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Solubilization and reconstitution of the sarcolemmal Na^+–Ca^{2+} exchange system of vascular smooth muscle

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The Na^+–Ca^{2+} exchange system of the sarcolemma of rat mesenteric artery was solubilized and reconstituted in soybean phospholipid vesicles. In the reconstituted system, the exchange process showed about 4-fold higher specific activity compared to that of native vesicles. The inhibitory effect of monensin and the stimulatory effect of valinomycin in the presence of K^+ on Na^+ gradient-dependent Ca^{2+} uptake were preserved and were pronounced in the reconstituted system. The stimulation by valinomycin indicates that the exchange process is electrogenic. Thus, the stoichiometry, the characteristics and the mechanism of action which were difficult to study in the native vesicles can now be determined conveniently using the reconstituted system. Also, solubilization and reconstitution of the exchange system confirms its existence in vascular smooth muscle.

The existence of a sodium–calcium exchange system in cell membranes of vascular smooth muscle has been reported [1]. The activity of this system was found to be very low compared to that of cardiac muscle cell membrane [2–4] and as such poses a problem in the study of many of the characteristics of this putative system in vascular smooth muscle. The objective of the present study was to determine whether a Na^+–Ca^{2+} exchange process exists in vascular smooth muscle cell membrane and, if so, if it can function with high specific activity when reconstituted in lipid vesicles.

Soybean phospholipid was obtained from Associated Concentrates, Woodside, NY and was recrystallized before use. $^{45}CaCl_2$ was obtained from New England Nuclear, Boston, MA. Valinomycin, monensin, 4-morpholinepropanesulfonic acid (Mops) and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO. All other chemicals used in this study were the purest commercially available grade. Male Sprague-Dawley rats weighing about 275–325 g were obtained from Harlan Industries, Inc., Indianapolis, IN.

Cell membrane vesicles from rat mesenteric arteries were isolated by a procedure described previously [1]. The membranes used in the present study were 12–15-fold enriched compared to tissue homogenate with the cell membrane markers 5′-nucleotidase activity and [^{3}H]nitrendipine binding [5]. The contamination by mitochondrial membranes was negligible as judged by the cytochrome *c* oxidase activity in the cell membrane fraction compared to that of mitochondrial fraction. The isolated cell membranes were quick-frozen in liquid nitrogen and stored at −70 °C. No loss of

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Na^+–Ca^{2+} exchange activity was observed in membranes frozen up to four months. The frozen membranes were thawed and about 5 mg protein was pooled and a portion of which (about 4 mg) was used for reconstitution experiments. The reconstitution of Na^+–Ca^{2+} exchange activity was carried out according to the procedure described previously for cardiac cell membranes [6,7]. Briefly, cell membrane vesicles were suspended at 2 mg protein/ml of a solution containing 25 mg soybean phospholipids/ml of 2% sodium cholate in 500 mM NaCl/20 mM Mops (pH 7.4). After 20 min incubation at 0 °C, the suspension was centrifuged at 180000 × *g* for 30 min. The supernatant was diluted into 5 volumes of ice-cold 160 mM NaCl/20 mM Mops (pH 7.4). Proteoliposomes were then collected by centrifugation (180000 × *g*; 60 min), resuspended in 160 mM NaCl/20 mM Mops (pH 7.4), centrifuged again (180000 × *g*; 60 min) and finally resuspended in 160 mM NaCl/20 mM Mops (pH 7.4). Na^+–Ca^{2+} exchange activity in both the native and the reconstituted lipid vesicles was measured as described previously [8]. Briefly, 2 μl of native vesicles or reconstituted vesicles were suspended in 100 μl of 160 mM KCl or NaCl, 20 mM Mops (pH 7.4) containing 14.45 μM $^{45}CaCl_2$. The uptake of $^{45}Ca^{2+}$ was terminated by quickly diluting the suspension with 5 ml of 200 mM KCl/20 mM Mops, 0.1 mM EGTA (pH 7.4). The vesicles were harvested on Whatman GF/A filters and washed twice with 5 ml of terminating medium. The filters were suspended in scintillation fluid and radioactivity was counted in a scintillation counter. The data represent the average of two experiments performed in triplicate unless indicated otherwise in the legend to figure. Protein concentration in the native membrane vesicles and in the reconstituted vesicles was determined by the amido black staining procedure of Schaffner and Weissmann [9] as modified by Newman et al. [10].

Before solubilization, the Na^+–Ca^{2+} exchange activity was determined in native sarcolemmal vesicles. In the Na^+-loaded vesicles, a time-dependent uptake of Ca^{2+} was observed when a Na^+ concentration gradient was created across the membrane by suspending the vesicles in KCl medium (Fig. 1, solid squares). The maximum uptake of Ca^{2+} was achieved in about 2 minutes.

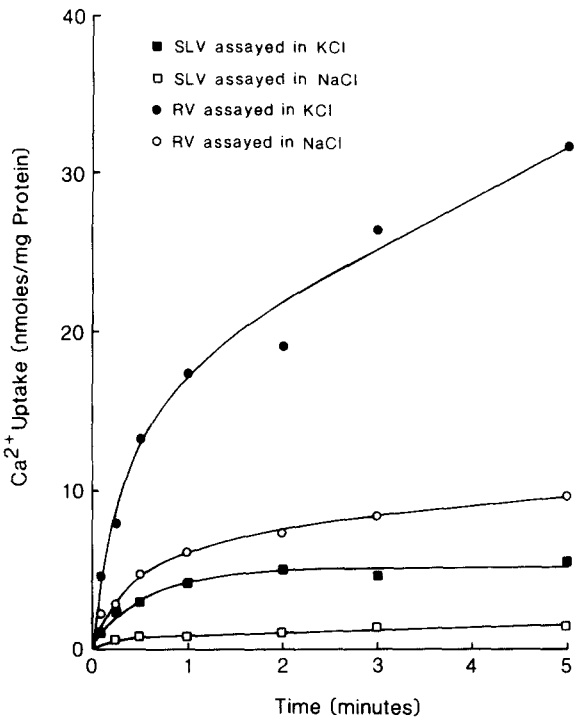

Fig. 1. Time-course of Ca^{2+} uptake in isolated sarcolemmal vesicles (SLV) and reconstituted vesicles (RV). The assay was carried out in media as indicated. ■———■ and ●———● 160 mM KCl, 20 mM Mops (pH 7.4); □———□ and ○———○, 160 mM NaCl, 20 mM Mops (pH 7.4).

When these vesicles were suspended in NaCl medium to decrease the Na^+ gradient, a small uptake of Ca^{2+} was observed (Fig. 1, open square). The sarcolemmal vesicles were then solubilized and proteins were re-incorporated into soybean phospholipid vesicles. A rapid uptake of Ca^{2+} was observed in the reconstituted Na^+-loaded phospholipid vesicles when they were suspended in KCl medium (Fig. 1, solid circle). The initial rate of Ca^{2+} uptake increased by 4-fold in these vesicles compared to that of native vesicles under identical conditions. As expected, the rate and magnitude of Ca^{2+} uptake in reconstituted vesicles was lower in the NaCl medium than it was in the KCl medium (Fig. 1, open circle).

In order to test if the uptake of Ca^{2+} occurred against a Na^+ gradient across the membrane, the effect of monensin, a monovalent cation ionophore [11], was studied. Monensin decreased Ca^{2+} uptake in native and reconstituted vesicles to about 33% of the respective control values in KCl

medium (data not shown). No effect of monensin was observed on Ca^{2+} uptake in the NaCl medium. These data indicate that the uptake of Ca^{2+} occurred due to a Na^+ concentration gradient across the membrane and that the decreased Ca^{2+} uptake in NaCl medium was due to decreased Na^+ concentration gradient.

The exchange of Na^+ for Ca^{2+} across the membrane may generate a negative potential inside the vesicles if more than two Na^+ ions are exchanged for each Ca^{2+} ion. If this is the case, then the process will be inhibited due to retardation of the Na^+ efflux from the vesicles. Valinomycin, a K^+-ionophore [11], in the presence of K^+ should neutralize the negative potential inside and thus should facilitate the Na^+–Ca^{2+} exchange activity. Valinomycin increased Ca^{2+} uptake slightly after 15 s in native vesicles suspended in KCl medium (Fig. 2A). However, a more pronounced effect of valinomycin on Ca^{2+} uptake was observed in the reconstituted vesicles even at 5 s (Fig. 2B). These data indicate that the exchange system is electrogenic. The stoichiometry of $3Na^+$ per Ca^{2+} has been observed in cardiac muscle membrane vesicles [3,12]. The stoichiometry of the vascular smooth muscle system remains to be determined. In cardiac muscle cell membrane vesicles, the operation of the Na^+–Ca^{2+} exchange process generates a negative inside membrane potential [4,13]. Under this condition, valinomycin in the presence of K^+ was found to enhance Ca^{2+} uptake in Na^+-loaded vesicles [4–13]. The effect of valinomycin on Na^+–Ca^{2+} exchange activity in cell membrane vesicles of vascular smooth muscle was very small. However, a pronounced effect of valinomycin on Ca^{2+} uptake was observed in reconstituted vesicles probably because higher rates of exchange activity resulted in more negative potential inside the membrane and/or due to more tightly sealed vesicles.

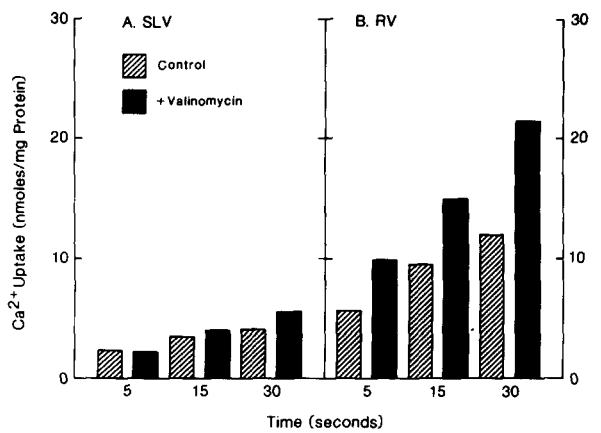

Fig. 2. Effect of valinomycin on Ca^{2+} uptake in (A) sarcolemmal vesicles and (B) reconstituted vesicles. The vesicles were loaded with Na^+ and the assay was carried out in 160 mM KCl, 20 mM Mops (pH 7.4) in the absence (hatched bars) and in the presence of 1 μM valinomycin (solid bars). The values represent Ca^{2+} uptake at the time indicated in the abscissa. The figure is representative of data of three similar experiments carried out in triplicate.

The results of the study suggest that the Na^+–Ca^{2+} exchange process of vascular smooth muscle cell membranes can be reconstituted in soybean phospholipid vesicles. The 4-fold increase in activity observed in reconstituted vesicles may be due to (a) partial purification of the process, (b) loss of an endogenous inhibitor during reconstitution, or (c) different lipid environment. Preliminary experiments on polyacrylamide gel electrophoresis of the reconstituted proteins in the lipid vesicles did not convincingly indicate enrichment of a specific protein-band (unpublished observations). However, this aspect of the reconstituted proteins requires more detailed study before any conclusions can be drawn.

There was considerable uncertainty regarding the existence of a Na^+–Ca^{2+} exchange process in smooth muscle (for a review, see Ref. 14). Lately, a Na^+–Ca^{2+} exchange process has been demonstrated in isolated cell membrane vesicles of vascular smooth muscle [1], uterine smooth muscle [15], ileal longitudinal smooth muscle [16]. However, the activities were very low compared to that of cardiac muscle [2–4]. Therefore, there is still some doubt about its existence in smooth muscle. Successful reconstitution of the process in liposomes is a clear indication of its existence in vascular smooth muscle cell membrane. In the past, many of the characteristics of the process in vascular smooth muscle could not be studied because of low specific activity. The reconstituted process in liposomes appears to be a more active system which can be used to determine its characteristics, the stoichiometry, the mechanism of action and the possible physiological role in vascular smooth muscle.

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