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Characterization of Two Novel Cholecystokinin Tetrapeptide (30–33) Analogues, A-71623 and A-70874, That Exhibit High Potency and Selectivity for Cholecystokinin-A Receptors

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SUMMARY

Based on their relative affinities for cholecystokinin octapeptide(26–33) (CCK-8), cholecystokinin tetrapeptide(30–33) (CCK-4), desulfated CCK-8, and gastrin, cholecystokinin (CCK) receptors have been classified as CCK-A (alimentary) and CCK-B (brain). Selective nonpeptide antagonists of CCK-A and CCK-B receptors, as well as highly selective CCK-A and CCK-B peptide agonists, have been described. We report here the characterization of two novel CCK-4-based peptides, A-71623 and A-70874. In radioligand binding assays, the IC_{50} values for A-71623 and A-70874 were 3.7 and 4.9 nM in guinea pig pancreas (CCK-A) and 4500 and 710 nM in cerebral cortex (CCK-B), respectively. Both were agonists in stimulating pancreatic amylase release, and their stimulatory effects were potently inhibited by the CCK-A antagonist L-364,718. A-71623 was a full agonist and A-70874 was a partial agonist (~80%) in stimulating phosphoinositide breakdown in pancreas. Both peptides also were potent agonists in stimulating CCK-A receptors in the ileum. They were, however, weak and behaved as partial agonists in calcium studies in NCI-H345 cells, which possess CCK-B/gastrin receptors. In guinea pig gastric glands, the affinities of A-71623 and A-70874 for the CCK-B/gastrin receptor were 11 and 1.6 μM, respectively. These results demonstrate that A-71623 and A-70874 are potent and selective agonists at CCK-A receptors. The preferential interaction of these novel CCK-4 analogs with CCK-A receptors is in contrast to other CCK-4-based peptides, which are primarily selective for CCK-B receptors. In addition, A-71623 and A-70874 are the first two examples of potent CCK-A agonists that do not contain a tyrosine residue whose sulfation is required for potent CCK-A agonist activity of larger peptides.

CCK is a brain-gut peptide that mediates many important biological functions in the periphery and brain (1, 2). In the periphery, it has been shown to be involved in pancreatic enzyme release, gall bladder contraction, intestinal motility, gastric emptying, and satiety. In brain, CCK has been implicated as a neurotransmitter or neuromodulator, and studies have indicated that CCK plays modulatory roles in dopamine function, memory, opiate analgesia, anxiety, and satiety. Based on their relative affinities for the CCK-8, CCK-4, gastrin, and the desulfated form of CCK-8, CCK receptors have been classified into two classes, CCK-A and CCK-B (3, 4). CCK-A (alimentary) receptors are located primarily in the periphery. They recognize CCK-8 with high affinity and possess low affinities for CCK-4, gastrin, and the desulfated form of CCK-8. In contrast, CCK-B (brain) receptors are located predominately in CNS tissues such as the cortex and hippocampus. They are less discriminating than CCK-A receptors and bind CCK-8, CCK-4, desulfated CCK-8, and gastrin with high affinity.

In recent years, much progress has been made in the development of selective CCK ligands. Potent and specific nonpeptide antagonists for CCK-A and CCK-B receptors, as well as specific peptide agonists for the CCK-B receptors, have been reported (5–9). Recently, we described the characterization of a CCK-7 analog, A-71378, that is potent and selective for CCK-A receptors (10). These developments have facilitated our understanding of the physiological significance of multiple CCK receptors. We report here the development and characterization of a new class of potent and selective CCK-A agonists, based on the replacement of Met^{31} in CCK-4 with a substituted lysine. The two most potent compounds are A-71623 and A-70874 (see Fig. 1 for structures). Both are devoid of the tyrosine residue of CCK, whose sulfation is required for potent CCK-A activity of larger peptides (1, 2). Compared with that of other CCK-4 analogs, which are CCK-B receptor selective, the selectivity of

ABBREVIATIONS: CCK, cholecystokinin; CCK-8, cholecystokinin octapeptide(26–33), Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; CCK-4, cholecystokinin tetrapeptide(30–33), Trp-Met-Asp-Phe-NH_2; PI, phosphoinositide; BH, Bolton-Hunter; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazineethanesulfonic acid; Boc, *tert*-butyloxycarbonyl; A-71623, Boc-Trp-Lys(ε-*N*-2-methylphenylaminocarbonyl)-Asp-(*N*-methyl)-Phe-NH_2; A-70874, Boc-Trp-Lys(ε-*N*-4-OH-cinnamoyl)-Asp-(*N*-methyl)Phe-NH_2; A-57282, Boc-Trp-Lys(ε-*N*-carbobenzoxy)-Asp-Phe-NH_2; CNS, central nervous system.

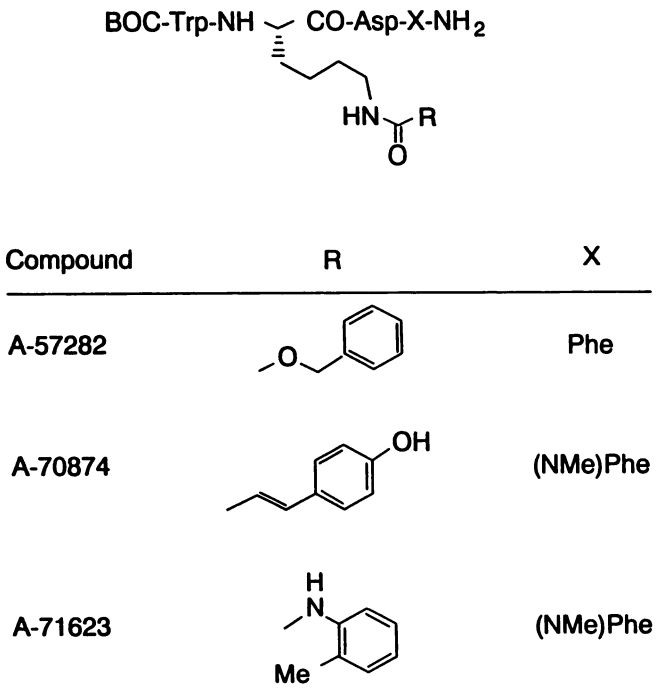

Fig. 1. Structures of A-57282, A-71623, and A-70874. *Phe*, phenylalanine; *Me*, methyl.

these two molecules for CCK-A receptors is unprecedented in the literature. These novel CCK-A agonists may provide new insights into the structural requirements for CCK-A receptor recognition and activation. A brief report describing the structure-activity relationship of these peptides has appeared (11).

Materials and Methods

CCK-8 was purchased from Cambridge Research Biochemicals (Valley Stream, NY). Phosphoramidon and bestatin were purchased from Peptide International (Louisville, KY). Heat-inactivated fetal calf serum, EGTA, HEPES, bovine serum albumin, type 1A collagenase, L-glutamine, minimum essential medium amino acids, vitamins, and bacitracin were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I-BH-CCK-8 (specific activity, 2200 Ci/mmol) and *myo*-[^{3}H]-inositol were from New England Nuclear (Boston, MA). Trichloroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee, WI). Collagenase (CLSPA) and soybean trypsin inhibitor were from Worthington Biochemical Corp. (Freehold, NJ). The acetoxymethyl ester of indo-1 was obtained from Molecular Probes (Eugene, OR). Male guinea pigs (225–300 g) were from Sasco (Omaha, NE). A-57282 and Boc-Trp-Lys(ε-*N*-carbobenzoxy)-Asp-(*N*-methyl)Phe-NH_2 were prepared by standard solution phase methods. The carbobenzoxy group in the latter compound was removed using a standard hydrogenolytic process, and the free amine was allowed to react with 2-methylphenylisocyanate, to form A-71623, or the *N*-hydroxysuccinimide ester of 4-hydroxycinnamic acid, to form A-70874. L-364,718 and (*R*)-L-365,260 were kindly supplied by Merck Sharp and Dohme Research Laboratories (Rahway, NJ). NCI-H345 cells were generously supplied by Dr. Terry W. Moody (George Washington University, Washington DC).

Radioligand binding assays. Binding assays using ^{125}I-BH-CCK-8 in guinea pig cerebral cortical and pancreatic membranes were performed as described (12, 13). Bestatin (100 μM) and phosphoramidon (3 μM) were used as protease inhibitors. The incubation time was 150 min at 30° for cortical membranes and 30 min at 37° for pancreatic membranes. The nonspecific binding, defined as binding of ^{125}I-BH-CCK-8 in the presence of 1 μM CCK-8, was 5–15% in pancreatic membranes and 10–25% in cortical membranes. Binding assays using guinea pig gastric glands and ^{125}I-BH-CCK-8 were performed as previously described (10). Bacitracin (0.025%) was used as a protease inhibitor. The nonspecific binding was 10–20% of the total binding.

Amylase release assay. Amylase secretion by guinea pig pancreatic acini was assayed as described (13, 14). Acini were incubated with peptides for 30 min at 37° in the presence of protease inhibitors (0.02% soybean trypsin inhibitor, 3 μM phosphoramidon, and 100 μM bestatin). Incubation was terminated by centrifugation. Basal amylase activity released into the medium, assayed by an Abbott VP Bichromatic analyzer, was 5–7% and, in the presence of CCK-8 (0.3 nM), 15–30%.

PI breakdown assay. PI breakdown assays using guinea pig pancreatic acini were performed as described (13, 14). Briefly, acini were incubated with [^{3}H]inositol (10 μCi/ml) for 1 hr, centrifuged, and resuspended in assay buffer containing 10 mM LiCl. Peptides were added for 30 min, and incubations were terminated by addition of methanol/chloroform. Total inositol phosphates were measured by elution from the Dowex AG1-X8 column with 1 M ammonium formate/0.1 N formic acid. The maximal stimulation by CCK-8 (100 nM) varied between 10- and 30-fold over control.

Ileal contraction assay. Segments of guinea pig ileum (~2 cm) were isolated, and isometric contractions were measured as described (15). Once isolated, ileal muscle strips were gassed with 95% O_2/5% CO_2 at 37°. After stable responses to CCK (10 nM) were obtained, the dose-response curves for peptides were generated. The maximum tension recorded for CCK-8 (100 nM) varied between 1.7 and 3.5 g.

Calcium mobilization studies in NCI-H345 cells. Procedures for studying agonist-induced changes in intracellular calcium levels in NCI-H345 cells, using the fluorescence probe indo-1, have been described (10). The basal levels of calcium were 200 ± 6 nM (nine experiments) and, following the addition of CCK-8 (1 μM), 530 ± 20 nM (eight experiments).

Results

Radioligand binding studies. The compound that served as a lead to develop the novel CCK-A-selective agonist series was A-57282 (Fig. 1). As shown in Table 1, the IC_{50} value of A-57282 in radioligand binding assays in guinea pig pancreatic membranes was 510 ± 50 nM (five experiments). In cortical membranes, its IC_{50} was 4.9 ± 1.2 μM (four experiments). Compared with Boc-CCK-4, which has an IC_{50} value of 1.8 ± 0.6 μM (five experiments) in the pancreas and 25 ± 4.5 nM (six experiments) in brain, A-57282 is 10-fold selective for pancreatic CCK-A receptors, in contrast to the type B selectivity of Boc-CCK-4. The fact that an aromatic residue on the lysine resulted in selective interaction with CCK-A receptors provided the impetus to design compounds with enhanced selectivity and potency at CCK-A receptors. Detailed structure-activity studies resulted in A-71623 and A-70874 (Fig. 1). As shown in Table 1, the IC_{50} values of A-71623 and A-70874 were 3.7 and

TABLE 1

Binding properties of CCK-8, Boc-CCK-4, A-57282, A-71623, A-70874, and A-71378 in guinea pig pancreatic and cerebral cortical membranes

Values are means ± standard errors of the number of determinations in parentheses. Each determination was done in duplicate, with <5% sample variability. IC_{50} values for each peptide were determined as the concentration of peptide that inhibited 50% of the specific binding of ^{125}I-BH-CCK-8 in each tissue.

Compound	IC_{50}		IC_{50} ratio, cortex to pancreas
	Cortex	Pancreas	
	nM		
CCK-8	8 ± 1 (52)	0.4 ± 0.04 (27)	20
Boc-CCK-4	25 ± 4.5 (6)	1800 ± 630 (5)	0.014
A-57282	4900 ± 1200 (4)	510 ± 50 (5)	9.6
A-71623	4500 ± 770 (4)	3.7 ± 0.85 (8)	1220
A-70874	710 ± 38 (5)	4.9 ± 1.6 (5)	140

4.9 nM in the pancreas and 4500 and 710 nM in the cortex, respectively. Compared with Boc-CCK-4, which was 70-fold selective at CCK-B receptors, A-71623 and A-70874 were about 1200- and 140-fold selective at CCK-A receptors, respectively. A-71623 was about 10 times less potent than CCK-8 at CCK-A receptors but was 60 times more selective than CCK-8 at CCK-A receptors.

Functional studies in the pancreas. Fig. 2A shows the efficacies of CCK-8, A-71623, A-70874, A-57282, and Boc-CCK-4 for CCK-A receptor-mediated pancreatic amylase release. Relative to CCK-8, all three new CCK-4 analogs induced the same maximal level of amylase secretion. The EC_{50} values for A-71623, A-70874, and A-57282 were 0.5 ± 0.2 nM (three experiments), 3.1 ± 0.4 nM (four experiments), and 200 ± 29 nM (five experiments), respectively. In contrast to CCK-8 and A-71623, neither A-70874 nor A-57282 exhibited inhibition of amylase release at supramaximal concentrations. In fact, A-70874 reversed the inhibition of amylase release by supramaximal concentrations of CCK-8 (Fig. 2B).

Fig. 3A shows the efficacies of CCK-8, A-71623, A-70874, A-57282, and Boc-CCK-4 in stimulating PI hydrolysis in guinea pig pancreatic acini. Compared with CCK-8, A-71623 was a full agonist in the PI assay, with an EC_{50} of 3.4 ± 0.65 nM (four experiments), but A-70874, A-57282, and Boc-CCK-4 were partial agonists, with ~80%, 40%, and 40% of the response produced by CCK-8, respectively.

Fig. 3B shows the abilities of 100 μM A-70874 and 100 μM A-57282 to inhibit the maximal response produced by 100 nM CCK-8. These results suggest that the observed PI responses for CCK-8, A-70874, and A-57282 are mediated through the same receptor.

Fig. 4 shows that the amylase-releasing properties of A-71623 (10 nM) were potently inhibited by the CCK-A antagonist L-364,718 [IC_{50} = 8.2 ± 0.8 nM (three experiments)]. Using the Cheng-Prusoff equation, the calculated K_i for L-364,718 was 0.15 ± 0.018 nM (three experiments). The stimulatory effect of A-71623 was also reversed by the CCK-B-selective antagonist L-365,260, but at much higher concentrations than those of L-364,718. The IC_{50} and calculated K_i were 3.6 ± 0.7 μM (three experiments) and 66 ± 11 nM (three experiments), respectively. In PI breakdown assays using pancreatic acini, the IC_{50} and K_i of L-364,718, competing against 3 nM A-71623, were 2.7 ± 0.7 nM (three experiments) and 1.25 ± 0.19 nM (three experiments), respectively.

Guinea pig ileal contraction assay. The efficacies of A-71623 and A-70874 were studied on the presynaptic CCK-A receptors in the ileum. Both were full agonists in eliciting ileal contractions, with EC_{50} values of 8.6 ± 1.5 nM (nine experiments) and 9.1 ± 3.6 nM (three experiments), respectively. The EC_{50} of CCK-8 was 2 ± 0.5 nM (10 experiments). This study demonstrates that, in addition to the pancreas, these two CCK-4-based agents also behave as CCK-A agonists in the ileum. In other studies, it was determined that the stimulatory effects of 10 nM A-71623 were inhibited by 40 ± 7.5% (seven experiments) and 66 ± 7% (five experiments) with 10 and 30 nM L-364,718, respectively.

Calcium studies in NCI-H345 cells. Activation of CCK-B/gastrin receptors by A-71623 and A-70874 was assessed by their ability to mobilize intracellular calcium in NCI-H345 cells. As shown in Fig. 5, both peptides possessed low potency and were partial agonists in activating calcium release. At 30 μM, A-71623 produced about 60% of the maximal response of CCK-8, whereas 30 μM A-70874 only stimulated 30% of the response produced by 1 μM CCK-8. At concentrations higher than 30 μM, A-71623 produced a higher calcium response than CCK-8 (1 μM); however, because high concentrations (10 μM) of the CCK-B-selective antagonist (*R*)-L-365,260 did not prevent the calcium response, it was concluded that the calcium signal observed at concentrations of >30 μM was not related to activation of the CCK-B/gastrin receptor. In contrast, the response produced by 30 μM was inhibited by ~90% with 300 nM (*R*)-L-365,260.

Binding studies in gastric glands. The affinities of A-71623 and A-70874 for CCK-B/gastrin receptors labeled by ^{125}I-BH-CCK-8 in guinea pig gastric glands were determined. The IC_{50} values of A-71623 and A-70874 were 11 ± 6 μM (three

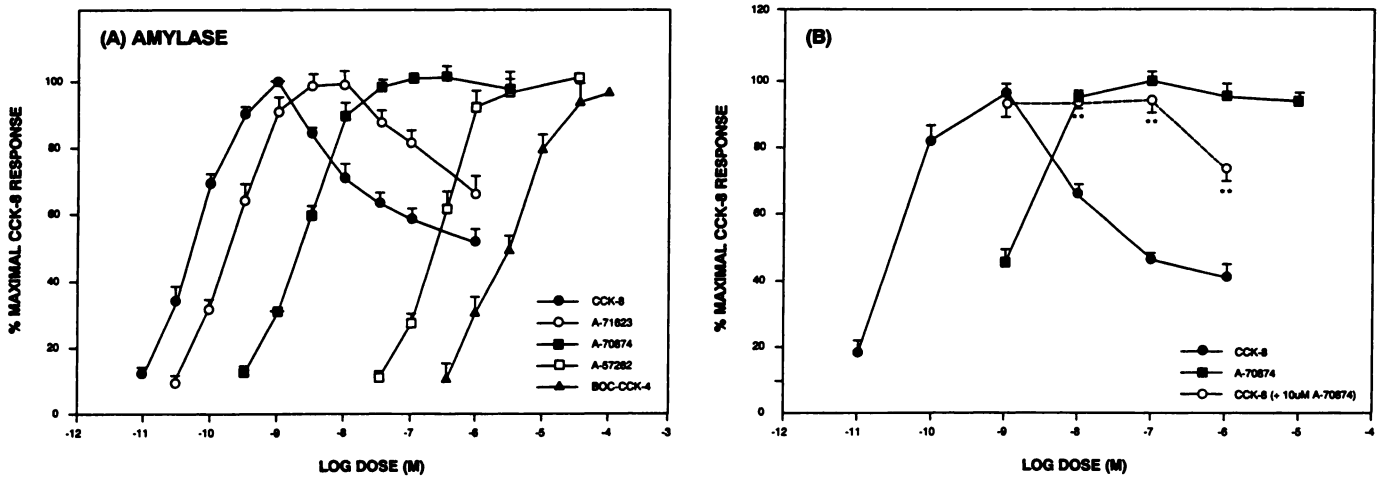

Fig. 2. A, Activation of the guinea pig pancreatic CCK-A receptors by A-71623, A-70874, and A-57282 in the amylase release assay. Protocols for measuring amylase activity were as described in Materials and Methods. Results shown are the mean ± standard error taken from at least three experiments, conducted in duplicate, with <5% sample variability. For the CCK-8 dose-response curve, the average value of amylase activity in the medium at the beginning of the peptide addition was 3.4 ± 0.3% (seven experiments) of the total amylase activity in the acini. After 30 min, the amylase activity was 7.6 ± 0.7%. In the presence of CCK-8 (1 nM), the amount of amylase was 25.3 ± 2.1%. B, Antagonism of CCK-8-induced high dose inhibition by A-70874. **, $p < 0.01$, CCK-8 alone versus CCK-8 plus 10 μM A-70874 (Student's paired *t* test).

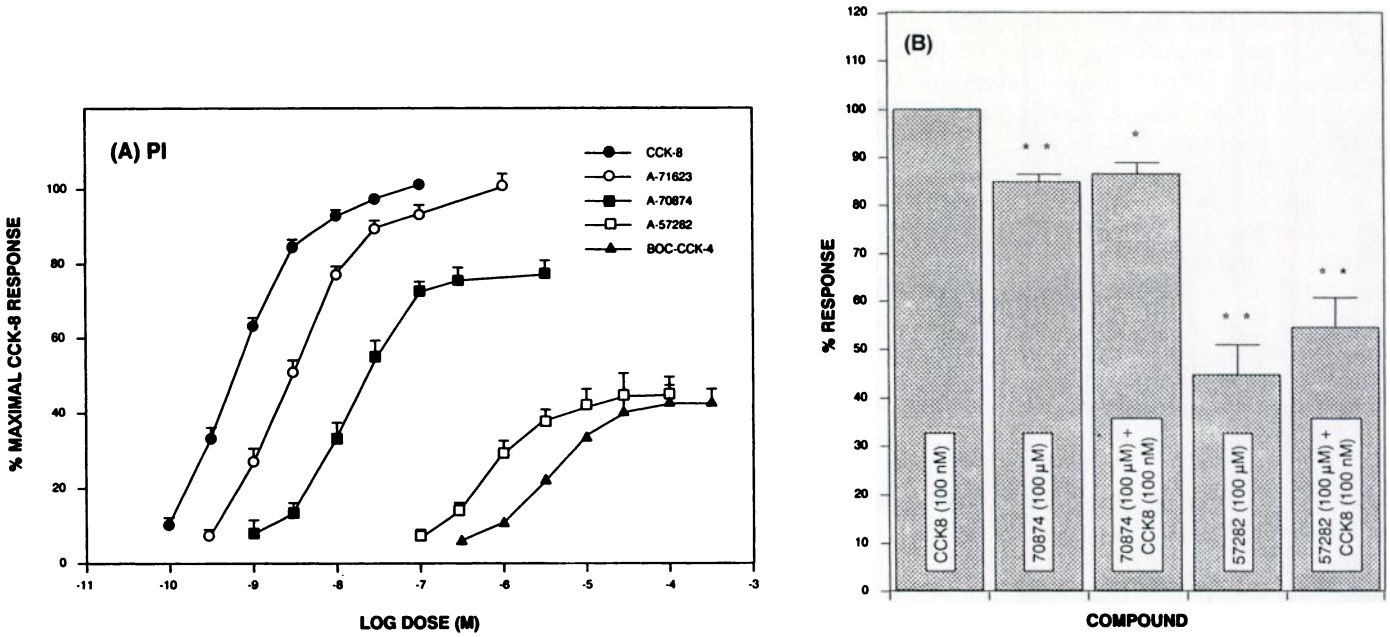

Fig. 3. A, Efficacies of CCK-8, A-71623, A-70874, A-57282, and Boc-CCK-4 in stimulating PI hydrolysis in guinea pig acini. Protocols for measuring PI hydrolysis were as described in Materials and Methods. Results shown are the mean ± standard error taken from at least three experiments, conducted in duplicate, with <5% sample variability. B, Effects of high concentrations of A-70874 and A-57282 on the PI response induced by 100 nM CCK-8. The amount of inositol phosphates in the absence of CCK-8 was 420 ± 57 dpm (three experiments) and, in the presence of CCK-8, 6100 ± 1100 dpm (three experiments). *, $p < 0.05$; **, $p < 0.01$, versus 100 nM CCK-8 alone (Student's paired *t* test).

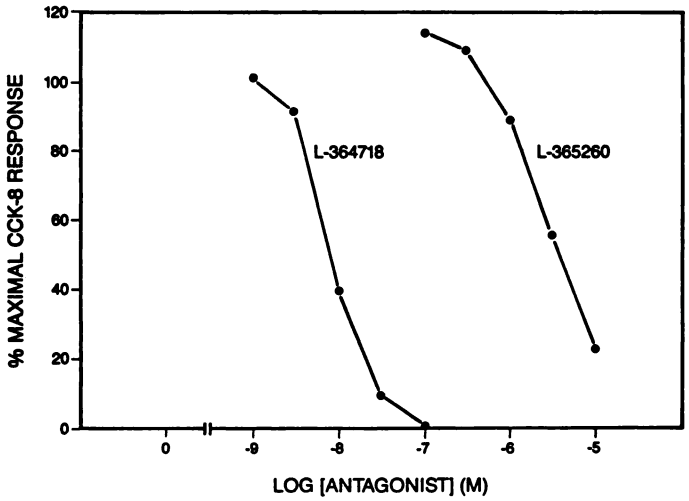

Fig. 4. Inhibition of amylase-releasing properties of A-71623 (10 nM) by L-364,718 and L-365,260. Results shown are the mean ± standard error from three experiments, conducted in duplicate, with <5% sample variability.

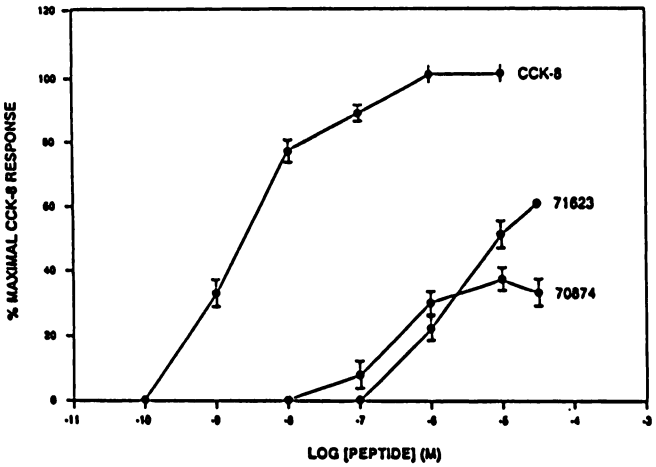

Fig. 5. Functional activities of CCK-8, A-71623, and A-70874 at CCK-B/gastrin receptors on NCI-H345 cells. Protocols for measuring calcium concentrations using indo-1 were as described in Materials and Methods. Results shown represent the mean ± standard error from at least three experiments. The basal levels of calcium were 200 ± 6 nM (nine experiments) and, in the presence of CCK-8 (1 μM), 530 ± 20 nM (eight experiments).

experiments) and 1600 ± 560 nM (three experiments), respectively. In contrast, the IC_{50} values of CCK-8 and gastrin-17-I were 2.0 ± 0.2 nM (four experiments) and 20 ± 2.9 nM (four experiments), respectively. Therefore, A-71623 and A-70874 exhibited low affinities for gastrin receptors, which have binding characteristics similar to those of the CCK-B receptors (12).

Discussion

Since the discovery of CCK and the identification of CCK binding sites in the brain, there have been many studies aimed at characterizing and understanding the roles of CCK receptors in the CNS. It has been shown that the majority of CCK receptors in brain are of the CCK-B subtype. CCK-A receptors also exist in discrete regions of the brainstem and nucleus accumbens, but in much smaller amounts than CCK-B receptors (16, 17); however, the physiological roles of these CCK-A and CCK-B receptors in the CNS remain largely unclear. In part, this is due to a lack of subtype-specific CCK agonists and antagonists. Within the last 5 years, many different classes of CCK-A-selective nonpeptide antagonists have been described and, recently, Merck and Parke-Davis scientists have reported on CCK-B-selective antagonists (5–7). Using these selective antagonists, many studies have been performed to address the

physiological significance of CCK-A and CCK-B receptors in the brain and periphery (2, 7, 18, 19). In the CCK agonist arena, selective CCK-B agonists based on CCK-4 or CCK-7 analogs, cyclic and linear, have been described (8, 9). Most recently, we have reported on a *N*-methyl-Asp[32] derivative of CCK-7, A-71378, that proves to be a potent and selective CCK-A agonist (10). However, to date, there have been no reports of any potent and selective CCK-A agonists that are devoid of sulfated or polar groups on the tyrosine of CCK peptides. Until now, all CCK-related peptides lacking the sulfated group on tyrosine have been shown to be 100–1000-fold weaker at the CCK-A receptor than their sulfated homologs (1, 2, 20). These observations have lead to the conclusion that a sulfated moiety on the tyrosine is essential for high affinity and potency at the CCK-A receptor. Here, we have described two CCK-4-based peptides with high potency and selectivity at CCK-A receptors but that are devoid of the tyrosine sulfated moiety. As shown in Table 1, the lead for the synthesis of these compounds comes from studies with A-57282. Its selectivity at the pancreatic CCK-A receptor was unexpected. After detailed structure-activity studies, A-71623 and A-70874 were subsequently developed. As shown in Table 1, these two compounds are about 1200- and 140-fold selective for the pancreatic CCK-A over the cortical CCK-B receptor, respectively. Compared with CCK-8, both peptides are agonists in stimulating amylase secretion (Fig. 2A), but they possess different efficacies in inducing PI hydrolysis in guinea pig pancreatic acini (Fig. 3A). A-71623 and CCK-8, which are full agonists in eliciting PI hydrolysis, show inhibition of amylase secretion at supramaximal concentrations, whereas A-57282 and A-70874, both partial agonists in eliciting PI response, do not exhibit inhibition of amylase release with supramaximal concentrations. Furthermore, A-70874 is able to reverse the inhibition produced by supramaximal concentrations of CCK-8 in the amylase assay (Fig. 2B), as well as the maximal response of CCK-8 in PI hydrolysis (Fig. 3B). The amylase secretion profile of A-70874 is, therefore, very similar to that of CCK-JMV-180, except that A-70874 possesses greater efficacy in eliciting PI responses (21–23). At present, the mechanism for the reduced secretion at supramaximal agonist concentrations is unclear. Whether it represents a population of low affinity CCK receptors mediating inhibition of amylase secretion (22) or a negative feedback process secondary to excessive receptor activation remains to be determined. It should be noted that, similar to CCK-JMV-180, there are species differences in the *in vitro* amylase-releasing properties of A-70874 in rodents (23). In contrast to the findings with guinea pigs, A-70874 shows significant inhibition of amylase secretion at supramaximal concentrations in rat pancreatic acini (data not shown).

Although A-71623 and A-70874 possess similar binding potencies, A-71623 is 6 times more potent than A-70874 in amylase assays. The reason for such discrepancy is unclear but may be related to their different efficacies in the PI response (Fig. 3A).

The stimulatory activity of A-71623 in inducing pancreatic amylase release is potently reversed by the CCK-A antagonist L-364,718 (Fig. 4), as are its stimulatory effects on other CCK-A-containing tissues such as ileum and gall bladder (data not shown).

In contrast to their high potencies and efficacies at the pancreatic CCK-A receptor (Figs. 2 and 3), A-71623 and A-70874 show low potencies and partial agonist activities in eliciting calcium mobilization at CCK-B/gastrin receptors on NCI-H345 cells (Fig. 5). In addition, both A-71623 and A-70874 exhibit low affinities for gastrin receptors on gastric glands. Taken together, these two novel CCK-4 peptides behave as potent and selective CCK-A agonists, while exhibiting low affinities and efficacies at CCK-B/gastrin receptors. These observations also are in agreement with our previous conclusion that CCK-A and CCK-B/gastrin receptors have distinct requirements for activation (13). The selectivity for CCK-A receptors shown by these two analogs is in direct contrast to that described to date for other CCK-4-based analogs, which are selective at CCK-B receptors. In fact, the ability of CCK-4 to display high affinity for CCK-B sites and low affinity for CCK-A receptors has been used as a criterion for the definition of CCK-B receptors (4). A-71623 and A-70874 also represent the first two examples of potent CCK-A agonists lacking the sulfated tyrosine residue on CCK-related peptides. These novel CCK-4 analogs, therefore, represent important breakthroughs in the areas of CCK-A agonists. Of paramount interest is the determination of structural and conformational relationships among the novel CCK-4 analogs, the CCK-A-selective CCK-7 analog A-71378, and the nonpeptide antagonists. Such information should enhance our understanding of CCK-A receptor recognition and activation.

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