Vol. 88, No. 3, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 13, 1979 Pages 1132-1139

UPTAKE AND RELEASE OF CALCIUM BY MICROSOMES OF NONVASCULAR SMOOTH MUSCLE

Arvind K. Chaturvedi, Susan Fox, B. V. Rama Sastry and Erwin J. Landon
Department of Pharmacology, Vanderbilt University School of Medicine
Nashville, Tennessee 37232

Received May 3,1979

Summary: Microsomes prepared from guinea-pig ileum longitudinal smooth muscle and rat uterus continuously sequester calcium for a one hour period in the presence of Mg-ATP as an energy source and oxalate anion as a trapping agent. Dithiothreitol is essential for maximal calcium uptake activity of the rat uterus microsomes. On sucrose density gradients, calcium uptake of the smooth muscle microsomes appears to be associated with intracellular membrane (sarcoplasmic reticulum). Release of sequestered calcium from the longitudinal muscle microsomes is very slow (20% in 50 minutes). A small labile fraction (20%) is released by EGTA (1 mM) in 10 minutes. Rapid release of sequestered calcium (90% in 10 minutes) occurs in presence of the calcium ionophore A23187 (2 μM) or in the presence of chlorpromazine (1 mM).

We have previously carried out studies examining calcium uptake of microsomes prepared from vascular smooth muscle (1-3). These microsomal preparations continuously sequestered calcium over a one hour time period in the presence of Mg-ATP as an energy source and oxalate anion as a calcium trapping agent. These microsomal preparations were examined on sucrose density gradients (1,2). Plasma membrane derived membrane vesicles localized principally to the top of the gradient. Intracellular membrane vesicles (sarcoplasmic reticulum) were found in all fractions but were more concentrated toward the bottom of the gradient. The Mg-ATP and oxalate dependent calcium uptake was most abundant in the lower part of the gradient (1,2). This observed oxalate dependent calcium uptake appeared to be associated with the sarcoplasmic reticulum. There remained, however, some ambiguity concerning the upper part of the gradient because a strong calcium uptake activity of the sarcoplasmic reticulum would obscure a relatively weak calcium sequestering activity of the plasma membrane.

The vascular muscle microsomal calcium uptake activity was greatly stimulated by adding dithiothreitol, a potent disulfide reducing agent, to the incubating microsomes (3). Somewhat greater stimulus was achieved by

0006-291X/79/111132-08$01.00/0

incubation of dithiothreitol with the vascular strips prior to the preparation of the homogenate. Addition of dithiobis-2-nitrobenzoate, a sulfhydryl oxidizing agent, to the microsomes depressed the calcium uptake.

The present report extends this study to nonvascular smooth muscle microsomes. It examines the distribution of microsomal calcium uptake activity on sucrose density gradients and also looks at release of the sequestered microsomal calcium.

Methods: The small intestine was removed from guinea-pigs weighing 450-550 g. Dissection of the longitudinal muscle layer was performed by making an initial incision along the site of the mesentery. The longitudinal muscle was separated from underlying structures by gentle tension with forceps in a direction parallel to the underlying circular muscle fiber. Horns and corpus of rat uterus were removed from each of four female Sprague-Dawley strain rats weighing approximately 240 grams. Excised uterine tissue was stripped to the muscle layer. The endometrium was removed with the edge of a glass slide. The rats had been estrogen primed for three days prior to the experiment with 100 µgrams of diethylstilbesterol.

Isolated tissues were washed three times with 20 ml of ice cold sucrose solution (0.25 M). The washed tissue was minced very fine with scissors and then was homogenized with a Potter-Elvejhem homogenizer (Thomas type C) and a teflon pestle of relative clearance 0.975" O.D. The I.D. of the homogenized glass tube was designated as 1.000". The uterine tissue was homogenized in 20 ml of the isotonic sucrose with twelve strokes of the homogenizer driven by an electric motor at 1900 rpm. The longitudinal muscle tissue was homogenized in 20 ml sucrose at 1000 rpm (6 strokes up and down). A cooling interval was employed every three strokes. Procedures were carried out in a cold room with an ice bucket.

Microsomes from guinea-pig ileum longitudinal smooth muscle were prepared as follows. The homogenate was centrifuged for 10 minutes at 1500 g in a Sorvall RC-2 refrigerated centrifuge. The supernatant was centrifuged at 27,000 g for 10 minutes. The new supernatant was centrifuged at 105,000 g in a Beckman Spinco L-65 ultracentrifuge for 60 minutes. The final pellet was resuspended in 4 ml of isotonic sucrose and used immediately. Microsomes from rat uterus were prepared as follows. The homogenate was centrifuged at 1500 g for 10 minutes in a Sorvall RC-2 refrigerated centrifuge. The supernatant from this spin was centrifuged at 10,500 g for 20 minutes in the same centrifuge. The second supernatant was centrifuged one hour at 105,000 g in a Beckman Spinco L-65 ultracentrifuge. The microsomal pellet that was obtained was resuspended in 4 ml of 0.25 M sucrose and was used immediately. All centrifugations were at 2°.

Calcium uptake and release: Calcium uptake was measured in the following standard assay medium - 100 mM KCl, 30 mM imidazole-histidine buffer (pH 6.8), 5 mM ATP, 5 mM sodium azide, 5 mM ammonium oxalate, 20 µM $CaCl_2$, 0.1 µCi/ml $(^{45}Ca)Cl_2$ and deionized distilled water. The reaction was started by addition of 0.4 ml of microsomal preparation in a total volume of 4 ml at 37°.

Aliquots of 500 µl of the incubation mixture were removed at selected time intervals for filtration through 0.45 µ membrane filters (Millipore Corpora-

tion). The filters had been prepared with a wash of 0.25 M KCl (2 ml) followed by water (10 ml). The samples were filtered with the aid of a vacuum apparatus and were washed with 0.25 M sucrose (2 ml). Filters were dried and (^{45}Ca) determined by liquid scintillation spectrophotometry in 2,5-diphenyloxazole (6 g/l) in toluene.

The release of accumulated calcium was determined as follows: microsomal vesicles were allowed to take up (^{45}Ca) for 30 minutes in the standard medium for calcium uptake except that twice as much of the microsomal preparation was added. After accumulation of calcium, the reaction mixture was put in ice to stop the uptake and aliquot removed to determine the uptake. An aliquot of the calcium loaded vesicles was diluted 10-fold into the release medium which contained 0.4 ml of imidazole-histidine buffer (30 mM, pH 6.8), 0.4 ml of sucrose (0.25 M) and 0.8 ml of deionized water and/or an agent. Aliquots of 0.5 ml were removed at time intervals from the release medium and (^{45}Ca) was measured by Millipore filtration.

Density gradient studies: These were carried out in a continuous 20 to 70 percent sucrose gradient (w/v) containing 3 mM imidazole-histidine buffer, pH 6.8. The microsomal fraction was layered on a 5 ml gradient and was centrifuged at 130,000 g for 16 hours at 0° in a Beckman SW50L rotor. After the gradient centrifugation, seven 0.7 ml fractions were collected from top to bottom. For each fraction, measurements were made of calcium uptake activity, 5' nucleotidase activity (4) as a marker for plasma membrane, rotenone insensitive NADH-cytochrome c reductase activity (5) as marker for sarcoplasmic reticulum and the protein concentration of the fraction. Protein concentration was measured by the method of Sutherland et al (6). Bovine serum albumin was used as protein standard.

Electron microscopy: Microsomes were examined with a Hitachi HV 11-B electron microscope. The microsomal pellet was fixed for 90 minutes in a phosphate-buffered 2% OsO_4, pH 7.5. The pellet was dehydrated in ethanol and propylene oxide and embedded in araldite. Sections were stained with uranyl acetate and lead citrate. Examination revealed membrane vesicles of uniform size free of visible mitochondrial contamination. Enzymatic assay of microsomal fractions for cytochrome c oxidase activity (7) confirmed the absence of any significant mitochondrial contamination.

Results: Calcium uptake of guinea pig ileum longitudinal smooth muscle microsomes is depicted in Fig. 1. The initial rate of calcium uptake was approximately twice that of dithiothreitol stimulated vascular muscle microsomes (3). The calcium uptake activity was not further enhanced by dithiothreitol. About 80% of the calcium sequestered in one hour was sequestered in the first thirty minutes. The optimal pH for calcium uptake was 6.8. The calcium uptake was unaffected by sodium azide, a compound that inhibits mitochondrial calcium uptake. If oxalate was omitted, the calcium uptake was less than 20% of that found in the presence of oxalate.

Fig. 2 shows the dithiothreitol stimulated calcium uptake activity of rat uterine microsomes. The calcium uptake activity of uterine microsomes

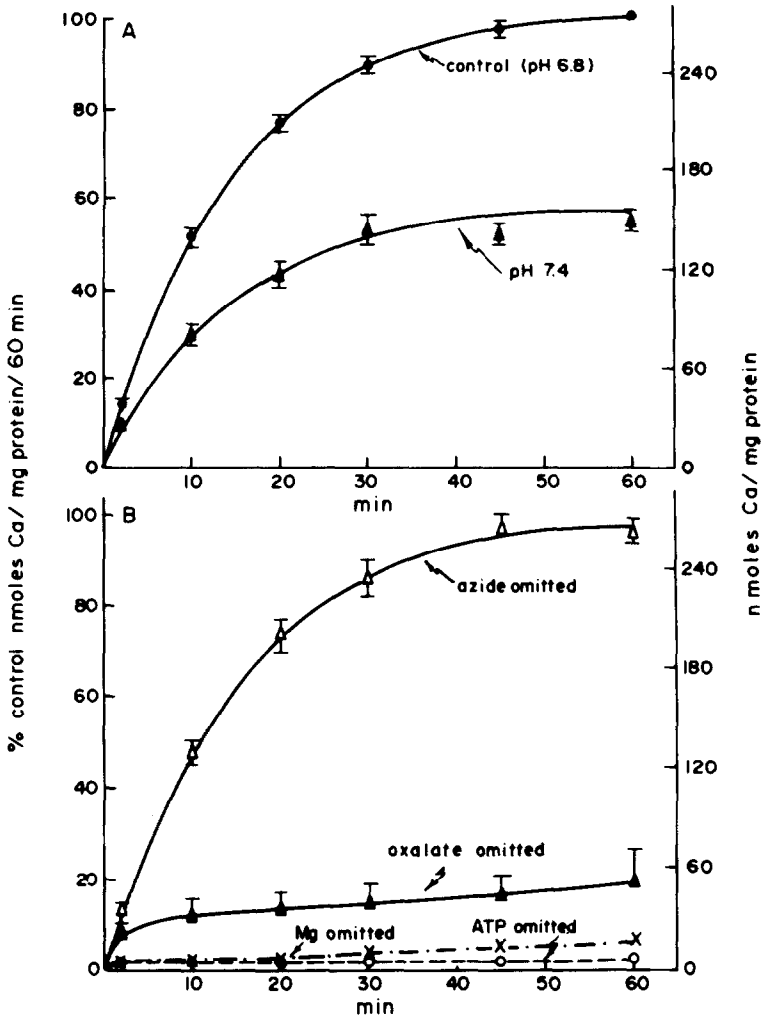
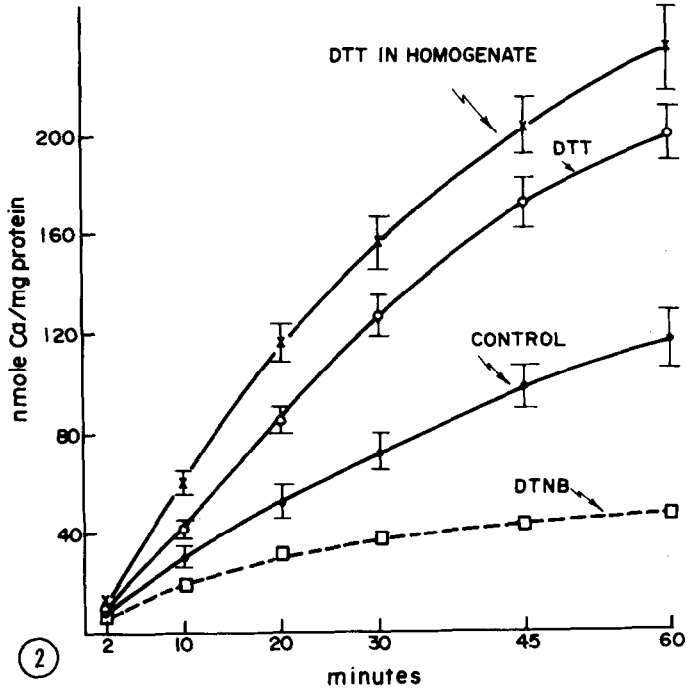


Fig 1A. Calcium uptake by microsomes of guinea-pig ileum longitudinal muscle. Microsomal fraction added to the incubation medium at zero time. Microsomal protein concentration was 39 µg/ml. Results are mean + S.E. Difference in uptake at 20 minutes between pH 7.4 (Tris buffer) and 6.8 (imidazole-histidine buffer) is significant ($P < 0.05$). Fig 1B. The effect of omission of sodium azide is not significantly different from control. Incubation pH is 6.8 (n=6)

was approximately identical to that of rat aorta microsomes (3). In addition to dithiothreitol stimulation, the preparation was inhibited by the sulfhydryl oxidizing agent, dithiobis-2-nitrobenzoate. In Fig. 3, the distribution of calcium uptake activity of the rat uterine microsomes on a sucrose density gradient is presented. With and without dithiothreitol, the activity was


DTT IN HOMOGENATE
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CONTROL
DTNB
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200
160
120
80
40
2
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minutes
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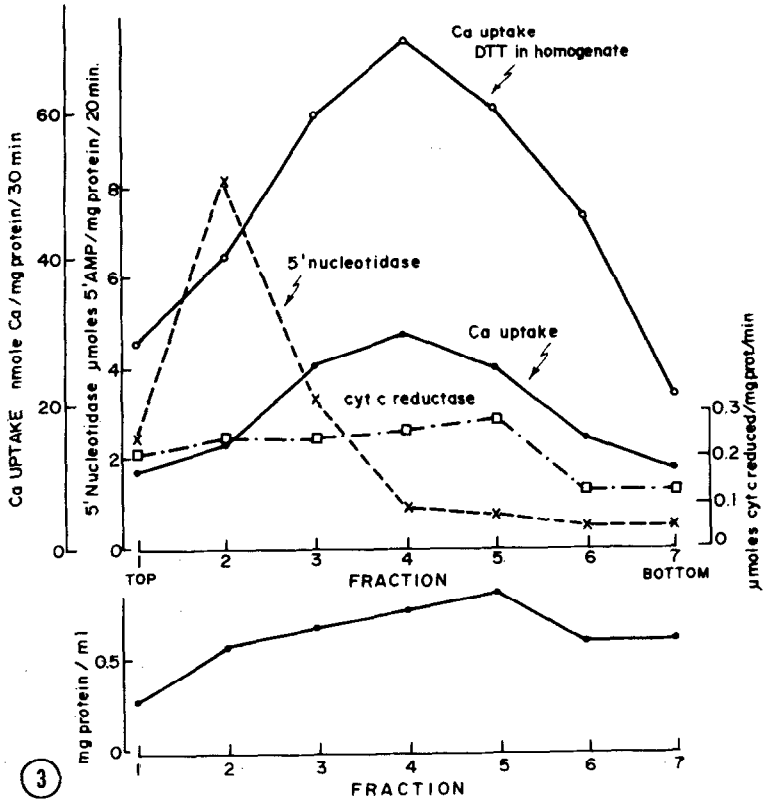
Ca uptake
DTT in homogenate
5'nucleotidase
Ca uptake
cyt c reductase
Ca UPTAKE nmole Ca/mg protein/30 min
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µmoles cyt c reduced/mg prot/min
TOP
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associated with the sarcoplasmic reticulum on the gradient. Similar gradient distribution was seen for calcium uptake activity of longitudinal muscle microsomes.

Release of sequestered calcium was examined following calcium accumulation by the highly active longitudinal muscle preparation (Fig 4). Release is normally very slow (20% in 50 minutes). EGTA (1 mM) removes in ten minutes an apparently superficial fraction representing about 20% of the calcium. The residual basal release rate is unaffected. The calcium ionophore A23187 causes release of 90% of the sequestered calcium within 10 minutes. In a separate study of chlorpromazine and guinea-pig ileum longitudinal smooth muscle (8), we obtained a similar rapid release of sequestered calcium from the microsomes in presence of 1 mM chlorpromazine. Two other agents, prostaglandin E_2 and p-chloromercuribenzoate modestly enhanced the release of calcium (Fig 4).

Discussion: Calcium uptake activity of guinea-pig ileum longitudinal smooth muscle microsomes was higher than activity previously obtained in dithiothreitol stimulated rat aorta microsomes (3). Gentle homogenization with the Potter homogenizer appeared to be a critical step. Somewhat less active preparations were obtained when a Polytron homogenizer was employed and all activity was lost if tissue was disrupted with a Sorvall omnimixer. Similar strong oxalate stimulated calcium uptake activity with guinea pig smooth muscle microsomes prepared by gentle homogenization procedures has been previously reported (9,10). Characterization of the calcium uptake of

Fig 2. Calcium uptake by microsomes of rat uterine smooth muscle. Dithiothreitol (DTT) 10 mM is added to microsomal incubation or is added to the homogenate before preparation of the microsomes. Dithiobis-2-nitrobenzoate (DTNB) 0.1 mM is added to the microsomes. Results are mean ± S.E. (n=6).

Fig 3. Density gradient characterization of rat uterine smooth muscle microsomes. Data is from a representative gradient. A separate gradient was prepared of microsomes derived from homogenate to which was added 10 mM dithiothreitol (DTT). Calcium uptake specific activity for this preparation is superimposed in the figure. Other parameters in the dithiothreitol pretreated preparation were similar to that for the controls. Data is presented as specific activity. Absolute protein concentration of each fraction is in lower part of the figure.

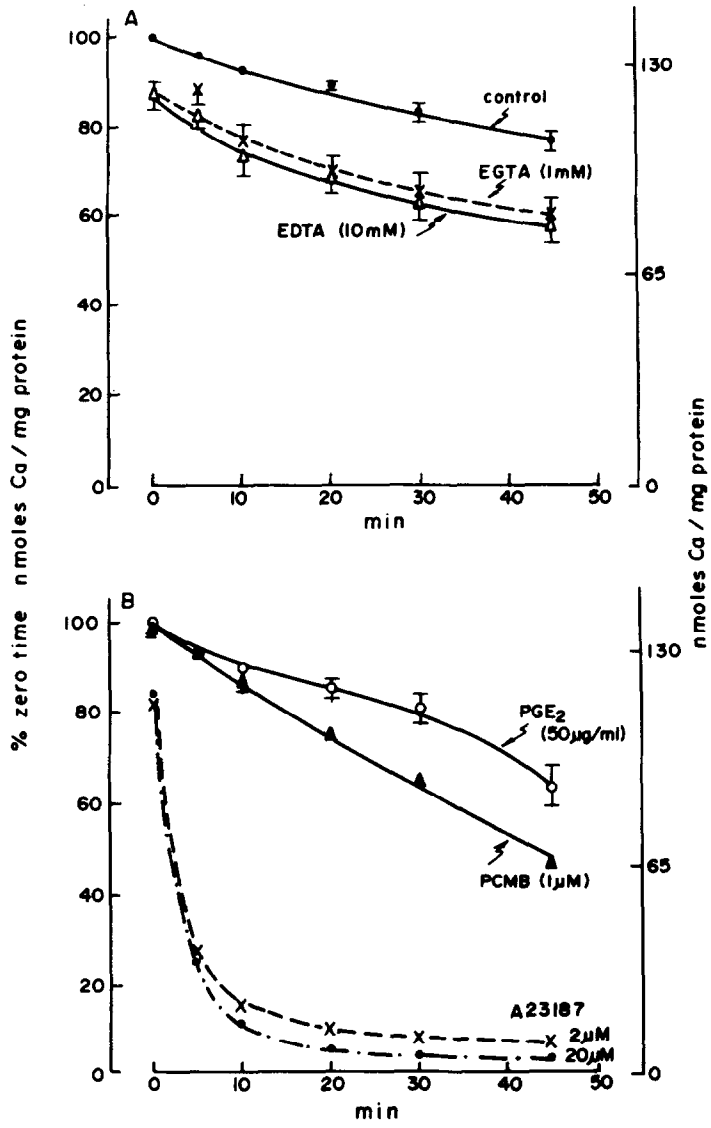

Fig 4. Release of (^{45}Ca) from preloaded microsomes of the guinea-pig ileum longitudinal muscle. There is a significant difference ($P < 0.05$) between control (n=41) and calcium released in presence of EDTA or EGTA at 45 min (n=8). For PCMB and PGE_2 (p-chloromercuribenzoate and prostaglandin E_2) n=3. Release in presence of the ionophore A23187 at 2 µM and 20 µM (n=2).

nonvascular smooth muscle microsomes on continuous sucrose density gradients has not been previously carried out. The apparent absence or very low level of calcium uptake activity in the plasma membrane component of the microsomes conceivably may be due to leakiness of microsomal vesicles formed from the disrupted plasma membrane during homogenization.

The calcium sequestering capability of the uterine microsomes was about 18 nmoles/min/gram wet weight of muscle and the activity of the longitudinal muscle was about 25 nmoles/min/gram wet weight of muscle. According to Goodford et al (11) a calcium influx of 0.12 nmole/min/gram wet weight of smooth muscle is sufficient for maximal activation of the muscle. The microsomes have a capacity more than 100-fold in excess to counter the influx and promote relaxation.

ACKNOWLEDGMENTS

This investigation was partially supported by NIH grants HL 14681, HD 08561 and HD 10607. Also in support was a grant from the Council for Tobacco Research, Inc. USA and Grant RR 05424 from Biomedical Research Support.

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