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The Na^+–Ca^{2+} exchange system in vascular smooth muscle cell membrane vesicles isolated from cultured cells and from tissue is similar

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The existence of a Na^+–Ca^{2+} exchange system was investigated in sarcolemmal vesicles isolated from cultured cells of dog mesenteric artery. When Na^+-loaded membrane vesicles were suspended in a Na^+-free KCl medium to create an outwardly directed Na^+ concentration gradient across the membrane, a time-dependent uptake of Ca^{2+} was observed. This uptake of Ca^{2+} was drastically reduced when the vesicles were suspended in NaCl medium to eliminate the Na^+ concentration gradient across the membrane. Monensin also decreased Ca^{2+} uptake in Na^+-loaded vesicles. The apparent K_m for Ca^{2+} was 2.97 μM and the apparent maximum velocity was 4.27 nmol/min per mg protein. The data indicate that a Na^+–Ca^{2+} exchange system exists in sarcolemmal membranes isolated from cultured cells and that it is similar to the system in membranes isolated from the tissue.

The existence of an active Na^+–Ca^{2+} exchange system in intact smooth muscles has been controversial [1–4]. However, Na^+–Ca^{2+} exchange activity has been demonstrated in isolated sarcolemmal membrane vesicles of several smooth muscles [5–8]. These tissues, although carefully cleaned, may contain variable amounts of non-muscle cells in addition to smooth muscle cells. Therefore sarcolemmal membranes isolated from these tissues may contain membranes from the contaminating non-muscle cells. It may be argued that the Na^+–Ca^{2+} exchange activity in such sarcolemmal membrane vesicles may have originated from the non-muscle cells. To nullify this doubt we have studied Na^+–Ca^{2+} exchange activity in sarcolemmal membranes prepared from isolated and cultured smooth muscle cells of dog mesenteric artery.

Plastic cultureware was obtained from Corning Glass Works, Corning, NY. Collagenase Type 1 (148 U/mg) and deoxyribonuclease Type 1 (2505 U/mg) were obtained from Worthington Biochemicals, Freehold, NJ. Elastase Type III (62 U/mg) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Sigma Chemical Co., St. Louis, MO. Fetal bovine serum (FBS), L-glutamine and antibiotics were obtained from Grand Island, NY. All other chemicals were the purest available from Sigma Chemical Co., St. Louis, MO, and Fischer Scientific Co., Cincinnati, OH.

A male mongrel dog (16 kg) was anaesthesized with sodium pentobarbital (35 mg/kg) and four segments of the second to third branches (external diameter less than 2 mm) of supramesenteric artery

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were obtained. The arterial segments were trimmed to remove fatty tissues and rinsed well in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin. Smooth muscle cells were obtained enzymatically according to a modified procedure of Ives et al. [9]. Briefly, arterial segments were cut open longitudinally and predigested for 1 h at 37°C in DMEM containing collagenase (1 mg/ml), deoxyribonuclease (2 mg/ml) and elastase (0.5 mg/ml). Tunica adventitia and internal elastic membrane were then carefully removed with a pair of forceps and the isolated medial layers were rinsed and digested for an additional 2 h at 37°C in a fresh DMEM-enzyme mixture. The cell suspension was sieved through sterile 100 μm^2 nylon mesh (Tetko, Elmsford, NY), washed once with DMEM containing 10% fetal bovine serum. Cells were then seeded in a 25 cm^2 plastic flask and grown in DMEM supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2/95% air in an incubator maintained at 37°C. When confluent, the cells were passaged at a density of 10^6 cells/75 cm^2 flask and fed twice a week with DMEM containing 10% fetal bovine serum. Cultures at the 5th and 6th passages were used to isolate membranes in the present study.

Cells were scraped from the culture flasks with a rubber policeman. The collected cells (approx. 10 million) were washed twice with a solution containing 250 mM sucrose, 10 mM 4-morpholinepropanesulfonic acid (Mops)-NaOH buffer (pH 7.4) and 0.05% bovine serum albumin (Sigma, fatty acid free). The cells were then homogenized with a glass-teflon homogenizer (size AA, Thomas Scientific, Philadelphia, PA) by making five passes five times with a motor driven pestle at a rheostat setting of 80% at 140 V. The homogenate was centrifuged and subcellular fractions were prepared according to a procedure described previously [10] with the slight modification that the homogenate was centrifuged at $12000 \times g$ instead of $1000 \times g$ for 10 min. Three membrane fractions, viz. homogenate (H), $12000 \times g$ pellet (12K-P) and microsomes (MIC) were obtained during differential centrifugation, and three others, viz. F1, F3 and F5, were obtained after sucrose density gradient centrifugation as described previously [10].

Protein concentrations were determined according to Lowry et al. [11] using bovine serum albumin as standard. 5′-Nucleotidase activity was determined as described previously [10]. Na^+–Ca^{2+} exchange activity was determined by measuring $^{45}Ca^{2+}$ uptake in Na^+-loaded membrane vesicles as described previously [6]. Briefly, after the membranes were sedimented during preparation, they were suspended in 150 mM NaCl and 10 mM Mops-NaOh buffer (pH 7.2), quick-frozen in liquid N_2 and stored at −70°C. Before the assay for Na^+–Ca^{2+} exchange, the membranes were quickly thawed and preincubated at 37°C for 30 min to ensure complete Na^+ loading. Two to three μl of the membrane vesicles (3 μg protein) were suspended in 100 μl of either 150 mM KCl, 10 mM Mops-KOH buffer (pH 7.2) and 14.5 μM $^{45}CaCl_2$ or 150 mM NaCl, 10 mM Mops-NaOH buffer (pH 7.2) and 14.5 μM $^{45}CaCl_2$. The uptake of $^{45}Ca^{2+}$ after a specific time period was terminated by quickly diluting the suspension with 5 ml of 150 mM KCl, 10 mM Mops-KOH buffer and 0.1 mM ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (pH 7.2). The membranes were harvested on Gelman GN6/A filters and washed three times with 5 ml of the above solution. The filters were suspended in scintillation fluid and counted for radioactivity in a Beckman scintillation counter. The concentration of free Ca^{2+} in the experiments described in Fig. 3 was maintained with a Ca-EGTA buffer system described by Robertson and Potter [12].

Fig. 1 shows the morphology of the primary culture cells under light microscope 8 days after isolation. The cells appeared spindle-shaped and growing towards confluency in array, characteristic of vascular smooth muscle cells [13]. After subculture, the cells grew in hills and valleys typical of smooth muscle cells [13]. Morphological characteristics of the cells in primary culture indicated absence of any detectable fibroblast cells.

To identify the sarcolemmal membranes, 5′-nucleotidase activity was measured in subcellular membrane fractions isolated from cultured smooth muscle cells of dog mesenteric artery. The highest specific activity (3.16 μmol P_i/mg protein per h) of the enzyme was observed in the F1 fraction formed at the interface between 8.5 and 30% sucrose after density gradient centrifugation.

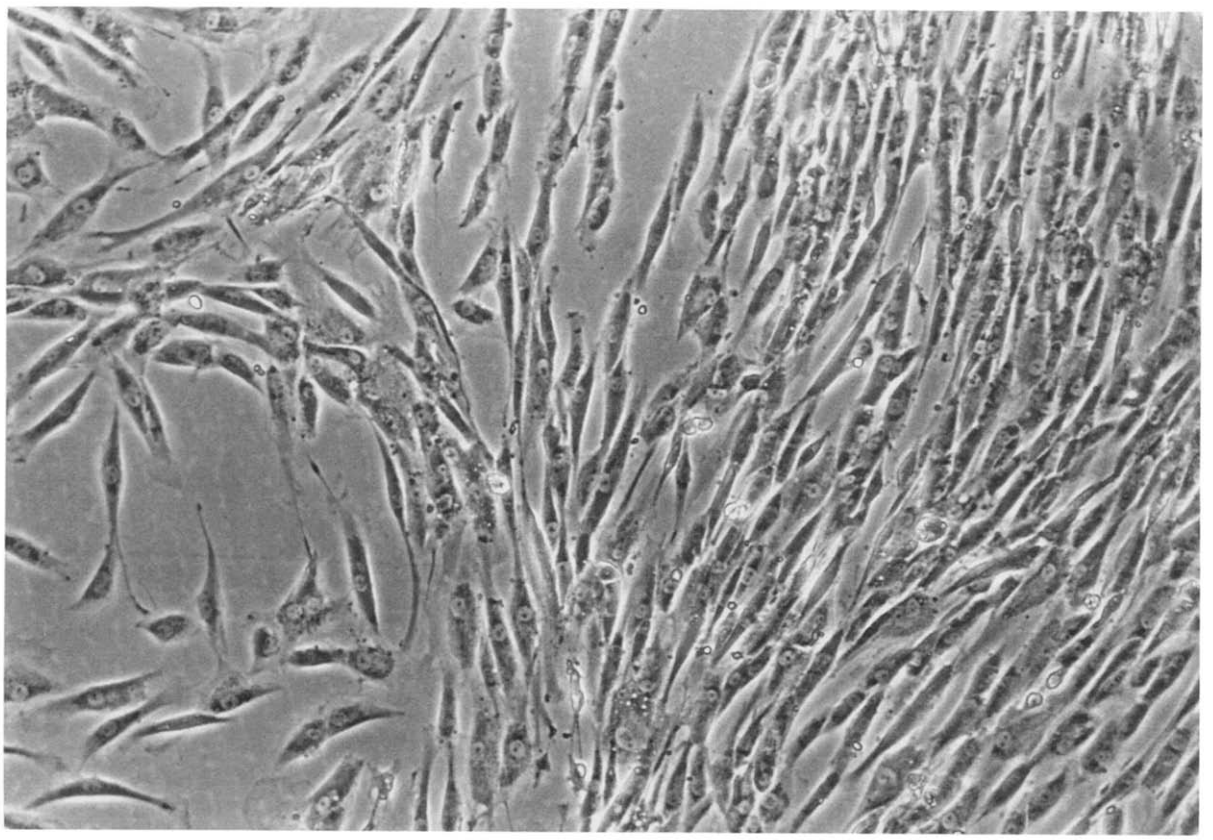

Fig. 1. Morphology of primary culture cells under light microscope. Areas showing sparsely distributed spindle-shaped cells and cells growing in array. Magnification: 106×.

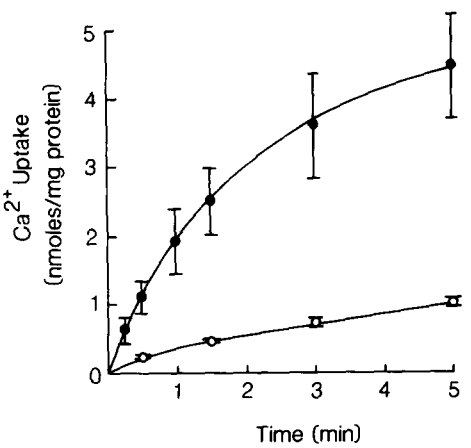

Fig. 2. Time-dependent uptake of Ca^{2+} in Na^+-loaded sarcolemmal vesicles. ●——●, vesicles suspended in high KCl medium; ○——○, vesicles suspended in high NaCl medium. The concentration of Ca^{2+} in the medium was 14.5 μM.

Compared to homogenate, the F1 fraction was 6.4-fold enriched with 5′-nucleotidase activity. The specific activity of the Na^+–Ca^{2+} exchange was also highest (0.91 nmol Ca^{2+}/mg protein in 15 s) in the F1 fraction. The pattern of distribution of these two activities was very similar in the subcellular fractions. In a previous study on sarcolemmal membranes prepared from rat mesenteric arteries, the highest specific activity of Na^+–Ca^{2+} exchange, insensitive to diltiazem, an inhibitor of Na^+–Ca^{2+} exchange of mitochondria, was also observed in the F1 fraction [6]. The F1 membrane fraction isolated from smooth muscle of several different tissues was found to be enriched with sarcolemmal membranes [6,10,14,15]. Since the F1 fraction prepared from cultured smooth muscle cells demonstrated the highest specific activity of a well-known cell membrane marker, it was con-

cluded that the Na^+–Ca^{2+} exchange activity observed in this fraction was localized in the cell membrane.

The Na^+–Ca^{2+} exchange activity in the F1 fraction enriched with sarcolemmal membranes was further characterized. When Na^+-loaded membrane vesicles were suspended in the high KCl medium to create an outwardly directed Na^+ concentration gradient, a time-dependent uptake of Ca^{2+} was observed (Fig. 2, closed circles). However, when the vesicles were suspended in the high NaCl medium to eliminate the Na^+ concentration gradient across the membrane, the uptake of Ca^{2+} was drastically reduced (Fig. 2, open circle). The sodium ionophore monensin, which also eliminates Na^+ concentration gradient, also reduced Ca^{2+} uptake by 31 ± 6% of the control in Na^+-loaded vesicles in high KCl medium. These data suggest that a Na^+ concentration gradient-dependent Ca^{2+} uptake system exists in the sarcolemmal membrane vesicles isolated from cultured smooth muscle cells of dog mesenteric artery.

The uptake of Ca^{2+} in Na^+-loaded vesicles was dependent on free Ca^{2+} concentration in the assay medium. The rate of Ca^{2+} uptake increased with increasing concentration of free Ca^{2+} (Fig. 3). The apparent maximum rate of Ca^{2+} uptake was observed at Ca^{2+} concentrations above 5 μM. The apparent K_m for Ca^{2+} calculated from Lineweaver-Burk plot was 2.97 μM and the apparent maximum velocity measured at 15 s was 4.27 nmol/min per mg protein. Sarcolemmal membranes isolated from dog mesenteric arteries according to the same procedure were enriched 11-fold with 5′-nucleotidase activity relative to the initial homogenate and exhibited an apparent K_m for free Ca^{2+} of 2.6 μM and an apparent velocity of 6.9 nmol/min per mg protein when assayed at 15 s for Na^+–Ca^{2+} exchange activity under conditions identical to those described in above. A more purified sarcolemmal membrane appears to be the reason why the apparent maximum rate of Ca^{2+} uptake was higher in the vesicles isolated from the tissue. We, therefore, concluded that the apparent K_m for Ca^{2+} and the apparent maximum velocity of Ca^{2+} uptake are similar to values observed in membranes isolated from the tissue of dog mesenteric artery. However, the observed K_m for Ca^{2+} and maximum velocity of Ca^{2+} uptake in isolated sarcolemmal vesicles should be taken with caution as they may not reflect the real values in vivo in the intact cell. The kinetics of the system may be altered in isolated membranes due to changes during isolation, differences in vesicle size or orientation of the membrane, and loss of the activators or inhibitors.

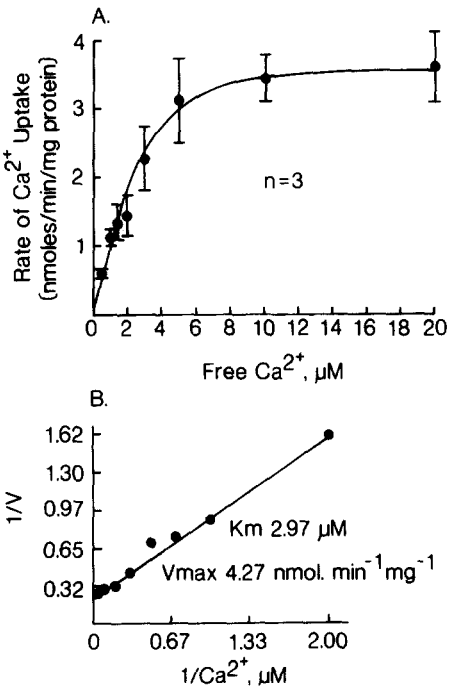

Fig. 3. (A) The rate of Ca^{2+} uptake in Na^+-loaded vesicles as a function of free Ca^{2+} concentration in the medium. Na^+-gradient independent Ca^{2+} uptake in 150 mM NaCl medium was subtracted to determine the Na^+-concentration gradient dependent Ca^{2+} uptake for each concentration of free Ca^{2+}. The rate of Ca^{2+} uptake was measured at 15 s. (B) Lineweaver-Burk plot of 1/(rate of Ca^{2+} uptake) vs. 1/(free Ca^{2+} concentration). Free Ca^{2+} concentrations were maintained with 0.1 mM EGTA and varying concentrations of $CaCl_2$ at pH 7.2 as described by Robertson and Potter [12].

The data presented here indicate that a Na^+–Ca^{2+} exchange system exists in the sarcolemmal membranes isolated from cultured smooth muscle cells of dog mesenteric artery. Although Na^+–Ca^{2+} exchange activity has been demonstrated in cultured vascular smooth muscle cells [16], this is the first demonstration of its existence in sarcolemmal membranes isolated from cultured smooth muscle cells free from any contaminating non-muscle cells. Since the Na^+–Ca^{2+} exchange

activity in the sarcolemmal membranes isolated from cultured smooth muscle cells is similar to that of membranes isolated from tissue, the exchange activity observed in the latter cannot be exclusively due to contamination. If there is any minor contamination it does not appear to significantly alter the kinetics of the process. Thus the results of the present study argue against contamination by non-muscle cells affecting the Na^+–Ca^{2+} exchange activity of sarcolemmal membranes prepared from smooth muscle of dog mesenteric artery.

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