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EVIDENCE THAT RELAXATION OF HOG BILIARY MUSCLE IS MEDIATED BY THE INTERACTION BETWEEN THE PROTEIN INHIBITOR OF CYCLIC AMP DEPENDENT PROTEIN KINASE AND CHOLECYSTOKININ *C*-TERMINAL PEPTIDES

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Abstract—The interaction of various cholecystokinin (CCK) peptides with the protein inhibitor (PK-I) of cyclic AMP dependent protein kinase (A-PK) has been studied. The order of the affinities (ED_{50}) for relaxation of hog biliary muscle by a series of *C*-terminal peptides of CCK correlated with the order of the potencies for A-PK-catalyzed phosphorylation of the sarcoplasmic reticulum enriched fraction (SR-F) in the muscle. CCK-4 peptide inhibited the PK-I of A-PK. The apparent K_m value of the peptide was 11.5 μM. Scatchard plot analysis for the binding of [^{14}C]CCK-6 peptide to the PK-I showed a dissociation constant (K_d) of 11.2 μM. These results indicated that the K_d value agreed with the ED_{50} value of CCK-6 and the K_m value of CCK-4. It is proposed that a receptor for CCK *C*-terminal peptides is probably a component in the PK-I; binding of the peptides to this site usually results in the relaxation by these allosteric inhibitors of the PK-I.

In our previous paper [1] we reported that the *C*-terminal 4 peptide of cholecystokinin (CCK-4) activated cyclic AMP dependent protein kinase (A-PK) induced phosphorylation of the sarcoplasmic reticulum enriched fraction (SR-F) obtained from biliary smooth muscles. Activation induced by CCK-4 was demonstrated to correspond closely with the increase in Ca uptake by phosphorylated SR-F. These findings have suggested the possibility that CCK-4 peptide may act on the protein inhibitor (PK-I) of A-PK [1], because the increase both in A-PK-catalyzed phosphorylation and in Ca uptake of SR-F produced by CCK-4 peptide was inhibited by PK-I.

Following the report by Walsh *et al.* [2] describing purification of PK-I from skeletal muscle, many investigators have studied the properties of this inhibitor [3–9]. PK-I was also found as a heat-stable protein from cardiac muscle [10–12], but there are no reports concerning the PK-I isolated from smooth muscle. In the present studies, the PK-I was isolated from hog biliary muscle and its interaction with CCK-6 peptide was investigated to obtain additional evidence for the mechanism of A-PK activation of the peptide.

MATERIALS AND METHODS

Tension recording. Circular segments of bile ducts in domestic hogs (*ca.* 90 kg) were prepared by cutting ampullae after removing fatty tissue, connective tissue, vessels and mucosa. The segments (10 mm in length and 1–2 mm in width) were loaded with 1 g and mounted in an organ bath containing 5 ml of Tyrode's solution bubbled continuously with 95% O_2 and 5% CO_2 at 37°. Tension was isotonically recorded with a strain gauge for the relaxation induced by CCK *C*-terminal peptides.

A-PK assay. The SR-F was prepared by the method of Katz and Repke [13] with some modifications [1, 14]. The PK-I was prepared from terminal bile ducts by the procedures of Donnelly *et al.* [5] and Kuo and Kuo [12] with some modifications [1], and was shown as a single band in 7% polyacrylamide gel electrophoresis. The source of A-PK was the supernatant fraction obtained from homogenates of biliary muscles centrifuged at 105,000 *g* for 1 hr. The assay of A-PK was performed after the muscles were incubated with and without drugs in Tyrode's solution as described previously [1, 14]. The reaction medium (0.25 ml) consisted of 8 mM phosphate buffer (pH 7), 1.6 mM theophylline, 8 mM NaF, 12 mM Mg acetate, 1 μCi/ml [γ-^{32}P]ATP, 10 μM ATP, SR-F (26.4–40.2 μg protein), PK-I (0–3.2 μg protein) and A-PK (30.5–55 μg protein) with 4 μM cyclic AMP. The medium was incubated at 37° for 10 min. Protein concns of fractions were determined by the method of Lowry *et al.* [15] using bovine serum albumin as a standard.

Binding assay of CCK-6 peptide to PK-I. The binding assay was carried out as follows: PK-I (28.7 μg protein) was incubated at 37° in Tyrode's solution (0.25 ml) containing 1 μM [1-glycyl-^{14}C]CCK-6 with different concns of unlabelled CCK-6. After stopping the reaction by adding ice-cold Tyrode's solution (1 ml), ice-cold 35% trichloroacetic acid (TCA) (0.5 ml) containing 7.5 mg

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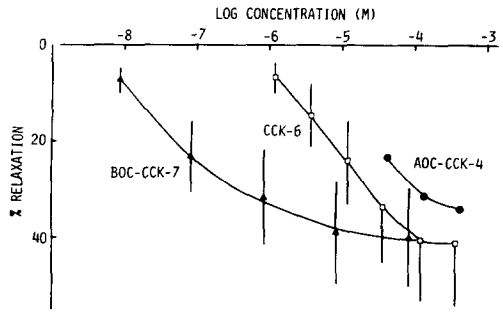

Fig. 1. Dose-dependent relaxation of *C*-terminal peptides of CCK on circular segments in hog biliary muscle. The % relaxation was presented with the mean ± S.E. as a percentage for the maximal relaxation induced by Ca-free Tyrode's solution. Note that the ED_{50} of these peptides were 22.3 μM (N = 2) for AOC-CCK-4, 9.3 μM (7.1–12.6) (N = 5) for CCK-6 and 0.105 μM (0.062–0.178) (N = 3) for BOC-CCK-7, respectively.

"Hyfrosuper celite" was added to aggregate small amounts of protein. The resulting pellets were centrifuged at 2000 *g* for 15 min and rinsed twice with ice-cold 10% TCA. The pellets were solubilized with 1 N NaOH and counted in a liquid scintillation counter.

Materials. The *C*-terminal peptides of CCK used were *t*-butyloxycarbonyl (BOC)-CCK-7, BOC-CCK-6, CCK-6 and [1-glycyl-^{14}C]CCK-6 (36.7 mCi/mmole). These were supplied by Dr G. Tsukamoto of the Kanebo Laboratory, CCK-33 (15% pure, 300 IDU/mg) from Dr S. Tachibana of the Eisai Laboratory, *t*-acetyloxycarbonyl (AOC)-CCK-4 from Nihon Kayaku Ltd, and CCK-8 from the Squibb Institute for Medical Research (Princeton, NJ). [γ-^{32}P]ATP (2 Ci/mmole) was commercially obtained from the Radiochemical Centre (Amersham, U.K.).

RESULTS

Correlation between relaxation and phosphorylation caused by CCK C-terminal peptides in the biliary muscle

The relaxing effects of CCK-peptides were measured and compared with their A-PK activating potency of the SR-F in hog biliary muscle. AOC-CCK-4, CCK-6 and BOC-CCK-7 caused a usual relaxation of the circular segments of the muscle; relative potencies of these peptides were 1, 2.4 and 212, respectively (Fig. 1). On the other hand, the peptides did not cause relaxation of the longitudinal segments of the muscle, but inhibited the contraction induced by 5.5 μM ACh. However, the peptides were equally potent in this inhibitory effect.

The relative potencies of A-PK activity with the saturable concn of cyclic AMP induced by AOC-CCK-4, BOC-CCK-6, CCK-6, CCK-33 and CCK-8 were 1, 10, 10, 17 and 30, respectively (Fig. 2). These results indicate that CCK-8 is perhaps 30 times more

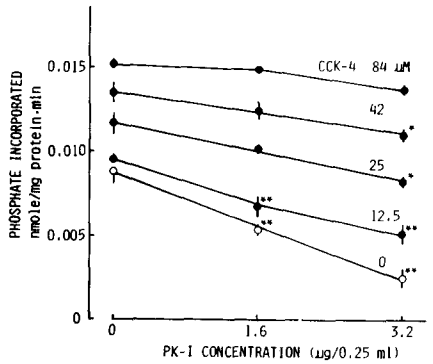

Fig. 3. Effect of the protein inhibitor on activation by CCK-4 peptide of cyclic AMP dependent protein kinase catalyzed phosphorylation of the sarcoplasmic reticulum enriched fraction. Protein kinase (50 μg/0.25 ml) was assayed at a saturable concn of cyclic AMP (4 μM) in the presence (●) or absence (○) of the peptide. These values were derived by subtracting values of phosphorylation in the absence of cyclic AMP (4 μM) (0.02194 ± 0.00021 nmoles/mg protein/min) (N = 3) from those in the presence of it. * and ** mean significantly different values in the presence of the inhibitor from those in the absence of it at P = 0.05 and 0.01, respectively.

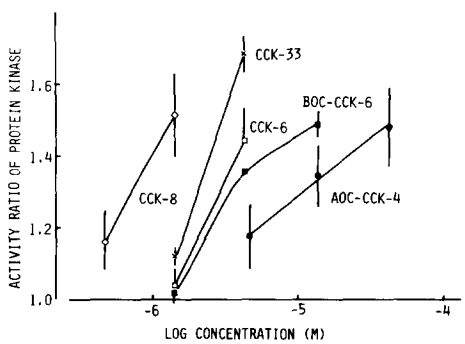

Fig. 2. Concn-dependent activation of *C*-terminal peptides of CCK on cyclic AMP dependent protein kinase catalyzed phosphorylation of the sarcoplasmic reticulum enriched fraction of hog bile ducts. The activity ratio of protein kinase (50 μg/0.25 ml) assayed under the saturable concn of cyclic AMP (4 μM) is expressed by the mean ± S.E. (N = 3) as the ratio of protein kinase activity incubated with drugs to the activity obtained without drugs (0.0125 ± 0.0010 nmoles/mg protein/min) (N = 27).

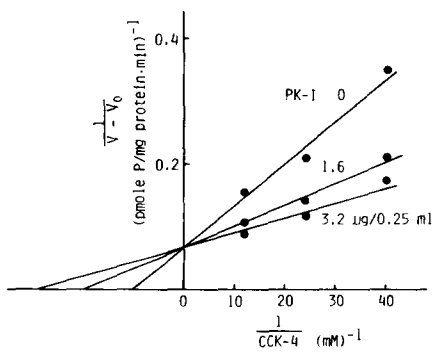

Fig. 4. Double-reciprocal plots for effects of the PK-I on CCK-4-induced phosphorylation by cyclic AMP dependent protein kinase. The increased rate of phosphorylation induced by CCK-4 peptide was calculated by subtraction of the rate of phosphorylation at zero CCK-4 (V_0) from data in Fig. 3. The inhibitor at 1.6 and 3.2 μg/0.25 ml activated the effect of CCK-4 peptide. Note that three lines cross the same point on the ordinate, indicating competitive interactions.

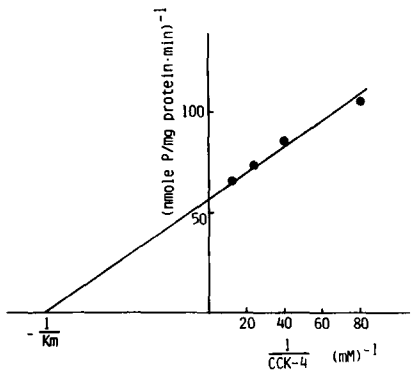

Fig. 5. Double-reciprocal plots for activating effects of CCK-4 peptide on cyclic AMP dependent protein kinase catalyzed phosphorylation of the sarcoplasmic reticulum enriched fraction. Data in the absence of the additional PK-I in Fig. 3 were replotted. Note that CCK-4 inhibited the endogenous PK-I activity and that its apparent K_m value was 11.5 μM.

potent than AOC-CCK-4 if one takes the approximate midpoint of the curves. Both BOC-CCK-6 and CCK-6 showed the same effect on A-PK activation, indicating that the protecting moiety (BOC) had no influence on A-PK activation.

Possible interaction between the PK-I and CCK-4 peptide

We have investigated the question whether the activation of A-PK by CCK-peptides is affected by the PK-I. As shown in Fig. 3, the phosphorylation of the SR-F was increased by increasing concns of CCK-4 peptide; the effect was inhibited by the PK-I in a concn-dependent fashion. When the data of Fig. 3 were replotted by the double-reciprocal method, the straight lines in Fig. 4 were obtained. Although the PK-I added exogenously inhibited CCK-4 peptide induced phosphorylation of the SR-F, there was no change in the V_{max} of the phosphorylation. The results indicate that CCK-4 peptide does interact with exogenously added PK-I resulting in an enhanced activity of the phosphorylation of the SR-F. This finding strengthens the concept that CCK-4 peptide interacts with endogenous PK-I in the fraction of A-PK (Fig. 5). The apparent K_m value of CCK-4 peptide for inhibition of the endogenous PK-I activity modulating A-PK was 11.5 μM.

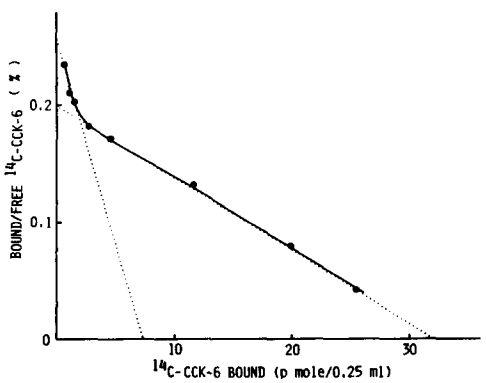

Fig. 6. Scatchard plot of binding of [1-glycyl-^{14}C]CCK-6 peptide to the protein inhibitor of cyclic AMP dependent protein kinase. The protein inhibitor (28.7 μg/0.25 ml) was incubated for 5 min at 37° with [^{14}C]CCK-6 (1 μM) added along with different concns of unlabelled CCK-6. Note that the inhibitor had two binding sites for CCK-6, one high-affinity (K_d = 11.2 μM) and the other low-affinity (K_d = 64.0 μM).

Binding of CCK-6 peptide to the PK-I

Direct evidence for interaction between CCK-peptides and the PK-I was obtained from binding studies. Maximum binding of the [^{14}C]CCK-6 peptide (1 μM) incubated with the PK-I at 37° in Tyrode's solution occurred within 5 min. Binding was both specific and non-specific as shown by displacement with unlabelled CCK-6. At 1 mM, 46.5% of radioactivity was displaced and thus 53.5% may be considered to be non-specifically bound. As shown in Fig. 6, Scatchard plot analysis of these data indicated one high- and one low-affinity site of CCK-6 peptide with values of 11.2 and 64 μM, respectively.

The apparent K_d values of the high- and low-affinity sites in the PK-I, the ED_{50} value observed by the relaxing assay of the muscle (Fig. 1) and the K_m value observed by the double-reciprocal plot (Fig. 5) are shown in Table 1. The results indicate that the K_d value in the high-affinity site of the PK-I for the peptide is in excellent agreement with the ED_{50} and K_m values, and suggest that the site of action *in vivo* of CCK-4 and CCK-6 peptides resembles the site on the PK-I.

DISCUSSION

The results of the present studies demonstrated that CCK C-terminal peptides usually caused relaxation only in the circular segments of terminal bile ducts. The minimal concns of these peptides that caused relaxation were similar to those which activated A-PK in the biliary muscle. In addition the order of affinities of both muscle relaxation and A-PK activation were parallel in a series of C-terminal peptide molecules structurally related to CCK. These findings provide further evidence supporting our previously suggested mechanism [1], by which CCK-4 peptide directly activates A-PK, followed by the phosphorylation of the SR-F to promote uptake of Ca ions, thereby inducing the relaxation of biliary muscle. Some reports showed that CCK-8 increased the level of cyclic AMP in the isolated sphincter of

Table 1. Dissociation constants (K_d) of CCK-6 for the protein inhibitor, K_m of CCK-4 in cyclic AMP dependent protein kinase activation, and ED_{50} of CCK-6 in producing relaxation of biliary smooth muscle

	Binding site	
	High-affinity	Low-affinity
K_d of CCK-6 (μM)	11.2	64
ED_{50} of CCK-6 (μM)	9.3 (7.1–12.6)	
K_m of CCK-4 (μM)	11.5	

Oddi of the cat, suggesting that a metabolic effect is important for the initiation of the relaxation by the hormone [16]. We demonstrated previously that CCK-4 peptide caused little increase of cyclic AMP compared with that by isoproterenol [1]. It would appear that an increase of cyclic AMP levels has little influence on A-PK activation induced by CCK *C*-terminal peptides, because in the present studies we also measured the A-PK activation by these peptides under a saturable concn of cyclic AMP (actually dibutyryl cyclic AMP) sufficient for maximal activation.

The evidence of mechanisms whereby CCK-4 or CCK-6 peptides directly interact with A-PK was obtained by the binding experiments using PK-I. The extracted PK-I caused the activation of phosphate incorporation of the SR-F induced by CCK-4 (Fig. 4), suggesting that CCK-4 peptide interacts directly with the PK-I in A-PK, irrespective of the increase in the level of cyclic AMP. The high-affinity binding site (11.2 μM) is consistent with an affinity constant (ED_{50}) of 9.3 μM (7.1–12.6) obtained from the *in vivo* experiment of the usual relaxation in circular segments of biliary muscle. These findings suggest that CCK-6 peptide causes the activation of A-PK by a mechanism which involves the high-affinity binding component in the PK-I of biliary muscle. On the other hand, the PK-I was found to have another binding recognition site of the low-affinity type for CCK-6 peptide. Recent studies have shown that the sphincter of Oddi has two receptors for CCK: one inhibitory, non-cholinergic, non-adrenergic, present in post-ganglionic neurons; the other excitatory, present in the circular muscle [17]. These findings do not seem to correspond with our findings, because these receptors mediate different types of responses. The low-affinity component may play a role in the allosteric site for CCK-6 peptide. Our finding also indicates that the apparent K_m of 11.5 μM for CCK-4 peptide binding to the PK-I (Fig. 5) agrees with the apparent K_d of 11.2 μM for CCK-6 peptide. This suggests that a series of *C*-terminal peptide molecules related chemically to CCK may bind to the same site of the PK-I.

Our observation is perhaps the first finding that the PK-I is possibly the site of action of the biological substance. The possibility may be also supported by the following reports. Recent results indicate that the PK-I (type 1) may serve as a regulator of the phosphorylation catalyzed by the cyclic AMP dependent system in rat heart [18]. It is becoming increasingly evident that CCK-4 peptide and the fragments of CCK *C*-terminal peptides are endogenous substances which are found in large quantities in the CNS. A recent report [19] showed that benzodiazepines bound to an endogenous protein inhibitor. The action of these peptides in the CNS may also be associated with a site of the PK-I. In biliary muscle, we conclude that the receptor for CCK *C*-terminal peptides is probably some component in the PK-I; this finding is considered to offer a clue as to the control of the usual relaxation of this tissue.

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