Stimulatory Effects of Cholecystokinin on Isolated Perifused Islets Inhibited by L 364718 Potent and Specific Antagonist

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The influence of L 364718 on islet responsiveness to sulfated cholecystokinin (CCK-8S) was investigated. In islets whose inositol-containing phospholipids were prelabeled during a 2-h incubation period, subsequent exposure to L 364718 (1 nM) significantly impaired the secretion of insulin usually noted in response to 200 nM CCK-8S in the simultaneous presence of 7 mM glucose. A higher level of the antagonist (10 nM) completely abolished insulin secretion. L 364718 (1–10 nM) reduced the efflux of ^{3}H from *myo*-[2-^{3}H]-inositol prelabeled islets in parallel with the reduction in secretion. L 364718 (10 nM) significantly reduced the accumulation of ^{3}H-containing inositol phosphates usually noted with CCK-8S addition. L 364718, at levels 10- to 100-fold greater than those necessary to attenuate CCK-8S–induced insulin secretion, had no adverse effect on the insulin secretory response of freshly isolated islets to 10 mM glucose alone, 5 mM D-glyceraldehyde, 15 mM α-ketoisocaproate, or 50 ng/ml gastric inhibitory polypeptide. L 364718 (1000 nM) had no adverse influence on carbamylcholine (1 mM)–induced phosphoinositide hydrolysis. These results establish L 364718 as a potent and highly selective antagonist of cholecystokinin's stimulatory actions on β-cells. Because of its potency, selectivity, and oral effectiveness, in vivo studies with L 364718, aimed at unraveling the pleiotropic effects of CCK-8S on glucose and insulin homeostasis, seem feasible.
***Diabetes* 137:1432–37, 1988**

A major problem in precisely defining the in vivo effects of sulfated cholecystokinin (CCK-8S, the COOH-terminal 8 amino acid derivative, fragment 26–33 amide sulfated on the tyrosine residue) has been the unavailability of potent and specific CCK antagonists. Competitive antagonists of CCK binding to its membrane receptor have been described (1–3), and these compounds inhibit the actions of CCK. Because of the high levels necessary to demonstrate these inhibitory effects, however, in vivo studies have not proved feasible. Recently, several more potent competitive receptor antagonists of this peptide have been described (4–6). In particular, a nonpeptide derivative of benzodiazepine, L 364718, has been shown to be effective at picomolar levels in competitively blocking the binding of CCK to its membrane receptors (5). Because of the possible future deployment of this potent, competitive antagonist of CCK-receptor binding in defining the in vivo effects of CCK on glucose and insulin homeostasis, experiments were designed to determine the in vitro impact of this drug on collagenase-isolated islets. Herein we demonstrate that L 364718 antagonizes the stimulatory effect of CCK-8S on islet tissue. The inhibitory effect is rapid, highly specific, and reversible. As such, this drug may prove useful in delineating the in vivo effects of CCK-8S on glucose and insulin homeostasis.

MATERIALS AND METHODS

Male Sprague-Dawley rats purchased from Charles River were used in all studies. The animals were fed ad lib and weighed 300–400 g at death. After nembutal (50 mg/kg)–induced anesthesia, islets were isolated by collagenase digestion (7). Some groups of islets were then directly perifused to establish secretory responsiveness to various agonists. In other experiments, batches of 40–70 islets were loaded onto nylon filters and placed in small glass vials. They were incubated for 2 h in 200 μl of a *myo*-[2-^{3}H]inositol–containing solution prepared by adding 10 μCi *myo*-[2-^{3}H]inositol (initial sp act 16.6–19.0 Ci/mmol) to 250 μl of incubation medium to label their inositol-containing phospholipids. The medium used for this incubation procedure was similar to that employed during the islet perifusion and consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 24 mM $NaHCO_3$, and 0.17 g/dl bovine serum albumin. The solution was gassed with 95% O_2/5% CO_2. Glucose

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Received for publication 22 February 1987 and accepted in revised form 9 May 1988.

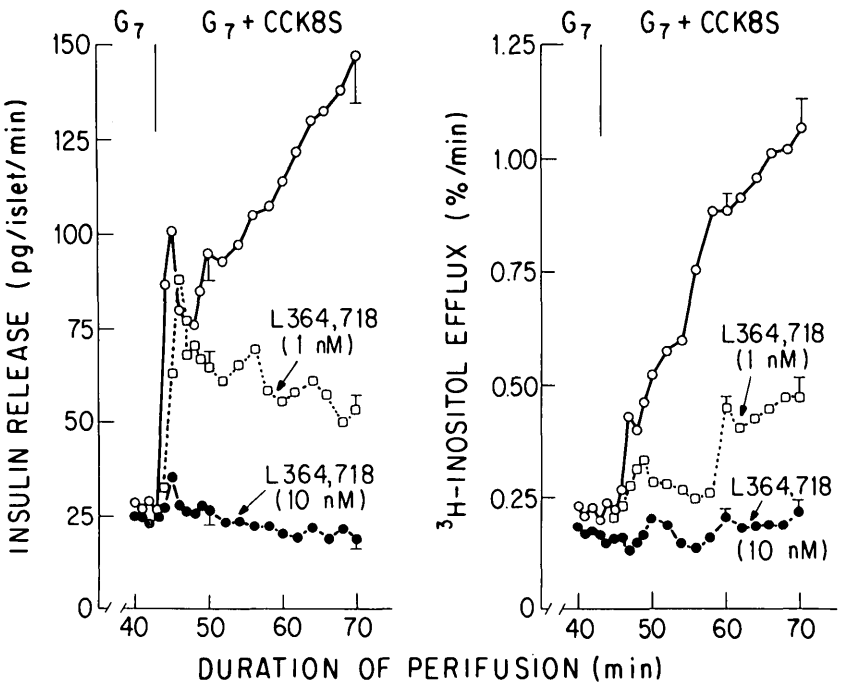

FIG. 1. L 364718 attenuates CCK-8S–induced insulin output and ^3H efflux from prelabeled islets. After 2-h period in *myo*-[2-^3H]inositol to label islet inositol-containing lipids, batches of islets were perifused to simultaneously assess insulin secretory responsiveness (*left*) and ^3H efflux (*right*) to 200 nM CCK-8S in presence or absence of various concentrations of L 364718. Fractional rate of [^3H]inositol efflux (%/min) was calculated as described previously (7a). In these studies, inhibitor was included in perifusion medium for 10 min before addition of CCK-8S. Mean values ± selected SEs for various time points are given. Figure has been corrected for dead space in perifusion system (~2.5 ml; 2.5 min with flow rate of 1 ml/min). ○, Controls. $n \geq 4$ experiments for each condition.

(2.75 mM) was also present during the incubation. We did not find it necessary to incubate islets in a higher glucose level to subsequently demonstrate agonist-induced phosphoinositide (PI) hydrolysis. After termination of the incubation, the islets, still attached to the nylon filters, were washed with 5 ml nonradioactive medium. Some of these islets were then perifused to assess ^3H efflux (7a), inositol phosphate accumulation, and insulin secretion in response to various treatments. Others were statically incubated and subsequently analyzed for the accumulation of labeled inositol phosphates under various conditions (8–10). Briefly, after neutralization with 0.25–0.28 ml 6 N KOH, the further addition of 5 ml water, and centrifugation, the supernatant was applied to columns. These columns were prepared by adding anion-exchange resin (AG1-X8, formate form, Bio-Rad, Richmond, CA) to Pasteur pipettes (to achieve a length of 3 cm). Further additions to the column included 10 ml water and 5 ml 5 mM Borax/60 mM sodium formate. Elution of the inositol phosphates was accomplished by the sequential addition of 10 ml 0.1 M formic acid/0.2 M ammonium formate (inositol 1-phosphate; IP_1), 0.1 M formic acid/0.4 M ammonium formate (inositol 1,4-bisphosphate; IP_2), and 0.1 M formic acid/1 M ammonium formate (inositol 1,4,5-trisphosphate plus inositol 1,3-4-trisphosphate; IP_3). Aliquots (0.4 ml) of the eluate were then analyzed for radioactive content.

For the perifusion studies, the pH of the medium was maintained at 7.4, the temperature at 37°C, and the flow at 1 ml/min. Islets were usually perifused for 30–40 min to establish stable insulin secretory rates and for an additional 30–40 min after the addition of a particular agonist. Perifusate samples were collected at time intervals indicated in the figures and 200-μl aliquots analyzed for ^3H content when appropriate and insulin (11) using rat insulin (615-D63-12-3, Lilly, Indianapolis, IN) as standard. In the [^3H]inositol experiments, cellular content of radioisotope after the perifusion was also determined. In those studies involving

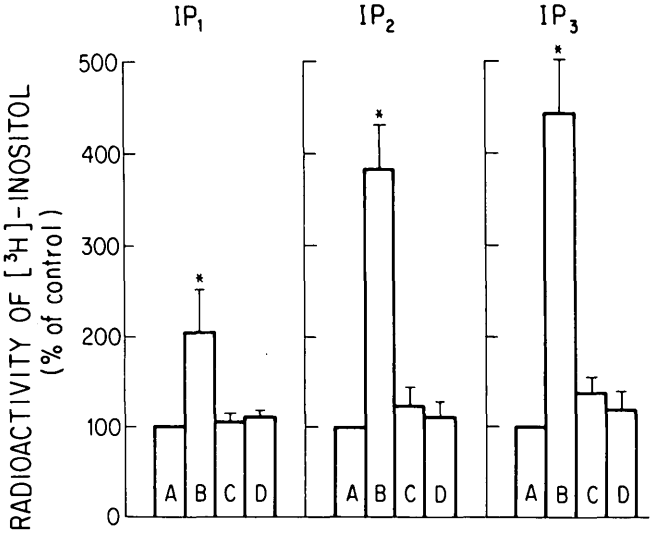

FIG. 2. L 364718 (10 nM) reduces labeled inositol phosphate accumulation in response to CCK-8S. After 2-h preincubation period with *myo*-[2-^3H]inositol to label their inositol-containing phospholipids, islets were washed with 5 ml of nonradioactive medium containing 10 mM lithium chloride. After 10-min stabilization period in 200 μl of 7 mM glucose plus lithium chloride, islets were incubated for 10 min with 7 mM glucose (*A*), 7 mM glucose plus 200 nM CCK-8S (*B*), 7 mM glucose plus 200 nM CCK-8S plus 10 nM L 364718 (*C*), or 7 mM glucose plus 10 nM L 364718 (*D*). CCK antagonist was present for 10 min before CCK-8S addition. $n \geq 4$ for each condition. IP_1, inositol 1-phosphate; IP_2, inositol 1,4-bisphosphate; and IP_3, inositol 1,4,5-trisphosphate plus inositol 1,3,4-trisphosphate. $P < .05$ vs. control response noted with 7 mM glucose alone. Control values averaged (mean ± SE): IP_1, 2056 ± 108 cpm/40 islets; IP_2, 285 ± 41; and IP_3, 128 ± 13.

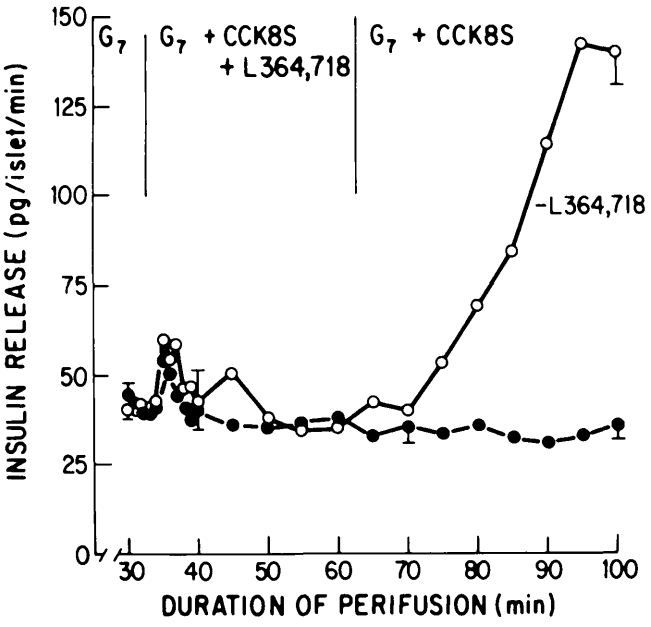

FIG. 3. Reversible nature of L 364718 inhibition. Freshly isolated islets were perifused for 30 min with 7 mM glucose. L 364718 (10 nM) was present during final 10 min. For next 30 min, both groups of islets were stimulated with 200 nM CCK-8S in continued presence of inhibitor. After this, 1 group was maintained for additional 40 min with CCK-8S minus inhibitor. $n \geq 3$ experiments for each condition. Note that, whereas removal of L 364718 was accompanied by significant elevation in insulin output, release rates subsequently measured were lower than those obtained if islets were not exposed to L 364718. For example, islets perifused for 60 min with 7 mM glucose alone before CCK-8S exposure (200 nM), released insulin at rates of 251 ± 17 pg · islet^{-1} · min^{-1} after 35–40 min of stimulation (n = 4, results not shown). Value for L 364718-treated islets at this time is ~125 pg · islet^{-1} · min^{-1}, significantly ($P < .05$) different. Figure has been corrected for dead space in perifusion system (~2.5 ml; 2.5 min with flow rate of 1 ml/min).

L 364718, new perifusion tubing was employed for each experiment. The radioisotope used to measure insulin release (^{125}I-labeled insulin) was purchased from New England Nuclear (Boston, MA) and the *myo*-[2-^{3}H]inositol from Amersham (Arlington Heights, IL). CCK-8S, (fragment 26–33 amide, sulfated on the tyrosine residue, lot 124F-0445), carbamylcholine chloride, D-glyceraldehyde, and α-ketoisocaproate (KIC; sodium salt) were purchased from Sigma (St. Louis, MO). Gastric inhibitory polypeptide (GIP) was purchased from Peninsular (Belmont, CA). L 364718 (3*S*-(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide) was the generous gift of V. Lotti (Merck, Sharp and Dohme, West Point, PA). It was dissolved in DMSO before use. Similar levels of DMSO (never >0.1%) were added to the medium in the control experiments.

Statistics. Where appropriate, statistical significance was determined with analysis of variance in conjunction with the Newman-Keuls multiple comparison test and $P < .05$ taken as signifcant. Values presented in the figures represent means ± SE of the specified number of observations.

RESULTS

In the simultaneous presence of 7 mM glucose, 200 nM CCK-8S induced a rapid and biphasic pattern of insulin output from islets previously incubated for 2 h to label their inositol-containing lipids. This insulin stimulatory effect was attenuated by L 364718 in a dose-dependent fashion. In these experiments, the drug was present 10 min before CCK-8S addition and during the 30-min stimulatory period with the polypeptide (Fig. 1). Under these experimental conditions, 0.1 nM L 364718 was without any significant inhibitory effect (results not shown), 1 nM L 364718 had a moderate but significant inhibitory effect, and secretion was virtually abolished with 10 nM of the drug. Note that the process of incubating isolated islets for 2 h to prelabel their inositol-containing phospholipids was accompanied by a reduced secretory response to CCK-8S in the presence of 7 mM glucose. For example, in response to 100 or 200 nM CCK-8S (approximately equipotent stimulant levels), second-phase release rates after 30 min of stimulation averaged ~250–300 pg · islet^{-1} · min^{-1} with freshly isolated islets (Fig. 1; 11). In contrast, after 2 h of incubation, the value at this time averaged ~140–150 pg · islet^{-1} · min^{-1}. Basal insulin release rates after 30 or 40 min with 7 mM glucose alone were also reduced by this preincubation period. Consistent with its inhibitory effect on CCK-8S–induced insulin output, L 364718 also significantly reduced, in a dose-dependent fashion, the CCK-8S–induced increases in [^{3}H]inositol efflux from prelabeled islets (Fig. 1). The highest level (10 nM) of

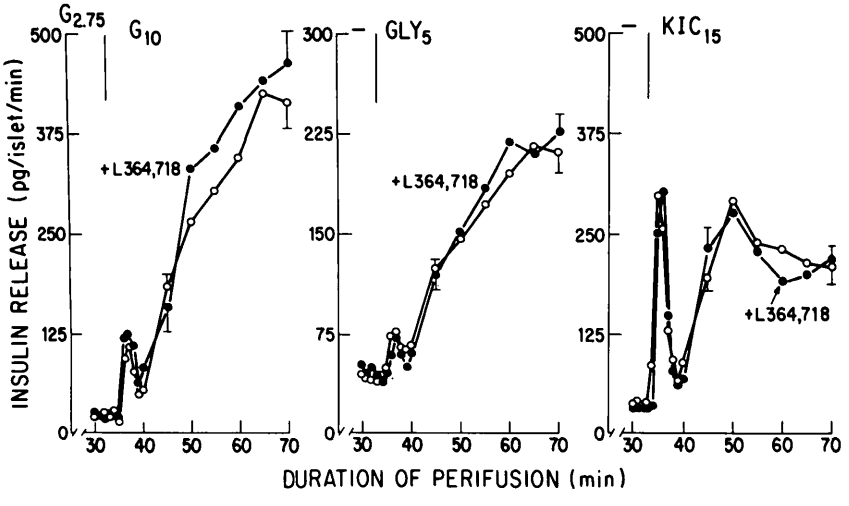

FIG. 4. L 364718 does not adversely affect glucose-, glyceraldehyde-, or α-ketoisocaproate–induced release. Freshly isolated islets were stimulated with 10 mM glucose, 5 mM glyceraldehyde, or 15 mM α-ketoisocaproate ± 1000 nM L 364718. CCK-8S antagonist was present during 10-min period before stimulant addition. L 364718 at this exorbitant level has no effect on the secretory response evoked by these secretagogues ($n \geq 3$ for each condition). Figure has been corrected for dead space in perifusion system (~2.5 ml; 2.5 min with flow rate of 1 ml/min).

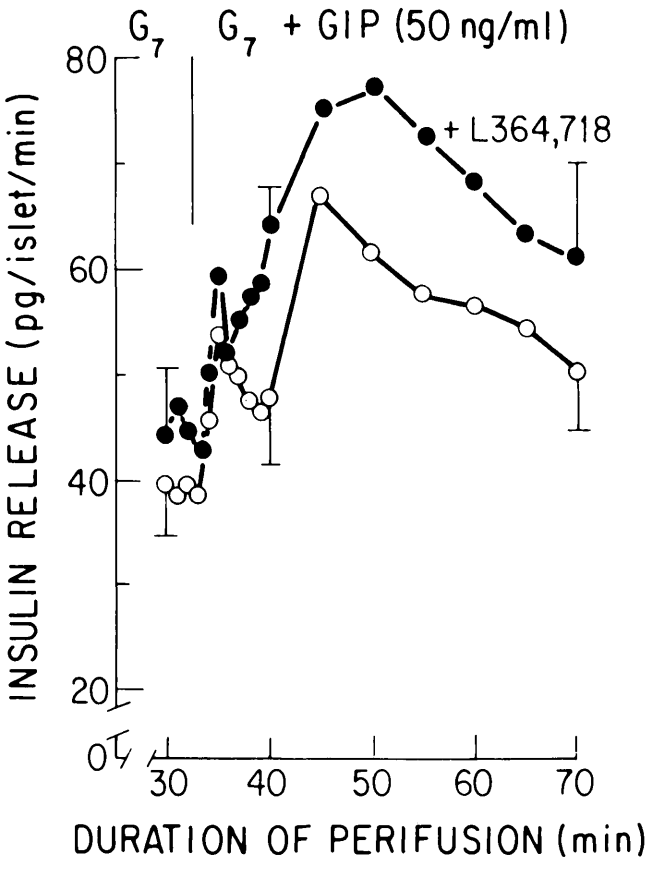

FIG. 5. L 364718 does not influence gastric inhibitory peptide (GIP)-induced insulin output. Freshly isolated islets were perifused for 30 min to establish basal insulin secretory rates, then stimulated for 40 min with 50 ng/ml GIP ± 100 nM L 364718 (n = 3 for each condition). The inhibitor was present during final 10 min with 7 mM glucose alone. Figure has been corrected for dead space in perifusion system (~2.5 ml; 2.5 min with flow rate of 1 ml/min).

L 364718 used in these studies significantly reduced the increase in ^3H efflux usually noted with CCK-8S addition.

Whereas the efflux of ^3H from prelabeled islets represented a convenient and dynamic, albeit indirect, method to monitor PI hydrolysis (9,10), the impact of CCK-8S on inositol-containing phospholipid hydrolysis was directly measured (Fig. 2). CCK-8S (200 nM) increased the amounts of all [^3H]inositol-containing phosphates measured during a static incubation, an effect that was significantly attenuated by 10 nM L 364718. L 364718 had no adverse impact on the accumulation of these phosphates observed with 7 mM glucose alone.

The issue of reversibility of L 364718 action was next addressed (Fig. 3). In these studies, freshly isolated islets were perifused for 30 min with 7 mM glucose. L 364718 (10 nM) was present during the final 10 min. The islets were then stimulated with 200 nM CCK-8S in the continued presence of the inhibitor. When compared to control islets, the insulin response was reduced. Removal of the inhibitor was accompanied by a delayed, but significant, increase in the insulin secretory response. This response, however, was less than that observed from control islets not exposed to L 364718 (Fig. 3).

Experiments were next designed to assess the specificity of L 364718. Freshly isolated islets were stimulated with 10 mM glucose alone, 5 mM D-glyceraldehyde or 15 mM KIC in the presence or absence of 1000 nM L 364718, a level 100-fold greater than that necessary to abolish the CCK-8S stimulatory effect (Fig. 3). This exorbitant level of the inhibitory compound had no adverse impact on the secretory response evoked by these diverse compounds (Fig. 4). Similarly, 10–100 nM L 364718 was also without any inhibitory effect on these stimulants (results not shown). In addition, L 364718 at 100 nM (Fig. 5) or 1000 nM (results not shown) had no adverse effect on the small insulin secretory response to 50 ng/ml GIP.

The possible influence of L 364718 (1000 nM) on carbamylcholine-induced PI hydrolysis was also examined. This cholinergic agonist resulted in a dramatic increase in ^3H efflux from [^3H]inositol-prelabeled islets and parallel increases in the levels of labeled inositol phosphates (Fig. 6). For example, in the presence of 2.75 mM glucose alone, levels of IP_1 in islets averaged 388 ± 25 cpm/40 islets (mean ± SE, n = 10). IP_2 and IP_3 averaged 101 ± 11 and 74 ± 6 cpm/40 islets, respectively. After 30 min in the presence of 1 mM carbamylcholine (in the continued presence

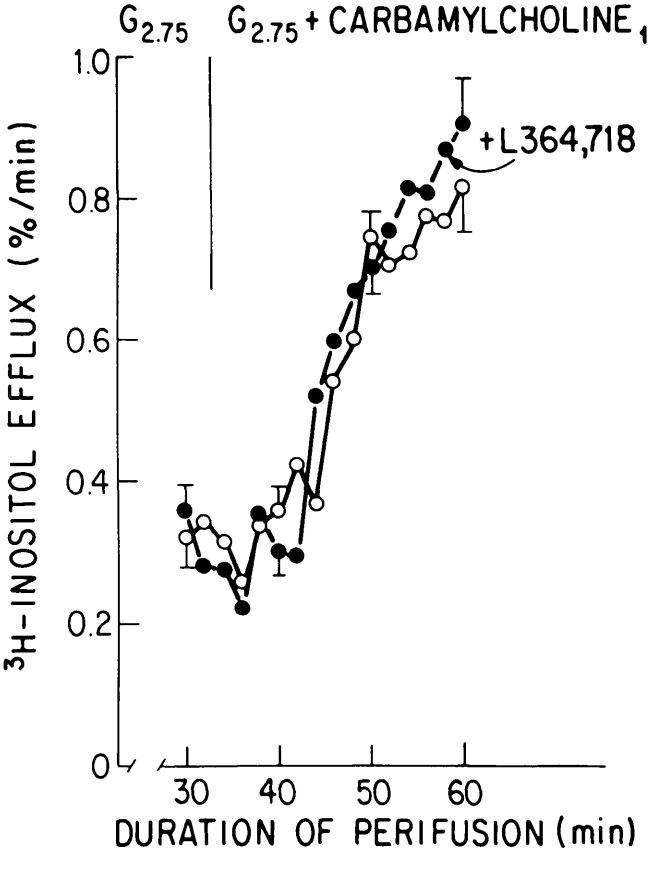

FIG. 6. L 364718 does not influence carbamylcholine-induced increases in [^3H]inositol efflux. Groups of islets (n = 4 for each condition) were incubated for 2 h with *myo*-[2-^3H]inositol to label their inositol-containing lipids, washed with 5 ml of fresh medium, and then perifused. After 30-min stabilization period with 2.75 mM glucose, both groups were stimulated for 30 min with 1 mM carbamylcholine chloride ± further addition of 1000 nM L 364718. This compound was included in perifusion medium 10 min before carbamylcholine addition. After perifusion, levels of labeled inositol phosphates in these islets were measured (see RESULTS). Figure has been corrected for dead space in perifusion system (~2.5 ml; 2.5 min with flow rate of 1 ml/min).

of 2.75 mM glucose), levels of IP_1, IP_2, and IP_3 averaged 783 ± 59, 273 ± 36, and 129 ± 7 cpm/40 islets, respectively (n = 4 for each measurement). All of these values were significantly different from those obtained in the presence of 2.75 mM glucose alone. In agreement with the ^3H-efflux data, L 364718 (1000 nM) did not influence this inositol phosphate response pattern to carbamylcholine. IP_1, IP_2, and IP_3 levels then averaged 758 ± 46, 258 ± 30, and 119 ± 12 cpm/40 islets, respectively (n = 4 for each measurement) when measured 30 min after exposure to carbamylcholine. Despite its stimulatory action on PI hydrolysis, carbamylcholine, with or without L 364718, had no stimulatory effect on insulin secretion (results not shown).

DISCUSSION

A major problem in precisely defining the possible in vivo contribution of CCK to insulin-mediated fuel homeostasis has been the lack of specific and potent antagonists of the peptide. Recently, however, the development of two highly specific competitive antagonists of CCK binding to its membrane receptor has been described (4–6). We recently reported that asperlicin, a fungal derivative, inhibited the direct stimulatory effects of CCK-8S on isolated perifused islets (12). At levels of 10–25 μM, this compound abolished to a large extent the stimulatory effects of CCK-8S on both insulin release and ^3H efflux from [2-^3H]inositol-prelabeled islets. Similar studies on L 364718, a nonpeptide competitive CCK-receptor antagonist, on islet tissue are presented here and warrant further comment.

First, L 364718 inhibits in a parallel fashion both the increase in insulin release normally noted with 200 nM CCK-8S in the presence of 7 mM glucose, the increase in ^3H efflux from prelabeled and subsequently perifused islets, and the accumulation of labeled inositol phosphates. The increase in membrane PI hydrolysis that accompanies CCK-8S addition to islets appears to account, to a large extent, for the subsequent insulin secretory response (9,12). Conditions that reduce CCK-induced PI hydrolysis also abolish its insulin stimulatory effect. Presumably by interacting with receptors in the β-cell membrane (13,14), CCK-8S induces the activation of phospholipase C, the subsequent hydrolysis of membrane PIs, and the accumulation of labeled inositol phosphates (9,12,15). Similar changes in inositol phosphate levels also accompany both glucose and cholinergic stimulation of islets and insulin-containing tumoral RIN cells (15–19). It is not clear how the inositol phosphates generated contribute to the insulin secretory response, although a role for inositol 1,4,5-trisphosphate in intracellular calcium mobilization has been suggested (20). The lipid-soluble diacylglycerol (DG), presumably generated stoichiometrically with inositol phosphates, appears to participate in the activation of the calcium and phospholipid-dependent kinase, termed protein kinase C (21). That the metabolic products of DG also contribute to β-cell activation also has to be considered, although events distal to DG generation remain for the most part undefined in islets. When compared to asperlicin, L 364718 appears to be 1000–10,000 times more effective in antagonizing the insulin stimulatory effects of 100–200 nM CCK-8S on perifused islets (12).

L 364718 exhibits a remarkable degree of inhibitory selectivity. At levels 10- to 100-fold greater than those necessary to abolish the insulin stimulatory effect of 200 nM CCK-8S (with 7 mM glucose) on islets, this compound is without any influence on release provoked by 10 mM glucose alone, 5 mM glyceraldehyde, 15 mM KIC, or 50 ng/ml GIP (Figs. 4 and 5). Lower levels of L 364718 were also without any effect on secretion evoked by these chemically disparate stimulatory compounds (results not shown). Futhermore, L 364718 (1000 nM) had no effect on the activation of PI hydrolysis in response to carbamylcholine.

The levels of CCK-8S (200 nM) used in the present experiments are considerably greater than circulating levels, suggesting perhaps that its actions noted here are more pharmacological than physiological (22). It is, however, possible to evoke insulin secretion from collagenase-isolated islets with lower CCK levels (5–10 nM) by elevating the glucose level (14,23). The exquisite glucose dependency of CCK-induced insulin secretion has been previously noted (24). Collagenase exposure may, in large part, be responsible for this observed alteration in CCK-8S sensitivity. A similar reduction in responsiveness of collagenase islets to various agents, including peptide hormones, has been previously documented (25,26). Furthermore, the physiological involvement of CCK in meal-induced insulin secretion in unstressed rats has been emphasized in a recent report (27). Finally, recent human studies also support an incretin role for CCK in amino acid–stimulated insulin secretion (28).

Note that even though CCK-8S induces a glucose-independent increase in PI hydrolysis (9), it amplifies insulin secretion only in the presence of moderate glycemia. Consequently, an increase in PI hydrolysis is an event insufficient in and of itself to significantly increase insulin output. A similar situation exists with carbamylcholine. A logical question concerns the possible physiologic role of this increase in PI hydrolysis that is not accompanied by any appreciable increase in insulin output. We recently suggested that CCK-8S–induced PI turnover in islets may represent an important futile cycle of sorts (29). Increased rates of PI hydrolysis induced by a variety of agonists, including CCK-8S, persist long after stimulant removal from the medium and induce a heightened state of secretory responsiveness to glucose (10,29–31). The persistent increase in PI hydrolysis in response to brief CCK exposure might participate in the incretin effect of peptide.

Another interesting facet of CCK-8S action on the β-cell is the dramatic synergistic interaction between it and GIP. This latter peptide, also postulated to be an important incretin factor (32), induces a glucose-dependent increase in insulin secretion, an effect thought to be mediated by its ability to generate cAMP (33). We have demonstrated that forskolin-induced elevations in islet cAMP levels dramatically amplified the insulin stimulatory effect of CCK-8S on secretion, an effect that occurs even in the presence of a hypoglycemic glucose level (9). Most recently we have demonstrated that in isolated perifused islets, CCK-8S and GIP interact synergistically to increase insulin output (34). We have attributed this positive interaction, at least in part, to the capacities of these separate incretins to generate disparate second-messenger molecules; GIP increases cAMP while CCK increases PI hydrolysis, with subsequent generation of many possible second-messenger molecules. If this synergy functions in vivo (and limited studies support this concept; 35),

the release of these incretin factors may represent a remarkable homeostatic mechanism to ensure the release of insulin in amounts commensurate with both the quantity and quality of food intake. Note also that this synergistic interaction between CCK-8S and GIP is abolished by L 364718 (34).

Together, these results indicate that L 364718 is a potent and specific antagonist of the stimulatory effect of CCK-8S on islet tissue. Considering the possible role of CCK-8S as both an incretin (32) and satiety factor (36), in vivo studies with this potent CCK-8S antagonist are not only feasible but most desirable. Consequently, studies to assess the precise contribution of CCK on glucose and insulin homeostasis seem a reasonable experimental objective at this time.

ACKNOWLEDGMENTS

The expert secretarial assistance of Patricia Cross and Joan Fettes is gratefully acknowledged.

These studies were supported by NIH Grant 34381 and by Grant 84-709 from the American Heart Association.

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