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MICROSOMAL CALCIUM-ACCUMULATING ABILITY OF BOVINE CORONARY ARTERY AND AORTA

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Abstract—Calcium uptake and binding activities of microsomal fractions from bovine coronary artery and aorta were examined. The isolated microsomal fraction of the coronary artery and aorta showed 7- to 8-fold higher glucose-6-phosphatase activity and 4- to 6-fold higher NADPH-cytochrome *c* reductase activity as compared with the corresponding values for the homogenate fraction. Coronary artery and aorta microsomal calcium uptake activities were 118 and 159 nmoles Ca^{2+}/mg protein/10 min in the presence of 100 μM $CaCl_2$, respectively. These activities for bovine vascular smooth muscle microsomes are higher than those of other species investigated. The calcium uptake activities were dependent on calcium concentrations ranging from 5 to 50 μM in the assay medium. The onset of the reaction for aorta microsomal calcium uptake was faster than that for the coronary artery. The calcium uptake activity was also dependent on ATP, but it was practically independent of oxalate ions in the assay medium. Microsomal calcium binding activities of the coronary artery and aorta were maximal at 20 min of incubation under the present experimental conditions. A lower K_m value of the aortic calcium binding for ATP was obtained as compared with that for the coronary artery. The present experiment explored several characteristics of the microsomal calcium-accumulating ability of vascular smooth muscle, which provides meaningful information for further study on cellular calcium movements in vascular smooth muscle.

Sarcoplasmic reticulum is capable of accumulating calcium in the cell. This accumulation is recognized as playing an important role in the process of contraction and relaxation of skeletal as well as cardiac muscles [1–4]. In contrast, such a definitive role for sarcoplasmic reticulum of vascular smooth muscle has not been fully established. Several reports have shown the ability to accumulate calcium ions into sarcoplasmic reticulum-enriched microsomal fractions isolated from vascular smooth muscles, such as rat aorta [5, 6] and rabbit aorta [7]. However, there is little information in the literature concerning a calcium-accumulating ability of the sarcoplasmic reticulum from coronary artery. Zelck *et al.* [8] and Wuytack *et al.* [9] have reported 20–33 nmoles Ca^{2+}/mg/10 min of calcium uptake activity in microsomal fraction from pig coronary artery. Such calcium uptake activity is very low as compared with that of cardiac microsomal fractions from various animals [10–12], though the low activity of the calcium uptake is probably due to physiological properties of the vascular smooth muscle cells. The present study was designed to explore a method for isolating the microsomal fraction with a high calcium-accumulating ability from bovine coronary artery, which may indirectly provide a subcellular basis for the intracellular free calcium movements in vascular smooth muscle cells. Furthermore, it is generally believed that responsiveness of vascular smooth muscle to physiological conditions and pharmacological interventions is different depending upon their intrinsic function. For example, it is well known that the adrenergic fibers to the coronary vessels exert a vasodilator effect but, in other locations, the adrenergic fibers are vasoconstrictor in function [13]. Moreover, van Breemen and Siegel [14] have demonstrated a different contractile response of the coronary artery and the aorta after exposure to norepinephrine. They have provided evidence that the difference in behavior of these arteries results from the release, or not, of intracellular Ca^{2+} upon exposure to norepinephrine. This raises a possibility that intracellular calcium movements of coronary artery differ from those of other vascular smooth muscles. The present study was undertaken to compare the calcium-accumulating abilities of the microsomal fractions from bovine coronary artery and aorta.

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METHODS

Isolation of microsomal fraction

Bovine heart and aorta were obtained from a slaughterhouse. It took 5–15 min to isolate these tissues from the calf after bleeding. The heart and aorta were put into cold saline and transferred to the laboratory. Fat and connective tissues were trimmed away from the aorta. About two-thirds of the aorta was separated from the adventitia to obtain the intima-media of the vascular smooth muscle. The coronary artery was carefully isolated from the myocardium, weighing 2.2 to 2.8 kg. This was made by dissecting several branches of the coronary artery from the myocardium, such as left circumflex, left ventricular anterior descending, and right coronary artery, with the aid of acrylic bars and scissors.

Isolated tissues thus obtained, after estimating their wet weight (6–8 g of each tissue), were finely minced with scissors. The minced tissues were suspended in 3 vol. (estimated from their wet weight) of a medium containing 120 mM NaCl, 5 mM $MgCl_2$ and 20 mM imidazole, pH 7.4. The suspension was preincubated at 37° for 5 min, then protease (Alkaline protease; Nagase Sangyo Co.) was added to the suspension to make up a 0.1% protease solution. The suspension was incubated for 5 min at 37° and then chilled in an ice-cold bath. The supernatant fraction was decanted, and the tissue was washed with 0.25 M sucrose–10 mM imidazole, pH 7.0. The suspension was immediately centrifuged at 1500 *g* for 2 min and then the supernatant fraction was discarded. The resulting minced tissue was washed once with 0.25 M sucrose–10 mM imidazole, pH 7.0, and again centrifuged at 1500 *g* for 2 min. The washed tissue was suspended in 5 vol. of 0.25 M sucrose–10 mM imidazole, pH 7.0, and homogenized for two 5-sec periods by an Ultraturrax (Janke Werk) at two-thirds maximal speed. The homogenate was filtered through two layers of gauze, and then the residual tissue was rehomogenized under the same conditions mentioned above. The homogenate was combined with the previous filtrate. The combined homogenate (homogenate fraction) was centrifuged at 12,000 *g* for 20 min. The supernatant fraction was filtered again through four layers of gauze to eliminate fat depots. The filtrate was centrifuged at 59,000 *g* for 60 min. The sediment (F-1) was gently suspended in 0.6 M KCl–20 mM imidazole, pH 7.0, and centrifuged again at 110,000 *g* for 30 min to obtain fraction 2(F-2) while the supernatant fraction was centrifuged at 110,000 *g* for 30 min to yield the pellet (F-3). The resulting pellets (F-2 and F-3) were finally suspended in 0.25 M sucrose–10 mM imidazole, pH 7.0, at concentrations of 1.0 to 1.5 mg/ml and used for an assay of the microsomal activities. The isolation was carried out at 0–4°. Protein was estimated according to the method of Lowry *et al.* [15].

Measurements of marker enzyme activity

Marker enzyme activities were measured according to methods described elsewhere. Each fraction was well homogenized again to explore latent enzyme activities of the fractions, before the assay. Glucose-6-phosphatase activity was measured according to the method of Nordlie and Arion [16]. NADPH-cytochrome *c* reductase activity was determined by the method of Masters *et al.* [17]. 5′-Nucleotidase activity was measured by the method of Heppel and Hilmoe [18]. Cytochrome *c* oxidase activity, expressed as the estimated first-order velocity constant, was estimated by the method of Wharton and Tzagoloff [19]. Measurements of potassium-stimulated, EDTA ATPase activity were conducted in the presence of 2 mM ATP according to the method of Martin *et al.* [20]. Measurements of potassium-stimulated phosphatase [21] and (Na^+-K^+)-ATPase activities [22] were performed as markers for cell membrane fraction of the smooth muscle.

Measurements of calcium-accumulating activity

Calcium uptake activity was measured for 10 min at 37° in medium containing 100 mM KCl, 20 mM Tris/HCl, 5 mM $MgCl_2$, 4 mM Na_2-ATP, 0.1 mM $^{45}CaCl_2$, 4 mM potassium oxalate and about 0.1 mg protein, pH 6.8, unless otherwise mentioned. Calcium binding activities were measured for 10 min at 25° in medium containing 100 mM KCl, 20 mM Tris/HCl, 5 mM $MgCl_2$, 4 mM Na_2-ATP, 0.1 mM $^{45}CaCl_2$ and about 0.1 mg protein, pH 6.8. The reaction was initiated by adding $^{45}CaCl_2$ and terminated by Millipore filtration methods described elsewhere [12]. Calcium uptake activity was also estimated in the presence of 5 mM NaN_3 or in the absence of ATP. Furthermore, both calcium binding and uptake activities were measured under various concentrations of ATP (0.3 to 4 mM), and different incubation intervals (2 to 30 min).

Measurements of ATPase activity

ATPase assay was performed according to the method described elsewhere [22]. Prior to the assay, the isolated membrane fraction was rehomogenized with ten vigorous strokes of a glass–Teflon homogenizer. Magnesium-dependent ATPase activity was measured at 37° in medium containing 100 mM KCl, 20 mM Tris/HCl, 5 mM $MgCl_2$, 0.2 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA), 5 mM NaN_3, 4 mM Tris-ATP and about 0.1 mg protein, pH 6.8. After a 3-min preincubation, the reaction was initiated by adding 4 mM ATP and, after 5 min of incubation, the reaction was terminated by adding 12% tricholoracetic acid. The resulting mixture was centrifuged under cooling at 1000 *g* for 10 min, and the supernatant fraction was sampled for an estimation of inorganic phosphate liberated according to the method of Taussky and Shorr [23]. Non-specific ATP-hydrolyzing ability was estimated in medium containing Tris/HCl, EGTA, Tris-ATP, NaN_3 and protein, and the activity was subtracted from each enzyme activity measured. Total ATPase activity was measured by the same procedure used for magnesium-dependent ATPase activity except that 0.1 mM $CaCl_2$ was substituted for 0.2 mM EGTA. The difference between the activities of total ATPase and magnesium-dependent ATPase was taken to be calcium-stimulated, magnesium-dependent ATPase activity. Both magnesium-dependent ATPase and calcium-stimulated, magnesium-dependent ATPase were also measured in the absence of 5 mM NaN_3 to determine azide-sensitivity of magnesium-dependent ATPase.

Statistical methods and reagents employed

Values are expressed as mean ± S.E.M. Student's *t*-test was used to compare the values for coronary artery with those for the aorta. Differences at the 95% confidence level were considered significant. Reagents employed in the present experiments were protease (alkaline protease; Nagase Sangyo Co.) and $^{45}CaCl_2$ (Japan Radioisotope Reagent Association). All other chemicals were special grade quality.

RESULTS

In the first set of experiments, several marker enzyme activities of subcellular fractions obtained during the isolation procedure were examined

Table 1. Marker enzyme activities of various subcellular fractions from bovine coronary artery and aorta*

	NADPH-cytochrome *c* reductase	Glucose-6-phosphatase	5′-Nucleotidase	Cytochrome *c* oxidase	Succinate dehydrogenase	Potassium-stimulated phosphatase	(Na^+-K^+)-ATPase	$(K^+$-EDTA)-ATPase	Protein yield
Coronary artery									
Homogenate	67 ± 7	14 ± 3	33 ± 1	290 ± 3	20 ± 3	5 ± 2	14 ± 4	0.93 ± 0.10	
F-1	122 ± 10	51 ± 5	126 ± 17	1260 ± 17	25 ± 2	11 ± 2	17 ± 2	1.02 ± 0.10	0.063 ± 0.004
F-2	244 ± 19	118 ± 10	374 ± 20	322 ± 5	22 ± 2	22 ± 4	17 ± 3	1.04 ± 0.15	0.040 ± 0.002
F-3	97 ± 7	32 ± 3	171 ± 15	0	4 ± 1	10 ± 3	3 ± 1	1.46 ± 0.11	0.052 ± 0.003
Aorta									
Homogenate	56 ± 6	15 ± 3	32 ± 3	251 ± 3	35 ± 5	4 ± 2	12 ± 2	1.09 ± 0.10	
F-1	303 ± 27	51 ± 6	137 ± 12	1490 ± 10	29 ± 3	12 ± 1	15 ± 2	1.41 ± 0.12	0.105 ± 0.007
F-2	372 ± 31	105 ± 9	270 ± 30	396 ± 7	25 ± 3	24 ± 3	14 ± 2	1.75 ± 0.15	0.062 ± 0.002
F-3	150 ± 12	17 ± 3	89 ± 10	0	7 ± 3	7 ± 1	4 ± 2	2.35 ± 0.21	0.096 ± 0.004

* Units of activities: NADPH-cytochrome *c* reductase (nmoles cytochrome *c*/min/mg protein); glucose-6-phosphatase (nmoles P_i/min/mg protein); 5′-nucleotidase (nmoles P_i/min/mg protein); cytochrome *c* oxidase (μg cytochrome *c*/min/mg protein); succinate dehydrogenase (nmoles succinate/min/mg protein); K^+-stimulated phosphatase (nmoles nitrophenol/min/mg protein); (Na^+-K^+)-ATPase (nmoles P_i/min/mg protein); and $(K^+$-EDTA)-ATPase (nmoles P_i/min/mg protein). Protein yield is expressed as a percentage of mg protein yielded, to initial tissue wet weight (N = 12). Each value represents the mean ± S.E.M. of four to five experiments.

(Table 1). Glucose-6-phosphatase and NADPH-cytochrome *c* reductase activities were measured as markers for the microsomal fraction [16, 17]. Glucose-6-phosphatase activities of fraction 2 (F-2) from coronary artery and aorta were about 8- and 7-fold higher compared with those of the corresponding homogenate while NADPH-cytochrome *c* reductase activities were about 4- and 6-fold higher respectively. These two enzyme activities of fraction 1 (F-1) and fraction 3 (F-3) were lower than those of F-2. 5′-Nucleotidase activity is considered to be a marker for microsomal and/or sarcolemmal fractions [18, 24]. 5′-Nucleotidase activities of F-2 derived from the coronary artery and the aorta were 11- and 9-fold higher compared with those of the homogenate, whereas the activities of F-1 and F-3 were about 4- to 7-fold and 3- to 5-fold higher respectively. These results suggest that F-2 is mostly enriched with the microsomal fraction of the vascular smooth muscles. Mitochondrial marker enzyme activities such as cytochrome *c* oxidase and succinate dehydrogenase were similar to those of each homogenate, suggesting negligible amounts of contamination of the mitochondrial fraction in F-2. Although potassium-stimulated phosphatase activities of F-2 from the coronary artery and aorta were increased 4- to 5-fold by the isolation procedure, (Na^+-K^+)-ATPase activities of F-2 were almost similar to those of the homogenate. This indicates considerably lower contamination of the sarcolemmal fraction in F-2. Potassium-stimulated, EDTA ATPase activity was also negligible in F-2 and F-3, suggesting less contamination of smooth muscle myofibrils in these fractions [20]. Thus, we used F-2 as the microsomal fraction from the bovine vascular smooth muscles in the following experiments.

In a preliminary study, we observed that the microsomal fraction isolated by the pretreatment with 0.1% protease exhibited high calcium uptake (89% high) and NADPH-cytochrome *c* reductase (47% hig) activities compared with those of the microsomal fraction isolated without any enzyme treatment. Furthermore, pretreatment of the tissues with 0.1% protease for a longer period (10 min) resulted in a significant reduction of the microsomal calcium uptake and NADPH-cytochrome *c* reductase activities. Instead of pretreatment of the tissue with protease, we preliminarily used 0.1% collagenase and found that the resulting F-2 showed about a 3-fold increase in glucose-6-phosphatase activity as compared with that of the homogenate, and about two-thirds of the microsomal calcium uptake activity as compared with that obtained by the treatment with protease.

Calcium uptake activity

Calcium uptake activity at different concentrations of $CaCl_2$. Microsomal calcium uptake activities were measured in the assay medium containing different concentrations of $CaCl_2$ (5–100 μM) (Fig. 1). The calcium uptake activity of the coronary artery was almost the same as that of aorta at calcium concentrations ranging from 5 to 50 μM. Higher calcium concentration of the assay medium (100 μM) increased the calcium uptake activity for the aorta (159 ± 18 nmoles Ca^{2+}/mg protein/10 min), but did

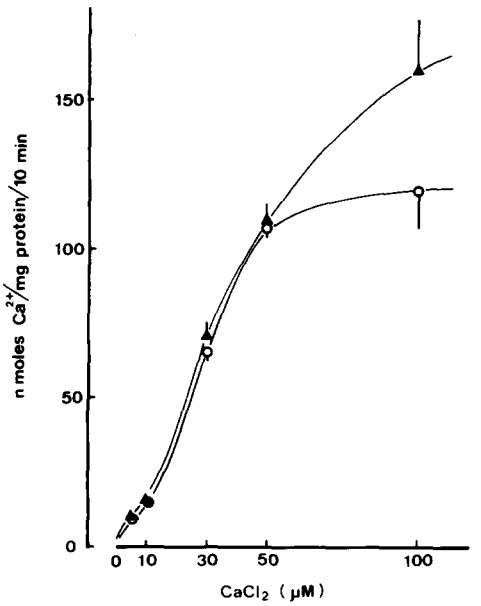

Fig. 1. Calcium uptake activities of microsomal fractions (F-2) isolated from bovine coronary artery (○) and aorta (▲) at different concentrations of calcium chloride (nmoles Ca^{2+}/mg protein/10 min). The assay conditions were described in Methods. Each value represents the mean ± S.E.M. of five experiments.

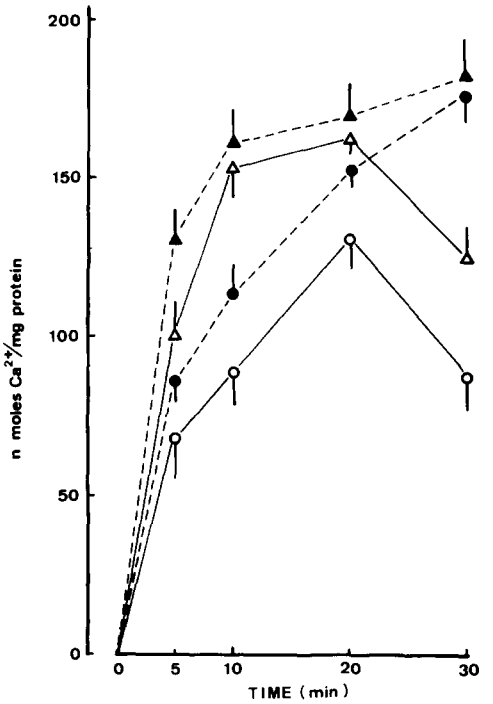

Fig. 2. Calcium uptake and calcium binding activities of microsomal fractions (F-2) isolated from bovine coronary artery and aorta at different incubation intervals (5–30 min). Key: calcium uptake of coronary artery (●), calcium uptake of aorta (▲), calcium binding of coronary artery (○), and calcium binding of aorta (△). Activities were measured in the presence of 4 mM ATP and 0.1 mM $CaCl_2$ with or without 4 mM potassium oxalate. Each value represents the mean ± S.E.M. of five experiments.

not increase that for the coronary artery appreciably (118 ± 13 nmoles Ca^{2+}/mg protein/10 min).

Time-course changes in the calcium uptake activity. To further examine different abilities between the coronary artery and the aorta in accumulating calcium, microsomal calcium uptake activities were measured at different incubation intervals in medium containing 100 μM $CaCl_2$ (Fig. 2). The calcium uptake activities of the coronary artery tended to be lower than those of the aorta throughout the whole incubation intervals (up to 30 min). Marked differences in the activities between the coronary artery and the aorta were seen at 5 and 10 min of incubation, but the activity of the coronary artery at 30 min of incubation was similar to that of the aorta.

Calcium uptake activity in the presence of sodium azide. Microsomal calcium uptake activities were also measured in medium containing 50 μM $CaCl_2$ and 5 mM NaN_3 (Table 2). The calcium uptake activity of the microsomal fraction from the coronary artery was 102 ± 5 nmoles Ca^{2+}/mg protein/10 min, and that from the aorta was 107 ± 8 nmoles Ca^{2+}/mg protein/10 min. ATP-independent calcium uptake activity (the activity in the absence of ATP) was less than 10% of the whole calcium uptake activity, indicating high dependency of the microsomal calcium uptake ability on a substrate, ATP. Sodium azide (5 mM) depressed the calcium uptake activity by about 25%.

Calcium uptake activities at different concentrations of ATP. Calcium uptake activities were measured in medium containing 50 μM $CaCl_2$ and different concentrations of ATP (0.3 to 4 mM) (Fig. 3). The activities of the coronary artery were essentially similar to those of the aorta at all the concentrations of ATP employed. The V_{max} and K_m values for the activities of the coronary artery, when estimated by the analysis of Lineweaver–Burk plots, were essentially similar to those of the aorta (125 nmoles Ca^{2+}/mg protein/10 min and 0.5 mM ATP respectively).

Calcium binding activity

Microsomal calcium binding activities of the coronary artery and the aorta were measured at different incubation intervals (5 to 30 min) in the presence of 100 μM $CaCl_2$ (Fig. 2). The calcium binding activities

Table 2. Microsomal (F-2) calcium uptake activity of bovine coronary artery and aorta in the presence and absence of ATP, and in the presence of NaN_3*

	Calcium uptake (nmoles Ca^{2+}/mg protein/10 min)	
	Coronary artery	Aorta
With ATP	102 ± 5	107 ± 8
Without ATP	10 ± 2	12 ± 2
NaN_3	75 ± 5	81 ± 4

* The assay was performed in the presence of 50 μM Ca^{2+} and 4 mM ATP (with ATP; control), 50 μM Ca^{2+} (without ATP), and 50 μM Ca^{2+}, 4 mM ATP and 5 mM sodium azide (NaN_3). The details for the assay are described in Methods. Each value represents the mean ± S.E.M. of five experiments.

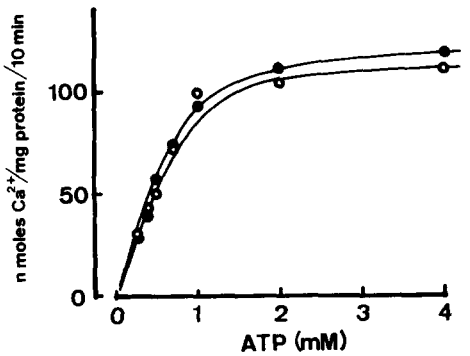

Fig. 3. Microsomal (F-2) calcium uptake activity of coronary artery (○) and aorta (●) measured in medium containing 50 μM $CaCl_2$ and different concentrations of ATP (0.3 to 4 mM). Activities were expressed as nmoles Ca^{2+}/mg protein/10 min. The results are typical of three experiments.

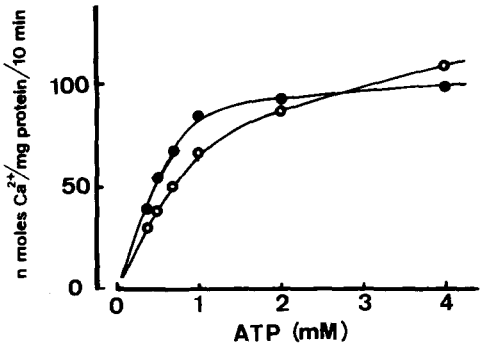

Fig. 4. Microsomal (F-2) calcium binding activity of coronary artery (○) and aorta (●) measured in medium containing 50 μM $CaCl_2$ and different concentrations of ATP (0.4 to 4 mM). Activities are expressed as nmoles Ca^{2+}/mg protein/10 min. The results are typical of three experiments.

of the coronary artery and the aorta were found to be maximum (128 ± 14 and 161 ± 19 nmoles Ca^{2+}/mg protein respectively) at 20 min of incubation, followed by a decline to about 70% of the maximal value at 30 min of incubation. This appeared to reveal partial calcium release from the microsomal fraction into the medium after 20 min. The calcium binding activity of the aorta was significantly higher than that of the coronary artery throughout the whole incubation intervals monitored. It should be pointed out that the calcium uptake activities of both tissues were essentially similar to the calcium binding activities of the corresponding tissue preparations except for the values at 30 min of incubation.

The calcium binding activity was also measured in medium containing 50 μM $CaCl_2$ and different concentrations of ATP (Fig. 4). The calcium binding activity of the aorta was highly responsive to ATP concentrations as compared with that for the coronary artery. The V_{max} and K_m values for the activities of the coronary artery and the aorta, when estimated by the analysis of Lineweaver–Burk plots, were 135 and 110 nmoles Ca^{2+}/mg protein/10 min, and 1.1 and 0.7 mM ATP respectively.

ATPase activity

Microsomal ATPase activities of bovine coronary artery and aorta were also measured. Prior to the assay, the isolated membrane fraction was rehomogenized with a glass–Teflon homogenizer. Magnesium-dependent ATPase activity of the aorta was about 2-fold higher than that of the coronary artery (Table 3). The magnesium ATPase activities of both coronary artery and aorta were further stimulated by the presence of Ca^{2+}, whose absolute values were almost equal.

In the microsomal fractions, sodium azide sensitivity to magnesium ATPase was found to be about 25%. This suggests some contamination of mitochondrial fraction in F-2, which is incompatible with the results on mitochondrial marker enzyme activity for F-2. To examine the degree of mitochondrial contamination in the microsomal fraction, we attempted to compare the influence of various mitochondrial inhibitors on the microsomal magnesium-dependent ATPase activity of the aorta with those on isolated mitochondria. The latter fraction was isolated according to the method of Kalra and Brodie [25], which consists of a differential centrifugation technique without any enzyme treatment to disrupt smooth muscle cell membranes. The results indicate that sodium azide significantly depressed magnesium-dependent ATPase activities of both mitochondria and microsomes; the agent at 5 mM

Table 3. Magnesium-dependent ATPase (Mg^{2+}-ATPase) and calcium-stimulated, magnesium-dependent ATPase (Ca^{2+}-ATPase) activities of microsomal fractions (F-2) from bovine coronary artery and aorta*

	Activities (μmoles P_i/mg protein/hr)			
	Mg^{2+}-ATPase		Ca^{2+}-ATPase	
	$-NaN_3$	$+NaN_3$	$-NaN_3$	$+NaN_3$
Coronary artery	31.4 ± 4.7	23.6 ± 2.0	2.9 ± 0.5	2.3 ± 0.4
Aorta	66.0 ± 3.3	48.2 ± 3.6	2.5 ± 0.4	2.6 ± 0.5

* The assay was performed in the presence of ATP, $MgCl_2$ and EGTA (Mg^{2+}-ATPase, $-NaN_3$), ATP, $MgCl_2$, EGTA and NaN_3 (Mg^{2+}-ATPase, $+NaN_3$), ATP, $MgCl_2$ and $CaCl_2$ (Ca^{2+}-ATPase, $-NaN_3$), and ATP, $MgCl_2$, NaN_3 and $CaCl_2$ (Ca^{2+}-ATPase, $+NaN_3$). The details for the assay are described in Methods. Each value represents the mean ± S.E.M. of six experiments.

Table 4. Magnesium-dependent ATPase activities of the microsomal and the mitochondrial fractions from bovine aorta in the presence of various metabolic inhibitors*

	Mg^{2+}-ATPase activity (μmoles P_i/mg protein/hr)	
	Microsomes	Mitochondria
Control	74.4 ± 6.9	20.3 ± 3.1
NaN_3, 1 mM	57.7 ± 5.9†	6.4 ± 0.7†
5 mM	52.7 ± 5.2†	4.5 ± 1.1†
10 mM	47.2 ± 4.1†	2.7 ± 1.2†
DNP, 10 μM	69.9 ± 5.1	17.0 ± 1.8
20 μM	71.0 ± 6.1	15.0 ± 1.2†
100 μM	73.0 ± 6.6	14.4 ± 1.4†
Oligomycin, 0.2 μM	69.9 ± 6.5	13.9 ± 2.0†
0.5 μM	72.0 ± 6.9	13.8 ± 2.3†
2 μM	70.1 ± 8.4	12.4 ± 2.1†
KCN, 50 μM	69.0 ± 6.6	15.0 ± 2.0†
100 μM	69.0 ± 7.4	16.2 ± 2.1†
500 μM	69.9 ± 7.4	16.1 ± 2.1†

* ATPase activities were measured in the presence of 4 mM $MgCl_2$, 4 mM ATP and various metabolic inhibitors (sodium azide, dinitrophenol, oligomycin and potassium cyanide). Each value represents the mean ± S.E.M. of four experiments.

† Significantly different from the control values.

inhibited these activities by 78 and 29% respectively. Furthermore, the mitochondrial magnesium-dependent ATPase activity was also depressed significantly with 20–100 μM dinitrophenol, 0.2–2 μM oligomycin and 50–500 μM KCN, whereas any significant depression in the microsomal magnesium-dependent ATPase activity was not seen at any concentrations of the metabolic inhibitors employed (Table 4).

DISCUSSION

In the present study, we isolated the microsomal fraction from bovine coronary artery and aorta using pretreatment of the tissues with protease. Protease was used to finely disrupt vascular smooth muscle cells, since it is generally admitted that vascular smooth muscle cells are embedded in a dense network of connective tissue, and thus they cannot be readily disrupted [26, 27]. We observed that the microsomal fraction isolated by the pretreatment with protease exhibited high calcium uptake and NADPH-cytochrome *c* reductase activities as compared with those of the microsomes isolated without any enzyme treatment. Although we cannot rule out the possibility of a significant effect of the protease treatment on the biochemical activity of the isolated organelles, we employed in the present experiment the isolating method by which the highest microsomal calcium-accumulating activity was assessed.

Marker enzyme studies on glucose-6-phosphatase, NADPH-cytochrome *c* reductase and 5′-nucleotidase activities indicate that F-2 is enriched with microsomes more than any other subcellular fraction examined. Mitochondrial marker activities such as cytochrome *c* oxidase and succinate dehydrogenase also suggest that F-2 is contaminated with the mitochondrial fraction less than any other subcellular fraction. On the contrary, we observed about 25% of azide sensitivity to the microsomal calcium uptake and 23–25% to the magnesium-dependent ATPase activities. Sodium azide is well recognized as being a potent inhibitor for mitochondrial biochemical activity [28]. Thus, it appears that the microsomal fraction isolated from the vascular smooth muscles in the present experiments may be, more or less, contaminated with fragments of mitochondria. It is generally believed that a microsomal fraction isolated by a differential centrifugation technique is heterogeneous. Furthermore, it has been demonstrated that a highly purified cardiac sarcolemmal membrane fraction isolated using a sucrose density gradient method [29] and the cardiac sarcolemma isolated by hypotonic shock-LiBr treatment [30] exhibit about 50 and 34% of azide-sensitive, magnesium-dependent ATPase activity respectively. This may indicate that care must be taken for the validity of sodium azide in the assessment of its specificity to biochemical activities of differentiated subcellular membranes. This may be supported by our findings of divergent effects of various metabolic inhibitors and a consistent inhibition of sodium azide on the microsomal and the mitochondrial magnesium-dependent ATPase activities, as shown in Table 4. We have observed that 6.4 to 7.7 mg cytochrome *c*/mg/min of cytochrome *c* oxidase activity for the mitochondria-enriched fraction derived from either bovine coronary artery or aorta [31]. If this mitochondrial fraction is absolutely pure, F-2, which reveals less than 0.4 mg cytochrome *c*/mg/min of cytochrome *c* oxidase activity, may be contaminated with less than 7% of the mitochondria. This is apparently incompatible with the ratio of azide sensitivity of the magnesium-dependent ATPase of the isolated microsomal fraction, if we assume that the azide sensitivity of magnesium-dependent ATPase absolutely represents the mitochondrial activity. Nonetheless, even though we could not detect the cytochrome *c* oxidase activity comparable with the ratio of azide sensitivity of the magnesium-dependent ATPase in the microsomal fraction, it is obvious that F-2 may be contaminated, to some extent, with other subcellular organelles such as fragmented mitochondria, cell membranes and/or myofibrils, as suggested in our findings of the marker enzyme activities.

Cardiac microsomal fractions, which are highly enriched with the sarcoplasmic reticulum, have been shown to exhibit about 450–700 nmoles Ca^{2+}/mg/10 min for the guinea pig heart [10] and 1500 nmoles Ca^{2+}/mg/10 min for the rabbit heart [9], when the activity was measured in the presence of 100 μmoles Ca^{2+}. In contrast, the microsomal fractions from various vascular beds, such as pig coronary artery, rat aorta, bovine aorta and rabbit aorta, have been demonstrated to exhibit 20–33 nmoles Ca^{2+}/mg/10 min [8, 9], 20 nmoles Ca^{2+}/mg/10 min [6], 42 nmoles Ca^{2+}/mg/8 min [32] and 110 nmoles Ca^{2+}/mg/10 min [3] of the calcium uptake activities respectively. Although we must consider different methods for isolating microsomal fractions and different assay conditions, the calcium uptake activities of the microsomes derived from bovine coronary artery and aorta were higher than those of any other species demonstrated, but notably low as compared with those of cardiac microsomal fraction as

described above. This suggests that the microsomal fraction of vascular smooth muscle is less potent in accumulating intracellular calcium, and possibly compatible with the recognized concept that sarcoplasmic reticulum of vascular smooth muscle cells is poorly developed [33–35].

The microsomal fractions isolated from bovine coronary artery and aorta also showed about 2.4 μmoles Pi/mg/hr of calcium-stimulated, magnesium-dependent ATPase activity, which was similar, in terms of the magnitude of the activity, to that of the microsomes from the cattle thoracic aorta [32] as well as that of the porcine coronary arterial microsomes (1.2 μmoles Pi/mg/hr) [36, 37]. However, these values were extremely lower than those for the cardiac and skeletal microsomal fractions [10, 11, 38]. This agrees with our findings of lower calcium uptake activity in the microsomal fraction from vascular smooth muscle compared with skeletal and cardiac muscles, since it is generally admitted that calcium uptake activity of the sarcoplasmic reticulum is coupled with the calcium-stimulated, magnesium-dependent ATPase activity [38–40].

Calcium uptake activity of the coronary artery was essentially similar to that of the aorta in terms of the dependency on calcium (5–50 μM) as well as ATP concentrations (0.3 to 4 mM). However, in the presence of 100 μM $CaCl_2$ (Figs. 1 and 2), coronary arterial microsomal fraction revealed a lower value and a slower rate of rise for the calcium uptake activity than the aortic microsomal fraction. Such reduced ability was also observed in the calcium binding activity of the coronary arterial microsomes, which consistently showed low activities at different incubation intervals monitored and high K_m value for the concentration of ATP, in comparison with those of the aorta. Since marker enzyme study did not show any distinct differences in the purity of the microsomal fractions between the coronary artery and the aorta, the present results suggest different properties in the calcium-accumulating abilities between these tissues.

It is customary to use oxalate as a permeant ion in the incubation medium for the measurement of calcium uptake activity. Oxalate is believed to facilitate an accumulation of calcium ions into the microsomal vesicles. In fact, calcium uptake ability of sarcoplasmic reticulum-enriched microsomal fraction of the cardiac muscle was 5- to 10-fold higher than its calcium binding ability which was measured in the absence of oxalate [11, 22]. The oxalate dependency of the calcium uptake was also seen in the microsomal fractions from various vascular smooth muscle cells [7, 32, 41, 42]. For example, Ford and Hess [32, 41] have demonstrated it in the bovine aortic microsomes isolated using differential centrifugation without any enzyme treatment. On the contrary, in the microsomes from the same tissue as above, oxalate did not elicit an appreciable increase in the calcium-accumulating ability, as shown in Fig. 2. A relative lack of oxalate dependency for the microsomal calcium uptake of vascular smooth muscle has been demonstrated in the pig coronary artery [8] and the rat aorta [6]. Several factors underlying these differences in oxalate dependency of microsomal calcium uptake are: differences in tissues, animals, methods for the isolation of the membrane fractions, and experimental conditions employed. Wuytack *et al.* [9] have demonstrated the oxalate dependency of the microsomal calcium uptake of the porcine coronary artery, whereas Zelck *et al.* [8] have shown the lack of oxalate dependency in the microsomal calcium uptake as well as the low facilitation of oxalate of the mitochondrial calcium uptake activities in the same tissue and animal as above. Enzyme treatment was not employed in these methods. Thus, it is unlikely that the differences in oxalate dependency are due to differences in tissues and animals. We also observed a relative lack of oxalate dependency of the calcium uptake in the mitochondrial preparation isolated from the bovine aorta with collagenase pretreatment under conditions similar to the present protease treatment [31]. Furthermore, the lack of oxalate dependency has also been demonstrated in mitochondria isolated from the same tissue and animal as above without any enzyme pretreatment [32]. This appears to show that pretreatment of the tissue with an enzyme for a short period may not induce a significant effect on the properties of the calcium uptake of the intracellular organelles. Though a definitive conclusion concerning the lack of oxalate dependency cannot be reached from our results or those of others, it is likely that the discrepancy seen between oxalate dependencies of the calcium uptake in the smooth muscle subcellular fractions may be due to differences in isolating methods or experimental conditions employed, including an effect of enzyme pretreatment.

The present study has provided several characteristics in the calcium-accumulating ability of the microsomal fraction of the coronary artery and the aorta. Obviously, it would be premature to correlate the present results with demonstrated evidence for the differences in the sensitivity of both tissues to norepinephrine or actions through the adrenergic fibres [13, 14]; they may, however, provide meaningful information for further physiological and pharmacological studies on calcium movements in the vascular smooth muscle cells.

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