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In-vitro Bioassays for Cholecystokinin

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Abstract—Two in-vitro methods for the bioassay of cholecystokinin (CCK) are described. They should be useful for determinations of potencies of pharmaceutical formulations of CCK. Both are based on the hormone's ability to stimulate exocrine pancreas. The stimulatory effect on ^{45}Ca efflux from preloaded suspended acinar cells or on release of amylase from pancreatic acini from the guinea-pig has been recorded. The assay based on stimulation of ^{45}Ca outflux is more specific and precise than the method using measurements of amylase secretion. Therefore, the calcium assay was further validated against the conventional guinea-pig gall-bladder contraction assay. Both methods gave similar potency readings but the results from the in-vitro assay were more precise.

Cholecystokinin was originally described by Ivy & Oldberg (1928) as an intestinal hormone which induces contraction of the gall-bladder. Later, Harper & Raper (1943) extracted a material from the small intestine which caused secretion of enzymes from the pancreas and which they called pancreozymin. In 1964, cholecystokinin was isolated from the upper porcine intestine in a pure form by Jorpes et al. During the purification of cholecystokinin, it was found that pancreozyminic activity (Harper & Raper 1943) also increased and it was demonstrated that pure cholecystokinin displayed high pancreozyminic activity (Mutt & Jorpes 1967). Thus, it was suggested that cholecystokinin and pancreozymin were different activities of one single hormone (Jorpes & Mutt 1966). Due to historic precedence, the hormone is nowadays usually called cholecystokinin (CCK).

The first molecular form of CCK to be characterized was a 33 amino acid peptide (Jorpes & Mutt 1973). Later a 39 amino acid variant, CCK-39, was also isolated from porcine intestinal extracts (Mutt 1976). These peptides have been demonstrated to be equipotent in their effects on acinar cells from guinea-pig pancreas (Sjödin & Gardner 1977) as well as on the isolated perfused rat pancreas (Kato et al 1984). A c-terminal octapeptide fragment, CCK-8, has been synthetized and reported to be more potent than the larger forms (Rubin et al 1969).

The classical bioassay for CCK is the gall-bladder contraction test in the anaesthetized dog described by Ivy & Oldberg (1928). A more practical test based on recording gall-bladder contraction in anaesthetized guinea-pigs was developed in this laboratory by Ljungberg (1969) and was also introduced in the Nordic pharmacopoea (1975). An alternative assay has been based on measurement of contraction of gall-bladder strips in-vitro (Hedner et al 1967; Rubin et al 1969; Marshall et al 1978; Giannoulis & Barry 1982). Recently, Liddle et al (1985) described an in-vitro bioassay for CCK based on its pancreozyminic action on rat isolated pancreatic acini. In addition, a number of radioimmunassays for CCK have been reported. However, it has been difficult to develop reliable radioimmunoassays for CCK (Cantor 1986). One reason is crossreactivity of CCK-antisera, directed towards the biologically active part of the molecules, with similar portions of peptides from the gastrin family (Cantor & Rehfeld 1985). Another difficulty relates to the lack of stability of CCK-molecules during conventional iodination by oxidizing methods (Cantor & Rehfeld 1985). Thus, there is a need for sensitive and precise but not too resource-demanding bioassays for CCK. CCK increases cytosolic free calcium in acinar cells in parallel with an activation of inositol phospholipid metabolism (Ochs et al 1985; Pandol et al 1985; Streb et al 1985). Mobilization of cellular calcium leads to stimulation of enzyme release from such cells (Jensen & Gardner 1981). Calcium mobilization by CCK also leads to an increased efflux of calcium from the cells (Gardner et al 1975).

In the present paper, two in-vitro bioassays for CCK preparations are described. Both are based on the interaction of CCK with pancreatic acinar cells from guinea-pigs. Either secretion of amylase or outflux of ^{45}Ca from preloaded cells in response to CCK stimulation is recorded. They have been designed as three-dose assays in which the results are subjected to normal pharmacopoeial tests for validity of the assay (European Pharmacopoeia 1971).

The assay based on calcium efflux was found to give more precise results than the amylase assay as well as the guinea-pig gall-bladder assay (Ljungberg 1969) and it also allows more samples to be tested during a working day than the in-vivo method.

Materials and Methods

Male guinea-pigs (150–200 g) were obtained from Sahlins försöksdjursfarm AB, Malmö, Sweden. Crude collagenase (E.C.3.4.24.3) and crude hyaluronidase (E.C.3.2.1.35) and purified soybean trypsin inhibitor were from Sigma Chemical Co, St Louis, Mo. Purified collagenase was purchased from Serva Feinbiochimica, Heidelberg, Germany. ^{45}Ca (12·5 mCi mg^{-1}) was from New England Nuclear Corp., Boston, Mass. Hepes (2 hydroxy-ethyl-piperazine-*N*-sulphonic acid) from Kebo, Stockholm, and Phadebase amylase test tablets from Pharmacia, Uppsala, Sweden. Eagle's minimal essential medium amino acid solution (50 times concentrated) and vitamin mixture (100 times concentrated) were from Gibco Bio-Cult Ltd, Paisley, Scotland. Bovine serum albumin was purchased from Miles Laboratories Inc., Elkhart, Indiana. All other chemicals were of the highest grade commercially available. Commercial preparations of

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natural CCK, containing a mixture of CCK-33 and CCK-39, were from Kabi-Vitrum AB, Stockholm or Ferring AB, Malmö, Sweden. As standard preparation, a material from Gastrointestinal Hormone Research Unit, Karolinska Institutet, Stockholm, Sweden, was used. 3 Ivy Dog Units of CCK-33 corresponds to 1 μg of pure CCK-33 (Mutt & Jorpes 1967).

Commercial synthetic CCK-8 (Kinevac) was from Squibb AB, Lidingö, Sweden. Material from a batch of Sincalide (CCK-8) from the same source was used as standard preparation.

All preparations of CCK were dissolved when not stated otherwise in 0·15 M NaCl with 0·1% bovine serum albumin.

Preparation of dispersed pancreatic acinar cells and acini

Guinea-pigs were deprived of food overnight and killed by a blow to the head and the pancreas was dissected free from mesentery. Dispersed acinar cells were prepared according to the method of Amsterdam & Jamieson (1972). Cells were suspended in a Krebs-Ringer bicarbonate buffer (pH 7·4) equilibrated with 95% O_2 and 5% CO_2 and containing 2% (w/v) amino acid mixture, 1% (w/v) bovine serum albumin, 0·01% trypsin inhibitor and 0·5 mM calcium. Mean cell concentration was $17{\cdot}6 \times 10^6 \pm 0{\cdot}4 \times 10^6$ (mean ± s.e.) cells mL^{-1}. Acini were prepared as described by Peikin et al (1978). They were incubated in a buffer solution containing (mM) Hepes (pH 7·4) 24·5, NaCl 98, KCl 6, NaH_2PO_4 2·5, $CaCl_2$ 0·5, $MgCl_2$ 1·0, Na pyruvate 5, Na fumarate 5, Na glutamate 5, glutamine 2, theophylline 5, 1% (w/v) albumin, 0·01% (w/v) trypsin inhibitor, 2% (v/v) amino acid and 1% (v/v) vitamin mixture. The solution was equilibrated with 100% O_2.

Calcium efflux

Cellular loss of ^{45}Ca was measured according to Gardner et al (1975). Cells were preincubated with ^{45}Ca for 60 min and the loss of cellular radioactivity during a 5 min incubation period at 37°C in the presence of 5 mM ethylenediamine tetraacetate (EDTA) was determined by taking dupli- or triplicate 100 μL samples which were added to microcentrifuge test tubes containing 300 μL of iced (4°C) buffer. After centrifugation for 15 s at 9000 *g* in a Beckman model B centrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) the cell pellet was washed four times with iced buffer. Perchloric acid 100 μL (10% v/v) was added to washed cells. After thorough agitation, the mixture was centrifuged for 30 s at 9000 *g*. The supernatant was mixed with 17 mL of a liquid scintillation solution containing 15 parts of toluene, 5 parts of Triton X-100 (New England Nuclear) and 1 part of Permafluor (New England Nuclear). The samples were counted for radioactivity in a Packard Tricarb 2000 CA liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Amylase secretion

Following a preincubation period of 30 min at 37°C, acini were incubated with hormones. After 0 and 30 min, an aliquot of the suspension of acini was centrifuged at 9000 *g* for 15 s in a microcentrifuge. Supernatant was assayed for amylase activity according to Ceska et al (1969) with the Phadebas reagent as substrate. Total amylase content of acini was measured by diluting an aliquot of the suspension of acini with 10 volumes of a solution containing 0·01 M sodium phosohate (pH 7·8), 0·1% (w/v) bovine albumin, and 0·1% (w/v) sodium dodecyl sulphate and determining amylase activity with the substrate. The amount of amylase released was measured as the difference between the activity present in the supernatant at the start of and after 30 min of incubation and was expressed as percentage of total amylase activity.

Potency determinations

Three equally spaced concentrations of CCK from the linear part of the log dose-response curve for calcium efflux or amylase secretion were chosen. In each incubation, tubes with test and standard preparations were processed in parallel. For control purposes tubes without hormones were included. Results from three separate incubations in one single experiment were employed for calculation of potency of a test preparation in per cent of the standard preparation. The estimated potency was calculated by analysis of variance for a three-dose assay according to general pharmacopoeial principles (European Pharmacopoeia 1971). The statistical weight is defined as the reciprocal value of the variance of the log potency estimate (European Pharmacopoeia 1971). The index of precision is calculated by dividing the standard deviation of responses by the slope of the dose-response relationship (Loraine & Bell 1966).

In-vivo assay of cholecystokinin

CCK activity of commercial preparations of the hormone was determined in-vivo by the method of Ljungberg (1969) measuring CCK elicited contraction of the guinea-pig gallbladder.

Results

Basal outflux of ^{45}Ca from preloaded dispersed acinar cells incubated with EDTA for 5 min at 37°C was 5·9 ± 0·3%/min (mean ± s.e.m.) of ^{45}Ca originally present. Increasing concentrations of CCK-8 caused a dose-related stimulation of calcium efflux from such cells (Fig. 1) or release of amylase from suspended acini (Fig. 2). 10^{-10} M of CCK-8 was a threshold concentration for induction of ^{45}Ca efflux (Fig. 1), while 10^{-11} M of CCK-8 sufficed to elicit an increased amylase secretion (Fig. 2). Approximately one order of magnitude higher concentrations produced maximal responses. Somewhat higher concentrations of CCK-33/39 were required to produce similar dose-related stimulation of ^{45}Ca efflux or secretion of amylase (data not shown).

Three concentrations of CCK-8 or CCK-33/39 on the linear part of the dose-response curves were chosen for a three dose assay of CCK formulations against standard preparations based on their ability to stimulate calcium outflux (Fig. 3) or amylase secretion (Fig. 4). In a number of experiments, CCK preparations were assayed against themselves. Table 1 shows results from such assays based on determinations of either efflux of ^{45}Ca or secretion of amylase. Overall, the individual assays based on ^{45}Ca efflux had on average a greater statistical weight than the amylase tests. The mean statistical weight of an individual ^{45}Ca assay was 1566 ± 199 (s.e., n = 26) and of an individual amylase assay 648 ± 116 (s.e., n = 9). Furthermore, index of precision

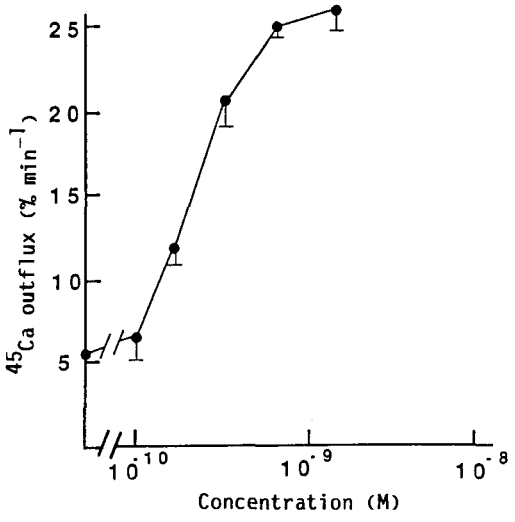

FIG. 1. Effect of CCK-8 on ^{45}Ca efflux from dispersed pancreatic acinar cells. Acinar cells were preincubated in a Krebs-Ringer bicarbonate buffer containing 0·5 mM ^{45}Ca for 60 min. Varied concentrations of CCK-8 were added and ^{45}Ca efflux was calculated from the loss of cellular ^{45}Ca during a 5 min incubation with 5 mM ethylenediaminotetraacetate (EDTA). Each point represent the mean from three separate incubations with duplicate determinations of each sample. Vertical bars in this and following figures denote s.e.m.

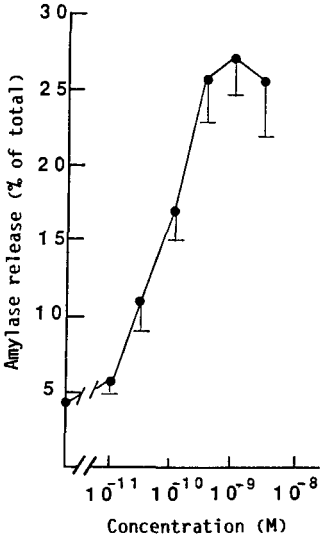

FIG. 2. Effect of CCK-8 on amylase release from dispersed pancreatic acini. The release of amylase was calculated as the difference in percentage of total cellular amylase which was present in the supernatant at the beginning and end of 30 min incubation in a Hepes buffer at 37°C. Each point represents the mean from three separate incubations.

for the calcium assays was on average 0·061 ± 0·006 (mean ± s.e.) and 0·084 ± 0·010 for amylase assays suggesting a higher precision for the former assay (Loraine & Bell 1966). Therefore, further in-vitro assays were based on measurements of efflux of ^{45}Ca from isolated acinar cells.

A preparation of CCK-8 diluted to various concentrations was tested against undiluted material using the ^{45}Ca assay, (Table 2). The results obtained were accurate. One pharmaceutical formulation of CCK-8 and two preparations of

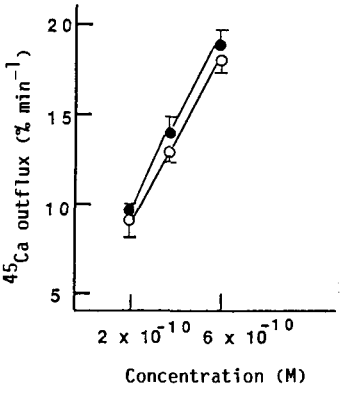

FIG. 3. Bioassay based on ^{45}Ca-efflux from pancreatic acinar cells of a commercial preparation (closed symbols) of CCK-8 against the manufacturer's CCK-8 standard (open symbols). Each point represents the mean from three separate incubations. Estimated potency of test preparation was 109% of the standard with limits of error ($P = 0.95$) of 91–110%.

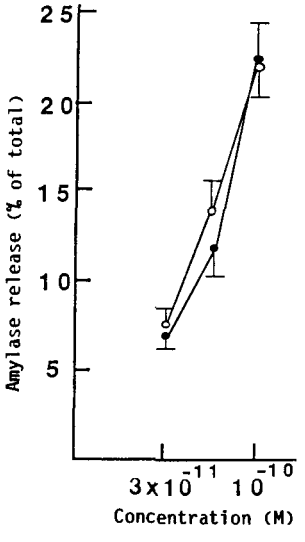

FIG. 4. Bioassay based on amylase release from suspended pancreatic acini of a preparation of CCK-8 (closed symbols) against a standard (open symbols). Each point represents the mean from three separate incubations. Estimated potency of the test preparation was 95% of the standard with limits of error ($P = 0.95$) of 85–117%.

CCK-33/39 were assayed against respective standards. Results obtained with the ^{45}Ca efflux method were compared with results using the in-vivo method based on measurement of gall-bladder contraction in-situ in the guinea-pig (Ljungberg 1969). The results (Table 3) were similar with both methods but the assays with the in-vitro method had greater statistical weights (Table 3). Particularly, when formulations of CCK-33/39 were tested, it was important to include albumin in the solvent, since the commercial preparations appeared to lose more activity than the standard preparation in the absence of albumin in the solvent (Table 4). Presumably, loss of activity was due to adsorption of CCK-33/39 to plastic surfaces.

In separate experiments, the standard preparation of CCK-33/39 dissolved in saline containing 1% albumin was assayed with the ^{45}Ca-efflux method against the same

Table 1. Potency estimates obtained with ^{45}Ca efflux or amylase secretion assays of two CCK-8 and one CCK-33/39 preparations tested against similarly diluted solutions of the same preparations used as standards.

Assay	Test = Standard Preparation	Potency (% of standard)	Limits of error ($P=0.95$)	Statistical weight	No. of assays
^{45}Ca-efflux	CCK-8-a	93	93–107	4566	6
^{45}Ca-efflux	CCK-8-b	104	94–106	5710	4
^{45}Ca-efflux	CCK-33/39-a	96	93–108	4008	4
Amylase	CCK-8-a	95	92–109	3057	4
Amylase	CCK-8-b	98	91–110	2719	2

Table 2. Potency estimates of one CCK-8 preparation diluted to various concentrations and assayed by measurement of ^{45}Ca efflux.

Potency (% of standard)		Limits of error	Statistical	Index of	No. of
Nominal	Estimated	($P=0.95$)	weight	precision	assays
100	105	87–114	1459	0.070	1
90	93	92–108	4009	0.042	1
80	77	90–111	2362	0.052	1

Table 4. Potency estimates obtained with gall-bladder assay of one commercial preparation of CCK-8 and two of CCK-33/39 dissolved in saline with and without 0.1% bovine serum albumin.

Preparation	Dissolved in albumin	Potency (% of standard)	Limits of error ($P=0.95$)	Statistical weight	No. of assays
CCK-8-c	–	81	84–120	698	2
CCK-8-c	+	81	83–121	611	2
CCK-33/39-b	–	115	87–114	1206	3
CCK-33/39-b	+	187	72–139	205	2
CCK-33/39-c	–	86	81–124	501	2
CCK-33/39-c	+	143	86–117	910	3

Table 3. Potency estimates of one CCK-8 and two CCK-33/39 preparations assayed by measurement of ^{45}Ca efflux in-vitro or by measurement of gall-bladder contraction in-vivo.

	^{45}Ca efflux				Gall-bladder contraction			
Preparation	Potency (% of standard)	Limits of error ($P=0.95$)	Statistical weight	No. of assays	Potency (% of standard)	Limits of error ($P=0.95$)	Statistical weight	No. of assays
CCK-8-c	108	93–107	4588	2	81	83–121	611	2
CCK-33/39-b	202	87–114	1319	2	187	72–139	205	2
CCK-33/39-c	158	90–111	1998	5	143	86–117	910	3

preparation with 0.1% albumin in the solvent. When 1% albumin was used, the potency of the preparation was found to be 124% (limits of error 93–108% $P=0.95$) of that for CCK-33/39 dissolved in saline with 0.1% albumin. Thus, 1% albumin may be optimal for reduction of adsorption of CCK-33/39 to glass and plastic surfaces.

Discussion

The present results demonstrated that an accurate, sensitive, and precise bioassay for CCK can be developed on the basis of the stimulatory effect of CCK on efflux of ^{45}Ca from pancreatic acinar cells from guinea-pigs. The calcium outflux assay tended to be more precise than the assay based on stimulation of amylase release. It is also more specific than the amylase assay, since amylase secretion from pancreatic acini is also stimulated by some other gastrointestinal hormones such as vasoactive intestinal peptide (VIP) and secretin (Peikin et al 1978) which do not affect the calcium homeostasis of these cells (Gardner et al 1975). CCK-like peptides, such as gastrin have been reported to be 50 000 times less potent in stimulating calcium efflux than CCK (Gardner et al 1975) and would therefore interfere with the assay only at very high concentrations. Substance P and other tachykinins stimulate outflux of calcium from pancreatic acinar cells (May et al 1978; Sjödin et al 1980). However, substance P and CCK-like peptides, do not generate parallel dose-response curves (May et al 1978; Sjödin et al 1980). On the other hand, bombesin and acetylcholine are two agents which evoke similar effects on calcium outflux as CCK (Gardner et al 1975; May et al 1978). The stimulatory effect of these substances could be antagonized by the bombesin receptor antagonist (D-Phe^{12}) bombesin (Heinz-Erian et al 1987) and atropine (Gardner et al 1975) without affecting the response to CCK. On the other hand, addition of a specific CCK-receptor antagonist, such as dibutyryl cyclic GMP (Peikin et al 1979) or L364718(3*s*(—)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-1H-indole-2-carboxamide) (Chang & Lotti 1986), should block the effect of CCK. Therefore, even if the stimulating effect on calcium efflux from acinar cells is not specific for CCK, tools are available to elucidate whether recorded effects are due to CCK in cases when that is uncertain.

Secretion of amylase is sensitive to somewhat lower concentrations of CCK than that required to stimulate calcium outflux (Sjödin et al 1980; Jensen & Gardner 1981). Therefore, a bioassay based on amylase secretion is the method of choice for determining picomolar concentrations of biologically active CCK in plasma (Liddle et al 1985) but is less advantageous for potency assessments of pharmaceuti-

cal formulations where stress is on precision and specificity rather than on maximal sensitivity.

The calcium efflux bioassay appears to be a practical alternative to the in-vivo gall-bladder contraction method since the two methods gave similar results when commercial preparations of CCK-8 or CCK-33/39 were assayed for potency. Results from the calcium efflux experiments had greater statistical weights than the average in-vivo results. Furthermore, the capacity for CCK bioassays is increased by using the in-vitro method by at least a factor of three. Thus, the presented bioassay has advantages for a control laboratory with limited resources. It also means that the need for laboratory animals for such assays is reduced.

To obtain valid estimates of the potency of CCK preparations, it is important to reduce the tendency for CCK-33/39 and to a lesser extent CCK-8 to adsorb to glass and plastic surfaces which has earlier been discussed by Solomon et al (1984). Thus, the in-vivo bioassay of Ljungberg (1969) which was introduced in the Nordic Pharmacopoeia (1975) should be modified to include bovine serum albumin or other suitable carrier proteins in the solvent for CCK. The reason why the commercial preparation of CCK lost approximately 1·6 times more activity than the standard when dissolved in the absence of albumin is not clear. The formulations differ from the standard only by containing a stabilizing additive, mannitol, at a concentration 20 mg per 75 Ivy Dog Units of CCK.

In conclusion, two in-vitro bioassays useful for pharmaceutical formulations of CCK based on the ability of the hormone to stimulate an increased calcium outflux from suspended pancreatic acinar cells or secretion of amylase from pancreatic acini are described. The ^{45}Ca efflux method is more precise and less resource demanding than the established in-vivo method for similar purposes.

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