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The activation of cyclic 3′, 5′-adenosine monophosphate-dependent protein kinase on sarcoplasmic reticulum fractions of various smooth muscles and its related novel relaxants

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The mechanisms of smooth muscle relaxants have been found to be connected with an increase in the cyclic AMP (cAMP) level of the tissues concerned, partially caused by an inactivation of phosphodiesterase (for example, papaverine) or as a consequence of stimulation of adenylcyclase (for example, isoproterenol). Recently Nillson *et al.* [1] have suggested that the basic mechanism by which drugs, increasing the cAMP levels, exert their relaxing effect connects with stimulation of the protein kinase system. We studied on the phosphorylation of sarcoplasmic reticulum fractions (SR-F) in various smooth muscles of domestic pigs using cAMP-dependent protein kinase (PK) of cardiac or tracheal muscles or bile ducts, in order to clarify the site of action of a potent relaxant different from papaverine for Oddi's sphincters.

The relaxant, a novel compound 3-(2′-hydroxy-4′, 5′-diethoxybenzoyl) propionic acid (AA373), did not have any inhibitory effect on the intestinal phosphodiesterase of guinea-pigs, and also the response on adrenergic β-receptor, because of no inhibition by β-adrenergic antagonist on bile ducts. The fact was observed, however, that PK was activated by its compound both in the presence of and in the absence of saturated dose of cAMP on a substrate histone.

Recently Katz *et al.* [2] have shown the possibility of controlling calcium transport in the myocardium by PK system. This paper is presented on the possible mechanism of relaxing the Oddi's sphincter by AA373, by which the phosphorylation of biliary sarcoplasmic reticulum may accelerate relaxation in the smooth muscles of bile ducts.

Microsomal fractions (M-F) and SR-F were prepared by the method of Katz and Repke [3] as follows. After a removal of most of the fat and vessels, 16 g of every tissue from domestic pigs were minced and then homogenized in a Waring Blender for 90 sec with 2.5 vol. of ice cold 0.3 M sucrose, 5 mM Tris–oxalate and 5 mM histidine at pH 7.4. After centrifugation for 30 min at 12,000 *g*, the supernatant was filtered through four layers of gauze, if necessary, and centrifuged for 90 min at 105,000 *g*. The pellet was taken up in 3 ml of 0.3 M sucrose, 1 mM Tris–oxalate and 5 mM histidine at pH 7.4 and homogenized by ten gentle strokes of a Teflon–glass Potter homogenizer. This M-F diluted with a 0.3 M sucrose to contain about 60 μg of protein were used to the assay of protein kinase. Homogenate (0.25 ml), containing approximately 3.6–2.1 mg protein of crude microsomes, was applied to the top of a sucrose gradient consisting of 2 ml of 35% sucrose and 2 ml of 20% sucrose, layered in a polyallomer tube. The gradient tubes were centrifuged for 2 hr at 25,000 rpm in RPS 40 swinging rotor (Hitachi Co. Ltd.). The purified microsomes (corresponding to SR-F) appeared in one prominent middle layer, of which we used about 2 ml. These SR-F had been reported to show a lower contamination by both soluble and mitochondrial enzymes one ninth of crude microsomes and to have Ca^{2+} uptake capacity and Ca^{2+}-activated ATPase activity [3]. These fractions were heated to 78–84° for 2 min to inactivate several interfering enzymes [4].

The preparation of protein kinase was supernate fractions obtained from 0.9 g cardiac muscles, terminal bile ducts and gall bladders of domestic pigs minced, incubated in 9 ml Tyrode solution with or without drugs for 10 min, homogenized for 3 min, and supercentrifuged at 105,000 *g* for 1 hr.

Protein kinase assay was as follows: the reaction medium (0.25 ml) consisted of 8 mM phosphate buffer (pH 7.0), 1.6 mM theophylline, 8 mM NaF, 12 mM Mg acetate, 1 μCi/ml ATP-γ-^{32}P (The Radiochemical Centre Ltd., England), 10 μM ATP, SR-F (20–48 μg protein) or M-F (53–81 μg protein) and PK (13–68 μg protein) with 4 μM cAMP incubated at 37° for 5, 10 and 15 min. The phosphorylation reactions at the concentrations of SR-F, M-F and PK were ascertained to be almost linear. The reaction was stopped by the addition of 5 ml of 10% cold trichloracetic acid (TCA) containing 20 mg 'Hyflosuper celite'. The mixture was filtered through a paper ('Toyo' No. 2) and a thin layer of 50 mg 'Hyflosuper celite'. The residue was washed with 15 ml of 10% TCA, 3 ml acetone, 10 ml of

Table 1. The comparison of contents of phosphates incorporated by cardiac protein kinase with cyclic AMP into sarcoplasmic reticulum fractions from various muscles of domestic pigs

	Yield ratio % (mg protein)$_{SR\text{-}F}$ / (mg protein)$_{M\text{-}F}$ (A)	Observations of phosphates incorporated for 10 min into* M F n mole/(mg PK · mg protein)) (B)	SR-F (C)	Calculations of those into SR-F† (n mole)$_{M\text{-}F}$ / (mg protein)$_{SR\text{-}F}$ · mg PK (B/A)	Phosphorylation ratios of SR-F % (n mole)$_{SR\text{-}F}$ / (n mole)$_{M\text{-}F}$ (AC/B)
Diaphragm	31.4	2.43*	6.41*	7.74†	82.8
Heart	16.7	3.06	5.03	18.3	27.5
Bile duct	30.4	4.61	6.87	15.2	45.2
Aorta	17.7	4.51	8.14	25.5	31.9
Trachea	18.6	3.97	5.90	21.3	27.7
Taenia coli	16.4	3.50	4.64	21.3	21.8
Uterus	15.7	5.90	5.52	37.6	14.7

* These values subtracted those without substrates and without cAMP.

† The phosphorylation of microsomal fractions (M-F) was assumed to come only from that of sarcoplasmic reticulum fractions (SR-F) contained within the M-F.

95% ethanol and 3 ml of ether, and dried. After the dried residue was solubilized by 0.5 ml of hyamine hydroxide, 10 ml phosphor solution was added. The final solution was counted with a liquid scintilation spectrometer. Proteins were measured according to Lowry's method [5].

The contents of phosphate incorporated by PK into SR-F were estimated as a ratio of observations of phosphates incorporated into SR-F to calculations obtained by assumption that observations of phosphates incorporated into M-F may only be dependent on all SR-F contained within its M-F.

Table 2. Relative potencies of relaxing action on various muscles induced by papaverine-related compounds

	Pap	AA373	AA149
Oddi's sphincter*	1‡	1.5	0.15
Tracheal m.†	1.13	0.60	0.057
Taenia-coli†	0.10	0.35	0.039

* domestic pig, † guinea-pig, ‡ ED_{50} of papaverine 1.3 μM as unity.

The direct relaxing actions of these compounds were assayed by (*) the increase of flow rates through the Oddi's sphincter and isotonically by (†) a modified Magnus method [6], in the bath containing 35 ml or 5 ml Tyrode or Lock–Ringer solution at 37° or 26° bubbled through by air.

From the last columns in Table 1, it was found that the phosphorylation ratio of SR-F existing in M-F for terminal bile ducts, tracheal muscles and taenia coli was almost 1:0.61:0.48. These ratios are almost parallel with the order of pharmacological relaxing effects on the corresponding smooth muscles for AA373 and its 2'-ethoxy derivative (AA149), but not for papaverine, which is shown in Table 2.

Table 3 showed that the relaxing effect of cAMP on taenia coli is potentiated both by papaverine and by AA373, and that the potentiative effect of AA373 is less than that of papaverine, in striking contrast to the relaxing effect on the bile duct. From these findings, the site of

Table 3. Relaxing effects of cyclic AMP potentiated by relaxants on isolated guinea pig taenia coli

Drugs	Dose (μM)	Effect of cAMP ED_{50} (μM)	Relative potency
without		20	1
AA373	1	5.9	3.4
Pap	1	3.4	5.9

The direct relaxations were obtained isotonically by a Magnus method in which the tissues were immersed in Locke–Ringer solution at 26° bubbled by air. The concentration of AA373 and Pap used induced 10.4 and 27.2% relaxation by themselves.

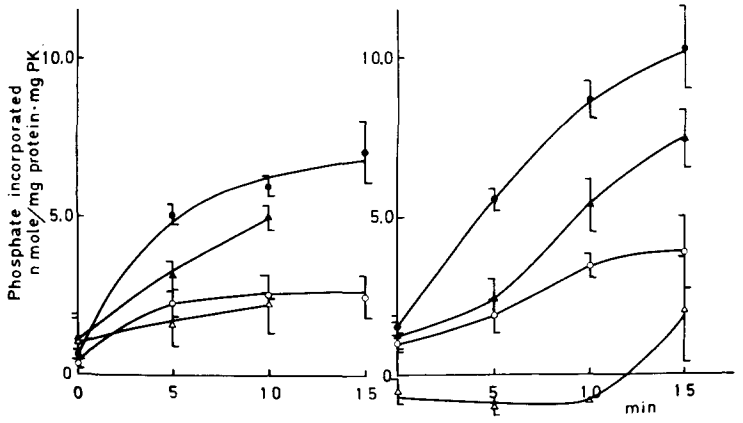

Fig. 1. The phosphorylations of crude microsomal fractions (left) and sarcoplasmic reticulum fractions (right) by cAMP-dependent protein kinase as a function of incubation time with (●,▲) or without cAMP (○,△). Circles; crude microsomal fractions, sarcoplasmic reticulum fractions and protein kinase from bile ducts of domestic pigs. Triangles; the same kind of preparations were obtained from gall bladders of domestic pigs. Note that the degree of phosphorylation of crude microsomal fractions and sarcoplasmic reticulum fractions of bile ducts are greater than those of gall bladder, and almost the same as those by the cardiac protein kinase as shown in Table 1.

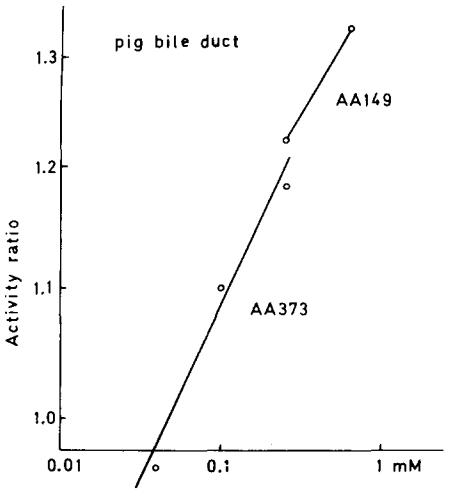

Fig. 2. Effects of incubation for 5 min with drugs on the phosphorylation of sarcoplasmic reticulum fractions from bile ducts by cAMP-dependent protein kinase from the same tissues with cAMP. Activities are expressed as the ratio of protein kinase activities after incubation with drugs to the activities obtained in the absence of drugs. Note that both compounds have almost the same activities, indicating that there are some different responses in the experimental levels between for the protein kinase and for the cells of smooth muscles.

action of AA373 seemed to be closely associated with the post-process of the formation of cAMP, that is the phosphorylation of SR-F caused by cAMP-dependent protein kinase. This assumption was supported in Fig. 2 by finding that AA373 and AA149 clearly activated effects of cAMP-dependent PK on SR-F in the bile ducts shown in Fig. 1, while that, in the presence of cAMP, PK was not activated by papaverine or isoproterenol.

These results suggested that the mechanisms of action of AA-compound series might be partly explained by the linked system of cAMP-dependent PK-SR. 2, 4, 6-Trihydroxy-1-propiophenone (THPP), which is similar to AA-compound series except for a carbonic acid group, has almost no activating effect, indicating that the carbonic acid group plays an important role to join AA-compound series with endogenous substrates in the linked system of cAMP-dependent PK. The more specific effects of AA compound series on bile ducts than on the other smooth muscles also might be explained by the distribution of the activity of PK.

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