2-Naphthalenesulphonyl L-aspartyl-(2-phenethyl)amide (2-NAP)—a selective cholecystokinin CCK_A-receptor antagonist

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1 The *in vitro* pharmacological characterization of the sodium salt of 2-naphthalenesulphonyl L-aspartyl-(2-phenethyl)amide [2-NAP], a hydrophilic compound derived from the C-terminal aspartate-phenylalanine dipeptide of cholecystokinin (CCK), is described.

2 2-NAP behaved as a competitive antagonist of sulphated cholecystokinin octapeptide (CCK-8) at CCK_A-receptors in both intact tissue bioassays (guinea-pig gall bladder, pancreas and ileum, human and rabbit gall bladder) and a radioligand displacement assay (guinea-pig pancreatic cells). The mean pK_B, over assays, was 6.5.

3 Compared to the other assays, the rabbit gall bladder assay gave a significantly higher pK_B estimate [7.0] for 2-NAP and a significantly lower estimate [8.9] for devazepide (formerly L-364,718 and MK-329), a well-characterized CCK_A-receptor antagonist; these anomalous results suggest that a different class of CCK_A-receptors may be involved.

4 2-NAP, was found to be highly selective, having at least 300 fold greater affinity for CCK_A-receptors than for 50 other pharmacological loci, including gastrin/CCK_B, as estimated by bioassay or radioligand displacement.

Keywords: Receptor, cholecystokinin; muscle, smooth, gall bladder, ileum; pancreas; cerebral cortex; stomach; 2-NAP

Introduction

The principle, that the properties of hormone efficacy and affinity are chemically-separable in hormone analogues, has been exploited in the discovery of antagonists for, *inter alia*, histamine H₂-receptors (Black *et al.*, 1972), β -adrenoceptors (Black & Stephenson, 1962) and angiotensin II-receptors (Marshall *et al.*, 1970). Thus, the retention and subsequent enhancement of the receptor recognition features of a hormone and the emasculation of its efficacy has resulted in the identification of highly specific, competitive antagonists. In common with other groups (Morley *et al.*, 1965; Spanarkel *et al.*, 1983; Horwell *et al.*, 1990) we have been trying to apply this principle to the cholecystokinin (CCK) family of hormones.

Two main classes of CCK/gastrin receptors have been identified: CCK_A and CCK_B/gastrin (see Woodruff & Hughes, 1991). We have used guinea-pig gall bladder contraction (Bishop *et al.*, 1992a) to assay CCK_A-receptor interactions and acid secretion in the isolated, lumen-perfused, stomach of the mouse (Black & Shankley, 1985) to assay CCK_B/gastrin receptor interactions. Using these assays to evaluate analogues of CCK fragments we found that the dipeptide, N-tert-butyloxycarbonyl-aspartyl-phenylalanine-amide (Boc-Asp-Phe-NH₂), representing the two amino acid-fragment C-terminus common to both CCK and gastrin, was a low affinity partial agonist at CCK_B/gastrin receptors (log [A₅₀] – 5.58 ± 0.15, max. effect 64.5 ± 10.4% of max. effect to pentagastrin, *n* = 5, unpublished observations) but inactive at CCK_A-receptors (100 μ M, in both guinea-pig gallbladder and pancreatic fragments, *n* = 4, unpublished observations). The latter result accords with the finding of Jensen *et al.* (1983) who reported that 300 μ M Boc-Asp-Phe-NH₂ was required for significant CCK_A-receptor antagonism in guinea-pig pancreatic tissue. The selective activity we found was abolished by removal of the C-terminal amide of Boc-Asp-Phe-NH₂. Replacement of the N-tert-butyloxycarbonyl group in this compound with an analogue, the 2-naphthalene sulphonyl group, to give 2-naphthalenesulphonyl L-aspartyl-(2-

phenethyl)amide (2-NAP, Figure 1) now produced significant affinity for CCK_A-receptors without the expression of efficacy. The efficacy at CCK_B/gastrin receptors was restored and the affinity for CCK_A-receptors was lost by introduction of the C-terminal-amide into 2-NAP.

The interesting symmetry of these and other structure-activity relations is being reported elsewhere. The *in vitro* pharmacological characterization of 2-NAP, as a selective, competitive antagonist of CCK_A-receptors is described here.

Methods

Bioassays based on measuring changes in contractility are often complicated by the responses 'fading', by the development of 'spontaneous' contractions or by large 'amplitude' variation between preparations. Fade and spontaneity can often be controlled by varying temperature and Ca²⁺ concentration; amplitude variation can be offset by normalising to an independent maximal stimulus. An attempt was made to optimise each of the bioassays by modifying experimental conditions and protocols to improve response stability and reproducibility both within and between preparations.

CCK_A-receptor bioassays

Guinea-pig gall bladder The assay was performed as described by Bishop *et al.* (1992a) which uses low Ca²⁺ Krebs-

Figure 1 Structure of 2-NAP (2-naphthalenesulphonyl L-aspartyl-(2-phenethyl)amide).

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Henseleit solution at 29°C with the modification that contractile responses were normalized to a maximal muscarinic response produced by 10 µM 5-methylfurfumethide (5-MeF).

Rabbit gall bladder The assay was performed as for the guinea-pig gall bladder on tissues from male, New Zealand white rabbits (1.5–2 kg). 5-MeF did not contract this preparation and a maximal response to CCK-8 could not be readily reversed by washout, therefore no normalization procedure was used.

Human gall bladder Gall bladders were obtained from four patients undergoing gallbladder surgery. The assay was performed as for the guinea-pig gall bladder assay except that the [Ca²⁺] was normal (2.5 mM) and the temperature 25°C. Muscle strips (~3 × 10 mm) were cut from the luminal portion of the gall bladder. Following preliminary studies, which showed that addition of 2-NAP to a stable, CCK-8-evoked contraction produced a relaxation, a single-shift, competitive interaction study was conducted on gall bladder tissue from a 71 year old male with pancreatitis diagnosed 2 weeks prior to surgery.

Guinea-pig ileum Longitudinal strips of guinea-pig ileum were set up for isotonic recording of muscle contraction under a 0.35 g load in Krebs-Henseleit buffer. In contrast to the gallbladder, CCK contracts guinea-pig ileum indirectly, by releasing acetylcholine (Vizi *et al.*, 1972). Therefore, sensitivity to CCK_A-receptor stimulation was increased by adding physostigmine (0.2 µM) to the bath. The resulting increase in spontaneous activity was reduced by lowering the temperature to 23°C. As CCK_B/gastrin receptors are also present on the vagal nerve terminals (Lucaites *et al.*, 1991) a selective concentration of the CCK_B/gastrin antagonist L-365,260 (0.2 µM, pK_i at CCK_B/gastrin receptors ~8.8, pK_B at CCK_A receptors ~6.5; Lotti & Chang, 1989) was added to avoid confounding effects. Preparations were washed twice during an initial stabilization period of 20 min, and then exposed to three 20 min cycles of 5-MeF (1 µM incubated for 10 min followed by washout).

Guinea-pig pancreas fragments—amylase release assay

The assay was based on the method described for the rat pancreas by Scarpignato *et al.* (1989). For each assay the pancreas was removed from three guinea-pigs (Dunkin Hartley 200–300 g), washed in buffer and the glandular tissue cut into fragments (~1 mm³); 0.3 ml of tissue was placed in a flow cell (made from 2 ml polythene syringes), superfused with the buffer at 37°C (1.5 ml min⁻¹) and gassed with carbogen. Amylase in the superfusate was measured with the Phadebas Amylase Kit (Pharmacia Diagnostics).

[¹²⁵I]-Bolton Hunter-CCK-8 displacement from guinea-pig pancreas

The pancreas was removed from two male guinea-pigs (Dunkin Hartley, 200–300 g) and homogenized in 20 volumes of ice-cold HEPES buffer (10 mM; brought to pH 7.2 with ~0.5 ml 10 M NaOH l⁻¹ and containing 130 mM NaCl, 4.7 mM KCl, 1 mM EGTA and 0.25 g l⁻¹ bacitracin) in a Polytron (Brinkmann, PT10, setting 7) for 6s. The homogenate was centrifuged at 39 800 g for 15 min at 4°C. The supernatant was discarded and the pellet resuspended in 20 volumes of buffer with a Teflon-in-glass homogenizer and recentrifuged as above. The final pellet was resuspended in the Teflon-in-glass homogeniser to a tissue concentration of 1 mg wet weight of original tissue ml⁻¹ and filtered sequentially through 500 µm and 200 µm (poresize) Nytex mesh. Membrane homogenates (400 µl) were incubated for 150 min at 21°C, in triplicate, with nominally 20 pM [¹²⁵I]-Bolton Hunter-CCK-8 ([¹²⁵I]-BH-CCK-8) and unlabelled ligands to a

final volume of 0.5 ml buffer. The buffer also contained 0.3 µM CI-988 (also known as PD134308) to inhibit binding to any CCK_B gastrin receptors (pK_B ~8.8, Hughes *et al.*, 1990) which may be present (Da-Hong *et al.*, 1987). The exact concentration of the radioligand was calculated for each experimental run by counting the gamma emission of an aliquot of the diluted radiolabel and converting this to a concentration on the basis that the specific activity was 2200 Ci mmol⁻¹. Non-specific binding was that obtained in the presence of a selective concentration (10 nM) of the CCK_A-receptor antagonist devazepide (formerly L-364, 718 and MK-329) (pK_i ~10; Chang & Lotti, 1986). Following incubation, free ligand was removed by rapid vacuum-filtration (Brandell Cell Harvester) through Whatman GF/B filters, pre-soaked in ice-cold 50 mM Tris HCl (pH 7.4). The filters were washed immediately, three times, with 3 ml ice-cold 50 mM Tris HCl (pH 7.4) and bound radioactivity determined by counting in an LKB Clinigamma counter.

The assay was validated by use of the CCK_A-receptor antagonists devazepide and loxiglumide which yielded pK_i values of 10.05 (95% c.l. 9.76, 10.35) and 7.00 (95% c.l. 6.82, 7.19), respectively, in accord with previously published results (Chang & Lotti, 1986; Setnikar *et al.*, 1987; Bishop *et al.*, 1992a).

Selectivity studies

Selectivity of 2-NAP at CCK_A- over CCK_B/gastrin receptors was assessed. CCK_B/gastrin receptor activity was measured by use of the isolated, lumen-perfused, stomach of the mouse; guinea-pig stomach fundic muscle strip and mouse cerebral cortex CCK_B radioligand binding assays. Selectivity of 2-NAP over several non-CCK-receptor assays was measured with both bioassays and radioligand binding assays (for details see Table 2).

Experimental protocols

A single agonist concentration-effect (E/[A]) curve was obtained in each assay in the absence or presence of antagonist pre-incubated for 60 min. Where CCK-8 produced sustained responses, single cumulative E/[A] curves were obtained. In the guinea-pig ileum, CCK-8 produced spike and fade responses. Therefore sequential curves were obtained using a 15 min cycle; the tissues were exposed to CCK-8 for ~30s, followed by washout and re-application of 2-NAP. Cumulative CCK-8 E/[A] curves were obtained on the pancreas fragments assay with a 16 min time cycle in which, following addition of CCK-8 to the reservoir, a 12 min collection period was allowed (void perfusate) and the following 4 min of superfusate collected for amylase analysis.

Data analysis

Logistic curve-fitting E/[A] data were fitted to a three parameter logistic function to provide estimates of the mid-point slope parameter index (p), midpoint location (log[A₅₀]) and upper asymptote (α) of the curves. These parameters, expressed as mean ± s.e.mean were used for subsequent analysis (Student's *t* test and one-way analysis of variance, ANOVA, as appropriate) and display of the data (Black & Shankley, 1985). On the guinea-pig pancreas fragment assay it was not possible to fit E/[A] data to the logistic function because the curves were bell-shaped and asymmetric about the log [A₅₀]. Therefore, ANOVA tests were not performed on the slopes and asymptotes of the curves and log [A₅₀] values were measured graphically from plots of the E/[A] data. These values were subsequently fitted to the model of competitive antagonism.

Competitive analysis Competitive analysis was performed as described previously (Black *et al.*, 1985b). Antagonist equili-

brism dissociation constants were estimated as $-\log K_B$ (i.e. pK_B). Where only a single antagonist concentration was examined, simple competition was assumed provided there was a parallel shift of the CCK-8 E/log[A] curve with no significant difference in α . The ability of compounds to inhibit competitively the specific binding of [125 I]-BH-CCK-8 to CCK_A sites was analysed by the programme ALLFIT which provided estimates of the IC₅₀ values for a test ligand. The pK_i values of test compounds were calculated from the Cheng-Prusoff equation. The pK_i values are quoted with the s.e.mean. The 95% confidence limits are calculated using $(n-1)$ d.f., where n is the number of experiments (i.e. pK_i determinations) performed.

Combined dose-ratio analysis This analysis was performed according to a procedure (Shankley *et al.*, 1988) based on the method of Paton & Rang (1965). In brief, when two antagonists act at the same site, then their combined dose-ratio is given by:

$$r_{(B+C)} = r_B + r_C - 1,$$

where r_B and r_C are the dose ratios obtained independently in the presence of the antagonists B and C, respectively. When two antagonists act independently their combined dose-ratio is given by:

$$r_{(B+C)} = r_B \cdot r_C$$

Two reference CCK_A-receptor antagonists have been used: the non-peptide, benzodiazepine-related devazepide and 4-CTP which is a derivative based on the C-terminal tetrapeptide sequence of CCK and gastrin, (i.e. N-tert-butyloxycarbonyl-L-tryptophyl-L-leucyl-L-aspartyl-2(4-chlorophenyl)ethylamide).

Compounds

The sulphated C-terminal octapeptide of cholecystokinin (CCK-8) and pentagastrin were obtained from Cambridge Research Biochemicals Ltd. CCK-8 was dissolved in 10% absolute ethanol to provide a final stock solution of 2 mM concentration. Pentagastrin was dissolved in 100% dimethylformamide (DMF) to give a 100 mM stock. [125 I]-BH-CCK-8 was purchased from DuPont, Stevenage, Herts. Some of the experiments used the free acid formulation of 2-NAP which behaved quantitatively in an identical manner to the sodium salt. 2-NAP was dissolved in 100% DMF to give a 20 mM stock.

Devazepide, L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N'-(3-methylphenyl)urea), CI-988 ([[(3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[(tricyclo[3.3.1.1^{3,7}]dec-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenyl-ethyl]amino]-4-oxobutanoic acid) and 4-CTP, loxiglumide (all prepared by James Black Foundation chemists) were dissolved in DMF to give 2 mM stock concentrations. Caerulein, bacitracin, atropine, (+)-tubocurarine, cyproheptidine, methysergide, (−)-propranolol, (−)-salbutamol, (−)-isoprenaline, histamine, acetylcholine and 5-hydroxytryptamine were purchased from Sigma. Angiotensin II was purchased from Cambridge Research Biochemicals. The following compounds were obtained as gifts: 5-methylfurfurmethide iodide (Wellcome Research Plc), mepyramine (May and Baker), tiotidine (ICI Pharmaceuticals plc), DuPont 753 (1-(2'-(5-tetrazoyl)phenyl)benzyl 2-butyl-4-chloro-5-methanolimidazole, K⁺ salt; DuPont-Merck Inc.). All compounds were subsequently diluted in distilled water. The maximum volume of solvents added to any one 20 ml organ bath was 20 μ l ethanol, 20 μ l DMF or 500 μ l distilled water. Neither the vehicles nor the antagonists were found to produce significant effects on baseline activity.

Figure 2 Analysis of competitive antagonism (a) CCK-8 E/log[A] curves ($n = 6/8 \pm$ s.e.mean) obtained on the guinea-pig gall bladder assay in the absence (●) and presence of (○) 0.5, (■) 1.5, (□) 5, (▲) 15 μ M 2-NAP. Ordinate scale: contraction as a percentage of response to 5-methylfurfurmethide (5-MeF, 10 μ M). (b) CCK-8 E/log[A] relationships ($n = 4/8 \pm$ s.e.mean) obtained on the guinea-pig pancreas assay in the absence (●) and presence of (○) 1, (■) 9, (□) 36 μ M 2-NAP. Ordinate scale: Effect as amylase release in arbitrary units. (c) Schild plots for the interaction between CCK-8 and 2-NAP in (●) guinea-pig gall bladder, (○) rabbit gall bladder, (■) guinea-pig pancreas, (□) guinea-pig ileum. For comparison the Schild plot values for the interaction between caerulein and 2-NAP (10 μ M) in the guinea-pig gall bladder (▲) and for CCK-8 and 2-NAP (30 μ M) in the human gall bladder (Δ) are also shown. dr: dose ratio.

Results

CCK_A-receptor antagonism by 2-NAP

Guinea-pig tissue assays We have used devazepide as a standard reference CCK_A-receptor antagonist on the assays. The pK_B estimates were as follows: guinea-pig gall bladder 9.98 (95% c.i. 9.77, 10.17) (Bishop *et al.*, 1992a), pancreas 9.86 (95% c.i. 9.62, 10.10) and ileum 10.0 (95% c.i. 9.46, 10.54). A pK_i value of 10.05 (95% c.i. 9.76, 10.35) was estimated on the pancreatic radioligand binding assay.

2-NAP behaved as a simple competitive antagonist of CCK-8 on the guinea-pig gall bladder, pancreas (Figure 2) and ileum bioassays and on the pancreatic radioligand binding assay. The dissociation constant values estimated were not significantly different from each other and ranged from 6.21 to 6.65 (Figure 3 and Table 1). As interpreted by combined dose-ratio analysis (Table 2), 2-NAP acted syntopically with CCK-8 and the benzodiazepine derivative, devazepide, and with CCK-8 and the peptide analogue 4-CTP. Antagonism was independent of the agonist. Thus, on the guinea-pig gall bladder assay, a single concentration of 2-NAP (10 μ M) produced a parallel rightward shift of caerulein E/[A] curves and a pK_B value of 6.65 (95% c.i. 6.37, 6.93) was estimated (Table 1).

Rabbit gall bladder In the rabbit gall bladder, although 2-NAP behaved as a simple competitive antagonist, the pK_B value estimated was 6.95 (95% c.i. 6.52, 7.38) (Table 1). A pK_B value of 8.87 (95% c.i. 8.48, 9.25) was estimated for devazepide.

Human gall bladder In the human gall bladder preparation (8 tissues from one tissue sample), a parallel rightward shift of the CCK-8 E/log[A] curve of 1.32 ± 0.39 log units was observed in the presence of 2-NAP (30 mM) equivalent to a pK_B value of 5.82 (95% c.i. 4.58, 7.06) (Table 1).

Analysis of 2-NAP antagonism between CCK_A-receptor assays

We wanted to know whether the estimated dissociation constants for 2-NAP given in Table 1 formed a homogeneous group. Conventionally, one-way ANOVA would be used;

Figure 3 Graphical representation of the estimates of the 2-NAP pK_B (or pK_i), together with 95% confidence limits, from the CCK_A-receptor assays listed in Table 1. The broken lines show the range of the weighted and unweighted global mean values of the complete data sets.

^a vs. caerulein; ^b pK_i

Table 2 Combined dose-ratio analysis

B:	Devazepide (3 nM)	4-CTP (30 μ M)
C:	2-NAP (10 μ M)	2-NAP (10 μ M)
Observed log r_B	1.14 ± 0.28	1.10 ± 0.38
Observed log r_C	1.41 ± 0.24	1.65 ± 0.32
Multiplication:		
Expected log $r_{(B+C)}$	2.55	2.76
Addition:		
Expected log $r_{(B+C)}$	1.58	1.75
Observed log $r_{(B+C)}$	1.69 ± 0.09	1.50 ± 0.27
Test Statistics:		
Devazepide $\pm$ 2-NAP		
$S_M \pm$ s.e.mean	$-0.86 \pm 0.37^*$ (95% c.i. $-1.64, -0.08$)	
$S_A \pm$ s.e.mean	0.11 ± 0.20 (95% c.i. $-0.31, 0.53$)	
4-CTP + 2-NAP		
$S_M \pm$ s.e.mean	$-1.26 \pm 0.47^*$ (95% c.i. $-2.15, -0.27$)	
$S_A \pm$ s.e.mean	-0.25 ± 0.25 (95% c.i. $-0.78, 0.28$)	

*Significantly different from zero, $P < 0.05$.

Table 1 Analysis of CCK-8/caerulein v 2-NAP interactions

A Intact tissue bioassays				
Assay	b (s.e.mean)	pK_B (s.e.mean)	N	95% c.i.
Guinea-pig:				
gall bladder	0.94 (0.12)	6.55 (0.14)	29	6.26, 6.84
gall bladder ^a	ND	0.65 (0.13)	11	6.37, 6.93
pancreas	0.94 (0.06)	6.45 (0.04)	24	6.36, 6.54
ileum	0.84 (0.08)	6.21 (0.12)	28	5.96, 6.46
Rabbit				
gall bladder	0.84 (0.20)	6.95 (0.21)	22	6.52, 7.38
Human				
gall bladder	ND	5.82 (0.39)	8	4.58, 7.06
B Radioligand displacement assay				
Assay	b (s.e.mean)	pK_i (s.e.mean)	N	95% c.i.
Guinea-pig pancreatic cells	0.83 (0.06)	6.45 (0.07)	5	6.24, 6.65

pK_B and pK_i values obtained from CCK_A-receptor assays. N represents the number of agonist dose-response curves used in the experiment or, in the case of radioligand displacement assay, the number of repeated experiments (where each experiment used triplicate samples) performed to obtain a pIC_{50} .

b is the Schild plot slope parameter determined in the functional tissue assays and the mid-point slope of the 2-NAP concentration-displacement curve of specific [¹²⁵I]-BH-CCK-8 binding in the radioligand studies.

^aCaerulein as agonist.

ND: not determined.

however, this is not applicable in this case as there are clear differences in the size of the variances associated with the pK_B/pK_i estimates. Consequently, we have used the method of Cochran (1954) based on a weighted F -test which indicated that the data are significantly ($P < 0.05$) non-homogeneous. That this is due apparently to the high pK_B estimate from the rabbit gall bladder assay is shown from the following analyses. Firstly, a global mean was calculated from all the pK_B (and pK_i) estimates in Table 1 and then each assay pK_B value was tested against this value using the individual 95% confidence limits. The global mean was calculated in three ways: the unweighted arithmetic mean of the estimates, a weighted mean using the number of experiments (N in Table 1) as a weighting factor and a weighted mean using $1/se^2$ as a weighting factor (Cochran, 1954). These gave very similar values of 6.44, 6.48 and 6.46, respectively. Only the 95% confidence intervals of the rabbit gall bladder pK_B value excluded these global mean values. Secondly, the weighted F -test was not significant when performed without the data from the rabbit gall bladder experiment. The global average pK_B values without the rabbit gall bladder result was 6.36, 6.39 and 6.46, respectively, when calculated by the methods above.

Selectivity of 2-NAP

CCK_B/gastrin-receptor selectivity 2-NAP (100 μ M) had no significant effect on pentagastrin-stimulation of gastric acid secretion from the mouse stomach and smooth muscle contraction of the guinea-pig fundus muscle assays. In the mouse cortex CCK_B-radioligand binding assay, a pK_i value of 4.16 was estimated (Table 3).

Non-CCK/gastrin-receptor selectivity 2-NAP was inactive at 100 μ M in several functional bioassays unrelated to the CCK/gastrin family of receptors with the exception of an antihistaminic action observed on the guinea-pig ileum equivalent to a pK_B value of ~ 4.4 (Table 3).

In addition, 37 radioligand binding assays 2-NAP had no significant activity.

Discussion

2-NAP behaved as a simple competitive antagonist of CCK-8 in a number of CCK_A-receptor assays ($pK_B \sim 6.5$). Previously, combined dose-ratio analyses, *inter alia*, have been used to establish that loxiglumide, lorglumide and devazepide interact with CCK-8 at a homogeneous popula-

tion of receptors in the guinea-pig gall bladder assay (Bishop *et al.*, 1992a). This conclusion is supported by the finding here that 2-NAP behaved competitively and syntopically at CCK_A-receptors with two different chemical classes of CCK_A-receptor antagonist (devazepide, a benzodiazepine-based non-peptide and the tetrapeptide-derived compound, 4-CTP). Additionally, on the guinea-pig gall bladder, the antagonism was shown to be independent of the choice of CCK_A-receptor agonist because 2-NAP had similar affinity against both CCK-8 and caerulein. In preliminary studies in rat pancreas fragments, prepared in the same way as the guinea-pig pancreas fragments, 2-NAP had no agonist action and antagonized the secretory effect of CCK-8 giving an estimated pK_B value of ~ 6.5 (data not shown).

Interestingly, the variance about the pK_B estimate made on the guinea-pig pancreatic fragment assay was considerably smaller than the others (Table 1). The inability to fit objectively the individual $E/[A]$ curves from this assay was compensated by a high degree of reproducibility between individual assay replicates as judged by the log $[A_{50}]$ estimates.

In the ileum assay the presence of CCK_B/gastrin-receptors coupled to smooth muscle contraction necessitated the inclusion of a selective CCK_B/gastrin-receptor antagonist (L-365,260) in the bathing solution. The concentration chosen was slightly less than the reported equilibrium dissociation constant for CCK_A-receptors (~ 300 nM, Lotti & Chang, 1989). According to the principle of combined dose-ratios (Paton & Rang, 1965), if the concentration used was equal to the K_B value for CCK_A-receptor blockade the error in the estimation of the affinity of 2-NAP would be expected to be 0.3 log units, which may not be experimentally-detectable. Notwithstanding this, the ileum pK_B estimate was 0.34 log unit lower than the value obtained on the guinea-pig gall bladder assay, although this difference was not significant.

Analysis of the equilibrium dissociation constants for all the CCK_A-receptor assays indicated that the rabbit gall bladder pK_B estimate was atypical. This preliminary suggestion of receptor heterogeneity was supported by the finding that the devazepide pK_B estimate (8.87) in this assay appears to be low; most other estimates of the equilibrium dissociation constant made in assays containing CCK_A receptors are approximately 0.1 nM (pK_B 9.98, guinea-pig gall-bladder, Bishop *et al.*, 1992a; pK_B 10.09, guinea-pig ileum, D'Amato *et al.*, 1991; pA_2 9.89 and 9.93, guinea-pig ileum and colon respectively, Chang & Lotti, 1986; pA_2 9.6, rat pancreatic acini amylase secretion, Louie *et al.*, 1988) although lower values have been reported (pK_A 9.28, rat solubilized pancreatic CCK receptors, Chang *et al.*, 1987; pK_i 8.4, guinea-pig pancreatic acini, Huang *et al.*, 1989). The fact that the

Table 3 Summary of selectivity studies with 2-NAP

Receptor	Assay	Agonist	Result
Gastrin	mouse stomach ^a	pentagastrin	IA 100 μ M
Gastrin	g-p fundus ^b	pentagastrin	IA 100 μ M
5-HT _{1C/1D/3}	rat fundus ^c	5-HT	IA 100 μ M
5-HT ₂	rabbit aorta ^d	5-HT	IA 100 μ M
Angiotensin II	rabbit aorta ^c	angiotensin II	IA 100 μ M
Histamine H ₁	g-p ileum ^f	histamine	$pK_B \sim 4.4$
Histamine H ₂	g-p right atrium ^g	histamine	IA 100 μ M
β_1 -Adrenoceptors	g-p right atrium ^h	isoprenaline	IA 100 μ M
β_2 -Adrenoceptors	g-p trachea ⁱ	salbutamol	IA 100 μ M
Muscarinic-ACh	g-p trachea ^j	5-mef	IA 100 μ M
Nicotinic-ACh	frog rectus ^k	ACh	IA 100 μ M
α_1 -Adrenoceptor	rabbit aorta ^l	Noradrenaline	IA 100 μ M
EDRF-release	rat aorta ^m	5-mef	IA 100 μ M
Chronotropy	g-p right atrium ⁿ		IA 100 μ M
CCK _B	g-p cerebral cortex ^o		$pK_i = 4.16 \pm 0.11$

The basic methods used were, as far as possible, based on the following references: ^aBlack & Shankley, 1985; ^bBishop *et al.*, 1992b; ^cFrankhuijzen & Bonta, 1974; ^dBlack & Shankley, 1990; ^eLiu *et al.*, 1992; ^fKenakin, 1980; ^gShankley *et al.*, 1988; ^hBlack *et al.*, 1985a; ⁱKenakin, 1980—using rings of trachea; ^jShankley *et al.*, 1988; ^kEdinburgh Staff, 1970; ^lBeckerlingh *et al.*, 1984; ^mBerg *et al.*, 1992; ⁿBlack *et al.*, 1986; ^oClark *et al.*, 1986 and Chang & Lotti, 1986.

IA: inactive.

affinity estimate of 2-NAP was higher and that of devazepide lower, than expected implies that the differences did not arise from differences in the ligand concentration, for example, due to loss of the antagonist from the domain of the receptors or saturable CCK-8 uptake.

Although the pK_B estimate for 2-NAP on the human gall bladder was low, the importance of this finding warrants further study. The limited number of experiments from a single tissue sample was associated with a large variance and consequently the pK_B estimate was not different from the global mean values. However, classification of the human gall bladder receptors mediating the contraction to CCK is unclear; for example, in one report, devazepide was found to be weakly active as an antagonist ($pK_B < 6$) of CCK-8-induced contractions (D'Amato *et al.*, 1991) while another study found that devazepide potently ($IC_{50} = 1$ nM) displaced [¹²⁵I]-D-Tyr-Gly[Nle^{28,31},pNO₂-Phe³³]CCK-26-33 binding to human gall bladder CCK binding sites (Schjoldager *et al.*, 1989).

The high selectivity of 2-NAP (> 300 fold) for CCK_A- over the hormone-family-related gastrin/CCK_B-receptors was shown, in functionally-intact tissues, by the failure of 2-NAP to antagonize the effects of pentagastrin in the guinea-pig

fundus and mouse stomach assays. This conclusion was reinforced by the radioligand displacement studies (CCK_B, cerebral cortex:CCK_A, pancreas) which gave an affinity ratio of 180 fold in comparison to other CCK_A-receptor antagonists, for example, lorglumide (100 fold) (Lotti & Chang, 1989), devazepide (3000 fold) (Chang & Lotti, 1986) and loxiglumide (30 fold) (Setnikar *et al.*, 1987). Studies using an extensive range of bioassays confirmed the high specificity of 2-NAP with respect to other pharmacological sites of action (Table 3).

The partition coefficient of 2-NAP (log P - 0.9) measured between chloroform and Krebs-Henseleit was found to be 100 000 fold and 23 fold respectively lower than the other CCK_A-receptor antagonists, devazepide (log P 4.1) and loxiglumide (log P 0.5). The lipophilicity of drugs is recognised as an important determinant of the rate at which they cross biological membranes such as the blood-brain barrier (Rall *et al.*, 1959). Therefore, we expect that the actions of 2-NAP will be predominantly confined *in vivo* to peripheral CCK_A-receptors. Therefore, 2-NAP might be a useful tool for future investigations of the physiology and pharmacology of CCK_A-receptors.

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