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CALCIUM INCORPORATION BY SMOOTH MUSCLE MICROSOMES

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SUMMARY

The purpose of the present work was to study the factors influencing calcium incorporation into a microsomal fraction prepared from the longitudinal smooth muscle of the guinea-pig ileum.

Calcium incorporation required the presence of both ATP and Mg^{2+} and was unaffected by azide. It was enhanced by oxalate; this effect was pH dependent and it was maximal at pH 6.6. The relation between calcium uptake with oxalate and free Ca^{2+} concentration in the medium was represented by a curve with an optimum for Ca^{2+} equal to $3 \cdot 10^{-5}$ M. The threshold concentration was comprised between $5 \cdot 10^{-7}$ and 10^{-6} M. The optimum calcium uptake rate was 4.5 nmol Ca^{2+}/mg protein per min. In the absence of oxalate, two distinct groups of binding sites were identified. Low affinity sites had a binding constant of $7 \cdot 10^{4}$ M^{-1} and a maximum binding capacity of 92 nmol Ca^{2+}/mg protein. High affinity sites had a binding constant of $0.6 \cdot 10^{6}$ M^{-1} and a binding capacity of 33 nmol Ca^{2+}/mg protein; their capacity was sensitive to pH changes. In the absence of oxalate, Ca^{2+} binding was depressed by Na^{+} with respect to K^{+} or choline^{+}. When the medium was supplemented with oxalate, the stimulation of ^{45}Ca incorporation was barely detectable in the presence of choline^{+} and it was lower in a medium containing Na^{+} instead of K^{+}.

The subcellular distribution profiles of calcium incorporation with and without oxalate indicate the microsomal location of both activities. However, the oxalate-stimulated calcium uptake activity sedimented faster than the calcium binding activity. The subcellular distribution of marker enzyme activities has been examined.

The present results indicate that Ca^{2+} incorporations with and without oxalate are the result of two processes likely related to two different structures. The role of microsomal calcium uptake in excitation-contraction coupling and its modification by the activity of the sodium pump is discussed.

INTRODUCTION

Calcium is required for excitation-contraction coupling in smooth muscle. Studies in whole muscle indicate that activator calcium might originate from several

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetra-acetic acid.

sources including extracellular calcium and intracellular calcium stores [1]. Plasma membrane [2], mitochondria [3] and sarcoplasmic reticulum [4] are possible intracellular calcium sequestering sites. It has recently been reported that microsomal fractions isolated from various kinds of smooth muscles were able to bind calcium in vitro [2, 4–10]. Divergent results were reported, concerning the presence [2, 5–7, 10] or the absence [4, 8, 9] of an oxalate-stimulated calcium uptake.

The purpose of the present work was to study the factors influencing calcium incorporation in a microsomal fraction prepared from the longitudinal smooth muscle of the guinea-pig ileum.

A preliminary report of part of this work has been presented to the Société Belge de Physiologie et de Pharmacologie [11].

METHODS

Preparation of the subcellular fractions of intestinal smooth muscle

Albino guinea-pigs weighing between 200 and 400 g were sacrificed by decapitation. The ileum was washed with Krebs solution. The longitudinal muscle of the ileum was separated from the circular layer and the mucosa as described by Rang [12] and by Paton and Zar [13]. The muscle strips were blotted and immersed into chilled 0.32 M sucrose buffered at pH 7.4 with 2 mM Tris/maleate and containing 10^{-4} M cysteine. 2 g of tissue were homogenized in 20 volumes of buffered 0.32 M sucrose at 0 °C using an all glass Potter-Elvehjem tube and a thermostatted Braun Potter-S apparatus. Three pestle strokes of 30 s at 1500 rev./min were sufficient for complete homogenization. 1 ml of this suspension maintained between 0 and 4 °C, was diluted 6-fold with buffered 0.25 M sucrose. The bulk of homogenate was spun at $500 \times g_{av}$* for 10 min in a refrigerated centrifuge (Christ Zeta equipped with rotor 8720). The nuclei pellet was suspended in 80 ml buffered 0.25 M sucrose and the supernatant was centrifuged at $20\,000 \times g_{av}$ during 30 min. The mitochondria pellet was suspended in 100 ml of buffered 0.25 M sucrose. The supernatant of the $20\,000 \times g_{av}$ spin was centrifuged at $100\,000 \times g_{av}$ for 60 min in an ultracentrifuge (Christ Omega II equipped with rotor 9720). The resultant pellet was gently resuspended in buffered 0.25 M sucrose using a glass-teflon Potter-Elvehjem tube. The post-microsomal supernatant was stored. Protein concentrations were measured by the method of Lowry et al. [14] using bovine serum albumin as a standard. The microsomal suspension was diluted to 0.5 mg/ml with buffered 0.25 M sucrose. All the fractions were kept in crushed ice, and used within 6 h after preparation.

Calcium incorporation studies

Unless otherwise stated, microsomes were incubated, under continuous magnetic stirring, at 37 °C, in 10 ml of a solution containing 20 mM maleate (adjusted with Tris at various pH values, as indicated under Results), 3 mM Tris/ATP, 5 mM $MgCl_2$, 100 mM KCl, 0 or 5 mM Tris/oxalate and 0.02 mM $^{45}CaCl_2$ (specific activity: 25 nCi/mol). In some experiments, the free Ca^{2+} concentration was controlled using Ca^{2+}-EGTA buffers (see below). Microsomal protein concentration was 0.05 mg/ml. After various incubation periods, 1 ml of the medium was filtered, under

* Gravitational acceleration in the middle of centrifugation tube.

suction, on a Millipore filter (HAWP 02500) or a Sartorius filter (SM 01386) disposed on a Millipore 3025 Sampling Manifold. After washing with 10 ml buffered 0.25 M sucrose the ^{45}Ca retained by the microsomes trapped on the filter discs was measured by liquid scintillation spectrometry in a Packard Tri-Carb liquid scintillation counter 3375. Filters were dissolved in the following scintillation solution: toluene/Triton X-100/ethyleneglycol-monoethylether (640 : 260 : 100, v/v) containing 2,5-diphenyl-oxazole (4 g/l) and *p*-bis-(*O*-methylstyril)-benzene (0.3 g/l). The radioactivity of the samples was counted as usual, with appropriate controls, and the efficiency was determined with internal standards.

The result of each determination was corrected for ^{45}Ca binding to filters after their washing with 1 ml of the incubation medium without microsomes and with 10 ml of sucrose. In order to reduce ^{45}Ca binding to filters, they were treated by a pre-filtration of 2 ml 1 M KCl followed by 10 ml buffered 0.25 M sucrose, 15 min before use [15]. Control filters bound less than 5 pmol Ca^{2+}. Microsomes were quantitatively retained by filters as no protein could be detected in the filtrate.

Ca^{2+}-EGTA buffers

In some experiments, the free calcium concentration in incubating solutions was controlled using Ca^{2+}-EGTA buffers. In a medium containing EGTA, ATP, Mg^{2+} and Ca^{2+}, the free Ca^{2+} concentration is a complex function of the total concentration of each species and of pH and temperature. Calculations were performed with a PDP-12 computer taking the following apparent equilibrium constants into account:

$[Mg^{2+}\text{-ATP}]/[Mg^{2+}] \cdot [ATP] = 10^{3.88}\ M^{-1}$ (pH 6.6, 37 °C) or $10^{4.11}\ M^{-1}$ (pH 7.4, 37 °C);
$[Ca^{2+}\text{-ATP}]/[Ca^{2+}] \cdot [ATP] = 10^{3.43}\ M^{-1}$ (pH 6.6, 37 °C) or $10^{3.66}\ M^{-1}$ (pH 7.4, 37 °C);
$[Ca^{2+}\text{-EGTA}]/[Ca^{2+}] \cdot [EGTA] = 10^{5.15}\ M^{-1}$ (pH 6.6, 37 °C) or $10^{5.72}\ M^{-1}$ (pH 7.4, 37 °C).
($EGTA = H_4EGTA + H_3EGTA^- + H_2EGTA^{2-} + HEGTA^{3-} + EGTA^{4-}$)
($ATP = ATP^{4-} + HATP^{3-} + KATP^{3-}$ or $NaATP^{3-}$)

Association constants of ATP complexes were reported by Nanninga [16, 17]. The Ca^{2+}-EGTA association constants were determined according to Murphy and Hasselbach [18] in solutions buffered as for calcium incorporation studies. The values were consistent with those reported by Ogawa [19]. The calculated free calcium concentrations were approx. 10 times higher than those which would be obtained by taking into account the Schwarzenbach's association constants which are, respectively, in the same conditions: $10^{5.75}$ and $10^{7.10}\ M^{-1}$ [20, 21].

In the present experiments, total calcium concentration was $2 \cdot 10^{-4}$ M. Total EGTA concentration was varied between 0.05 and 15 mM in order to obtain the free Ca^{2+} concentrations reported in Table I. As Mg^{2+} concentration in incubating solutions was in excess with respect to ATP, no significant pCa change occurred during hydrolysis of ATP, Ca^{2+} being displaced from ATP by Mg^{2+}.

Enzyme determinations

ATPase activities. ATPase activities were determined by measuring the rate of

TABLE I

Ca^{2+}-EGTA BUFFERS

Free Ca^{2+} concentration as a function of total EGTA concentration in solutions buffered with 20 mM Tris/maleate at pH 6.6 or 7.4 and containing 3 mM ATP, 5 mM Mg^{2+}, 0.2 mM Ca^{2+} and 100 mM KCl or NaCl.

Free Ca^{2+} concentration (M)	Total EGTA concentration (mM)	
	pH 6.6	pH 7.4
$1.0 \cdot 10^{-7}$	14.349	4.008
$3.0 \cdot 10^{-7}$	4.909	1.467
$5.0 \cdot 10^{-7}$	3.021	0.959
$1.0 \cdot 10^{-6}$	1.604	0.577
$1.5 \cdot 10^{-6}$	1.131	0.449
$2.0 \cdot 10^{-6}$	0.895	0.385
$3.0 \cdot 10^{-6}$	0.657	0.320
$5.0 \cdot 10^{-6}$	0.466	0.266
$1.0 \cdot 10^{-5}$	0.317	0.220
$3.0 \cdot 10^{-5}$	0.193	0.165
$5.0 \cdot 10^{-5}$	0.146	0.131
$1.0 \cdot 10^{-4}$	0.060	0.054

inorganic phosphate (P_i) liberation during incubation in the presence of ATP. Assay mixtures contained, in 1 ml, 20 mM Tris/maleate (pH 7.4), 3 mM Tris/ATP, various divalent and monovalent cations (see legend Fig. 6) and 50 μg microsomal protein. After 30 min incubation at 37 °C, the reaction was stopped by adding 1 ml of 10 % trichloroacetic acid. After centrifugation, inorganic phosphate was determined in the protein-free supernatant by a modified method of Fiske and SubbaRow [22].

To 1 ml of sample were added 1.6 ml of 1.25 % $(NH)_6Mo_7O_{24} \cdot 4\,H_2O$ in 0.88 M H_2SO_4 and 0.4 ml of Fiske and SubbaRow reagent. Absorbance was measured at 660 nm, exactly 15 min later, in a Kipp-Scalar digital photometer. The result of each determination was corrected for acid ATP hydrolysis measured in samples treated as above, but in the absence of microsomes.

5'-Nucleotidase (EC 3.1.3.5). 5'-Nucleotidase activity was determined by the estimate of the rate of inorganic phosphate liberation during incubation in the presence of 1 mM 5'-AMP and 5 mM $MgCl_2$ in 20 mM Tris/maleate (pH 7.0) [23]. Reactions were stopped after 30 min of incubation by adding trichloroacetic acid and samples were further treated as described above for ATPase activities.

Cytochrome oxidase (EC 1.9.3.1). Cytochrome oxidase activity was determined according to Cooperstein and Lazarow [24], by observing during 3 min the rate of oxidation of cytochrome *c*, at 25 °C, in a Beckman dual beam 26 K spectrophotometer.

Reagents

All solutions were prepared using distilled, deionized water. All chemicals used were analytical grade and purchased from E. Merck, Darmstadt. EGTA and bovine serum albumin were purchased from Sigma Chemical Co, St. Louis, Mo. ATP was purchased from Boehringer Gmbh, Mannheim. Tris/ATP was prepared by

passage of disodium ATP through Dowex 50 in the H^+ form, followed by neutralisation with Tris. Calcium stock solutions were prepared using $CaCO_3$ titrated by HCl. Radioactive ^{45}Ca was supplied as a $CaCl_2$ solution by the Radiochemical Centre, Amersham.

Statistical methods. Whenever possible, values are presented as means ± S.E. of *n* determinations. Significance of differences between means was checked by Student's *t*-test. Regression analysis was based on the least squares method.

RESULTS

Effect of Mg^{2+}, ATP, oxalate and pH on ^{45}Ca incorporation

The incorporation of ^{45}Ca in smooth muscle microsomes has been studied at pH 6.6, with and without oxalate in the medium. As illustrated in Fig. 1, ^{45}Ca incorporation in microsomes was markedly enhanced by 5 mM oxalate. Whereas, in the absence of oxalate, calcium incorporation reached a plateau after 20–30 min incubation, in the presence of oxalate, it increased linearly with time during the first 20 min of incubation and slowed down only slightly in the subsequent 10 min.

As shown in Table II, whether or not oxalate was added to the incubation medium, calcium incorporation required the presence of both ATP and Mg^{2+}, and was unaffected by azide, an inhibitor of mitochondrial calcium accumulation [6].

Microsomes have been incubated for 20 min, with or without oxalate, in solutions buffered by 20 mM Tris/maleate at pH ranging from 5.6 to 7.4. Fig. 2 shows that calcium incorporation in the absence of oxalate was only slightly influenced by pH. By contrast, the stimulation of calcium incorporation by oxalate was markedly pH dependent. It was very low at pH 5.7 and above pH 7.2; it was maximal at pH 6.6.

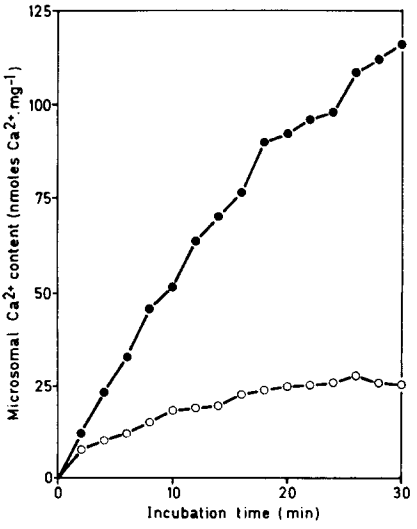

Fig. 1. ^{45}Ca incorporation by smooth muscle microsomes with (●) and without (○) oxalate. The experiment was carried out at 37 °C in the following solution: 20 mM Tris/maleate (pH 6.6), 5 mM $MgCl_2$, 3 mM ATP, 100 mM KCl, 0.02 mM $CaCl_2$, 0 or 5 mM Tris/oxalate. Each experimental point corresponds to one determination.

TABLE II

EFFECTS OF Mg^{2+}, ATP AND NaN_3 ON MICROSOMAL CALCIUM INCORPORATION

^{45}Ca content of smooth muscle microsomes incubated for 20 min with or without oxalate, at 37 °C, in solutions containing: 20 mM Tris/maleate (pH 6.6), 100 mM KCl, 0.02 mM $CaCl_2$, ATP, $MgCl_2$ and NaN_3 as indicated in the table. Data are means of three determinations ±S.E.

ATP, $MgCl_2$ and NaN_3 concentration in the medium (mM)		Incubation without oxalate (nmol Ca^{2+}/mg protein)	Incubation with oxalate (nmol Ca^{2+}/mg protein)
ATP	0		
$MgCl_2$	0	1.2±0.2	1.5±0.1
NaN_3	0		
ATP	0		
$MgCl_2$	5	1.0±0.1	1.0±0.1
NaN_3	0		
ATP	3		
$MgCl_2$	0	2.1±0.5	2.4±0.1
NaN_3	0		
ATP	3		
$MgCl_2$	5	22.5±0.2	76.0±0.5
NaN_3	0		
ATP	3		
$MgCl_2$	5	22.1±0.2	73.9±0.4
NaN_3	5		

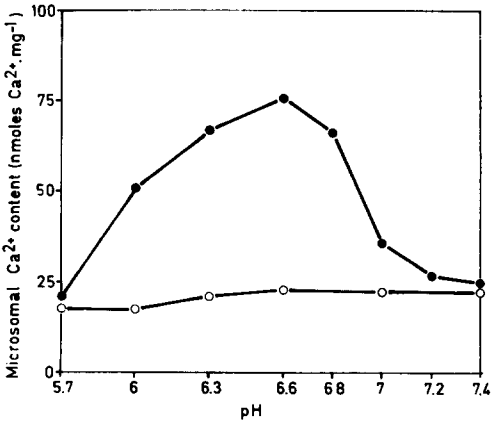

Fig. 2. ^{45}Ca incorporation by smooth muscle microsomes incubated with (●) and without (○) oxalate at different pH's. The experiment was carried out at 37 °C in the following solution: 20 mM Tris/maleate (pH varying from 5.7 to 7.4), 5 mM $MgCl_2$, 3 mM ATP, 100 mM KCl, 0.02 mM $CaCl_2$, 0 or 5 mM Tris/oxalate. Each result is the mean of six determinations. S.E. was comprised within the symbol's diameter.

The influence of Ca^{2+} concentration on ^{45}Ca incorporation with and without oxalate

The influence of Ca^{2+} concentration on ^{45}Ca incorporation has been studied at pH 6.6 in the presence of oxalate and at pH 6.6 and pH 7.4 in the absence of oxalate. The free Ca^{2+} concentration was varied from 10^{-7} to 10^{-4} M and controlled by Ca^{2+}-EGTA buffers as described under Methods.

The oxalate-sensitive ^{45}Ca incorporation was estimated by measuring the difference between ^{45}Ca contents of microsomes incubated for 20 min with and without oxalate. The relation between calcium uptake with oxalate and free Ca^{2+} concentration is represented by a curve with an optimum for a Ca^{2+} concentration equal to $3 \cdot 10^{-5}$ M. The threshold concentration is comprised between $5 \cdot 10^{-7}$ and 10^{-6} M (Fig. 3). The optimum calcium oxalate uptake rate, measured between 10 and 20 min incubation in the presence of $3 \cdot 10^{-5}$ M Ca^{2+}, was 4.5 nmol Ca^{2+}/mg protein per min. In the absence of oxalate, ^{45}Ca incorporation has been measured after 30 min incubation at pH 6.6 or pH 7.4. Fig. 4 shows, for one typical experiment at pH 6.6, the analysis according to Scatchard [25] of the relation between ^{45}Ca incorporated without oxalate and free Ca^{2+} concentration in the medium. Scatchard plots show two distinct linear components, an indication for the existence of two distinct groups of binding sites. Low affinity sites had a binding constant of $(7 \pm 1) \cdot 10^4$ M^{-1} and a maximum binding capacity of 92 ± 16 nmol Ca^{2+}/mg protein and high affinity sites had a binding constant of $(0.6 \pm 0.1) \cdot 10^6$ M^{-1} and a binding capacity of 33 ± 9 nmol Ca^{2+}/mg protein. In Table III we reported the means of binding constants and binding capacities measured in several experiments performed at pH 6.6 and 7.4. Increase in pH tended to reduce the binding capacity of the high affinity sites ($P < 0.01$) whereas the capacity of low affinity sites was not affected. The low affinity binding constant was higher at pH 6.6 than at pH 7.4 ($P < 0.01$); high affinity binding constants were less sensitive to pH change.

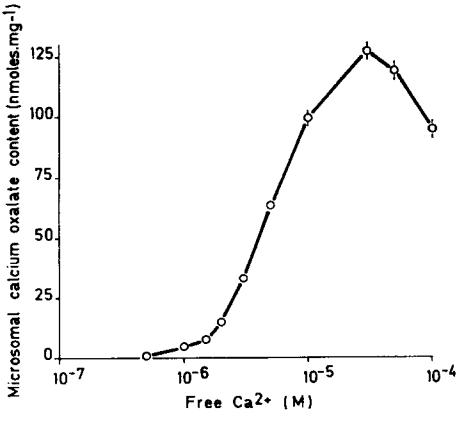

Fig. 3. Oxalate-sensitive ^{45}Ca incorporation as a function of free Ca^{2+} concentration in the medium. Calcium oxalate content was estimated by measuring the difference between ^{45}Ca contents of microsomes incubated for 20 min with and without oxalate. The experiment was carried out at 37 °C in the following solution: 20 mM Tris/maleate (pH 6.6), 5 mM $MgCl_2$, 3 mM ATP, 100 mM KCl, 0 or 5 mM Tris/oxalate; the free Ca^{2+} concentration was varied from 10^{-7} to 10^{-4} M using Ca^{2+}-EGTA buffers. Each result is the mean of three determinations ±S.E.

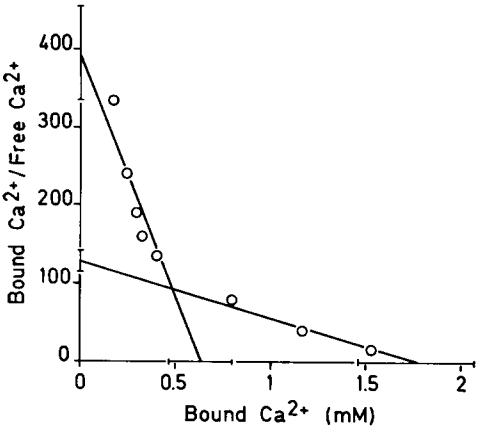

Fig. 4. Scatchard plot of microsomal calcium binding as a function of free Ca^{2+} concentration. The figured points were obtained from the means of bound Ca^{2+} at each experimented pCa. Regression lines were calculated from two groups of experimental data corresponding, respectively, to free Ca^{2+} concentrations ranging from $5 \cdot 10^{-7}$ to $3 \cdot 10^{-6}$ M and 10^{-5} to 10^{-4} M. Horizontal and vertical bars represent standard deviations of abscissa and ordinate intercepts, respectively. The binding capacities of high and low affinity sites were calculated from the abscissa intercepts. The binding constants were calculated from the slopes of regression lines.

TABLE III

HIGH AND LOW AFFINITY CALCIUM BINDING SITES

^{45}Ca content of microsomes was measured after an incubation of 30 min at 37 °C in the following solution: 20 mM Tris/maleate (pH 6.6 or 7.4), 5 mM Mg^{2+}, 3 mM ATP, 100 mM K^+. The free Ca^{2+} concentration was varied from 10^{-7} to 10^{-4} M using Ca^{2+}-EGTA buffers. The bound Ca^{2+} was measured in six or nine samples for each free Ca^{2+} concentration. The maximum binding capacities (n) and affinity constants (K) were calculated according to Scatchard. Data are the average of several determinations ±S.E.; the number of experiments is given in parentheses.

	Maximum binding capacity of high affinity binding sites (n) (nmol Ca^{2+}/mg)	High affinity binding constant (K) ($10^{-6} \times K$) (M^{-1})	Maximum binding capacity of low affinity binding sites (n') (nmol Ca^{2+}/mg)	Low affinity binding constant (K') ($10^{-4} \times K'$) (M^{-1})
pH 6.6	42±5 (5)	0.32±0.03 (5)	86±5 (5)	8.8± 0.4 (5)
pH 7.4	19±3 (3)	0.52±0.07 (3)	83±4 (3)	2.39±0.09 (3)

The influence of monovalent cations on ^{45}Ca incorporation

The influence of Na^+, K^+, and choline$^+$ on ^{45}Ca incorporation was examined at pH 6.6. Data reported in Table IV indicate that ^{45}Ca content of microsomes incubated for 20 min with and without oxalate was dependent upon the nature of the monovalent cations. In the absence of oxalate, Ca^{2+} binding was depressed by Na^+ with respect to K^+ or choline$^+$ ($P < 0.01$). When the medium was supplemented with oxalate, the stimulation of ^{45}Ca incorporation was barely detectable in the presence

TABLE IV

EFFECTS OF MONOVALENT CATIONS ON MICROSOMAL CALCIUM INCORPORATION

^{45}Ca content of smooth muscle microsomes incubated for 20 min with and without oxalate, at 37 °C, in solutions containing: 20 mM Tris/maleate (pH 6.6), 5 mM $MgCl_2$, 3 mM ATP, 0.02 mM $CaCl_2$ and either 100 mM choline chloride or 100 mM NaCl or 100 mM KCl. Data are means of six determinations ± S.E.

Monovalent cation	Incubation without oxalate (nmol Ca^{2+}/mg)	Incubation with oxalate (nmol Ca^{2+}/mg)
$Choline^+$	31.7 ± 0.9	37.3 ± 0.3
Na^+	25.4 ± 0.8	91.4 ± 2.2
K^+	29.5 ± 1.0	103.3 ± 3.3

of $choline^+$ and it was lower in a medium containing Na^+ instead of K^+ ($P < 0.01$). ^{45}Ca binding to high and low affinity sites was measured at pH 6.6 and pH 7.4 in Na^+ and K^+ medium. The results of a representative experiment carried out with one microsomal preparation are reported in Table V. In this experiment, observations made with K^+ were in the range of those reported in Table III. Experimental data show that the binding capacities of high and low affinity sites were lower with Na^+ than with K^+ at pH 7.4 ($P < 0.05$, $P < 0.01$, respectively). For pH 6.6, the large standard deviations did not allow to give statistical meaning to the observed differences. The affinity of binding sites was not significantly influenced by the nature of monovalent cations.

Subcellular distribution of azide-insensitive ^{45}Ca incorporation

^{45}Ca incorporation by the different subcellular fractions was measured after 20 min incubation, at pH 6.6, with and without oxalate. In every fraction, Mg^{2+} and ATP were required to support ^{45}Ca incorporation, whether or not oxalate was present (data are not shown). ^{45}Ca bound in the absence of oxalate and calcium oxalate incorporated by each fraction isolated from 1 g of smooth muscle are reported in Table VI to illustrate a representative experiment. Azide-insensitive calcium stores in smooth muscle homogenate were able to bind 0.55 μmol Ca^{2+}/g. When oxalate was present, ^{45}Ca uptake rate measured between 10 and 20 min incubation was 0.2 μmol Ca^{2+}/g per min. 65 % of ^{45}Ca binding activity and of calcium uptake activity measured in the homogenate were recovered in the isolated subcellular fractions. Respectively 29 and 13 % of both activities were found in the microsomal fraction. The distribution patterns of calcium binding and calcium oxalate incorporation activities were represented according to de Duve et al. [26]. In this representation the specific activity is taken to be 1 in the sum of all recovered fractions. The height of the blocks gives the extend of purification achieved over the homogenate and the surface area of the blocks is the percentage of the activity recovered in the corresponding fraction. The distribution profiles indicate the microsomal location of both activities (Fig. 5). They were, however, dissimilar. The oxalate-stimulated calcium uptake activity sedimented faster than the calcium binding activity as indicated by the higher contamination of the nuclei and mitochondrial fractions. The supernatant was free of calcium sequestering components.

TABLE V

Na^+ AND K^+ SENSITIVITY OF HIGH AND LOW AFFINITY CALCIUM BINDING SITES

Maximum binding capacities and affinity constants of high and low affinity calcium binding sites. ^{45}Ca incorporation was measured without oxalate using one microsomal preparation for pH 6.6 and 7.4. Experiments were carried out at 37 °C in the following solution: 20 mM Tris/maleate (pH 6.6 or 7.4), 5 mM $MgCl_2$, 3 mM ATP, 100 mM KCl or NaCl. The free Ca^{2+} concentration was varied from 10^{-7} to 10^{-4} M using Ca^{2+}-EGTA buffers. The bound Ca^{2+} was determined in six samples for each free Ca^{2+} concentration. The maximum binding capacities (n) the affinity constants (K) and their standard deviations were calculated, respectively, from the abscissa intercepts and the slopes of Scatchard plots.

pH	Monovalent cation	Maximum binding capacity of high affinity binding sites (n) (nmol Ca^{2+}/mg)	High affinity binding constant (K) ($10^{-6} \times K$) (M^{-1})	Maximum binding capacity of low affinity binding sites (n') (nmol Ca^{2+}/mg)	Low affinity binding constant (K') ($10^{-4} \times K'$) (M^{-1})
6.6	K^+	41±14	0.24±0.08	73±12	7.8±0.8
	Na^+	29±10	0.28±0.09	69± 7	6.6±0.5
7.4	K^+	15± 3	0.50±0.08	76± 5	2.2±0.1
	Na^+	7± 1	0.60±0.08	47± 4	2.0±0.1

TABLE VI

AZIDE-INSENSITIVE ^{45}Ca INCORPORATION IN SUBCELLULAR FRACTIONS

Subcellular fractions were incubated, at 37 °C, in a medium containing: 20 mM Tris/maleate (pH 6.6), 5 mM $MgCl_2$, 3 mM ATP, 100 mM KCl, 0.1 mM $CaCl_2$, 10 mM NaN_3 and 0 or 5 mM Tris/oxalate. After 20 min incubation, ^{45}Ca contents are measured by the Millipore filtration method. Calcium oxalate content was estimated as the difference between ^{45}Ca bound with and without oxalate. Data represent calcium binding and calcium oxalate uptake activities of different subcellular fractions isolated from 1 g of smooth muscle. Each result is the mean of six determinations ±S.E.

Subcellular fractions	^{45}Ca bound without oxalate (nmol Ca^{2+}/g)	Oxalate-sensitive ^{45}Ca content (nmol Ca^{2+}/g)
Homogenate	546±17	3668±147
Nuclei	122± 4	1084± 65
Mitochondria	80± 5	844± 13
Microsomes	158± 2	460± 7
Supernatant	0	0

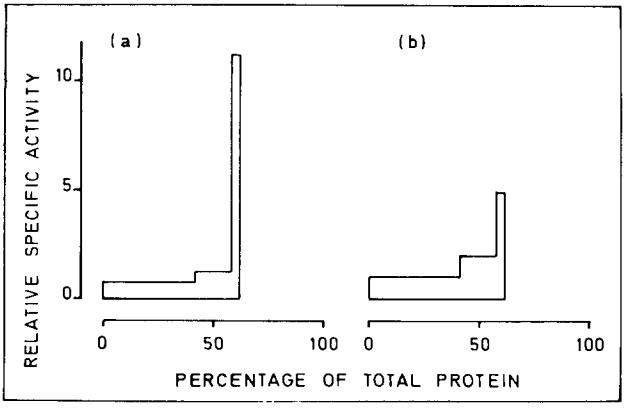

Fig. 5. Distribution profiles of calcium binding without oxalate (a) and oxalate-stimulated calcium uptake (b). On the abscissa are represented the relative protein contents of each fraction in the order in which they were isolated, i.e. from left to right: nuclei, mitochondria, microsomes, supernatant. On the ordinate, we represented the relative specific activities, i.e. the relative activity of each fraction divided by the relative protein content of the fraction.

Subcellular distribution of marker enzyme activities

The subcellular distribution of different enzymic activities was examined. Cytochrome *c* oxidase and 5′-nucleotidase were determined as reported under Methods. Mg^{2+}-ATPase was determined as the activity elicited in the presence of 5 mM $MgCl_2$ and 100 mM KCl. $(Na^+ + K^+)$-ATPase was estimated as the 1 mM ouabain-sensitive activity in the presence of 5 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl and 1 mM EGTA. Ca^{2+}-activated extra-ATPase activities were estimated as the activities inhibited by 1 mM EGTA in the presence of 100 mM KCl, 0.1 mM $CaCl_2$ with or without 5 mM $MgCl_2$ and were, respectively, called $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+}-ATPase.

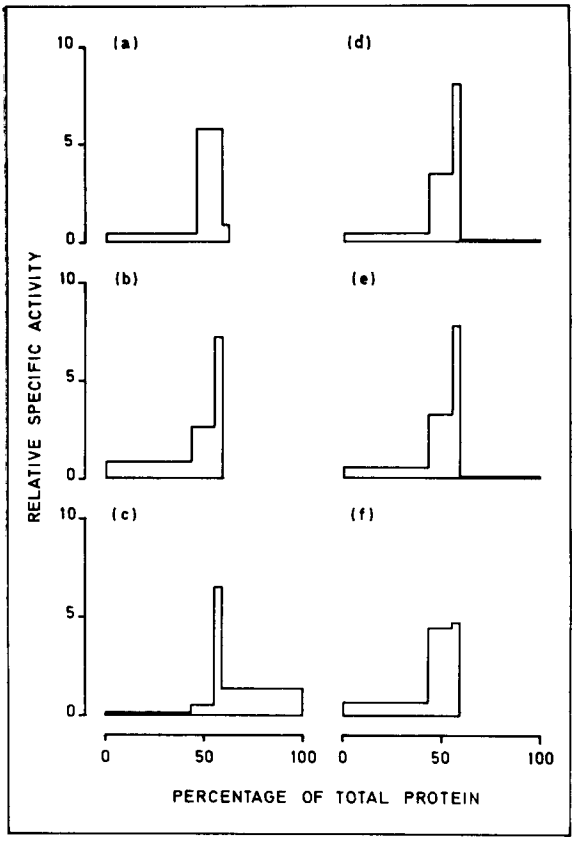

Fig. 6. Distribution profiles of enzymic activities. On the abscissa, are represented the relative protein content of each fraction in the order in which they were isolated, i.e. from left to right; nuclei, mitochondria, microsomes, supernatant. On the ordinate, are represented the relative specific activities, i.e. the relative activity of each fraction divided by the relative protein content of the fraction. a, cytochrome *c* oxidase; b, $(Na^+ + K^+)$-ATPase; c, 5′-nucleotidase; d, Mg^{2+}-ATPase; e, Ca^{2+}-ATPase; f, $(Ca^{2+} + Mg^{2+})$-ATPase.

The distribution profiles of the different enzymic activities presented in Fig. 6 illustrate a representative experiment. Cytochrome *c* oxidase was located in the mitochondrial fraction and was barely detectable in the microsomes. $(Na^+ + K^+)$-ATPase and 5′-nucleotidase, which are plasma membrane marker enzymes [27, 28], presented a microsomal location. Mg^{2+}-ATPase was distributed like $(Na^+ + K^+)$-ATPase. The Ca^{2+}-activated ATPase activities were differently distributed. Ca^{2+}-ATPase, determined in the absence of Mg^{2+}, was distributed like $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase. $(Ca^{2+} + Mg^{2+})$-ATPase was equally distributed in the mitochondrial and the microsomal fraction.

DISCUSSION

Experimental data reported here confirm that microsomes prepared from

intestinal smooth muscle do incorporate calcium from the incubating medium. This incorporation requires the presence of Mg^{2+} and ATP. Experimental results indicate that ^{45}Ca accumulation without oxalate is due to a mechanism different from the one responsible for ^{45}Ca accumulation enhanced by oxalate. This is shown by the observation that changes in factors such as pH, pCa and monovalent cations affected differently the two processes.

It is unlikely that mitochondrial contamination was responsible for the present observations. The preparations did not contain cytochrome *c* oxidase, a specific marker of mitochondria [27]. Furthermore, ^{45}Ca incorporation was insensitive to azide a suppressing agent of calcium accumulation in mitochondria [6]. It is likely that the preparation here studied was constituted by fragments of plasma membrane and of sarcoplasmic reticulum. This is first suggested by the presence of enzymic activities linked to these cell components. $(Na^+ + K^+)$-ATPase and 5'-nucleotidase are generally considered as plasma membrane marker enzymes [27, 28]. A Ca^{2+}-ATPase has been described in sarcolemmal preparations of heart muscle [29] and of intestinal smooth muscle [30]. $(Ca^{2+} + Mg^{2+})$-ATPase on the other hand has been reported to be associated with sarcoplasmic reticulum in striated and cardiac muscle [31, 32].

The distribution profiles show that azide-insensitive calcium binding without oxalate was associated with the microsomal fraction containing $(Na^+ + K^+)$-ATPase and Ca^{2+}-ATPase. On the other hand, the azide-insensitive oxalate-stimulated calcium uptake was more equally distributed between the mitochondrial and the microsomal fractions. A similar distribution was found for $(Ca^{2+} + Mg^{2+})$-ATPase. This could indicate that sarcoplasmic reticulum sedimented faster than plasma membranes and that it contained most of the calcium uptake activity stimulated by oxalate.

In comparison with smooth muscle mitochondria [3, 33], isolated microsomal components present higher affinity but lower capacity in the calcium binding process.

Hurwitz et al. [2] have studied the distribution of the calcium sequestering microsomal components by centrifugation in a sucrose density gradient. They have found that the specific activity of calcium uptake in each fraction rose and fall in parallel with $(Na^+ + K^+)$-ATPase. This seems to be contradictory with our findings. It should be pointed out that the former authors made their observations at pH 7.4 in the presence of oxalate whereas we found that the calcium oxalate uptake phenomenon was barely detectable in those conditions. Hurwitz et al. [2] concluded from their results that the longitudinal muscle of the guinea-pig ileum was deficient in calcium pump activity associated with sarcoplasmic reticulum. Our results do not support their assumption. Electron micrographs of the longitudinal smooth muscle show the presence of sarcoplasmic reticulum located close to the plasma membrane in association with mitochondria and caveolae [34]. They may be considered as divalent cations stores as shown by their ability to accumulate strontium substituted for calcium in the physiological solution. The results here reported regarding the relative influence of Na^+ and K^+ on calcium binding and calcium uptake are consistent with observations done on whole muscle. It has been shown that a rise in $[Na^+]_i$, due to inhibition of the sodium pump, was followed by a decrease of total calcium content suggesting that intracellular calcium stores were less effective, in the presence of high $[Na^+]_i$, which, however, increased trans-membrane calcium influx [35].

There is no information on calcium influx related with contraction in the

longitudinal smooth muscle of the guinea-pig ileum. In rat aortae, ^{45}Ca influx in the La^{3+}-resistant calcium fraction is an estimate of the biologically important component of calcium exchange across smooth muscle cell membrane. Maximal activation by noradrenaline is associated with an influx of 0.03 mmol Ca^{2+}/kg per min [36]. In the taenia coli, Goodford's [37] estimation based on electrophysiological measurements indicates that an influx of 0.12 mmol Ca^{2+}/kg per min would cause a maximal activation of the muscle. Azide-insensitive calcium binding and uptake activities measured in smooth muscle homogenate were, respectively, 0.55 mmol Ca^{2+}/kg and 0.2 mmol Ca^{2+}/kg per min in the presence of 10^{-4} M Ca^{2+}. If those activities occur in whole muscle, and if they are due to microsomal components as demonstrated by distribution studies, they might play a major role as a calcium regulatory factor during excitation and relaxation of the smooth muscle cell.

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