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EFFECTS OF CYCLIC AMP AND PROTEIN KINASE ON CALCIUM UPTAKE IN A MICROSOMAL FRACTION FROM GUINEA PIG TAENIA CAECUM

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Abstract—A microsomal fraction was isolated from guinea pig taenia caecum by differential centrifugation. Activities of ouabain-sensitive (Na^+, K^+)-ATPase, 5′-neucleotidase and NADPH-cytochrome *c* reductase were enriched in the microsomal fraction. On the other hand, less cytochrome *c* oxidase and monoamine oxidase were contained in this fraction. These results suggest that the microsomal fraction used in this study was derived from both sarcolemma and sarcoplasmic reticulum. Ca^{2+} uptake by this fraction was strictly dependent on the presence of ATP and was facilitated by oxalate. An ATP-regenerating system was required for the determination of Ca^{2+} uptake, when a lower concentration of ATP (e.g. 0.25 mM) was used. Phosphorylation of the microsomal fraction was doubled when these membranes were incubated in the presence of cyclic AMP plus cyclic AMP-dependent protein kinase (protein kinase). When the microsomal fraction was pretreated with cyclic AMP plus protein kinase, Ca^{2+} uptake was stimulated. The increases in microsomal phosphorylation and Ca^{2+} uptake were significantly correlated ($P < 0.01$). This stimulation of Ca^{2+} uptake by microsomal phosphorylation was observed only in the presence of protein kinase, oxalate, and low Ca^{2+} and Mg^{2+} concentrations. The results suggest that stimulation of Ca^{2+} uptake may be the mechanism by which cyclic AMP is involved in β-adrenergic relaxation of smooth muscle.

It is now accepted that myoplasmic free calcium ions play a central role in controlling the contraction–relaxation processes of smooth muscle. β-Sympathomimetic agents relax smooth muscle by activation of receptors located on sarcolemma which, in turn, leads to a decrease in myoplasmic Ca^{2+} concentration [1]. The complete mechanism, however, of the β-adrenergic effect is unclear [1]. Cyclic AMP has been postulated to be a second messenger for β-adrenergic agents in many tissues, including smooth muscle [2].

It has been demonstrated that, in cardiac and slow skeletal muscles, phosphorylation of sarcoplasmic reticulum by protein kinase stimulates Ca^{2+} uptake into this membrane system, resulting in a decrease in cytoplasmic Ca^{2+} concentration [3]. Although evidence has been obtained recently that this mechanism also operates in smooth muscle in the expression of β-adrenergic action [2, 4–10], there are contradictory reports [11–16]. The present study was undertaken to investigate the effects of cyclic AMP and protein kinase on phosphorylation and Ca^{2+} uptake in the microsomal fraction of guinea pig taenia caecum, which is one of the tissues most extensively studied for elucidation of β-adrenergic relaxation in smooth muscle [1].

METHODS AND MATERIALS

Preparation of the microsomal fraction. Guinea pigs of either sex, weighing 300–500 g, were killed, and taeniae caeci were rapidly dissected and trimmed of connective tissues. A microsomal fraction (hereafter called microsomes for brevity) was prepared as follows, and all operations were performed at 0–4°. Tissues were minced in a chilled 0.30 M sucrose solution containing 10 mM Hepes†/KOH (pH 7), 1 mM DTT and 0.1 mM PMSF (a buffered sucrose solution). Thereafter, homogenization was carried out in 20 vol. of a buffered sucrose solution with a Potter–Elvehjem homogenizer (6 strokes), followed by three bursts of 5 sec each in a Polytron with intermittent pauses of 25 sec. Mitochondria and other large particles were removed by centrifugation, first at 2500 *g* for 10 min and then at 15,000 *g* for 20 min. The supernatant fraction was then centrifuged at 100,000 *g* for 1 hr. The pellet obtained was subjected to treatment with 0.6 M KCl for 2.5 hr and was resedimented by centrifugation. Microsomes thus obtained were stored on ice, and used within a day.

Activation of latent enzymic activities. Prior to enzymic assays, the preparations were treated with detergents to unmask latent activities according to a modification of the method of Jørgensen [17]. An aliquot of the homogenate or of the microsomal suspension was added to a tube containing an equal volume of 0.3% (v/v) Triton X-100 (for cytochrome *c* oxidase assay) or 0.2% (w/v) sodium deoxycholate dissolved in a buffered sucrose solution. After incubation for 20 min at 25°, the mixture was diluted 30- to 60-fold (with a portion of the reaction medium prewarmed to the desired temperature) and then assayed for enzymic activity as described.

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† Abbreviations: Hepes, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; and EGTA; ethyleneglycol-bis-(β-aminoethyl *N*,*N*′-tetraacetic acid.

5′-Nucleotidase, ouabain-sensitive (Na^+, K^+)-*ATPase, and basal ATPase.* These activities were assayed by measuring the inorganic phosphate that was liberated during 30 min at 37° from 5′-AMP (5′-nucleotidase) and ATP (ATPases). The released phosphate was estimated by the method of Fiske–Subbarow [18]. The reaction medium for 5′-nucleotidase has already been described [19]. ATPase activities were measured in 30 mM Tris/HCl (pH 7.4 at 37°), 140 mM NaCl, 14 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 3 mM Na_2ATP, and 1 mM ouabain (where indicated). Ouabain-sensitive activity was defined as the difference between the activities in the presence and absence of ouabain. ATPase activity in the presence of ouabain was designated as basal ATPase activity.

Monoamine oxidase. Activity was assessed according to the method of Harada and Nagatsu [20].

Cytochrome c oxidase. This enzymic activity was assayed by the method of Orii and Okunuki [21].

NADPH-cytochrome c reductase. Activity was determined according to Omura and Takesue [22], with the slight modification that 2 μM rotenone was included in the reaction medium.

Ca^{2+} *uptake.* Generally, Ca^{2+} uptake was carried out in a solution composed of 30 mM Tris/HCl (pH 7.0 at 32°), 100 mM KCl, 0.25 mM Na_2ATP, 0.25 mM $MgCl_2$, 10 mM Na_2 creatine phosphate, 40 μg/ml creatine kinase, 5 mM NaN_3, 5 mM K_2 oxalate, 0.1 mM $CaCl_2$, 0.2 μCi/ml $^{45}Ca^{2+}$, and 0.35 mM EGTA. The concentration of free Ca^{2+} ions in the reaction medium was calculated to be 0.3 μM from the apparent binding constant between Ca^{2+} and EGTA ($1.26 \times 10^6\ M^{-1}$ at pH 7.0 from Ref. 23). The bindings among other ligands and cations were not taken into account for the calculation. After preincubation of the reaction medium for 5 min at 32°, a microsomal suspension and protein kinase were added and incubated for 10 min. Thereafter, a small amount of solution containing Ca^{2+} buffer + $^{45}Ca^{2+}$ and K_2 oxalate was added to give final concentrations of 0.3 μM Ca^{2+}, 0.2 μCi/ml $^{45}Ca^{2+}$ and 5 mM oxalate, and the reaction was permitted to proceed for 10 min. To stop the reaction, an aliquot of the mixture was withdrawn and filtered through a Millipore filter (HAWP) followed by two quick washings with chilled buffered sucrose solution. The radioactivity retained on a disc was counted as described in Ref. 19.

Preparation of cyclic AMP-dependent protein kinase. Partially purified protein kinase was prepared through the DEAE-cellulose step, according to the method of Rubin *et al.* [24]. The active material was further purified by gel filtration chromatography (Sephadex G-200, 2.5 × 90 cm), to give a final purification ratio of about 400. The enzyme activity was assayed by the method of Rubin *et al.* [24], and the protein kinase preparations had phosphorylating activities of about 200 units/mg protein (one unit will transfer 1.0 nmol of phosphate from (γ-^{32}P)ATP to protamine per min at pH 7.0 and 30°). (γ-^{32}P)ATP was synthesized by the method of Glynn and Chappell [25].

Membrane phosphorylation by cyclic AMP and protein kinase. Reactions were carried out in 0.2 ml of much the same reaction mixtures used in the Ca^{2+} uptake experiment, except that the ATP-regenerating system and $^{45}Ca^{2+}$ were omitted and ATP was replaced with (γ-^{32}P)ATP. Microsomal concentration was increased up to 0.5 mg-ml.

Protein assay. Protein concentration was determined by the method of Lowry *et al.* [26], using bovine serum albumin as a standard.

Statistical analysis of data. The differences between the values of Ca^{2+} uptake or ^{32}P incorporation, which were observed under various specified conditions (shown in Tables 3, 4 and 5), were compared with an analysis of variance for paired and grouped data. For evaluation of the data of the other experiments, Student's *t*-test was used.

Materials. Materials used in the present study were as follows: sodium deoxycholate, 5′-AMP (sodium salt), Na_2ATP, cyclic AMP (sodium salt) (Sigma Chemical Co.), ouabain, creatine phosphate (disodium), creatine kinase (Boehringer-Mannheim Co.), Triton X-100, $^{45}CaCl_2$ (16 mCi/mg) (New England Nuclear), and ^{32}P-orthophosphate (Japan Atomic Energy Institute). Other chemicals used were of analytical grade.

RESULTS

Characterization of microsomal fraction. Table 1 shows that the microsomal fraction was rich in (Na^+, K^+)-ATPase, 5′-nucleotidase (markers for sarcolemma) and NADPH–cytochrome *c* reductase (a putative marker for sarcoplasmic reticulum). The specific activity of NADPH–cytochrome *c* reductase was increased as much as 11-fold (approximately) as compared with that of a muscle homogenate. Similarly, (Na^+, K^+)-ATPase and 5′-nucleotidase activities were 18- and 14-fold higher than those measured in the homogenate. Basal ATPase activity was also increased (13-fold) over the homogenate. On the other hand, the activities of cytochrome *c* oxidase (a marker for mitochondrial inner membrane) and monoamine oxidase (a putative marker for micochondrial outer membrane) were increased, at most, 3 to 4-fold. Consequently, we used this $10^5\ g$ pellet washed with 0.6 M KCl or a microsomal fraction in the experiments described below.

Ca^{2+} *uptake in the presence and absence of an ATP-regenerating system.* There have been several reports that some microsomes from smooth muscle take up Ca^{2+} linearly with regard to time (e.g. Refs. 4, 6, 8, 9, 14 and 27). In the microsomes of other smooth muscles, Ca^{2+} uptake has been reported to level off as the reaction proceeds and, finally, to reach a plateau [7, 10]. In the course of investigating these discrepancies, we checked the possibility that the appearance of the plateau depends on the ATP concentration in the reaction mixture. The results are presented in Table 2. When 0.25 mM ATP was included in the mixtures, Ca^{2+} uptake almost reached a steady state within 20 min. Addition of an ATP-regenerating system (10 mM creatine phosphate + 40 μg/ml creatine kinase) resulted in a marked enhancement of Ca^{2+} uptake at 40 min (a 5-fold increment), while no change was observed at 1 min. At 5 mM ATP, the effect of the ATP-regenerating system was also apparent, but less pronounced. Creatine kinase alone had no effect on ATP-depen-

Table 1. Characterization of a microsomal fraction used in this study by marker enzyme determination*

	Protein (mg)	Basal ATPase	Ouabain-sensitive (Na^+, K^+)-ATPase	5'-Nucleotidase	NADPH-cyt. *c* reductase	Monoamine oxidase	Cyt. *c* oxidase
Homogenate	363	25.9 ± 0.29 9,403	3.71 ± 0.29 1,347	5.13 ± 0.15 1,863	1.43 ± 0.55 519	0.67 ± 0.01 242	46.8 ± 1.8 17,000
10^5 *g* Pellet	11.4	296 ± 3 3,377	29.3 ± 3.8 334	44.1 ± 1.8 503	8.80 ± 0.61 100	3.27 ± 0.01 37.3	58.8 ± 2.6 670
KCl-washed 10^5 *g* pellet	4.6	334 ± 4 1,523	67.4 ± 5.7 307	71.5 ± 2.4 326	16.2 ± 1.4 74.1	2.92 ± 0.04 13.3	146 ± 7 670

* Tissue of 3.0 g wet weight as a starting material was used for the experiments (N = 3–5). Upper and lower lines for each fraction indicate specific and total activities, expressed as nmoles/min/mg protein and nmoles/min respectively.

dent Ca^{2+} uptake, and 10 mM creatine phosphate could not be substituted for ATP as an energy source (data not shown). These results suggest the possibility that the kinetics of Ca^{2+} uptake may deviate from the theoretical, particularly when relatively low concentrations of ATP are used, unless an ATP-regenerating system is added. Subsequent experiments were carried out under conditions where an ATP-regenerating system was included.

Table 3 shows that Ca^{2+} uptake by microsomes was ATP-dependent and facilitated by 5 mM oxalate. By omitting ATP, with or without oxalate, no Ca^{2+} uptake was observed.

Figure 1 shows the time course of Ca^{2+} uptake in the presence of 5 mM ATP and 1 μM Ca^{2+}. The rate of Ca^{2+} uptake into microsomes was constant for 10 min after the start of the reaction.

Microsomal phosphorylation. Table 4 shows a transfer of the phosphoryl group at the γ-position of ATP to microsomes for 1 min after the start of the reaction with cyclic AMP and/or protein kinase. Combined addition of cyclic AMP (10 μM) and protein kinase (1.6 units/ml) doubled ^{32}P incorporation into microsomes, whereas cyclic AMP or protein kinase alone had no stimulatory effect on microsomal phosphorylation. With incubation for more than 1 min, phosphorylation tended to decline, possibly due to the combined actions of ATPases and phosphatase(s), which brought about depletion of [γ-^{32}P]ATP and dephosphorylation of phosphorylated materials respectively. From these results, it follows that there exists in microsomes a substrate for protein kinase, and that microsomes contain insufficient endogenous cyclic AMP-dependent protein kinase or adenylate cyclase to activate protein kinase for 1 min (see Discussion).

Effects of cyclic AMP and protein kinase on Ca^{2+} uptake. The effects of cyclic AMP and protein kinase on Ca^{2+} uptake were examined (Table 5). When microsomes were pretreated with cyclic AMP plus protein kinase for 10 min, the rate of Ca^{2+} uptake at a Ca^{2+} concentration of 0.3 μM was increased by around 25%. Cyclic AMP-dependent protein kinase alone caused slight, but significant, stimulation of Ca^{2+} uptake. The pretreatment with cyclic AMP alone for 10 min had no detectable effect on Ca^{2+} uptake. To correlate protein kinase catalyzed microsomal phosphorylation with stimulated Ca^{2+} uptake, both phosphorylation and Ca^{2+} uptake were measured with several concentrations of protein kinase. A positive correlation was found ($r = 0.89$, $p < 0.01$), and the best fit line, calculated by the method of least mean squares, is shown in Fig. 2.

Effects of microsomal phosphorylation on Ca^{2+} uptake in the absence of oxalate. Ca^{2+} uptake, not supported with oxalate, was measured in the absence

Table 2. Effects of an ATP-regenerating system on Ca^{2+} uptake in the presence of oxalate by microsomes from guinea pig taenia caecum*

Reaction time (min)	Ca^{2+} uptake (nmoles/mg protein)		
	No addition	+10 mM CP	+10 mM CP + 40 μg/ml CPK
0.25 mM ATP			
1	2.46 ± 0.05	2.47 ± 0.11	2.83 ± 0.09
20	8.97 ± 0.04	22.2 ± 0.6	31.0 ± 0.6
40	10.0 ± 0.1	34.4 ± 0.5	49.7 ± 0.7
5.0 mM ATP			
1	4.75 ± 0.16	4.79 ± 0.2	5.12 ± 0.29
20	72.3 ± 0.2	88.0 ± 1.2	105 ± 2
40	112 ± 1	141 ± 0	164 ± 2

* Concentrations of Ca^{2+} and oxalate were 1 μM and 5 mM, respectively, and a molar ratio of $MgCl_2$ to ATP was selected to be unity. Each value represents a mean ± S.E. of three determinations. Abbreviations: CP, creatine phosphate; and CPK, creatine phosphokinase.

Table 3. Dependence of microsomal Ca^{2+} on ATP and oxalate*

Medium	Ca^{2+} uptake (nmoles/mg protein/10 min)
Complete	11.2 ± 0.2†‡
no oxalate	6.01 ± 0.13§
no ATP	0.03 ± 0.07
no oxalate ATP	0.14 ± 0.26

*Ca^{2+} uptake into microsomes was determined in the presence (complete medium) of ATP (0.25 mM) and oxalate (5 mM), and in the absence of either or both. The concentration of Ca^{2+} was 0.3 μM. Each value represents a mean ± S.E. of five determinations.

†, ‡, § Significantly different from the group without ATP, oxalate, or both respectively, at $P < 0.05$.

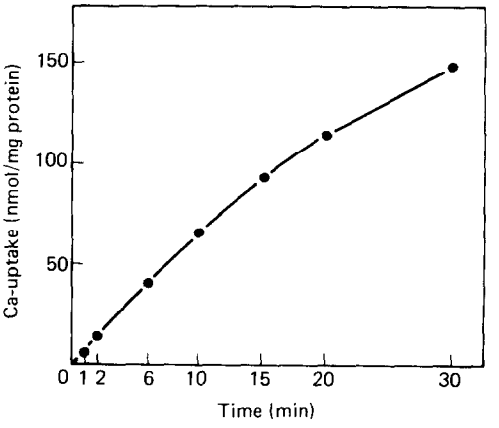

Fig. 1. Rate of Ca^{2+} uptake into microsomes at 32°. Concentrations of Ca^{2+} and ATP were 1 μM and 5 mM respectively. Each point is a mean of five determinations, and S.E. is within a point.

(control) and presence of cyclic AMP plus protein kinase. The calcium taken up in 10 min amounted to 9.89 ± 0.29 nmoles/mg protein (mean ± S.E., N = 5; control), whereas in the presence of cyclic AMP and protein kinase it was 9.97 ± 0.17 nmoles/mg protein. These results indicate that microsomal phosphorylation did not stimulate Ca^{2+} uptake in the absence of oxalate, as reported previously for cardiac microsomes [28].

Effects of ATP and $MgCl_2$ *on the stimulation of* Ca^{2+} *uptake by microsomal phosphorylation.* The rate of Ca^{2+} uptake was considerably increased by 10 μM cyclic AMP and protein kinase in the range of ATP concentrations indicated in Fig. 3, with an optimum at 250 μM ATP. At concentrations higher than this optimum, stimulation of Ca^{2+} uptake decreased, but it was significant even at 5 mM ATP. When the $MgCl_2$ concentration was increased at a fixed level of ATP (5 mM), the stimulatory effect of cyclic AMP plus protein kinase on Ca^{2+} uptake was diminished (Fig. 4). In addition, higher concentra-

Table 4. Effects of cyclic AMP and protein kinase on ^{32}P incorporation into microsomes from guinea pig taenia caecum*

Medium	^{32}P incorporation (pmoles/mg protein/min)
Control	10.8 ± 1.1
+ Cyclic AMP	8.1 ± 1.8
+ Protein kinase	9.1 ± 0.2
+ Cyclic AMP + protein kinase	18.4 ± 1.3†

* The effects of cyclic AMP (10 μM) and/or protein kinase (1.6 units/ml) on ^{32}P incorporation into microsomes (0.5 mg/ml) were examined. Each value represents a mean ± S.E. of five determinations.

† Significantly different from other groups at $P < 0.01$.

Table 5. Effects of cyclic AMP and protein kinase on Ca^{2+} uptake in the presence of oxalate by microsomes from guinea pig taenia caecum*

Medium	Ca^{2+} uptake (nmoles/mg protein/10 min)
Control	11.0 ± 0.2
+ Cyclic AMP	11.0 ± 0.1
+ Protein kinase	12.7 ± 0.2
+ Cyclic AMP + protein kinase	13.8 ± 0.2+‡

* The effects of cyclic AMP (10 μM) and/or protein kinase (1.6 units/ml) on Ca^{2+} uptake into microsomes (0.1 mg/ml) were examined. The concentration of Ca^{2+} was 0.3 μM. Each value represents a mean ± S.E. of five determinations.

†, ‡ Significantly different from control and protein kinase-treated groups, respectively, at $P < 0.05$.

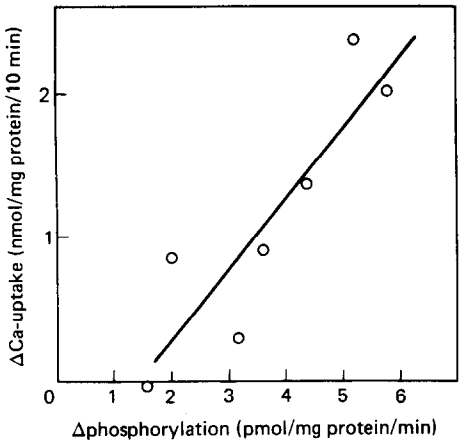

Fig. 2. Relationship between net stimulation of Ca^{2+} uptake by protein kinase and net increase in protein kinase-catalyzed phosphorylation. Ca^{2+} uptake was assayed following preincubations of microsomes with 10 μM cyclic AMP and various concentrations of protein kinase in five replicates. Concentrations of Ca^{2+}, oxalate and ATP were 0.3 μM, 5 mM and 0.25 mM respectively. Phosphorylation of the same microsomes was measured in parallel experiments. A positive correlation was found ($r = 0.89$, $P < 0.01$), and the best fit line is shown.

tions of $MgCl_2$ inhibited Ca^{2+} uptake itself. Consequently, the molar ratio of ATP to $MgCl_2$ was chosen to be 1.0 in all experiments except for those in Fig. 4.

Effects of Ca^{2+} on stimulation of Ca^{2+} uptake by microsomal phosphorylation. When Ca^{2+} uptake by microsomes pretreated with cyclic AMP plus protein kinase was measured at various Ca^{2+} concentrations, significant stimulation of Ca^{2+} uptake was seen below approximately 1 μM (Fig. 5). As calcium ion concentration was lowered, the extent of stimulation was increased.

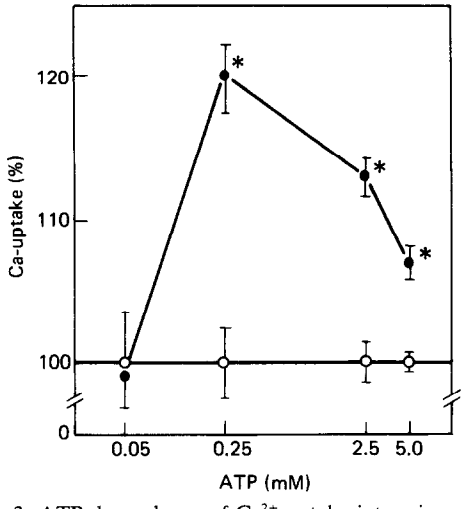

Fig. 3. ATP dependence of Ca^{2+} uptake into microsomes pretreated with (●) and without (○) cyclic AMP (10 μM) protein kinase (1.6 units/ml). Control uptake at each concentration of ATP was expressed as 100%. Each point is a mean of five determinations, and a vertical bar indicates S.E. The molar ratio of $MgCl_2$ to ATP was always kept at 1.0, and concentrations of Ca^{2+} and oxalate were 0.3 μM and 5 mM respectively. Key: (*) significantly different from each control at $P < 0.05$.

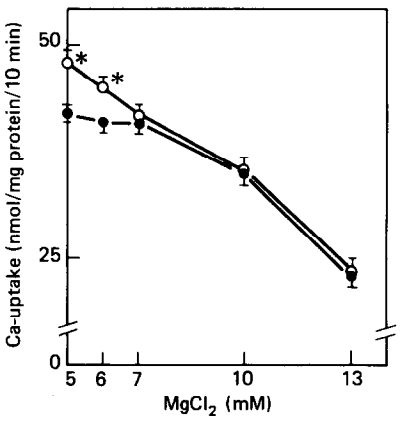

Fig. 4. $MgCl_2$ dependence of Ca^{2+} uptake into microsomes pretreated with (●) and without (○) cyclic AMP (10 μM) and protein kinase (1.6 units/ml). Each point is a mean of five determinations, and a vertical bar indicates S.E. Concentrations of ATP, oxalate and CA^{2+} were 5 mM, 5 mM and 0.3 μM respectively. Key: (*) significantly different from each control at $P < 0.05$.

DISCUSSION

Microsomes obtained from guinea pig taenia caecum accumulated Ca^{2+} in the presence of ATP, and this accumulation was facilitated by the addition of oxalate. This Ca^{2+} uptake was not derived from mitochondria, since sodium azide (5 mM) was included in the reaction mixture. The rate of Ca^{2+} uptake at a Ca^{2+} concentration of 10^{-6} M was about 60 nmoles-mg per protein per 10 min; this value is

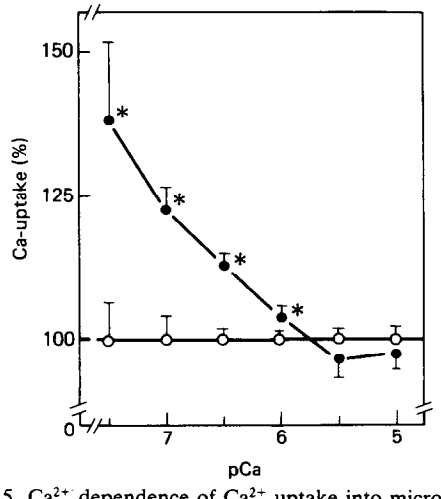

Fig. 5. Ca^{2+} dependence of Ca^{2+} uptake into microsomes pretreated with (●) and without (○) cyclic AMP (10 μM) and protein kinase (1.6 units/ml). Control uptake at each concentration of Ca^{2+} was expressed as 100%. Each point is a mean of five determinations, and a vertical bar indicates S.E. Concentrations of both ATP and oxalate were 5 mM. Key: (*) significantly different from each control at $P < 0.05$.

comparable to that reported by Raeymaekers *et al.* [27] using the same tissue. From the data presented in Table 2, it follows that introduction of an ATP-regenerating system into the assay medium is necessary to measure Ca^{2+} uptake in smooth muscle microsomes accurately, particularly when utilizing relatively low concentrations of ATP.

For a decade, the possible involvement of cyclic AMP in the effects of β-sympathomimetic drugs on smooth muscle relaxation has been studied [1, 2]. The hypothesis that cyclic AMP can induce smooth muscle relaxation by stimulating Ca^{2+} uptake into sarcoplasmic reticulum (or Ca^{2+} extrusion from plasma membrane) has been proposed [1, 2], since it is well accepted that cyclic AMP-dependent protein kinase catalyzes the phosphorylation of a protein called phospholamban in myocardial ventricular sarcoplasmic reticulum [3].

In this study, enhancement of phosphorylation of microsomes from taenia caecum was observed in the presence of cyclic AMP plus cyclic AMP-dependent protein kinase. Cyclic AMP or protein kinase alone did not stimulate ^{32}P incorporation into microsomes. The lack of effect of cyclic AMP indicated a substantial loss of endogenous protein kinase activity in microsomes from guinea pig taenia caecum. This result contrasts with those reported by Nishikori *et al.* [7], Tomioka *et al.* [10] and Bhalla *et al.* [9], using rat uterus, bovine trachea and rat aorta, respectively, but this discrepancy may be due to differences in the tissues used or in the microsomal preparation [29].

Concomitant preincubation of microsomes with cyclic AMP plus protein kinase for 10 min potentiated Ca^{2+} uptake by about 25% at relatively low concentrations of ionized calcium (below 10^{-6}M), as shown in Table 5 and Fig. 5. Cyclic AMP-dependent protein kinase alone also increased Ca^{2+} uptake slightly, but cyclic AMP alone was without effect. These findings are in agreement with the results of Fitzpatrick and Szentivanyi [8], using rabbit aortic microsomes. The extent of microsomal phosphorylation correlated significantly with the increase in the stimulation of the rate of Ca^{2+} uptake by microsomes when concentrations of protein kinase varied, and these data suggest the existence of a functional relationship between microsomal phosphorylation and an increased rate of calcium transport (Fig. 2).

In our studies, protein kinase alone did not catalyze phosphorylation but stimulated Ca^{2+} uptake into microsomes significantly. These results seem to be conflicting, if the suggestion discussed above is correct. This discrepancy, however, may be due to the differences in reaction time employed in the two assays. Microsomal phosphorylation was carried out for 1 min, which would be too short for protein kinase alone to give any detectable effect on phosphorylation, whereas in the Ca^{2+} uptake assays microsomes were pretreated with protein kinase for 10 min, which might be enough time for cyclic AMP to accumulate endogenously and, thus, to activate protein kinase by adenylate cyclase activity contained in microsomes, as discussed by other investigators [6,7,9,10].

Workers using microsomes derived from intestinal [4], vascular [5,8,9], and air way smooth muscles [10] and uterus [6,7] have claimed a positive effect of cyclic AMP on Ca^{2+} uptake either in the presence or absence of added protein kinase. On the other hand, other investigators have been unable to detect any effect of cyclic AMP and/or protein kinase on Ca^{2+} uptake by microsomal fractions from uterus [11], air way [14], vascular [13,15,16] and intestinal [12,13] muscles.

With respect to these conflicting effects of cyclic AMP on microsomal Ca^{2+} uptake reported by many investigators, Nilsson *et al.* [4] and Nishikori *et al.* [7] tried to explain the negative findings by utilization of the rather high ATP concentrations employed in these experiments. For example, Nishikori *et al.* assumed that endogenous cyclic AMP, which was formed when a higher concentration of ATP was added, would mask its stimulating effect [7], and this assumption is plausible, since protein kinase alone enhanced Ca^{2+} uptake slightly, as discussed above. In addition to this plausible effect of endogenously formed cyclic AMP, the concentration of Mg^{+} in the reaction mixture may be critical in the expression of protein kinase-induced enhancement of Ca^{2+} uptake, although its physiological significance is unclear. Since Maeno and his colleagues actually adopted 2 [7] or 4 [10] as a molar ratio of $MgCl_2$ to ATP in their reports, there is the possibility that Mg^{2+} suppresses the effects of cyclic AMP on Ca^{+} uptake when a high concentration of ATP is used.

Marker enzyme determinations suggested that the microsomes used in this study contained both sarcoplasmic reticulum- and sarcolemma-derived membranes. It has been reported that, in contrast to the behaviour of sarcoplasmic reticulum, Ca^{2+} uptake by the sarcolemmal vesicles is not stimulated by oxalate, in smooth muscles [30–32] and other muscles [33, 34]. In light of this view, Ca^{+} uptake into the vesicles derived from sarcolemma may not contribute to the enhancement of microsomal Ca^{2+} uptake by cyclic AMP plus protein kinase employed in our assay, if any, since such an enhancement of Ca^{2+} uptake was observed only when oxalate was added. In other words, this would indicate a major involvement of the sarcoplasmic reticulum in lowering the myoplasmic Ca^{2+} concentration through a Ca^{2+}-pumping process(es) in the expression of β-adrenergic action. Further studies, however, are needed.

Electrophysiological studies show that relaxation of taenia caecum by β-adrenergic agonists is accompanied by cessation of the Ca^{2+} spike [35]. But an absence of a correlation between Ca^{2+}-spike frequency and contractility was presented qualitatively [35, 36] and quantitively by Kimura *et al.* [37], who proposed that the relaxation induced by isoproterenol was not due to direct suppression of Ca^{2+} influx. Tomiyama *et al.* [38] also reported that isoproterenol and dibutyryl cyclic AMP did not inhibit $^{45}Ca^{2+}$ influx in taenia caecum. In addition, it was reported that the β-adrenergic effect is to decrease the amount of free Ca^{2+} available for contraction by increasing Ca^{2+} binding to an intracellular Ca^{2+} store [1, 39].

These results suggest that β-inhibitory action in taenia caecum is brought about by mechanisms other than a direct block of Ca^{2+} influx, such as an increase in Ca^{2+} uptake into Ca^{2+} store, and our results support this suggestion.

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