

Linogliride stimulated insulin release from the in vitro perfused rat pancreas. Linogliride had the same effect as glucose in that it stimulated insulin release both in the absence of any fuel and in the presence of a physiological mixture of amino acids, whether in the presence or absence of glucose. Yet linogliride only moderately enhanced insulin release if only 5 mM glucose was present. Zawalich et al. (2) in contrast to our finding reported that linogliride (100 μM) stimulated insulin release from perifused isolated islets in the presence but not absence of 5.5 mM glucose. However, they perifused the islets with 2.8 mM glucose and then switched to 0 or 5.5 mM glucose at the same time as they introduced linogliride.

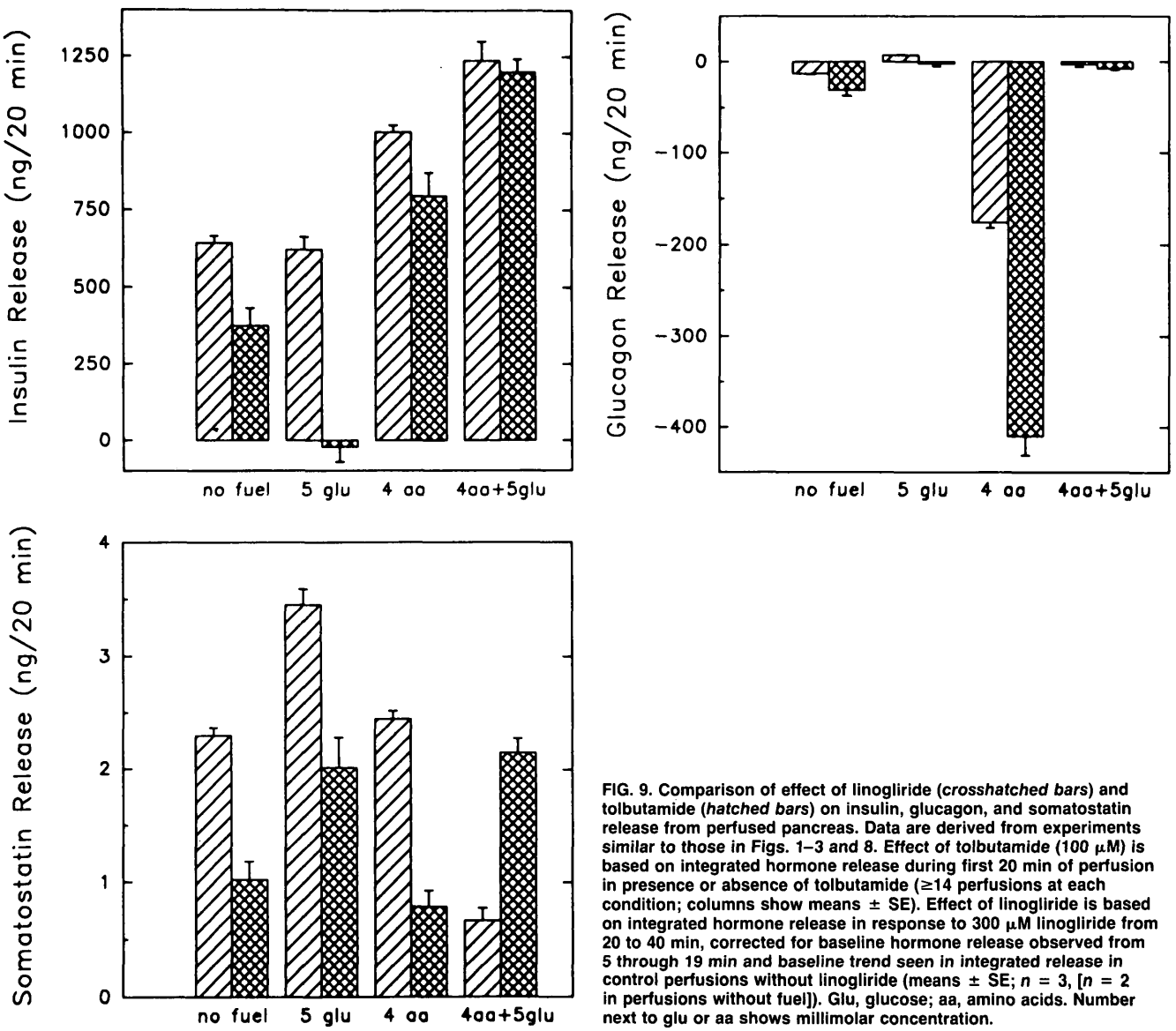

FIG. 9. Comparison of effect of linogliride (*crosshatched bars*) and tolbutamide (*hatched bars*) on insulin, glucagon, and somatostatin release from perfused pancreas. Data are derived from experiments similar to those in Figs. 1–3 and 8. Effect of tolbutamide (100 μM) is based on integrated hormone release during first 20 min of perfusion in presence or absence of tolbutamide (≥14 perfusions at each condition; columns show means ± SE). Effect of linogliride is based on integrated hormone release in response to 300 μM linogliride from 20 to 40 min, corrected for baseline hormone release observed from 5 through 19 min and baseline trend seen in integrated release in control perfusions without linogliride (means ± SE; $n = 3$, [$n = 2$ in perfusions without fuel]). Glu, glucose; aa, amino acids. Number next to glu or aa shows millimolar concentration.

Zawalich et al. (3) also claim that pirogliride, a congener of linogliride, has no effect on insulin release in the absence of glucose, although they do not present specific data.

Linogliride strongly inhibited amino acid–induced glucagon release in the absence of glucose. The half-maximally effective concentration (8 μM) was only one fifth that necessary for half-maximal stimulation of insulin release under the same conditions. Likewise, linogliride half-maximally inhibited glucagon release in the absence of any fuels at one third the concentration needed for half-maximal stimulation of insulin release. Whenever 5 mM glucose was present, glucagon release was very small (i.e., <3.1 ng/min) and not appreciably changed by linogliride. Our in vitro experiments suggest that glucagon release plays no role in the acute hypoglycemic effects of linogliride in vivo, provided blood glucose levels are normal or elevated; however, linogliride is expected to prevent glucagon release in vivo during periods of hypoglycemia.

Linogliride and tolbutamide appear to have similar effects on hormone release from the in vitro perfused rat pancreas (Fig. 9), except that linogliride was markedly ineffective in stimulating insulin release in the presence of 5 mM glucose. Although tolbutamide effects were determined only during the first 20 min and linogliride effects during the second 20 min of in vitro perfusion, the difference in timing is unlikely to affect this result qualitatively. Interestingly, similar mechanisms of action of linogliride and tolbutamide had previously been apparent from a study in diabetic patients (5).

Linogliride and tolbutamide may act on the same target. Thus, in pancreases that were preperfused with tolbutamide, the effects of linogliride were abolished or greatly reduced (Figs. 4–8); the same was true when pancreases were perfused with linogliride and then stimulated with tolbutamide (data not shown). The common target is in all likelihood an ATP-sensitive K^+ channel or a protein that regulates channel activity. As demonstrated by Ronner et al. (this issue, p. 885), linogliride indeed inhibits ATP-sensitive K^+ currents in rat pancreatic β-cells.

The fact that linogliride, like sulfonylureas, has similar effects to glucose, poses the question of whether these drugs do not interfere with a recognition site for the signal that emanates from the glucose-sensing mechanism.

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