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EFFECTS OF cAMP- AND cGMP-DEPENDENT PROTEIN KINASES, AND CALMODULIN ON Ca^{2+} UPTAKE BY HIGHLY PURIFIED SARCOLEMMAL VESICLES OF VASCULAR SMOOTH MUSCLE

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Sarcolemmal fractions of vascular smooth muscles were prepared from porcine thoracic aortae by differential and sucrose density gradient centrifugation. In these fractions, there was a high activity of 5'-nucleotidase, a putative marker enzyme of plasma membrane, and a low activity of rotenone insensitive NADH-cytochrome *c* reductase a marker of sarcoplasmic reticulum. In these fractions, the Ca^{2+} uptake was ATP-dependent. A low concentration of saponin which inhibited Ca^{2+} uptake by the plasma membrane but not by the sarcoplasmic reticulum, inhibited 65% of the Ca^{2+} uptake of this fraction. The Ca^{2+} uptake of this fraction was enhanced by cAMP- and cGMP-dependent protein kinases, and by calmodulin. The cAMP-dependent protein kinase enhanced the phosphorylation of 28 and 22 kDa proteins, while the cGMP-dependent protein kinase phosphorylated the 35 kDa protein. The phosphorylation of 100, 75, 65, 41 and 22 kDa proteins was enhanced by Ca^{2+} and calmodulin. These results indicate that cAMP- and cGMP-dependent protein kinases as well as calmodulin play important roles in Ca^{2+} transport in the sarcolemma, and that the phosphorylated proteins may be associated with an enhancement of Ca^{2+} transport in the sarcolemma.

Introduction

Cyclic nucleotides play important roles in the relaxation of vascular smooth muscles, as induced by some pharmacological agents. The relaxation induced by β stimulators and by nitrocompounds is associated with increases in levels of cAMP [1] and cGMP [2], respectively.

The ATP-dependent Ca^{2+} pump appears to be present in sarcolemmal membrane of smooth muscles [3–7], therefore, the possibility that these cyclic nucleotides may have an effect on the Ca^{2+} pump in sarcolemmal membrane has to be considered.

Calmodulin plays an important role in regulating intracellular Ca^{2+} concentration and stimulation of Ca^{2+} transport has been evidenced in various tissues [8–12].

We isolated sarcolemmal membrane from porcine thoracic aortae and the effects of cyclic nucleotides and calmodulin on the Ca^{2+} transport in this tissue were given attention.

Materials and Methods

Isolation of sarcolemmal membrane. Porcine thoracic aortae were obtained from a local slaughter house. Adventitia and adherent connec-

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Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

tive tissue were removed and the intima and media layer cut into small pieces with scissors were homogenized in medium containing 100 mM KCl, 20 mM Tris-maleate (pH 6.80), 5 mM $MgCl_2$, 5 mM NaN_3, 1 mM ATP with a Polytron PT 10 (three bursts of 15 s at half-maximal speed). All procedures were carried out between 0 and 4°C.

The homogenate was centrifuged at $1500 \times g$ for 10 min, followed by $27000 \times g$ for 20 min and $140000 \times g$ for 60 min. The resultant pellet was suspended in buffer containing 100 mM KCl, 20 mM Tris-maleate (pH 6.80) and 8%(*W/V*) sucrose. The suspension was layered on discontinuous sucrose density gradient consisting of 14, 24, 28 and 40% of sucrose, and centrifuged at $73000 \times g$ for 180 min. The subfractions F_1(8–14%), F_2(14–24%), F_3(24–28%). F_4(28–40%) were collected with a Pasteur pipette and centrifuged at $140000 \times g$ for 30 min. The pellets were suspended in buffer containing 100 mM KCl, 20 mM Tris-maleate (pH 6.80). The protein concentration was determined by the method of Lowry et al. [13], using bovine serum albumin as a standard.

Enzyme assay. 5′-Nucleotidase was measured, as described by Wattiaux de Coninck and Wattiaux [14]. The reaction medium contained 20 mM Tris-HCl (pH 7.40), 5 mM $MgCl_2$, and 50 μg of vesicle protein, in a final volume of 1.0 ml. The reaction initiated by adding 5 mM 5′-AMP, was allowed to continue for 30 min at 37°C and halted by adding 1 ml of 20% trichloroacetic acid. The liberated inorganic phosphate was determined by the method of Fiske and SubbaRow [15]. The blank value obtained using 2′(3′)-AMP instead of 5′-AMP was subtracted.

Rotenone insensitive NADH-cytochrome *c* reductase was measured by the method of Sottocasa et al. [16]. The reaction medium contained 0.1 mM NADH, 0.1 mM cytochrome *c*, 0.3 mM KCN, 50 mM potassium phosphate buffer (pH 7.5), 1.5 μM rotenone, and 50 μg/ml of vesicle protein, in a total volume of 3 ml. The enzyme activity was measured spectrophotometrically by the following reduction of cytochrome *c* at 550 μm.

Preparation of 'inside-out vesicles' of human erythrocytes and canine heart sarcoplasmic reticulum. 'Inside-out vesicles' of human erythrocytes were prepared by the method of Steck and Kant [17]. Canine heart sarcoplasmic reticulum was prepared by the method of Kitazawa [18].

Measurement of Ca^{2+} uptake. Ca^{2+} uptake was measured by the Millipore filtration method using ^{45}Ca. The reaction medium contained 100 mM KCl, 20 mM Tris-maleate (pH 6.80), 8 mM $MgCl_2$, 5 mM NaN_3, 0.1 mM $^{45}CaCl_2$(5 000–6 000 cpm/nmol), specific concentration of EGTA to obtain the desired free Ca^{2+} concentration and 40–60 μg/ml of vesicle protein in a total volume of 1 ml. The reaction was started by adding 5 mM ATP at 37°C and was halted by passing through a Millipore filter (0.45 μm of pore size). The filter was washed twice with the above mixture without ^{45}Ca, dried and counted in 10 ml of a scintillation cocktail (4 g 2,5-diphenyloxazole and 0.2 g 1,4-bis(5-phenyl-2-oxazolyl benzene) in 1 l toluene). Apparent binding constant of EGTA for Ca^{2+} was assumed to be $1 \cdot 10^6$ M^{-1} [19] at pH 6.80, and that of ATP for Mg^{2+} to be $4 \cdot 10^3$ M^{-1} [20].

Phosphorylation of sarcolemmal vesicles by cyclic nucleotide-dependent protein kinases and calmodulin. Phosphorylation of vesicle proteins was carried out at 37°C in 200 μl of the incubation medium containing 100 mM KCl, 20 mM Tris-maleate (pH 6.80), 8 mM (20 mM) $MgCl_2$, 40–60 μg of vesicle proteins. The reaction started by adding of 10 μM ATP containing 1 μCi [γ-^{32}P] ATP was allowed to continue for 10 min at 37°C and halted by the addition of 1 ml of 20% trichloroacetic acid. The mixture was centrifuged at 3000 rpm for 15 min, and the pellet was subjected to 0.1% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli [21]. The gel was stained and dried, and autoradiography was carried out with Fuji X-ray film for 7–8 days.

Materials. cAMP, cGMP, catalytic subunit of cAMP-dependent protein kinase, and bovine serum albumin were purchased from Sigma. Saponin was from I.C.N. $^{45}CaCl$, [γ-^{32}P]ATP were the products of New England Nuclear Inc. Other reagents were commercial preparations of reagent grades. cGMP-dependent protein kinase was prepared from pig lung by the method of Nakazawa and Sano [22]. Some of the cGMP-dependent protein kinase was a kind gift from Dr. M. Inagaki and Professor H. Hidaka (Department of Pharmacology, Mie University School of Medicine). Calmodulin was prepared from dog brain by the method of Yazawa et al. [23].

Results

Saponin-inhibitable Ca^{2+} uptake of sarcolemma-rich fraction

Fig. 1A shows the inhibition of Ca^{2+} uptake by saponin in the inside-out vesicles of human erythrocytes and canine heart sarcoplasmic reticulum. Saponin (20 μg/ml) inhibited over 90% of the Ca^{2+} uptake in inside-out vesicles of human erythrocytes while the same concentration of saponin inhibited less than 5% of the Ca^{2+} uptake in the canine heart sarcoplasmic reticulum. Fig. 1B shows the inhibition of Ca^{2+} uptake by saponin in subcellular fractions isolated by sucrose density gradient centrifugation. Saponin (20 μg/ml) inhibited 65% of the Ca^{2+} uptake in fraction F_2, while it inhibited only 40% and 5% of the Ca^{2+} uptake in fractions F_3 and F_4, respectively. This ATP-dependent Ca^{2+} uptake of fraction F_2 was not stimulated by 5 mM oxalate, while that of fraction F_4 was stimulated to the extent of 30% (data not shown).

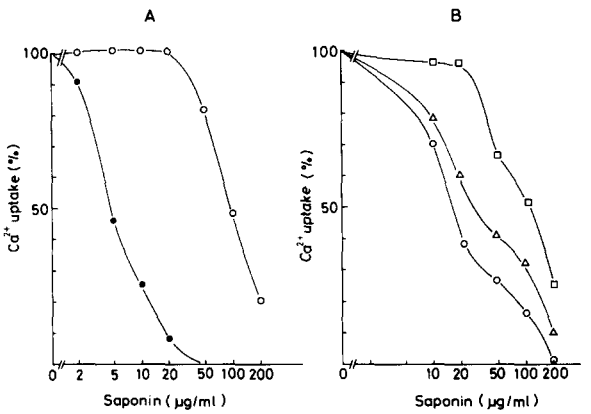

Fig. 1. Inhibition of Ca^{2+} uptake by saponin in the inside-out vesicles of human erythrocytes and canine heart sarcoplasmic reticulum (A), and subcellular fractions prepared from porcine aortic smooth muscle (B). Ca^{2+} uptake was assayed as described under Materials and Methods for 30 min. Vesicles were incubated with various concentrations of saponin. The free Ca^{2+} concentration was $2.4 \cdot 10^{-5}$M which was obtained with 0.12 mM $CaCl_2$ and 0.1 mM EGTA. (A) ●, inside-out vesicles of human erythrocytes (100% of Ca^{2+} uptake: 26.6 nmol/mg protein); ○, canine heart sarcoplasmic reticulum (50.2 nmol/mg protein); (B) ○, fraction F_2 (14/24%) (20.1 nmol/mg protein); △ fraction F_3 (24/28%) (16.4 nmol/mg protein); □, fraction F_4 (28/40%) (17.2 nmol/mg protein). The energy-independent Ca^{2+} uptake was carried out in the absence of ATP and subtracted from the value of Ca^{2+} uptake in the presence of ATP. The same applies for the following figures. Each point shows the mean for three independent experiments.

The purity of the subcellular membrane was routinely determined by measuring the activities of marker enzymes. Therefore, we examined the distribution of two types of enzyme activities in the subcellular fractions. 5′-Nucleotidase was the one of the marker enzymes of the plasma membrane and rotenone-insensitive NADH-cytochrome *c* reductase was a marker of sarcoplasmic reticulum. Activity of 5′-nucleotidase was highest in fraction F_2, and rotenone-insensitive NADH-cytochrome *c* reductase activity was highest in fraction F_4 (Table I). These results indicated that fraction F_2 was the fraction richest in sarcolemma, and fraction F_4 was rich in sarcoplasmic reticulum. Therefore, we used fraction F_2 as sarcolemma fraction for the following experiments.

Effects of cyclic nucleotide-dependent protein kinases and calmodulin on the Ca^{2+} uptake of sarcolemma-rich fraction

The cAMP-dependent protein kinase is a holoenzyme. The binding of cAMP to the regulatory subunit of the enzyme causes the dissociation of the catalytic subunit, which is the active form of the enzyme. Thus, we used the catalytic subunit of the enzyme instead of both cAMP and the enzyme, in the assay of Ca^{2+} uptake.

Fig. 2 shows the effect of a catalytic subunit of cAMP-dependent protein kinase on the Ca^{2+} uptake of the sarcolemma-rich fraction F_2. The catalytic subunit of cAMP-dependent protein kinase stimulated the Ca^{2+} uptake to the extent of 30% at 1 mM $MgATP^{2-}$. cAMP alone had no effect on Ca^{2+} uptake of fraction F_2. The catalytic subunit of cAMP-dependent protein kinase also stimulated the Ca^{2+} uptake in the sarcoplasmic reticulum-rich fraction F_4 (16.2 to 20.3 nmol/mg protein; mean for two experiments). Fig. 3 shows the Ca^{2+} uptake of the F_2 fraction stimulated by cGMP-dependent protein kinase. As in the case of cAMP, cGMP alone did not enhance the Ca^{2+} uptake. With the addition of both cGMP-dependent protein kinase and cGMP, there was a 20% enhancement in the maximal uptake. The extent of the stimulation was small, as compared with the effect of the catalytic subunit of cAMP-dependent pro-

TABLE I

ACTIVITIES OF MARKER ENZYMES IN SUBCELLULAR FRACTIONS OF PORCINE THORACIC AORTAE

Fractions were prepared as described under Materials and Methods. Values are means ± S.E. of four different preparations examined.

Enzyme	Fractions					
	1500 × *g* supernatant	140000 × *g* microsome	F_1 (8/14%)	F_2 (14/24%)	F_3 (24/28%)	F_4 (28/40%)
5'-Nucleotidase (μmol P_i/mg protein per h)	2.69 ± 0.32	20.5 ± 2.0	17.6 ± 2.5	38.1 ± 0.7	27.8 ± 1.4	20.5 ± 1.7
Rotenone-insensitive NADH-cytochrome *c* reductase (μmol/mg protein per min)	0.23 ± 0.02	0.84 ± 0.03	0.70 ± 0.06	1.15 ± 0.07	1.05 ± 0.05	1.32 ± 0.06

tein kinase. The cGMP-dependent protein kinase prepared by the method of Nakazawa and Sano [22] contained no calmodulin, as examined by the phosphodiesterase assay system reported elsewhere [24]. Therefore, the stimulatory effect of cGMP-dependent protein kinase on the Ca^{2+} uptake in fraction F_2 was not due to contaminating calmodulin. The Ca^{2+} uptake in the sarcoplasmic reticulum-rich fraction F_4 was not stimulated by the addition of either cGMP- or cGMP-dependent protein kinase.

Calmodulin also stimulated the Ca^{2+} uptake of fraction F_4 (Fig. 4) in a time- and free Ca^{2+} concentration-dependent manner. Calmodulin increased both the affinity for Ca^{2+} and the maximal uptake in fraction F_2. This stimulation was observed without exposing the membranes to EGTA solution to remove the endogenous calmodulin [7]. In the sarcoplasmic reticulum-rich

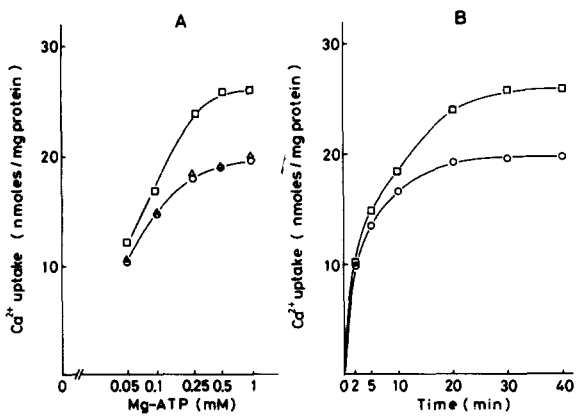

Fig. 2. Effect of a catalytic subunit of cAMP-dependent protein kinase on the Ca^{2+} uptake in fraction F_2. (A) Ca^{2+} uptake was assayed at various concentrations of $MgATP^{2-}$ for 30 min. The Ca^{2+} uptake capacity nearly reached the steady-state filling at 30 min as shown in (B). The concentration of $MgATP^{2-}$ was calculated as described under Materials and Methods. The Free Mg^{2+} concentration was 1 mM and the free Ca^{2+} concentration was $2.4 \cdot 10^{-5}$M. Creatine phosphate (2 mM) and creatine phosphokinase (50 μg/ml) were added as the ATP regenerating system. ○, control; △, 10 μM of cAMP; □, 1 μg/ml of catalytic subunit of cAMP-dependent protein kinase; (B). Time-course of Ca^{2+} uptake stimulated by the catalytic subunit of cAMP-dependent protein kinase. $MgATP^{2-}$ was 1 mM. ○, control; □, 1 μg/ml of catalytic subunit of cAMP-dependent protein kinase. Each point shows the mean for three independent experiments.

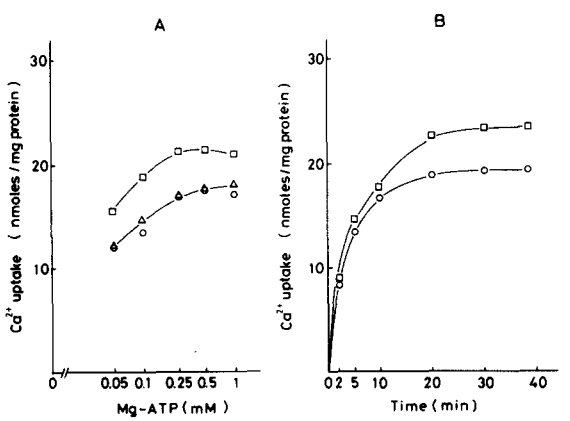

Fig. 3. Effect of cGMP-dependent protein kinase on Ca^{2+} uptake in fraction F_2. (A) Ca^{2+} uptake was assayed at various concentrations of $MgATP^{2-}$ for 30 min. The free Mg^{2+} concentration was 10 mM and the free Ca^{2+} concentration was $2.4 \cdot 10^{-5}$M. ○, control; △, 10 μM of cGMP; □, 10 μM of cGMP + 50 μg/ml of cGMP-dependent protein kinase. (B). Time-course of Ca^{2+} uptake stimulated by cGMP-dependent protein kinase. $MgATP^{2-}$ was 0.5 mM. ○, control; □, 10 μM of cGMP + 50 μg/ml of cGMP-dependent protein kinase. Each point shows the mean for three independent experiments.

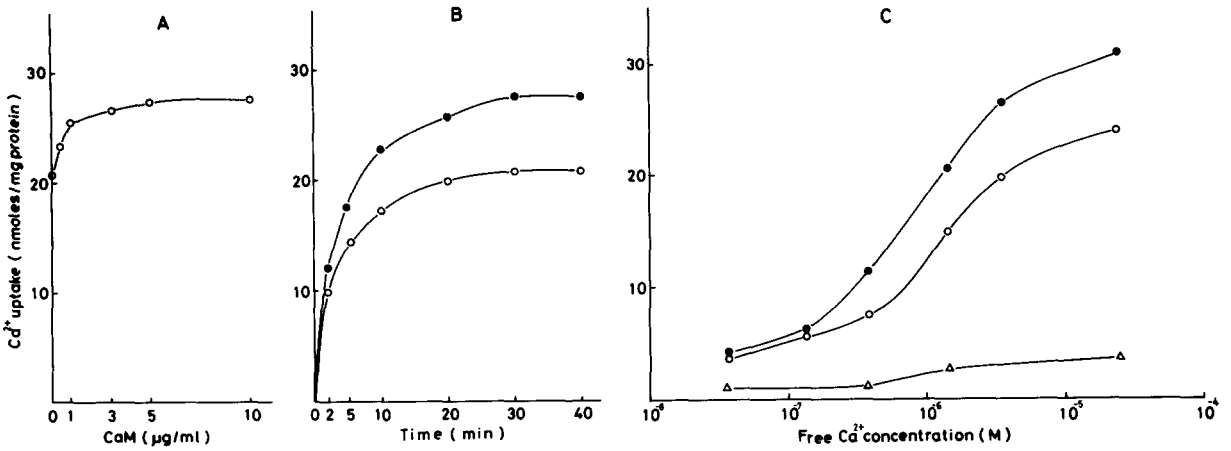

Fig. 4. Effect of calmodulin (CaM) on Ca^{2+} uptake in fraction F_2. (A) Ca^{2+} uptake was assayed at various concentrations of calmodulin for 30 min. The free Ca^{2+} concentration was $2.4 \cdot 10^{-5}$M. (B) Time-course of Ca^{2+} uptake stimulated by calmodulin. The free Ca^{2+} concentration was $2.4 \cdot 10^{-5}$M. ○, control; ●, 10 μg/ml of CaM. (C) Ca^{2+} uptake was assayed at various concentrations of free Ca^{2+} for 30 min. In this experiment, ATP-independent Ca^{2+} binding is shown, and was not subtracted from the ATP-dependent Ca^{2+} uptake. △, ATP-independent Ca^{2+} binding; ○, ATP-dependent Ca^{2+} uptake; ●, ATP-dependent Ca^{2+} uptake with 10 μg/ml of calmodulin. Each point shows the mean for three independent experiments.

fraction F_4, calmodulin also stimulated the Ca^{2+} uptake (17.2 to 22.0 nmol/mg protein; mean for three experiments).

These findings indicate that cAMP- and cGMP-dependent protein kinases, and calmodulin stimulate the ATP-dependent Ca^{2+} uptake of the sarcolemma-rich fraction F_2. On the other hand, ATP-independent Ca^{2+} binding of fraction F_2 was 2–3 nmol/mg protein at 30 min and this was not affected by cyclic nucleotide-dependent protein kinases and calmodulin.

Phosphorylation of the sarcolemma-rich fraction by cyclic nucleotide-dependent protein kinases and calmodulin.

It is generally considered that cyclic nucleotides affect various functions through protein phosphorylation by a specific protein kinase [25]. Therefore, we examined the protein phosphorylation of the sarcolemma-rich fraction F_2 by cyclic nucleotides and calmodulin.

The phosphorylation of 28 and 22 kDa proteins was increased with the addition of both cAMP- and cAMP-dependent protein kinase (Fig. 5D). The 65 kDa protein was also phosphorylated, however, this phosphorylation seemed to be due to the phosphorylatable contaminant of cAMP-dependent protein kinase because the phosphorylation was evenly observed when only the protein kinase without fraction F_2 was included. The addition of both cGMP-dependent protein kinase and

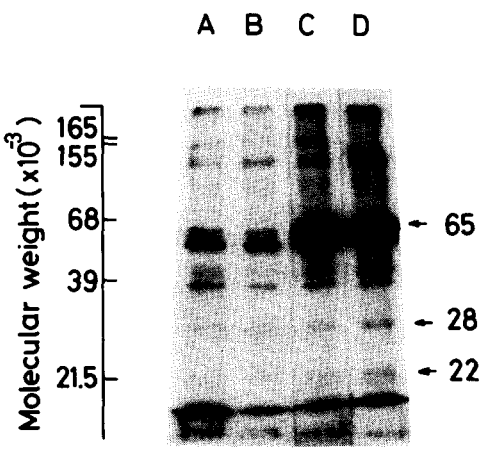

Fig. 5. Autoradiography of fraction F_2 vesicles phosphorylated by cAMP-dependent protein kinase, carried out as described under Materials and Methods with 12% of polyacrylamide gel. The apparent molecular weight of designated bands was estimated from several runs of membrane samples and protein standards. The molecular weight standards used in this experiment were trypsin inhibitor (M_r 21500), bovine serum albumin (68000), and RNA polymerase (α 39000; β 155000; γ 165000). $MgCl_2$ was 8 mM. A, control; B, 1 μM of cAMP; C, 20 μg/ml of cAMP-dependent protein kinase; D, 1 μM of cAMP + 20 μg/ml of cAMP-dependent protein kinase.

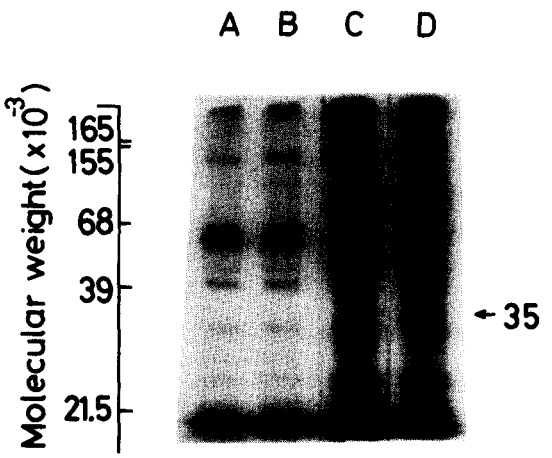

Fig. 6. Autoradiography of fraction F_2 vesicles phosphorylated by cGMP-dependent protein kinase. $MgCl_2$ was 20 mM. A, control; B, 1 μM of cGMP; C, 50 μg/ml of cGMP-dependent protein kinase; D, 1 μM of cGMP + 50 μg/ml of cGMP-dependent protein kinase. For the molecular weight standards see the legend to Fig. 5.

cGMP phosphorylated a new protein band equivalent to the 35 kDa (Fig. 6D). The phosphorylation of other proteins may be the phosphorylatable contaminants of enzyme preparation because they were evenly obseved when only cGMP-dependent protein kinase without fraction F_2 was included. Calmodulin increased the phosphorylation of five protein bands, equivalent to 100, 75, 65, 41 and 22 kDa, only in the presence of $2.4 \cdot 10^{-5}$M free Ca^{2+} (Fig. 7D). The phosphorylation by Ca^{2+} and calmodulin may be due to the calmodulin-dependent protein kinase which associated with fraction F_2, as demonstrated in *Torpedo* synaptic vesicles, heart sarcoplasmic reticulum and brain [26–28].

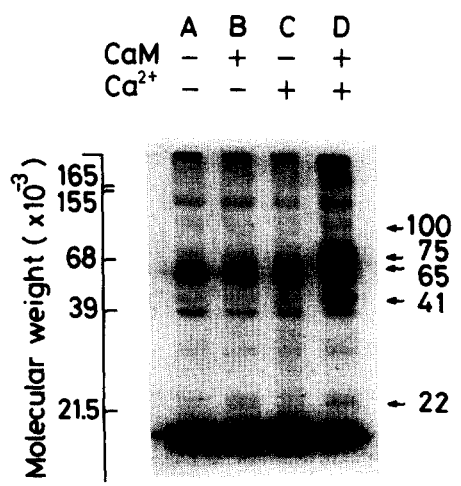

Fig. 7. Autoradiography of fraction F_2 vesicles phosphorylated by calmodulin (CaM). Phosphorylation was carried out at $1.5 \cdot 10^{-8}$M of free Ca^{2+} concentration (0.12 mM $CaCl_2$ and 8 mM of EGTA) (A and B) and $2.4 \cdot 10^{-5}$M of free Ca^{2+} concentration (C and D), with (B and D), or without (A and C) 10 μg/ml of calmodulin. $MgCl_2$ was 8 mM. For the molecular weight standards see the legend to Fig. 5.

Phosphorylation by protein kinases and calmodulin, as reported in fraction F_2, was observed to a much lesser extent in other fractions, indicating that the phosphorylated protein bands described above belong to sarcolemmal membrane. The extent of phosphorylation of these proteins roughly paralleled the stimulation of Ca^{2+} uptake.

Discussion

We prepared sarcolemma-rich fractions from porcine thoracic aortae using differential and sucrose density gradient centrifugation. This membrane fraction showed a high activity of 5'-nucleotidase and also ATP-dependent Ca^{2+} uptake. Furthermore, 65% of the Ca^{2+} uptake in the sarcolemma-rich fraction was inhibited by the addition of 20 μg/ml saponin, while only 5% of the Ca^{2+} uptake in the sarcoplasmic reticulum-rich fraction was inhibited by saponin. Saponin removes the cholesterol molecules from vesicle membrane [29] and these vesicles lose the ability to take up Ca^{2+}. Sarcolemmal membrane contains a larger amount of cholesterol than does the sarcoplasmic reticulum [6]. The extent of saponin-inhibited Ca^{2+} uptake may thus serve to elucidate the origin of Ca^{2+} uptake by the vesicles in smooth muscle.

At least two mechanisms are involved in the cAMP-dependent protein kinase mediation of the relaxation of smooth muscle by β-stimulators [1]. One is through the stimulation of membrane Ca^{2+} transport and the other is through the phosphorylation of myosin light chain kinase [30].

Some investigators reported that cAMP- and cAMP-dependent protein kinase enhanced the Ca^{2+} uptake by microsomes and sarcolemma-rich fraction [31–35], while others observed no stimulatory effects [36–38]. In the present study, the catalytic subunit of cAMP-dependent protein kinase activated the Ca^{2+} uptake of the sarco-

lemma-rich fraction, thereby indicating that the cAMP-dependent protein kinase may play an important role in the extrusion of Ca^{2+} through the sarcolemmal membrane. Furthermore, the phosphorylation of the 28 and 22 kDa proteins was enhanced by cAMP-dependent protein kinase, and these phosphorylated proteins may be associated with the stimulation of Ca^{2+} uptake by cAMP-dependent protein kinase in sarcolemmal membrane.

It has been suggested that the increase in cGMP levels is responsible for the relaxant effect of nitrocompounds [2]. In smooth muscle, cGMP-dependent protein kinase stimulated the microsomal Ca^{2+} uptake [39]. The 250, 130, 85 and 75 kDa proteins were phosphorylated by cGMP in sarcolemma of rabbit aortic smooth muscle [40], 130 and 80 kDa proteins in microsome of canine tracheal smooth muscle [41] and lower molecular weight substrates were phosphorylated in intact rat aortae [42]. In our study, cGMP-dependent protein kinase stimulated the Ca^{2+} uptake in the sarcolemma-rich fraction, and phosphorylated the 35 kDa protein, this substrate differing in molecular weight from the proteins phosphorylated by cAMP-dependent protein kinase.

Calmodulin plays a major role in cell functions [43]. (Ca^{2+}, calmodulin)-dependent protein phosphorylation in membranes from a variety of tissues suggests that such phosphorylation may be responsible for (Ca^{2+}, calmodulin)-mediated cell functions [44].

Calmodulin stimulated the Ca^{2+} uptake and phosphorylated the 100, 75, 65, 41 and 22 kDa proteins in the sarcolemma-rich fraction. The relation between phosphorylated proteins and stimulation of the Ca^{2+} uptake by calmodulin has been studied using other tissues. In *Torpedo* synaptic vesicles, for example, the 64, 58, 54 and 41 kDa proteins were phosphorylated and the Ca^{2+} uptake was stimulated by calmodulin [26]. In canine heart sarcoplasmic reticulum, the 22 kDa protein (phospholamban) was also detected [27]. Furthermore, the calmodulin-dependent protein kinase was also detected in bovine brain [28].

Calmodulin and cAMP-dependent protein kinase stimulated the Ca^{2+} uptake of both the sarcolemma-rich and sarcoplasmic reticulum-rich fractions, while cGMP-dependent protein kinase stimulated the Ca^{2+} uptake of the sarcolemma-rich, but not the sarcoplasmic reticulum-rich fraction. Thus, the stimulatory effect of cGMP-dependent protein kinase on the Ca^{2+} uptake may be specific for the sarcolemma-rich fraction.

In sarcolemma of aortic smooth muscle, the Ca^{2+} uptake is stimulated by cAMP- and cGMP-dependent protein kinases and by calmodulin. As the molecular weights of these phosphorylated proteins differed, the relaxation of smooth muscle may relate to different pathways in the Ca^{2+} pump process.

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References

1 Bär, H.P. (1974) Adv. Cyclic Nucleotides Res. 4, 195–237
2 Schultz, K.D., Schultz, K. and Schultz, G. (1977) Nature 265, 750–751
3 Janis, R.A., Crankshaw, D.J. and Daniel, E.E. (1977) Am. J. Physiol. 232, C50–C58
4 Kwan, C.Y., Garfield, R. and Daniel, E.E. (1979) J. Mol. Cell. Cardiol. 11, 639–659
5 Grover, A.K., Kwan, C.Y., Crankshaw, J., Crankshaw, D.J., Garfield, R.E. and Daniel, E.E. (1980) Am. J. Physiol. 239, C66–C74
6 Raeymaekers, L., Wuytack, F., Eggermont, J., De Schutter, G. and Casteels, R. (1983) Biochem. J. 210, 315–322
7 Morel, N., Wibo, M. and Godfraind, T. (1981) Biochim. Biophys. Acta 644, 82–88
8 Jarrett, H.W. and Penniston, J.T. (1977) Biochem. Biophys. Res. Commun. 77, 1210–1216
9 Gopinath, R.M. and Vincenzi, F.F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209
10 Caroni, P. and Carafoli, E. (1981) J. Biol. Chem. 256, 3263–3270
11 Pershadsingh, H.A., Landt, M. and McDonald, J.M. (1980) J. Biol. Chem. 255, 8983–8986
12 Colca, J.R., Kotagal, N., Lacy, P.E. and McDaniel, M.L. (1983) Biochim. Biophys. Acta 729, 176–184
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
14 Wattiaux de Coninck, S. and Wattiaux, R. (1969) Biochim. Biophys. Acta 183, 118–128
15 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
16 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell. Biol. 32, 415–438
17 Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172–180

18 Kitazawa, T. (1976) J. Biochem. 80, 1129–1147
19 Harafuji, H. and Ogawa, Y. (1980) J. Biochem. 87, 1305–1312
20 Hirata, M. and Koga, T. (1982) Biochem. Biophys. Res. Commun. 104, 1544–1549
21 Laemmli, U.K. (1970) Nature 227, 680–685
22 Nakazawa, K. and Sano, M. (1975) J. Biol. Chem. 250, 7415–7419
23 Yazawa, M., Sakuma, M. and Yagi, K. (1980) J. Biochem. 87, 1313–1320
24 Hirata, M., Suematsu, E. and Koga, T. (1983) Mol. Pharmacol. 23, 78–85
25 Greengard, P. (1978) Science 199, 146–152
26 Rephaeli, A. and Parsons, S.M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 5783–5787
27 Le Peuch, C.J., Haiech, J. and Demaille, J.G. (1979) Biochemistry 18, 5150–5157
28 Moskowitz, N., Glassman, A., Ores, C., Schook, W. and Puszkin, S. (1983) J. Neurochem. 40, 711–718
29 Ohtsuki, I., Manzi, M., Palade, G.E. and Jamieson, J.D. (1978) J. Microsc. Biol. Cell. 31, 119–126
30 Adelstein, R.S. and Eisenberg, E. (1980) Annu. Rev. Biochem. 49, 921–956
31 Webb, R.C. and Bhalla, R.C. (1976) J. Mol. Cell. Cardiol. 8, 145–157
32 Nishikori, K., Takenaka, T. and Maeno, H. (1977) Mol. Pharmacol. 13, 671–678
33 Thorens, S. and Haeusler, G. (1978) Biochim. Biophys. Acta 512, 415–428
34 Bhalla, R.C., Webb, R.C., Singh, D. and Brock. T. (1978) Am. J. Physiol. 234, H508–H514
35 Brockbank, K.J. and England, P.J. (1980) FEBS Lett. 122, 67–71
36 Kreye, V.A.W. and Schlicker, E. (1980) Br. J. Pharmac. 70, 537–544
37 Sands, H. and Mascali, J. (1978) Arch. Int. Pharmacodyn. 236, 180–191
38 Allen, J.C. (1977) Blood Vessels 14, 91–104
39 Sarmiento, J.G., Janis, R.A. and Lincoln, T.M. (1981) Fed. Proc. 40, 1831
40 Ives, H.E., Casnellie, J.E., Greengard, P. and Jamieson, J.D. (1980) J. Biol. Chem. 255, 3777–3785
41 Hogaboom, G.K., Emler, C.A., Butcher, F.R. and Fedan, J.S. (1982) FEBS Lett. 139, 309–312
42 Rapoport, R.M., Draznin, M.B. and Murad, F. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6470–6474
43 Cheung, W.Y. (1980) Science 207, 19–27
44 Schulman, H. and Greengard, P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5432–5436