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RELATIONSHIP BETWEEN CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVATION AND Ca UPTAKE INCREASE OF SARCOPLASMIC RETICULUM FRACTION OF HOG BILIARY MUSCLES RELAXED BY CHOLECYSTOKININ-C-TERMINAL PEPTIDES

MASAYASU KIMURA,* IKUKO KIMURA and SHINJIRO KOBAYASHI

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

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Abstract—In hog terminal bile duct cholecystokinin peptides caused an activation of cyclic AMP-dependent protein kinase (A-PK) with cyclic AMP, followed by increase in Ca uptake of sarcoplasmic reticulum fraction (SR-F). By contrast, papaverine showed no activation of A-PK-induced Ca uptake by SR-F with cyclic AMP. The Ca uptake by SR-F was dependent on ATP and Mg^{2+}, but the component phosphorylated was not the phosphoenzyme intermediate in Ca^{2+}-ATPase. The effect of Ca uptake was blocked by the inclusion of a protein inhibitor of A-PK. The correlation coefficient between cyclic AMP-dependent SR-F phosphorylation and stimulated Ca uptake by the phosphorylated SR-F was 0.731 ($P < 0.01$). These results suggest that one of the mechanisms by which CCK-4, CCK-8, and CCK-33 peptides relax isolated Oddi's sphincters of terminal bile ducts is activation of A-PK-induced Ca uptake by sarcoplasmic reticulum fraction and possibly also by plasma membrane.

Cholecystokinin (CCK), one of gastrointestinal hormones, also has an effect on biliary smooth muscles to accelerate bile flow into duodenum as a result of contraction of gallbladder and relaxation of sphincter of Oddi. The mechanisms, however, by which CCK causes the relaxation of Oddi's sphincter, remain unknown. At present, drug-induced relaxation of smooth muscles has been considered to result from a series of events: the stimulation of adenylate cyclase, an increase in cyclic AMP levels, the activation of cyclic AMP-dependent protein kinase (A-PK), the activation of Ca-ATPase, and a reduction in intracellular free Ca^{2+} level.

The effect of CCK was reported by Andersson *et al.* [1, 2] to be connected with changes in the intracellular concentration of cyclic AMP, which is probably increased by stimulation of adenylate cyclase. On the other hand, a close relationship between A-PK-catalysed phosphorylation and Ca uptake stimulation was reported in cardiac sarcoplasmic reticulum fraction (SR-F) [3] and in uterine microsomal fraction [4]. We also proposed that 3-(2′-hydroxy-4′,5′-di-ethoxybenzoyl) propionic acid, a biliary dilator, activates A-PK in biliary smooth muscles [5].

The aim in this report is to obtain evidence for a possible relation between A-PK phosphorylation and Ca uptake acceleration of the SR-F of hog bile ducts stimulated by CCK and its C-terminal peptides, and thereby to indicate that the A-PK has a vital role for the muscle relaxation induced by these peptides.

* Author to whom correspondence and reprint requests should be addressed.

MATERIALS AND METHODS

Assay for dilation of Oddi's sphincter and for contraction of gallbladder. Using techniques described by Crema and Berté [6] and by Kimura and Kagami [7], cumulative dose–response curves were obtained as a per cent of increase in the drop counts induced by 0.253 mM theophylline (ThP) through Oddi's sphincters of domestic hogs; this concentration of the drug produces a maximum response. Cumulative dose–isometric tension curves of gallbladders of domestic hogs were expressed as a per cent of maximum contraction induced by 0.55 mM acetylcholine.

Preparation of sarcoplasmic reticulum fraction. The SR-F was prepared by the procedure described in our previous paper [5]. This SR-F, when negatively stained with 5% uranyl acetate, had the same characteristics as SR-F obtained from rabbit skeletal muscles [8, 9] in the electron microscope (Fig. 1). The SR-F was heated to 78–84° for 2 min to inactivate several interfering enzymes [10] for the A-PK assay.

Preparation of protein kinase. A-PK was prepared from the supernatant fractions of 0.9 g of terminal bile ducts of domestic hogs. The ducts were minced, incubated in 9 ml Tyrode solution with or without drugs for 10 min at 37°, homogenized in a Potter homogenizer for 3 min at 4°, and centrifuged at 105,000 *g* for 1 hr at 4°. A-PK assay was carried out in 0.25 ml of the medium containing 12 mM Mg acetate, 1 μCi/ml ATP-γ-^{32}P, 10 μM ATP, 1.6 mM theophylline, 8 mM NaF, 8 mM phosphate buffer (pH 7.0), 4 μM cyclic AMP, SR-F and 38–55 μg A-PK, as previously described [5].

Assay for Ca uptake. Ca uptake procedure were those of Kirchberger *et al.* [3]. Ca–EGTA buffer

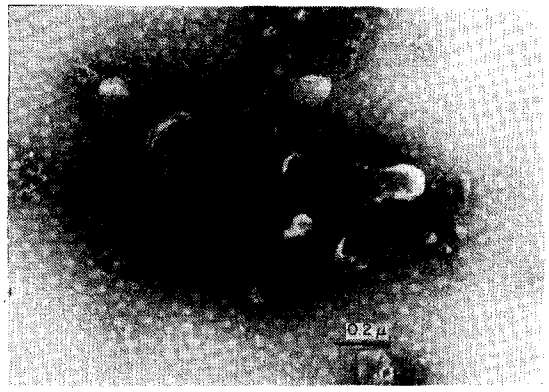

Fig. 1. Electron micrograph of sarcoplasmic reticulum fractions (SR-F) obtained from terminal bile ducts of domestic hogs, stained negatively with 5% uranyl acetate. The scale shows 0.2 μm. Note homogeneous vesicles of approximately 0.1–0.2 μm diameter with membranes of 20 nm in thickness.

solution was prepared as follows: $CaCl_2$-diethylamine solution was rapidly titrated with 10 mM EGTA-diethylamine solution in the presence of 0.4% calcon–methanol solution. The free Ca^{2+} concentration present in a mixture of 125 μM $CaCl_2$ and 391 μM EGTA at pH 6.8 was calculated using the association constant of $4.4 \times 10^5\ M^{-1}$ for the Ca–EGTA complex [11], using the assumption that the constant does not change with temperature (0–40°) [12]. One μM Ca^{2+} was reported by Tada *et al.* [13] to be a submaximal concentration inducing Ca^{2+}-activated ATPase activity. One ml of the A-PK assay medium together with unlabelled ATP was added to the following assay medium for measuring Ca uptake. The final composition was 40 mM histidine–HCl buffer (pH 6.8), 120 mM KCl, 13.2–22.2 μg SR-F, 5 mM ATP, 5 mM $MgCl_2$ and Mg acetate, Ca–EGTA buffer (Ca^{2+} = 1 μM; EGTA 391 μM, $CaCl_2$ 125 μM) containing $^{45}CaCl_2$ (0.08 μCi/ml), 0.4 mM theophylline, and 2 mM NaF. The solution was kept on ice until Ca–EGTA buffer and $^{45}CaCl_2$ were added to the reaction medium (total 0.5 ml), and then was incubated at 37° for 3 min. The mixture was filtered through a millipore filter (0.45 μm), and washed with 0.5 ml of 120 mM KCl in the histidine buffer solution. The filtrate was counted with a liquid scintillation spectrometer.

Assay for cyclic AMP level. Cyclic AMP levels in the terminal bile duct and in gallbladder were measured by the sensitive radioimmunoassay as described by Honma *et al.* [18].

Characterization of phosphorylation of SR-F. For trypsin treatment, the method of Tada [14] was followed. The SR-F (200–267 μg) was digested with trypsin at 37° in 0.75 ml of the histidine buffer, 120 mM KCl, and 1 M sucrose. The weight ratio of trypsin to SR-F was 1:20. The reaction was started by the addition of trypsin. Aliquots taken at various time intervals were placed in tubes containing trypsin inhibitors (2 times the weight of trypsin). The tubes were kept on ice until aliquots of this mixture were subjected to assay for Ca uptake and phosphorylation. This brief digestion with trypsin in the presence of 1 M sucrose did not significantly affect microsomal calcium transport activity [14].

Stability of phosphorylated protein. Stability in hydroxylamine solution of phosphorylated SR-F was studied as follows; the pellets obtained by centrifugation were treated with 10 ml of 0.8 M hydroxylamine and 50 mM sodium acetate (pH 5.4) as described by Lipmann *et al.* [15]. This treatment was terminated by the addition of 15 ml of 10% trichloracetic acid (TCA).

Preparation of protein inhibitor of protein kinase. The protein inhibitors of A-PK (PK-I) were prepared from bile ducts as described by Tada *et al.* [16] and Kuo *et al.* [17]. Hog bile duct segments (100 g) were finely dissected with scissors in 5 vol. of ice-cold 10 mM Tris–HCl buffer (pH 7.5) and homogenized in a Waring Blender for 3 min. The supernatant obtained by centrifugation (15,000 *g*, 15 min) was boiled for 10 min. To the supernatant 50% TCA (5% final concentration of TCA) was added. The resultant precipitate was centrifuged, resuspended in 100 ml distilled water, and adjusted to pH 7.5 prior to dialysis against 200 vol. of distilled water at 4°. The supernatant obtained by centrifugation (15,000 *g*, 15 min) was left overnight after adding solid ammonium sulfate (35 g/100 ml). The resultant precipitate was dissolved in 4 ml of 5 mM phosphate buffer (pH 7.0) and dialysed against 3 litres of the phosphate buffer for 48 hr. The supernatant was applied to a column (2.3 × 65 cm) of Sephadex G100 equilibrated with the phosphate buffer, and was eluted with the same buffer. Sixty ml of the eluent containing PK-I were pooled and were concentrated on a Dia-filter G-01 membrane (BEC) to 17 ml.

Materials. CCK-33 (15% pure, 300 Ivy dog unit/mg) and CCK-8 peptides were kindly supplied by Dr. S. Tachibana (Eisai) and by the Squibb Institute for Medical Research (Princeton, NJ), respectively. CCK-4 peptide (Nihon Kayaku) was dissolved in 0.1 N $NaHCO_3$ solution and diluted with Tyrode's solution. The other compounds used as follows: ATP·2 Na (Kowa), cyclic AMP (Kohjin), papaverine·HCl, and DL-isoproterenol·HCl (Nakarai), ATP-γ-^{32}P sodium salt in 50% ethanol solution (1.66–2.88 Ci/mmole, 1 mCi/ml, Radiochemical Centre) and $^{45}CaCl_2$ aqueous solution (0.479 Ci/mmole, 20.8 mCi/ml, Radiochemical Centre). Cyclic AMP, ^{125}I-succinyl cyclic AMP tyrosine methyl ester, and anticyclic AMP antiserum were commercially available from the Yamasa cyclic nucleotide assay kit.

RESULTS

Relation among effects of CCK-4 peptide on mechanical response, cyclic AMP levels, and A-PK-catalysed phosphorylation of biliary muscles

In the upper graph of Fig. 2, CCK-4 peptide-induced dose–response curves were compared for the relaxation of Oddi's sphincter and the contraction of gallbladder. The concentration-dependent range of these two effects exactly overlap.

These differences of both tissues in response to CCK-4 peptide did not seem to be brought out by the difference in cyclic AMP level. The effects of CCK-4 peptide on cyclic AMP levels in terminal bile ducts were increased much less by the addition of relatively high concentrations of CCK-4 peptide than

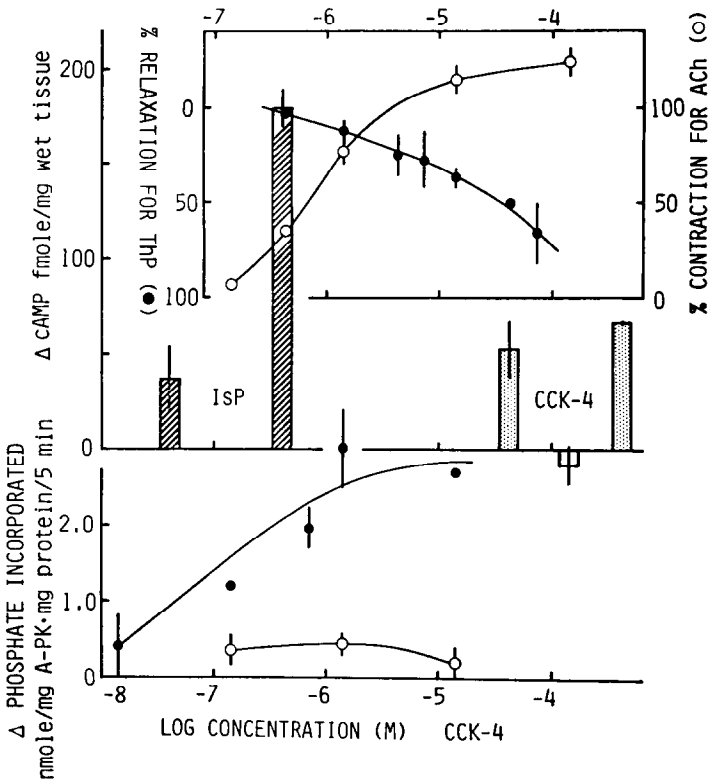

Fig. 2. The relationship among pharmacological responses, the changes in cyclic AMP levels and A-PK-catalysed SR-F phosphorylation with 4 μM cyclic AMP induced by CCK-4 peptide in terminal bile ducts and gallbladders ± S.E. (N = 3–6). Top: % relaxation (% decrease in flow rates (●) through Oddi's sphincters) as a per cent of the maximal response induced by 0.253 mM theophylline, and % contraction (○) in gallbladders of 0.55 mM acetylcholine-induced contraction. Middle: the changes in cyclic AMP levels of terminal bile ducts induced by isoproterenol (hatched columns) and CCK-4 peptide (dotted columns), and those of gallbladders induced by CCK-4 peptides (open columns). The unstimulated control values are 271 ± 18 fmole/mg wet tissue (N = 14) for terminal bile ducts, and 76 ± 21 fmole/mg wet tissue (N = 6) for gall bladders. Bottom: the changes in A-PK-catalysed SR-F phosphorylation with 4 μM cyclic AMP induced by CCK-4 peptide in terminal bile ducts (●) and gallbladders (○). The unstimulated control value was 4.42 ± 0.23 nmole/mg A-PK·mg protein/5 min (N = 9) for terminal bile ducts. The control value for gallbladders was 3.05 ± 0.34 nmole/mg A-PK·mg protein/5 min (N = 5). The horizontal axes in the top, middle and bottom panels represent log concentration of CCK-4 peptide. Experimental data in both the middle and bottom graphs were plotted as the differences in activities in the presence and absence of CCK-4 peptide.

by isoproterenol, despite the fact that at these concentrations both drugs markedly relaxed terminal bile ducts. In gallbladder CCK-4 peptide produced a very small decrease in cyclic AMP.

Concerning the activation of A-PK, the peptide was much more potent in terminal bile ducts than in gallbladders (the lower graph in Fig. 2). The graph shows the effect of CCK-4 on incorporation of phosphate into the SR-F by A-PK obtained from terminal bile ducts and gallbladders. The threshold concentration at which CCK-4 peptide induced relaxation of Oddi's sphincter was approximately 0.3 μM, slightly higher than the concentration at which CCK-4 peptide induced the activation of A-PK obtained from terminal bile ducts. In order to determine which kinase (that is, cyclic AMP-dependent or -independent) is affected by CCK-4 peptide, the protein kinase activity ratio [19] was measured. Incubation of the terminal bile duct with CCK-4 peptide resulted in a dose–dependent diminution in the ratio (Table 1), indicating that A-PK is activated by CCK-4 peptide.

The experiments in the next section on Ca uptake

Table 1. The protein kinase activity ratio of cyclic AMP-dependent protein kinase from terminal bile ducts pre-incubated with CCK-4 peptide

CCK-4 (μM)	Protein kinase activity ratio −cAMP/+cAMP (N)	
0.0	0.54 ± 0.02	16
0.7	0.54 ± 0.03	3
1.4	0.51 ± 0.03	3
14.2	0.47 ± 0.01*	3

The enzyme was assayed in the absence and presence of saturated exogeneous cyclic AMP (4 μM).
* $P < 0.05$.

Table 2. Effect of hydroxylamine on phosphorylation of SR-F by cyclic AMP-dependent protein kinase*

	A-PK activity†	
	Control	Hydroxylamine
SR-F	0.0208 ± 0.0024	0.0188 ± 0.0019‡
SR-F + A-PK	0.0414 ± 0.0009	0.0328 ± 0.0012
SR-F + A-PK with CCK-4	0.0510 ± 0.0021	0.0405 ± 0.0024

* The SR-F phosphorylated by A-PK from bile ducts preincubated with or without CCK-4 peptide (42 μM) was incubated for 10 min at 30° in the presence or absence of 0.8 M hydroxylamine.
† Unit; nmole phosphate incorporated/mg protein/min.
‡ Mean ± S.E. ($N = 3$).

of SR-F induced by A-PK could not be influenced by the external Ca^{2+} ions in the preincubation step of minced tissue (not shown in data).

In order to check the stability of phosphorylated SR-F, the SR-F was treated with 0.8 M hydroxylamine (Table 2). Treatment of control SR-F with the reagent resulted in complete recovery of the acid-precipitable ^{32}P-phosphate. Similarly, the recovery of acid precipitate of ^{32}P-phosphate formed in the presence of both cyclic AMP and A-PK was slightly reduced after treatment with the reagent (Table 2). The same effects were obtained in A-PK prepared from tissues previously incubated with CCK-4 peptide. The chemical stability of acid-precipitable ^{32}P-phosphate eliminated the possibility that ^{32}P-phosphate was incorporated into the acyl phosphate ATPase intermediate of the Ca transport system.

Confirmation of Ca uptake by SR-F obtained from terminal bile ducts

Further evidence for Ca uptake for SR-F obtained from terminal bile ducts was obtained by treatment with $MgCl_2$, ATP, and oxalate. Ca uptake was increased in a concentration-dependent manner by $MgCl_2$ and ATP in Ca^{2+} uptake assay (Fig. 3) and also by oxalate (Fig. 4). When not subsequently washed before assay of Ca uptake, SR-F preincubated with A-PK and cylic AMP showed enhancement of the initial rate of Ca uptake, depending on the concentration of oxalate. This is valid evidence of suitable experimental conditions for the Ca uptake by SR-F. On the basis of the time course of the oxalate effects (Fig. 4), 2.5 mM oxalate and an incubation time of 3 min were used in the subsequent experiment on Ca uptake.

Effect of CCK-C-terminal peptides on the A-PK phosphorylation—SR-F Ca uptake coupling compared with papaverine

Since CCK-4 peptide did not significantly increase cyclic AMP levels in the bile duct (Fig. 2), we investigated the possibility that the site of action of the peptide may be in the A-PK system. At a concentration of 20 μM dibutyryl cyclic AMP (not shown in data) or 4 μM cyclic AMP, 14.1 μM CCK-4 peptide caused activation of A-PK. This suggests that the effect of cyclic AMP on CCK-4 peptide-induced activation of A-PK can be produced both in the tissue and cell-free preparations. When terminal bile ducts were incubated in the presence of CCK-4 (4.65, 14.1 and 42.3 μM), CCK-8 (0.47 and 1.4 μM) and CCK-33 peptides (1.4 and 4.2 μM) in Tyrode's solution for 10 min at 37°, the following results were obtained: CCK-C-terminal peptides produced a

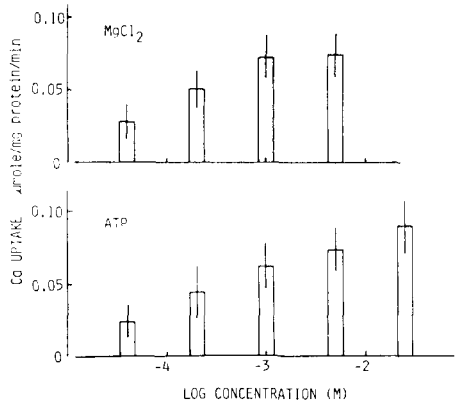

Fig. 3. Dependence on $MgCl_2$ and ATP concentrations of Ca uptake by SR-F obtained from terminal bile ducts. Five mM Mg^{2+} in the Ca uptake medium was used when ATP was varied and vice versa. The horizontal axes in the upper and lower parts represent log concentrations of $MgCl_2$ and ATP, respectively. The experimental data are absolute values ± S.E. ($N = 3$–4). Based on these results, we chose 5 mM $MgCl_2$ and 5 mM ATP as the reaction medium for Ca uptake in subsequent experiments.

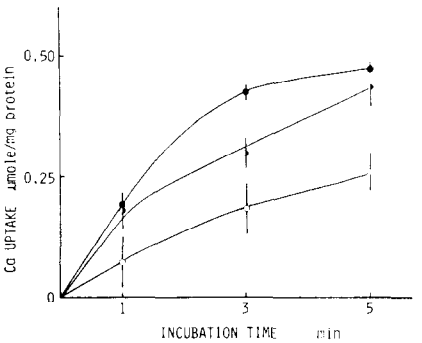

Fig. 4. A-PK-induced Ca uptake by SR-F for various incubation times in the presence of various concentrations of Tris–oxalate [1 mM (○), 2.5 mM (◑) and 5 mM (●)]. SR-F was preincubated with 0.2 mg/ml A-PK and 4 μM cyclic AMP. The experimental data are absolute values ± S.E. ($N = 3$–7). Based on these results, we chose an incubation time of 3 min and a concentration of 2.5 mM oxalate as the reaction medium for Ca uptake in subsequent experiments.

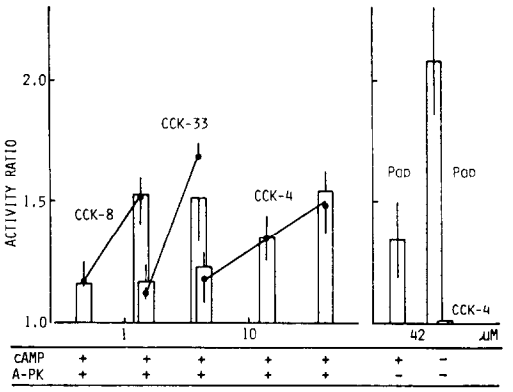

Fig. 5. Left: concentration-dependence of activation of both the phosphorylation (●) and the Ca uptake (columns) induced by A-PK prepared from tissue preincubated with CCK-33, CCK-8, or CCK-4 peptides. Activities are expressed as the ratio of A-PK activation or Ca uptake stimulation after incubatyon with CCK-peptides to those without CCK-peptides (± S.E., $N = 2$–4). Right: lack of activation of A-PK-independent (not induced by A-PK) Ca uptake by SR-F preincubated with 42 μM CCK-4 peptide for 10 min, and high degree of activation of Ca uptake by SR-F after incubation with 42 μM papaverine in the presence and absence of 4 μM cyclic AMP.

concentration-dependent increase (1.2–1.7-fold) in both A-PK-catalysed phosphorylation of SR-F and A-PK-induced Ca uptake by SR-F (Fig. 5). The order of potency was CCK-8 > CCK-33 > CCK-4 (Fig. 5). In contrast to CCK-4-induced Ca uptake by SR-F in the presence of A-PK, the Ca uptake by SR-F was not increased in the absence of A-PK. Papaverine (42.3 μM), which has been reported by us [5] to produce no activation of A-PK, increased Ca uptake by SR-F 2-fold without cyclic AMP and increased it 1.3-fold even with a saturated concentration (4 μM) of cyclic AMP (Fig. 5).

Effects of trypsin treatment and protein inhibitors of A-PK on A-PK-catalyzed Ca uptake by SR-F

The phosphorylation of unheated SR-F was increased to a lesser extent than that of pre-heated

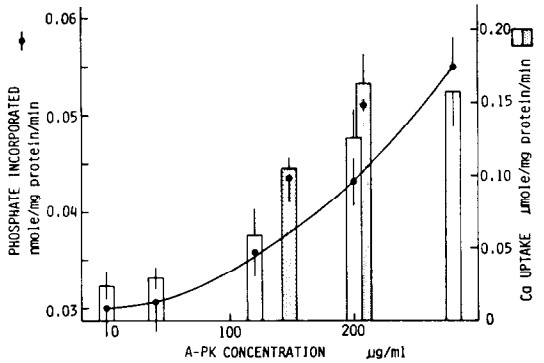

Fig. 6. Effect of A-PK and SR-F phosphorylation (●) and its catalysed Ca uptake (open columns) by native (unheated) SR-F. Activation of both the phosphorylation (upper ●) and Ca uptake (shaded columns) induced by A-PK prepared from tissues previously incubated with CCK-4 peptide (14 μM). The experimental data are absolute values ± S.E. ($N = 2$–5).

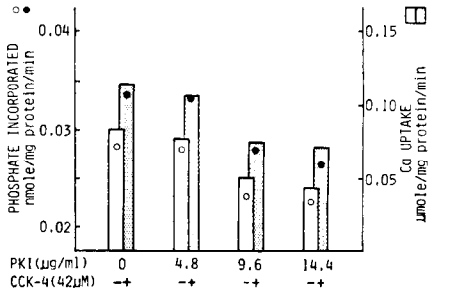

Fig. 7. Inhibition by PK-I of CCK-4 peptide-induced A-PK activation (circles) and A-PK stimulation of Ca uptake (columns) by the phosphorylated SR-F in terminal bile ducts of domestic hogs. Data without (open symbols) and with CCK-4 peptide (closed or shaded symbols) are absolute values ($N = 1$–4).

SR-F (data not shown), but the increasing phosphorylation of unheated SR-F induced by A-PK paralleled the increase in Ca uptake rate by unheated SR-F over a wide range of A-PK concentrations (Fig. 6). The A-PK preparation which was obtained from tissues preincubated in the presence of CCK-4 peptide (14.1 μM) was more highly activated in causing stimulation of both phosphorylation and Ca uptake than that incubated in the absence of CCK-4 peptide (Fig. 6).

Additional evidence of the correlation of A-PK-catalysed SR-F phosphorylation with A-PK-induced Ca uptake by SR-F was obtained by the effects of PK-I. Increasing amounts of PK-I incubated with A-PK and SR-F progressively inhibited A-PK-catalysed phosphorylation of SR-F. This was accompanied by a simultaneous decrease in the rate of Ca uptake stimulated by A-PK (Fig. 7). Increase in A-PK-catalyzed phosphorylation and Ca uptake of SR-F produced by 42 μM CCK-4 peptide were also inhibited by increasing amounts of PK-I, again suggesting that CCK-4 peptide may act on the PK-I.

Concerning the property of phosphorylation of SR-F, trypsin-treated SR-F loses most of its ability to be phosphorylated by A-PK (Fig. 8). In contrast to control SR-F where the rate of Ca uptake was

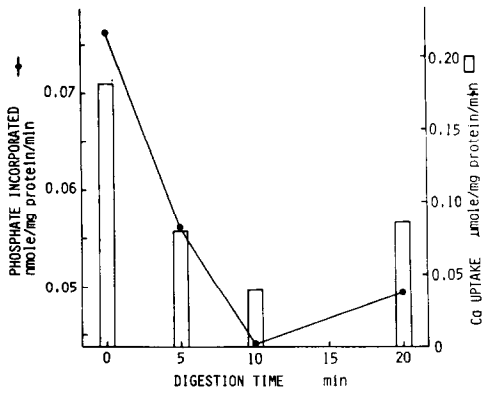

Fig. 8. Effect of trypsin treatment on SR-F. Note parallel decrease of phosphorylation (●) and Ca uptake (open columns) by A-PK (0.28 mg/ml).

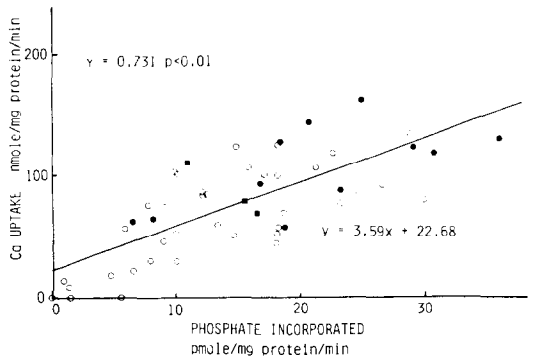

Fig. 9. Relationship between A-PK stimulation of Ca uptake and A-PK-catalysed phosphorylation without (○) and with CCK-4 (●) or CCK-33 peptides (■). Data were taken from Figs. 5 and 6 at each concentration of A-PK. The correlation coefficient (r) and the line of best fit were obtained by the least squares method.

greatly stimulated by treatment with A-PK and cyclic AMP, Ca uptake by SR-F after pre-treatment with trypsin was not stimulated by incubation with A-PK and cyclic AMP (Fig. 8). This brief digestion with trypsin was not considered to affect microsomal Ca transport activity [14]. Therefore, the phosphorylation of SR-F and then necessarily its coupled Ca uptake were impaired by this digestion.

The relationship between A-PK-catalysed SR-F phosphorylation and A-PK-stimulated Ca uptake by SR-F was investigated. For this purpose, the phosphorylation of SR-F was plotted against Ca uptake by phosphorylated SR-F at identical concentrations of A-PK over a wide range of A-PK concentrations. As shown in Fig. 9, a positive correlation was found with a correlation coefficient of $r = 0.731$ ($P < 0.01$).

DISCUSSION

One of the possible mechanisms for cyclic AMP-mediated relaxation of smooth muscles is an activation of A-PK [4, 5], which is thought to catalyse a protein phosphorylation resulting in a reduction in free Ca^{2+}. Cyclic AMP and cardiac A-PK have been reported to produce marked stimulation of Ca uptake as well as Ca^{2+}-activated ATPase activity of canine cardiac microsomes [3, 13]. Our present studies using SR-F and A-PK obtained from domestic hog terminal bile duct demonstrated that A-PK-catalysed SR-F phosphorylation showed a good correlation with A-PK-induced Ca uptake by SR-F, phosphorylation increasing in parallel with Ca uptake over a wide range of A-PK concentrations. In this respect we purposely used the term 'SR-F' instead of 'SR' because some component of the crude supernatant may subtly influence the above activation.

In the presence of 1 μM Ca^{2+} ion and 200 μg/ml A-PK, SR-F and A-PK obtained from terminal bile ducts demonstrated the same amount of Ca uptake (0.13 μmole Ca/mg protein/min) as cardiac microsomes and A-PK. However, the latter system showed a much lower extent of phosphorylation (0.44 nmole ^{32}P/mg protein/min [3]). In the report on cardiac microsomes, the contracting effect of *l*-epinephrine was also explained by the Ca uptake of phosphorylated SR-F. However, the reason that the same biochemical change produced the relaxation in smooth muscles, and on the other hand the contraction in cardiac muscles, remains uncertain.

One of the CCK-C-terminal peptides, CCK-4, was used instead of CCK-33 in this investigation. CCK-4 peptide was recently reported to be a possible endogeneous transmitter [20] because CCK-4 peptide predominated in all regions of the porcine gut, especially in the duodenum, in contrast to CCK-33 peptide.

The mechanism by which CCK-8 caused the relaxation of biliary tracts was considered by Andersson *et al.* [2] to depend on the increase in cyclic AMP levels. CCK-4 peptide elevated cyclic AMP levels in whole tissues of terminal bile ducts in normal Tyrode's solution, but was much less potent than isoproterenol. The observation that CCK-4 peptide relaxed Oddi's sphincters of domestic hogs in normal Tyrode's solution, hence, could not be explained sufficiently by the increase of cyclic AMP induced by CCK-4 peptide, but rather by acceleration of A-PK-induced Ca uptake by SR-F in the process after the increase of cyclic AMP levels. This is because CCK-4 peptide did not facilitate the Ca uptake in the absence of A-PK and cyclic AMP added externally (Fig. 5).

The phosphorylation reaction by A-PK obtained from terminal bile duct previously incubated with CCK-4 peptide was much greater than that from gallbladder. This observation is concluded with the finding that the phosphorylation ratio (45.2%) of SR-F of bile ducts [5] is greater than that (20.2%) of gallbladders (unpublished data). One of the conclusions of this paper is that some site of action of CCK-4 peptide as well as of CCK-33 in the terminal bile duct may be associated with A-PK-catalysed phosphorylation of SR-F as well as plasma membranes. The latter possibility is considered because membrane-associated and cytosolic A-PK are reported to have the same physiochemical, immunological, and affinity-labeling properties [21]. Although the CCK-4-induced diminution of the protein kinase activity ratio (Table 1) suggests that CCK-4 actually affected the A-PK system, the possibility still exists that CCK-4 might also affect a different, cyclic AMP-independent kinase in addition to A-PK.

This present results suggests that the mechanisms of relaxation of CCK-4 peptide may be quite different from those of papaverine. As previously reported [5], papaverine does not activate A-PK-catalysed phosphorylation. In addition, papaverine greatly facilitated Ca uptake even in the absence of A-PK added externally with and without cyclic AMP, suggesting that papaverine-induced relaxation may be caused by cyclic AMP-independent mechanisms as well as by the inhibition of cyclic AMP-phosphodiesterase. The former mechanisms of papaverine are also supported by studies previously carried out on *Taenia coli* of guinea pig [22, 23] or rat myometrical strips [24] under K^+-depolarized conditions.

Our observation that CCK-8 peptide is more potent than CCK-33 in promoting A-PK-catalysed phosphorylation as well as Ca uptake supports the suggestion by Rehfeld and Larsson [20] that the *in*

vivo action of CCK-33 may be mediated by low molecular weight peptide metabolites. We postulate that the effects of CCK-C-terminal peptides are caused by A-PK activation as a possible result of the association of CCK-4 peptide with PK-I.

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