Biochimica et Biophysica Acta 904 (1987) 92–104
Elsevier

BBA 73731

Sodium-calcium exchange in sarcolemmal vesicles from tracheal smooth muscle

Robert S. Slaughter, Ann F. Welton and Douglas W. Morgan

Allergy and Inflammation Research, Hoffmann-La Roche Inc., Nutley, NJ (U.S.A.)

(Received 27 January 1987)
(Revised manuscript received 15 June 1987)

Key words: Sodium–calcium ion exchange; Sarcolemmal vesicle; (Tracheal smooth muscle)

Sarcolemmal vesicles prepared by a new procedure from bovine tracheal smooth muscle were found to have a Na-Ca exchange activity that is significantly higher than that reported for different preparations from other types of smooth muscle. The exchange process system co-purified with 5′-nucleotidase, a plasma membrane marker enzyme, and was significantly enriched (over 100-fold) compared to mitochondria (cytochrome-*c* oxidase) but only slightly enriched (4-fold) compared to sarcoplasmic reticulum (NADPH–cytochrome-*c* reductase). The Na^+ dependence of Ca^{2+} transport was demonstrated through both uptake and efflux procedures. The uptake profile with respect to Ca^{2+} was monotonic with a linear v_0 vs. $v_0 \cdot S^{-1}$ plot. The resultant K_m of Ca^{2+} from the airway sarcolemmal vesicles (20 μM) was similar in magnitude to the K_m of cardiac sarcolemmal vesicles (30 μM). Tracheal vesicles demonstrated a V_{max} of 0.3–0.5 nmol $\cdot$ mg^{-1} $\cdot$ s^{-1} which is significantly higher than that reported in preparations from other smooth muscle types. Furthermore, two processes found to stimulate cardiac Na-Ca exchange, pretreatment with either a mixture of dithiothreitol and Fe^{2+} or with chymotrypsin, were ineffective on the tracheal smooth muscle. Thus, the Na-Ca exchanger identified in tracheal smooth muscle appears to be different from that observed in cardiac muscle, implying that regulation of this activity may also be different.

Introduction

The present study on Na-Ca exchange was based on an interest in defining the biochemical processes involved in the control of contractility in tracheal smooth muscle. It is well known that Ca^{2+} is involved in tension development in all muscle types. In smooth muscle the initial step in response to contractile mediators is the rise in internal free Ca^{2+} by a combination of influx of external Ca^{2+} and release of intracellular stores. This Ca^{2+} then binds calmodulin which activates myosin light chain kinase thus leading to phosphorylation of myosin with resultant contraction of the actomyosin complex [1].

In subsequent relaxation, the free internal Ca^{2+} levels must be reduced by returning Ca^{2+} to its place of origin, either the cell exterior or sarcoplasmic reticulum. There are two known sarcolemmal Ca^{2+} transport systems for Ca^{2+} removal, the Na-Ca exchanger and a Ca-pump ATPase. In sarcoplasmic reticulum there is a different Ca-pump ATPase which is involved in Ca^{2+} sequestration. These systems have been well studied in cardiac muscle [2–7] but only recently have been identified in smooth muscle [8–16] and heretofore have not been reported in tracheal smooth muscle.

Abbreviation: Mops, 4-morpholinepropanesulfonic acid.

Correspondence (present address): R.S. Slaughter, Department of Membrane Biochemistry, Merck Institute for Therapeutic Research, P.O. Box 2000, Rahway, NJ 07065, U.S.A.

In an attempt to differentiate the relative importance of these systems in tracheal smooth muscle we began with a study of the Na-Ca exchange process.

A Na-Ca exchange system would be of greatest significance in contractile systems which depend on external Ca^{2+}. External Ca^{2+} has been reported to be an important source of contractile Ca^{2+} in airway smooth muscle (for reviews see Refs. 17–21) when tissue is challenged with leukotrienes [22–25], histamine [22–24,26], acetylcholine [24,26] and potassium [24]. These conclusions were based on depletion experiments for external Ca^{2+} and the use of Ca^{2+} channel blockers with each of these agonists. Thus, for several contractile activators an important role for external Ca^{2+} is implicated in the mechanism by which many agents induce contraction in tracheal smooth muscle, and the Na-Ca exchange process could play an important role in removal of Ca^{2+} from the intracellular compartment during relaxation.

We report the isolation of sarcolemmal vesicles from a relatively homogeneous preparation of bovine tracheal smooth muscle cells. Na-Ca exchange activity has been identified in high quantity in these vesicles, 10-fold higher than reported for rat myometrium [9] and guinea pig intestinal smooth muscle [11]. This level of activity implies a much more significant role for the Na-Ca exchange system in tracheal smooth muscle than has been proposed for these other smooth muscle types [27]. In addition, this activity appears to be under different control than the Na-Ca exchanger from heart.

Methods

Cell preparation from tracheal smooth muscle

Large male bovine tracheae were obtained from an abattoir and were kept on ice until use. All cell preparation procedures were performed at 0–4 °C. Each trachea was cut open on the side opposite the fold where the smooth muscle strip was found. The smooth muscle was then exposed by careful removal of the epithelial layer from the luminal surface of the trachea. Then, strips of smooth muscle were carefully cut away from the adventitia and placed in a beaker on ice.

These smooth muscle strips (100 g) were washed once with 200 ml of a Mops-modified Tyrodes buffer (155 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2 \cdot 6H_2O$, 1 g/l glucose, and 5 mM Mops, pH 7.40 adjusted with Tris base), resuspended in 200 ml of the same buffer, passed through an electric meat grinder, filtered through two layers of cheesecloth, and split into three portions. These portions were placed in plastic bags (Tekmar Co., PO Box 371856, Cincinnati, OH 45222, U.S.A.) with 200 ml Mops-modified Tyrodes buffer and the tissue in these bags was homogenized for 20 s in a Tekmar Stomacher, and then filtered through one layer of cheesecloth. The filtrate was discarded. The remaining tissue was agitated again in the Stomacher in 200 ml of buffer for 3×30 s with 90 s intervals between each agitation. Filtrates were collected and examined under a light microscope and those filtrates with greater than 95% smooth muscle and highest cell density were combined.

Preparation of membrane fractions

All solutions and procedures used in the preparation of membrane fractions were at 0–4 °C. Procedures leading to sarcolemmal membrane (plasma membrane) preparation are described below. Cells were pelleted from the combined filtrate by centrifugation at $13\,000 \times g$ for 30 min and resuspended in 300 ml fresh sucrose buffer (250 mM sucrose, 20 mM Mops, adjusted to pH 7.40 with Tris base). The suspension was incubated in a Parr Nitrogen Cavitation Chamber at 1100 psi for 15 min and the cells were broken as they were slowly released from the chamber. This cavitated fraction was termed the crude homogenate.

The separation of the membrane fractions from the crude homogenate was based on procedures used by Grover et al. for rat myometrium [28,29], rat mesenteric arteries [30], and for canine trachealis [31]. The homogenate was centrifuged at $1000 \times g$ for 10 min. The resultant supernatant was centrifuged at $10\,000 \times g$ for 10 min to remove mitochondria. This supernatant was in turn subjected to centrifugation at $120\,000 \times g$ for 45 min (Beckman Type 35 rotor) to pellet the remaining membranes and to remove soluble proteins. The $120\,000 \times g$ pellet was resuspended in 8% sucrose and layered on a discontinuous gradient prepared from 30% and 40% sucrose. Four frac-

tions were collected after centrifugation at 180 000 $\times g$ for 90 min (Beckman Type 50Ti rotor): the interfaces between 8 and 30% sucrose and between 30 and 40% sucrose, the pellet, and the remaining noninterface supernatant. These fractions were diluted at least 3-fold in 160 mM NaCl solution (160 mM NaCl, 20 mM Mops, adjusted to pH 7.40 with Tris base) and centrifuged at 180 000 $\times g$ for 30 min.

The resultant pellets were resuspended in the 160 mM NaCl solution and homogenized with a teflon-glass homogenizer. Aliquots (0.25–1.0 ml) were placed in 1 ml Nunc cryotubes from InterMed, quick-frozen in liquid nitrogen, and stored at $-80\,^{\circ}C$.

Bovine cardiac sarcolemmal vesicle preparations [32] were the generous gift of Dr. John P. Reeves.

Assays for membrane marker enzymes

5′-Nucleotidase. An assay was developed for 5′-nucleotidase, a plasma membrane (sarcolemmal) marker, from a combination of the traditional 5′-nucleotidase assay [33] and an assay for phosphodiesterase which utilized a Dowex column procedure for analysis of products [34]. Samples were incubated with 1 ml of the reaction mixture (5 mM [^{3}H]AMP (New England Nuclear), 100 mM Tris-HCl (pH 7.50), 10 mM $MgSO_4$) at 37 °C for 10 min. Reactions were stopped by placing the tubes in boiling water for exactly 1 min and then the tubes were cooled in ice. Samples were centrifuged for 2 min in a Brinkman Microfuge and 400 μl of each sample were placed on a Dowex column to separate the product, adenosine, from the reactants. The [^{3}H]adenosine was determined by liquid scintillation.

Each minicolumn contained 1.5 ml bed volume of Dowex A1-2X anion exchange resin. Columns were prepared by washing with 10 ml H_2O, followed by 20 ml 1 M HCl. Immediately prior to addition of sample, the columns were washed twice with 1 ml of 5 mM adenosine in isopropanol/water (1 : 1), pH 5.0. After the application of the 400 μl sample, the [^{3}H]adenosine was eluted with three washes of 0.7 ml of the 5 mM adenosine isopropanol/water.

NADPH–cytochrome-c reductase. This enzyme was assayed as a marker for endoplasmic reticulum by a modification of the method described by Pederson et al. [35]. Membrane preparations were added to 1 ml of the reaction mixture (71.1 μM cytochrome *c* (Sigma, type VIII), 100 μM EDTA, 1 mM KCN, 50 mM potassium phosphate buffer, pH 7.40). The reaction was initiated with the addition of 10 μl of 10 mM NADPH (0.1 mM final concentration) and followed by the increase in absorbance at 550 nm. Cytochrome *c* and NADPH solutions were made immediately prior to use.

Cytochrome-c oxidase. The assay for this mitochondrial marker was based on the procedure of Cooperstein and Lazarow [36]. The reaction mixture contained 71.1 μM reduced cytochrome *c* (by $Na_2S_2O_4$) in 50 mM potassium phosphate buffer, pH 7.40. The reaction was initiated by the addition of protein and was followed by the decrease in absorbance at 550 nm.

Protein. Protein was measured by the amidoschwarz dye binding method of Schaffner and Weissmann [37] as modified by Newman et al. [38].

Na-Ca exchange assay

Some modifications of previous methods [39] for Na-Ca exchange assays were necessary to facilitate this study in membranes from tracheal smooth muscle. For Na^+-dependent Ca^{2+} uptake experiments, vesicles were preloaded with Na^+ either during formation in 160 mM Na buffer or by incubation for 30 min at 37 °C in the Na buffer. A 2 μl bead of these vesicles was placed on the side in the lower portion of a 12 × 75 mm polystyrene test tube. The bead was then mixed in with 100 μl of reaction mixture (10 μM $^{45}Ca^{2+}$ (Amersham) with 160 mM KCl or 320 mM glycerol and 20 mM Mops adjusted to pH 7.40 with Tris base) by stirring with a Fisherbrand Touch Mixer and the reaction was terminated by addition of 5.0 ml ice-cold stop solution (1 mM $LaCl_3$, 200 mM KCl, 5 mM Mops, pH adjusted to 7.40 with KOH). In experiments employing a postincubation step (see Results), the vesicles were incubated with the stop solution on ice for 3 min before collection on a glass fiber filter (Whatman GF/A). The tube was washed again with the termination solution and this wash was passed through the filter and the filter was then washed with 5 ml of

the termination solution. The filters were counted for $^{45}Ca^{2+}$ by liquid scintillation.

For Ca^{2+} efflux experiments, vesicles were loaded similarly, but with $^{45}Ca^{2+}$. Then they were diluted into Na^{+}-containing solutions and measured for loss of $^{45}Ca^{2+}$. Other modifications for specific experiments are listed in the Results section.

Results

The separation of smooth muscle cells from other tracheal cell types depends on a good tissue separation and then on an effective cell separation. The tissue separation was effected by the careful removal of the epithelium from the surface of the smooth muscle and then by the removal of the smooth muscle from the underlying adventitia. As determined by histological methods, this procedure resulted in relatively clean strips of smooth muscle with low levels of contaminating epithelium or connective tissue.

The cells freed from the tissue strips by the agitation procedure in the Tekmar homogenizer were 95% smooth muscle with less than 5% contaminating erythrocytes and fibroblasts as observed through light microscopy. The cells appeared to sustain some damage as determined by visual examination through a light microscope and by their uptake of trypan blue. However, they were sufficiently intact to require breakage by nitrogen cavitation, although relatively high pressure was necessary (1100 psi), probably because smooth muscle cells are relatively small and rigid. At this pressure with a 15 min incubation a few cells remained unbroken upon release, but greater than 97% were disrupted.

The distribution of membrane fraction marker enzymes in the various fractions generated from the separation procedure is described in Table I. These data are from one of two preparations for which marker enzymes were examined for every fraction. Comparison on a step-by-step basis revealed essentially the same fold purification for each marker throughout both preparations. Subsequent preparations have been monitored only by Na-Ca exchange activity and protein and follow the same pattern as established in Table II. From the data in Table I, it can be seen that mitochondria, as indicated by cytochrome-*c*

TABLE I

COMPARISON OF MARKER ENZYME ACTIVITIES IN MEMBRANE FRACTIONS PREPARED FROM DISRUPTED TRACHEAL SMOOTH MUSCLE CELLS

Tracheal smooth muscle cells were broken through nitrogen cavitation and the membranes were separated by differential and sucrose density gradient centrifugation. This separation procedure was monitored by enzyme activities representative of the membrane types of interest and by Na-Ca exchange activity. The specific activities are reported in this table relative to the specific activities measured in the homogenate derived from nitrogen cavitation.

Fraction	5′-Nucleotidase (sarcolemma)		NADPH–cytochrome-*c* reductase (sarcoplasmic reticulum)		Cytochrome-*c* oxidase (mitochondria)	
	spec. act. ($nmol \cdot mg^{-1} \cdot min^{-1}$)	enrichment (-fold)	spec. act. ($nmol \cdot mg^{-1} \cdot min^{-1}$)	enrichment (-fold)	spec. act. ($nmol \cdot mg^{-1} \cdot min^{-1}$)	enrichment (-fold)
Homogenate	18.5	1.00	1.79	1.00	102	1.00
$10^3 \times g$ supernatant	162	8.74	9.40	5.26	406	3.99
$10^3 \times g$ pellet	3.38	0.18	1.25	0.70	70.3	0.69
$10^4 \times g$ supernatant	172	9.31	7.69	4.30	40.7	0.40
$10^4 \times g$ pellet	85.4	4.62	6.70	3.75	782	7.67
$1.2 \cdot 10^5 \times g$ supernatant	–	–	0.32	0.18	–	–
$1.2 \cdot 10^5 \times g$ pellet	651	35.2	14.1	7.89	109	1.07
Gradient						
pellet	110	5.97	7.88	4.40	81.7	0.80
8–30% interface	2073	111	26.4	14.7	41.4	0.41
30–40% interface	1220	65.9	31.7	17.8	134	1.32
remaining supernatant	672	36.3	32.7	18.3	133	1.30

TABLE II

PURIFICATION TABLE FOR Na-Ca EXCHANGE ACTIVITY IN MEMBRANE FRACTIONS PREPARED FROM DISRUPTED TRACHEAL SMOOTH MUSCLE CELLS

Purification of the tracheal smooth muscle Na-Ca exchange activity was monitored by measurement of the specific activity and protein in each fraction.

Fraction	Net spec. act. ($pmol \cdot mg^{-1} \cdot s^{-1}$)	Total net activity ($nmol \cdot s^{-1}$)	Percent recovery from homogenate	Purification [a] (-fold)
Homogenate	5.42	4.33	100	1.00
$10^3 \times g$ supernatant	25.9	3.17	73.2	4.77
$10^3 \times g$ pellet	0.52	0.18	4.06	1.10
$10^4 \times g$ supernatant	24.1	1.94	44.7	4.43
$10^4 \times g$ pellet	12.7	0.67	15.5	2.34
$1.2 \cdot 10^5 \times g$ supernatant	0.75	0.04	0.83	0.14
$1.2 \cdot 10^5 \times g$ pellet	105.7	2.44	56.4	19.5
Gradient				
pellet	9.8	0.08	1.79	1.81
8–30% interface	329.2	0.99	22.8	60.6
30–40% interface	76.5	0.31	7.11	14.1
remaining supernatant	29.3	0.45	1.03	5.3

[a] Purification is with respect to homogenate.

oxidase, were pelleted in the $10^4 \times g$ centrifugation. While some of the marker enzyme activity for plasma membrane (5′-nucleotidase) and for endoplasmic reticulum (NADPH–cytochrome-*c* reductase) pelleted with the mitochondria, this step was very effective in removing mitochondria from the greater portion of these other two membrane fractions. Mitochondria are known to have a Na-Ca exchange activity [40] distinct from that found in plasma membrane, and for this reason the removal of mitochondria was of great importance.

Two of the differential centrifugation steps resulted in significant purification of the sarcolemmal vesicles (3–4-fold) compared to homogenate but did not significantly contribute to the separation of the major membrane components of interest from each other: the first centrifugation ($10^3 \times g$), which removed dense material such as nucleii and cellular debris; and the third centrifugation ($1.2 \cdot 10^5 \times g$), which removed soluble protein. The $1.2 \cdot 10^5 \times g$ centrifugation was performed primarily for the purpose of concentrating the membranes for application to the gradient.

In the sucrose density gradient step, most of the Na-Ca exchange activity was usually found to band in membranes at the top of the 30% level (8/30 interface), as shown in Table II. This 8/30 interface in five different preparations always had the highest sarcolemmal marker activity and always had the highest Na-Ca exchange activity. A 40% band (30/40 interface) was also included in the usual preparation procedure because some of the Na-Ca exchange activity was found here and because sarcoplasmic reticulum was at higher levels in this fraction. Thus, the 8/30 interface had the highest specific Na-Ca activity with the least sarcoplasmic reticulum. With respect to the 5′-nucleotidase activity (Table I), this fraction was enriched over 100-fold in sarcolemma when compared to the homogenate. Mitochondria were reduced to 0.41-fold of homogenate but sarcoplasmic reticulum was enriched 14.7-fold, which, while significant, was less than 15% of the sarcolemmal enrichment.

The sarcoplasmic reticulum marker NADPH–cytochrome-*c* reductase had its highest activity in the 30/40 interface fraction, although substantial activity was found in the 8/30 interface, the putative sarcolemmal fraction. Both the sarcolemmal marker, 5′-nucleotidase, and the Na-Ca exchange activity were reduced in the 30/40 interface compared to the 8/30 interface, indicating reduced proportion of sarcolemma to sarcoplasmic reticu-

lum in the 30/40 interface. However, Na-Ca exchange activity in the 30/40 interface was reduced to a greater extent than 5′-nucleotidase. This partial separation of these two activities suggests that they may reside in different sarcolemmal subfractions.

The specific activity of Na-Ca exchange was highest in the 8/30 interface fraction (329 pmol · $mg^{-1} \cdot s^{-1}$) and was over 60-fold improved compared to the homogenate (Table II). In addition, there was relatively high recovery (approx. 23%) of the exchange activity in this fraction, and thus the 8/30 fraction was utilized for all subsequent Na-Ca exchange characterization. Although the calcium uptake assay at 3 s slightly underestimates true initial velocities because of the curvature of the uptake time course (Fig. 2), these longer time points were necessary to achieve enough uptake to compensate for the large Ca^{2+} binding component of the background in the lower specific activity crude fractions prior to the gradient separation.

When using this assay to compare the fractions, the loss in each centrifugation step is only about 20% when the Na-Ca exchange activities in the supernatant and pellet are compared with the suspension prior to centrifugation. In comparing the Na-Ca exchange activity in Table II with 5′-nucleotidase in Table I, the exchanger showed greater loss in activity at each fractionation step, perhaps indicating that the exchanger is a more labile protein. Other major losses were in the $10^4 \times g$ mitochondrial pellet and at the 30/40 interface from the gradient. Total protein in the 8/30 interface was 3.0 mg compared to 800 mg in the initial homogenate.

The Na-Ca exchange uptake time course is significantly curved even at early times and comes rapidly to equilibrium after 2–3 min (Fig. 1, left panel, open triangles). Even at times above 1 s, as shown in Fig. 2 (filled squares), significant curvature is evident. Thus, in estimating initial velocities for characterizing kinetic parameters, it is imperative to perform assays at as short a time as practicable (usually 1–3 s, as indicated). In Fig. 1, the net Na^+-dependent Ca^{2+} uptake was calculated as the difference between the $^{45}Ca^{2+}$ uptake by Na^+-loaded vesicles diluted into 15 μM $^{45}Ca^{2+}$ solutions which contained either 160 mM KCl (open triangles) or 160 mM NaCl (filled triangles). The Na^+ is reported to block $^{45}Ca^{2+}$ entry via the Na-Ca exchange process in cardiac sarcolemmal vesicles by acting as a competitive inhibitor when present on the outside with $^{45}Ca^{2+}$ [39]. Thus external Na^+ should be an effective control for non-Na-Ca exchange-mediated transport.

Because of the known Ca-Ca exchange mode of the Na-Ca exchange system, it is not sufficient to demonstrate Na^+ dependence merely by preincubating vesicles in Na^+ or K^+ and then diluting them into a solution containing $^{45}Ca^{2+}$ in KCl. In the K^+-loaded vesicles, indigenous Ca^{2+} could exchange for external $^{45}Ca^{2+}$. For this reason it was necessary to incubate the vesicles in EGTA to remove internal Ca^{2+}.

In Fig. 1, experiments with vesicles preincubated in 50 μM EGTA (filled circles) for 4 h showed little change in uptakes compared to controls (open triangles). This observation, then, validates the use of EGTA for Ca^{2+} removal from these vesicles. Vesicles preloaded with 160 mM KCl without EGTA incubation showed significant $^{45}Ca^{2+}$ uptake above background. This uptake can be lowered by a 0.5 h EGTA incubation (filled squares) and reduced to near background levels (filled triangles) with a 4 h incubation. EGTA treatment of Na^+-loaded vesicles (open circles) lowered the background level in NaCl slightly, possibly due to removal of residual Ca-Ca exchange even in the presence of 160 mM Na_i^+. Further reduction of background was observed for EGTA-treated KCl-loaded vesicles diluted into NaCl (crosses). This last effect would be expected if the external Na^+ exchanges for $^{45}Ca^{2+}$ which had entered the vesicles by other means.

Thus, in these experiments with Na_i^+, K_i^+ and EGTA incubations we have demonstrated unequivocally the Na^+ dependence of $^{45}Ca^{2+}$ uptake in tracheal smooth muscle vesicles. In addition, we have shown a Ca-Ca exchange component which can be removed by EGTA incubation.

It was important to establish that the $^{45}Ca^{2+}$ taken up in Na-Ca exchange experiments actually had been internalized and not merely bound to the membrane. The Ca^{2+} ionophore A23187 is known to rapidly transport Ca^{2+} across lipid membranes. Thus when Na-loaded vesicles were preloaded by Na-Ca exchange with 20 μM $^{45}Ca^{2+}$ for 2 min and then treated with 1 ml of 2 μM

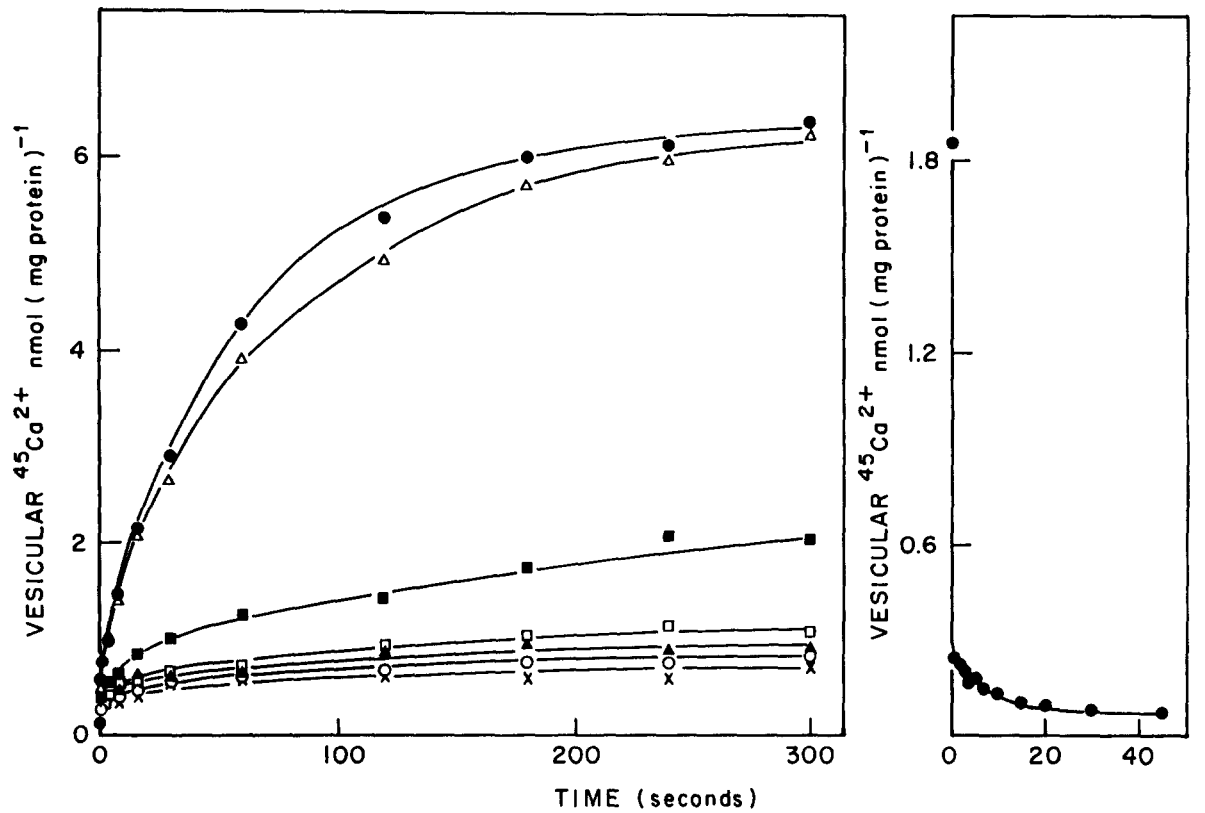

Fig. 1. Left panel. Time course for Na_i^+-dependent Ca_0^{2+} uptake. Net Na^+-dependent $^{45}Ca^{2+}$ uptake was typically measured using tracheal smooth muscle sarcolemmal vesicles which had been preincubated in 160 mM NaCl (buffered by 20 mM Mops, pH 7.40 with Tris base) for 1 h at 37 °C. These vesicles (3 μl) were then diluted into 200 μl 15 μM $^{45}Ca^{2+}$ in either 160 mM KCl (△) for uptake or 160 mM NaCl (▲) for background. Because of residual $^{45}Ca^{2+}$ uptake due to the Ca-Ca exchange mode of the Na-Ca exchanger when the vesicles were preloaded with KCl, it was necessary to treat these vesicles with EGTA by first washing with 1 mM EGTA in KCl and then incubating them at 37 °C with 50 μM EGTA in either 160 mM NaCl or KCl. Na-loaded, EGTA-treated vesicles were diluted into either KCl (●) or NaCl (○) and produced lines similar to the untreated controls. K-loaded vesicles, EGTA-treated for 0.5 h were diluted into KCl (■) showing some $^{45}Ca^{2+}$ uptake. EGTA treatment for 4 h lowered the $^{45}Ca^{2+}$ uptake to background when diluted into KCl (□) and not surprisingly reduced apparent background when diluted into NaCl (×). Right panel. Effect of the Ca^{2+} ionophore A23187 on vesicular $^{45}Ca^{2+}$ from Na-dependent Ca^{2+} uptake. Vesicles preloaded with NaCl were diluted into 15 μM $^{45}Ca^{2+}$ in KCl and allowed to transport for 2 min, at which time 1 ml 2 μM A23187 in 20 μM EGTA and 160 mM KCl was added. Vesicles were measured for $^{45}Ca^{2+}$ at the times indicated.

A23187 in 20 μM EGTA and 160 mM KCl, vesicle-associated $^{45}Ca^{2+}$ dropped to near background levels within 1 s (Fig. 1, right panel). In this experiment, then, $^{45}Ca^{2+}$ taken up by the Na-Ca exchange process was rapidly released by A23187, which indicates that the $^{45}Ca^{2+}$ was actually inside the vesicles.

Given these controls, in all other experiments reported in this paper, background was determined by dilution of the vesicles into $^{45}Ca^{2+}$ solution in 160 mM NaCl. Because the uptake was linear only for the first few seconds (Fig. 2, left panel), initial velocities were estimated from 1–3 s, as indicated in succeeding experiments.

An additional factor of importance in these measurements of Na-Ca exchange was that there appeared to be a significant level of $^{45}Ca^{2+}$ binding to the vesicles, depending upon the assay conditions. When the uptake assay was performed by diluting 3 μl 160 mM Na^+-preloaded vesicles into solution containing 15 μM $^{45}Ca^{2+}$ and either 320 mM glycerol or 160 mM KCl, $^{45}Ca^{2+}$ uptake was much lower in K^+ than in glycerol (Fig. 2, left panel). However, in a 3 min postincubation with

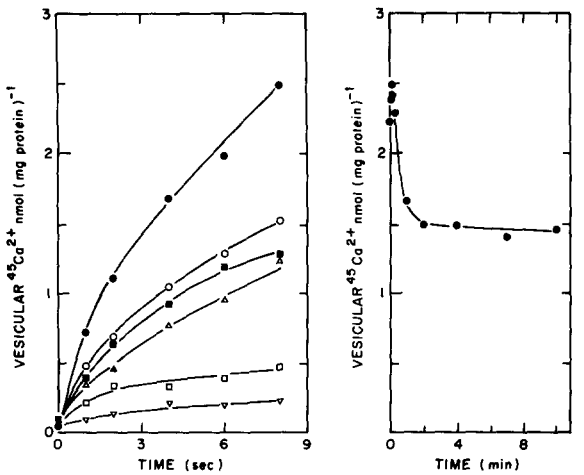

Fig. 2. Left panel. The effect of the postincubation procedure on the time course of Na-Ca exchange uptake. In experiments performed without postincubation, Na^+ (160 mM)-loaded vesicles were diluted into 320 mM glycerol (●), 160 mM KCl (○) or 160 mM NaCl (□), and at the times indicated were terminated by dilution with 4 ml cold stop solution immediately prior to filtration through glass fiber filters. For the postincubation procedure vesicles were similarly diluted into glycerol (■), KCl (△) and NaCl (▽). However after the addition of ice-cold termination solution to the reaction tube, the tubes were immediately placed in ice for a period of exactly 3 min, at which time the vesicles were collected on glass fiber filters as in the regular termination procedures. Right panel. Postincubation time course. Tracheal smooth muscle sarcolemmal vesicles (2 μl) which had been loaded with $^{45}Ca^{2+}$ were diluted with 4 ml of La^{2+} termination solution, incubated on ice, and collected by filtration at the times indicated.

stop solution (4° C), apparent uptake of $^{45}Ca^{2+}$ from the glycerol solution was considerably reduced. This 3 min time was set in an experiment where the postincubation was varied from 2 s to 10 min. There was a rapid loss of vesicle-associated $^{45}Ca^{2+}$ for up to 3 min, after which time further losses leveled off and became much slower (Fig. 2, right panel).

In this same 3 min period, vesicle-associated $^{45}Ca^{2+}$ was also reduced when the uptake was measured from KCl or from the NaCl background (Fig. 2, left panel). In the presence of these ions the losses were considerably less than were found in glycerol. It is also important to note that these losses in KCl and NaCl were of similar magnitude. Our interpretation of these data is that there is considerable nonspecific $^{45}Ca^{2+}$ binding to the vesicles in the course of these uptake experiments, and that K^+ and Na^+ can compete with Ca^{2+} for much of this binding. The postincubation of the vesicles in the stop solution reduced $^{45}Ca^{2+}$ counts. This solution contains 1 mM La^{3+} in 160 mM KCl (pH 7.40 by 20 mM Mops adjusted with Tris base) which effectively competes with Ca^{2+} binding in glycerol and the residual Ca^{2+} binding in KCl and NaCl. The La^{3+} is also known to block Ca^{2+} fluxes. Nevertheless it is important to note that even after the postincubation, experiments in K^+ always had somewhat lower apparent $^{45}Ca^{2+}$ uptake than those in glycerol. In addition, because of high $^{45}Ca^{2+}$ binding, even in K^+ solutions, to vesicles from those purification procedures which were utilized before the sucrose gradient separation, the postincubation was used for comparison of Na-Ca exchange activities in the various vesicle fractions from the purification. However, for other analyses involving the 8/30 interface vesicles diluted into only external K^+, no postincubation was performed.

Assessment of Na_0^+-dependent $^{45}Ca_i^{2+}$ efflux (Fig. 3) allowed the evaluation of exchange phenomenon in a way that obviated some potential alternative interpretations of the uptake study. Vesicles which had been washed twice with 320 mM glycerol solution and then preincubated with

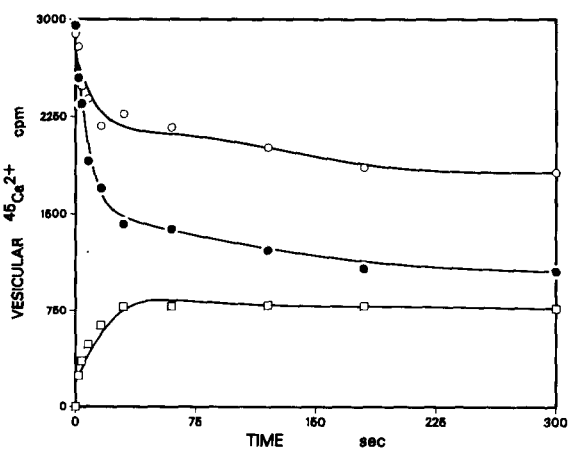

Fig. 3. Na^+-dependent Ca^{2+} efflux. Sarcolemmal vesicles (4 μl) which had been preloaded with 40 μM $^{45}Ca^{2+}$ in 160 mM KCl solution were diluted into 100 μl of either 160 mM NaCl (●) or KCl (○) containing 100 μM EGTA (to prevent backflux). Net $^{45}Ca^{2+}$ efflux (□) was determined from the subtraction of the efflux in the presence of Na^+ from the efflux in the presence of K^+.

40 μM $^{45}CaCl_2$ in glycerol solution for 2 h at 37°C were diluted into either 160 mM NaCl solution or 160 mM KCl solution. In both external solutions EGTA was present to prevent exogenous Ca^{2+} (nonradioactive) from driving $^{45}Ca^{2+}$ out via Ca-Ca exchange. The postincubation procedure was employed at the shorter time points in this experiment to prevent potential differential effects of Na^+ and K^+ in their competition for $^{45}Ca^{2+}$ binding. At longer points additional postincubation was not necessary since the presence of EGTA should have removed bound vesicular Ca^{2+}. In this case, bound $^{45}Ca^{2+}$ probably resulted from the preincubation procedure to load the vesicles with $^{45}Ca^{2+}$. Under these assay conditions Na_0^+ effectively drove $^{45}Ca_i^{2+}$ out of the vesicles and the net efflux ($^{45}Ca_i^{2+}$ into Na_0^+ subtracted from the efflux into K_0^+) time course was quite similar to the uptake time course. Because of the rapidity of the efflux and because of the multistep nature of the assay, it was not possible to determine initial velocities from single point analysis at such time as 3 s. Multiple-point v_0 determinations were possible but beyond the scope of this initial characterization.

However, for Na^+-dependent $^{45}Ca^{2+}$ uptake it was quite feasible to do initial velocity determinations based on time points at 3 s. The Michaelis plot of net $^{45}Ca^{2+}$ uptake at 3 s time points is shown in Fig. 4 with the v_0 vs. $v_0 \cdot S^{-1}$ plot of the same data shown in the inset. The K_m for Ca^{2+} derived from those points was 19.6 μM and varied only slightly from that determined in other tracheal smooth muscle vesicle preparations (10–25 μM). The K_m values determined for these vesicles were well within the range determined for cardiac tissue by the same procedures (30 μM). The maximum velocity, 0.33 nmol · s^{-1} · (mg protein)$^{-1}$ was from 10 to 50% of the activity that we were able to find in bovine cardiac sarcolemmal vesicle preparations which were determined concurrently.

External K^+ transported inside by the ionophore valinomycin will result in an inside positive potential. To determine the effect of this induced potential on Na-Ca exchange in the tracheal smooth muscle vesicles, Na^+-loaded vesicles were diluted into solutions with and without valinomycin in the presence of 15 μm $^{45}Ca^{2+}$ in 160 mM KCl or 320 mM glycerol (Fig. 5). While uptake in

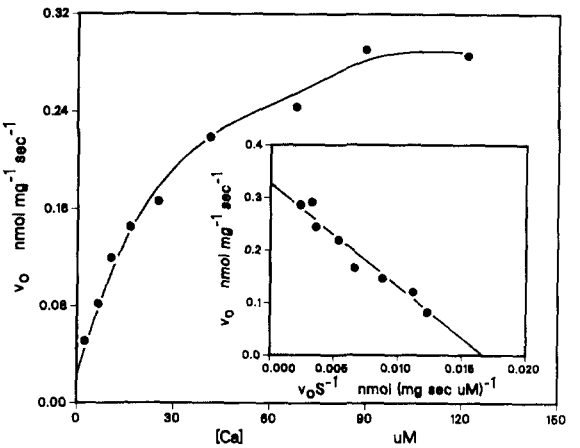

Fig. 4. Ca^{2+} dependence of Na-Ca exchange. Bovine tracheal smooth muscle sarcolemmal vesicles (3 μl) preloaded with 160 mM NaCl were diluted into 100 μl of 160 mM KCl containing the indicated levels of $^{45}Ca^{2+}$. Reactions were terminated at 3 s with no postincubation. Inset: linear transformation (v_0 vs. $v_0 \cdot S^{-1}$) of Ca^{2+} uptake data ($V_{max} = 0.33$ nmol · mg^{-1} · s^{-1}; $K_m = 19.6$ μM Ca^{2+}).

the presence of K_0^+ without valinomycin was less than that observed in glycerol, the addition of valinomycin stimulated Ca^{2+} uptake significantly

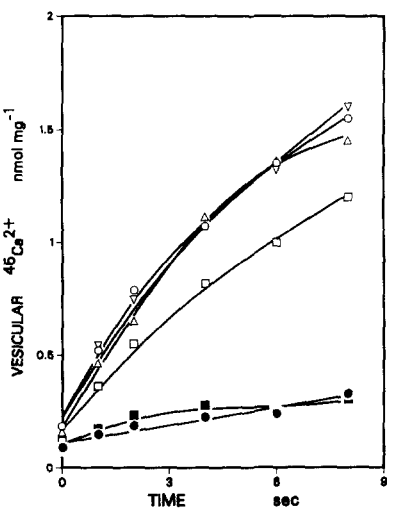

Fig. 5. Effect of valinomycin and K^+ on Na^+-dependent Ca^{2+} uptake into sarcolemmal vesicles from tracheal smooth muscle. Control time courses of Na-Ca exchange activity were determined by diluting 3 μl of 160 μM NaCl-loaded vesicles into 100 μl of 160 mM KCl (□), 320 mM glycerol (○), or 160 mM NaCl (●). The effect of 1 mM valinomycin (from a 2 mM solution in Me_2SO) was determined in these same solutions: KCl (▽), glycerol (△) and the NaCl background (■).

in the membranes assayed in K^+. In glycerol there was no significant effect of valinomycin.

These observations suggest that the tracheal smooth muscle exchanger may be electrogenic. Valiomycin added in the presence of external K^+ would cause a flow of K^+ into the vesicles resulting in a net influx of positive charges. In the presence of this inward flow of positive charge, influx of Ca^{2+} was stimulated in exchange for Na^+. Thus Na^+ must balance the flux of two positive charges from Ca^{2+} and the additional postive charges from the valinomycin-dependent K^+ influx, implying that more than two Na^+ exchange for each Ca^{2+}. This effect cannot be due to valinomycin alone since valinomycin has no effect on Na-Ca exchange in glycerol.

There are certain procedures known to modulate the activity of the exchanger in cardiac sarcolemmal vesicle preparations. Two procedures which appear to have some specificity for the exchanger and which markedly increase uptake activity are pretreatment with chymotrypsin [41] and pretreatment with a dithiothreitol and Fe^{2+} mixture [42]. Tracheal smooth muscle and cardiac vesicles were compared for relative stimulation under both of these procedures. The cardiac muscle sarcolemmal vesicles were prepared by the method of Kuwayama and Kanazawa [43] according to the modification of Slaughter et al. [32]. Vesicles from tracheal sarcolemma were prepared by a procedure similar to the method used with cardiac tissue which employed a 70 mM Tris/H_2SO_4 buffer (pH 7.4) containing 250 mM mannitol instead of the sucrose buffer described in this manuscript (see Methods). The specific activity and recovery of Na-Ca exchange activity did not differ significantly from that using the sucrose buffer.

For the chymotrypsin procedure, vesicles in 160 mM NaCl solution were pretreated with a final concentration of 0.1 mg/ml chymotrypsin for 30 min at 37 °C and the chymotrypsinization was terminated with phenylmethylsulfonyl fluoride at a final concentration of 1 mM. Vesicles were placed on ice and assayed for Na^+-dependent $^{45}Ca^{2+}$ uptake in the conventional manner. Cardiac vesicles were assayed for 1 s and smooth muscle vesicles for 3 s, at 10 μM Ca^{2+}. This treatment with chymotrypsin had negligible effects on the level of activity of the exchange system from tracheal smooth muscle (0.09 vs. 0.07 nmol $\cdot$ s^{-1} $\cdot$ (mg vesicle protein)$^{-1}$). In contrast, in heart, over 11-fold stimulation of activity at 10 μM $^{45}Ca^{2+}$ was observed (3.7 vs 0.32 nmol $\cdot$ s^{-1} $\cdot$ (mg vesicle protein)$^{-1}$).

In a similar experiment, vesicles in 160 mM NaCl solution were preincubated with a final concentration of 1 mM dithiothreitol and 1 μM $FeSO_4$ for 15 min at 37 °C and assayed immediately for 2 s uptakes. The net $^{45}Ca^{2+}$ uptake in smooth muscle vesicles showed no changes with the dithiothreitol plus Fe^{2+} treatment (0.33 vs. 0.40 nmol $\cdot$ s^{-1} $\cdot$ (mg protein)$^{-1}$) while heart vesicles were stimulated over 2-fold (1.2 vs. 0.56 nmol $\cdot$ s^{-1} $\cdot$ (mg protein)$^{-1}$). In addition, no stimulation was observed by either chymotrypsin treatment or dithiothreitol plus Fe^{2+} treatment applied to tracheal smooth muscle vesicles prepared by the original procedure in this paper.

Discussion

Two significant separations had to be accomplished to effect the preparation of sarcolemmal vesicles from tracheal smooth muscle cells and allow the study of Na-Ca exchange in these vesicles: (a) the smooth muscle cells had to be isolated from other cell types in the trachea, and (b) the sarcolemmal membranes had to be separated from other intracellular membranes. The cell separation was of paramount importance because many other cell types in surrounding tissue also depend on external Ca^{2+} for function, especially secretory cells found in the epithelium which might be expected to have Ca^{2+}-transporting systems similar to those in smooth muscle. Similarly, Ca^{2+}-transport systems might be expected to be found in other organelles of tracheal smooth muscle, the mitochondiral Na-Ca exchange being the most significant in relation to this study. However by the procedures described in this paper, a smooth muscle preparation of good purity (over 95%) and a sarcolemmal vesicle preparation greatly enriched compared to mitochondria were obtained which permitted this study of Na-Ca exchange activity.

In the vesicle subfractionation on the sucrose gradient there was a predominant partitioning of

sarcolemma into the 8/30 interface and of the sarcoplasmic reticulum into the 30/40 interface. A comparison of 5′-nucleotidase and Na-Ca exchange activities in these two gradient fractions revealed a differential between these two presumably sarcolemmal proteins. The Na-Ca exchange activity was markedly reduced in the 30/40 interface fraction to less than 25% of its activity in the 8/30 interface, while 5′-nucleotidase was only reduced by half. This observation suggests that the 8/30 fraction may contain a higher portion of a certain subfraction of the plasma membrane in which the Na-Ca exchange system resides.

The curved time courses observed in Figs. 1 and 3 for net uptake and efflux through Na-Ca exchange are most likely due to differing phenomena. In uptake, the most likely interpretation is that as internal $^{45}Ca^{2+}$ rises quickly to fill the small space inside the vesicles, back fluxes rapidly rise and become nearly equal to influx after 2 min. In the efflux experiments backflux should not have been a problem because of the EGTA in external solutions. Thus, the curvature was probably the loss of transport rate with decreasing internal free Ca^{2+} concentrations.

The high levels of vesicle-associated $^{45}Ca^{2+}$ in the presence of external Na^+ in background estimates for uptake experiments on the purification fractions before the sucrose gradient indicated that substantial amounts of $^{45}Ca^{2+}$ had been bound to these fractions. The main reason for development of the postincubation assay for smooth muscle vesicles was to reduce this high binding of external Ca^{2+}, making it possible to compare specific Na^+-Ca^{2+} exchange activities among various experiments.

In the valinomycin experiment, the implication of electrogenicity is that the tracheal smooth muscle exchanger has a stoichiometric ratio of greater than $2Na^+ : Ca^{2+}$. Such a stoichiometric ratio would mean that the exchanger would be functioning to drive Ca^{2+} out of the cells under most normal ionic concentrations and polarized conditions (see Reeves and Hale [44], Reeves [45], Eisner and Lederer [46] and Mullins [47] for complete discussion of the significance of stoichiometric ratios on the direction of flux for Na-Ca exchange). However, this idea awaits establishment of the exact stoichiometric ratio in smooth muscle, although it is now reasonably probable that the ratio in heart is $3Na^+ : Ca^{2+}$ as established by Reeves and Hale through thermodynamic perturbation methods [44].

While basal kinetics for Na-Ca exchange in both heart and smooth muscle appear similar, pretreatments with either chymotrypsin or dithiothreitol and Fe^{2+} fail to stimulate smooth muscle exchange under conditions which cause marked stimulation of cardiac Na-Ca exchange. These stimulation procedures are quite different from each other and while chymotrypsin may be somewhat less specific, the dithiothreitol and Fe^{2+} procedure could be indicative of a more significant difference in the way the Na-Ca exchange activity may be controlled in different tissues.

The role and even the existence of the Na-Ca exchanger in smooth muscle has been the subject of considerable debate. A.K. Grover, who did the pioneering work in plasma membrane isolation and in showing Na-Ca exchange activity from several smooth muscle types, assessed the significance of Na-Ca exchange compared to the sarcolemmal Ca^{2+}-ATPase activity [27]. Comparing these activities from rat myometrium [9] and guinea pig intestinal smooth muscle [11], he reports maximal initial rates of 2–4 $\mu mol \cdot g^{-1} \cdot min^{-1}$. These rates are only 20–60% of Ca^{2+}-ATPase activity in these same membranes and for this reason he states that ATP-dependent Ca^{2+} efflux is the major pathway for cellular Ca^{2+} extrusion. Grover's experiments [9,11] were performed at time points of 1 min, well after the region of linearity. It is possible that the actual Na-Ca exchange activity in those tissues would be much higher if measured at short time points. For kinetic analyses, our experiments on trachealis Na-Ca exchange were performed at short time points (3 s) to approximate true initial velocities as closely as possible, and the resulting V_{max} was 30 $\mu mol \cdot g^{-1} \cdot min^{-1}$ in the same units, or roughly 10-times greater. These data, then, would indicate a considerably larger role for the exchange system in tracheal smooth muscle, possibly to rapidly reduce tension by pumping Ca^{2+} out of the cell. However, the high exchange activity by itself, coupled with the physiological evidence for utilization of external Ca^{2+} in response to K^+, histamine, leukotrienes and acetylcholine [17–26], implies that

an important component of tracheal smooth muscle contraction and relaxation may be modulated through movements of Ca^{2+} across the sarcolemma.

In summary, the studies described in this paper have lead to the identification of a Na-Ca exchange process in sarcolemmal vesicles prepared from tracheal smooth muscle. This activity is present in significant amounts and may have a role in the movement of Ca^{2+} out of the smooth muscle cell during bronchodilation. The tracheal exchanger has different properties from the cardiac exchanger, suggesting that regulation of this activity in the two cell types is under differential control. Thus knowledge of the physiological mechanisms by which the exchanger in tracheal smooth muscle may be activated may have application in the development of unique bronchodilators for the treatment of pulmonary dysfunctions.

Acknowledgements

The authors wish to thank Dr. John P. Reeves for providing the bovine cardiac sarcolemmal vesicles. We also would like to thank Dr. David L. Feldman for examination of the tissue cell during the cell preparation procedure. In addition we would like to thank Dr. Carole Bailey for providing the cardiac vesicle stimulation procedures.

References

1 Kamm, K.E. and Stull, J.T. (1985) Annu. Rev. Pharmacol. Toxicol. 25, 593–620
2 Philipson, K.D. (1985) Annu. Rev. Physiol. 47, 561–571
3 Reeves, J.P. (1985) Curr. Topics Membranes Transport 25, 77–127
4 Trumble, W.R., Sutko, J.L. and Reeves, J.P. (1981) J. Biol. Chem. 256, 7101–7104
5 Carafoli, E. and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279–301
6 Ikemoto, N. (1982) Annu. Rev. Physiol. 44, 297–317
7 Inesi, G. (1985) Annu. Rev. Physiol. 47, 573–601
8 Grover, A.K., Kwan, C.Y. and Daniel, E.E. (1981) Am. J. Physiol. 240, C175–C182
9 Grover, A.K., Kwan, C.Y., Rangachari, P.K. and Daniel, E.E. (1983) Am. J. Physiol. 244, C158–C165
10 Aickin, C.C., Brading, A.F. and Burdyga, Th.V. (1984) J. Physiol. 347, 411–430
11 Morel, N. and Godfraind, T. (1984) Biochem. J. 218, 421–427
12 Matlib, M.A., Schwartz, A. and Yamori, Y. (1985) Am. J. Physiol. 249, C166–C172
13 Popescu, L.M. and Ignat, P. (1983) Cell Calcium 4, 219–235
14 Verbist, J., Wuytack, F., DeShutter, G., Raeymaekers, L. and Casteels, R. (1984) Cell Calcium 5, 253–263
15 Wuytack, F., Raeymaekers, L., Verbist, J., DeSmedt, H. and Casteels, R. (1984) Biochem. J. 224, 445–451
16 Grover, A.K. (1985) Cell Calcium 6, 227–236
17 Barbero, L.J., Anderson, W.H. and Weiss, E.B. (1985) in Bronchial Asthma, Mechanisms and Therapeutics (Weiss, E.B., Segal, M.S. and Stein, M., eds.) pp. 776–791, Little, Brown and Company, Boston
18 Barnes, P.J. (1983) Thorax 38, 481–485
19 Middleton, E., Jr. (1983) Eur. J. Respir. Dis. 64 (Suppl. 128) 123–132
20 Triggle, D.J. (1983) Allergy 38, 1–9
21 Vanhoutte, P.M., Rimole, T.J. and Rooke, T.W. (1984) in Advances in Cyclic Nucleotide and Protein Phosphylation Research, Vol. 17 (Greenwood, P., et al., eds.) pp. 596–573, Raven Press, New York
22 Findlay, S.R., Leary, T.M., Parker, C.W., Bloomquist, E.I. and Sheid, C.R. (1982) Am. J. Physiol. 243, C113–C139
23 Weiss, E.B. and Mullick, P.C. (1983) Prostaglandins Leukotrienes Med. 12, 53–66
24 Jones, T.R., Denis, D. and Comptois, P. (1984) Prostaglandins 27, 939–959
25 Sasaki, T., Takagi, K., Satuke, T., Sugiyama, S. and Ozawa, T. (1984) Lung 162, 369–380
26 Anderson, W.H., Krzanowski, J.J., Polson, J.B. and Szentivanyi, A. (1983) Naunyn-Schmiedeberg's Arch. Pharmacol. 322, 72–77
27 Grover, A.K. (1984) INSERM 124, 235–244
28 M.A., Crankshaw, J., Garfield, R.E., Crankshaw, D.J., Kwan, C.-Y., Branda, L.A. and Daniel, E.E. (1979) J. Biol. Chem. 254, 1834–1840
29 Grover, A.K., Kwan, C.Y., Crankshaw, J., Crankshaw, D.J., Garfield, R.E. and Daniel, E.E. (1980) Am. J. Physiol. 239, C66–C74
30 Kwan, C.Y., Garfield, R. and Daniel, E.E. (1979) J. Mol. Cell. Cardiol. 11, 639–659
31 Grover, A.K., Kannan, M.S. and Daniel, E.E. (1980) Cell Calcium 1, 135–146
32 Slaughter, R.S., Sutko, J.L. and Reeves, J.P. (1983) J. Biol. Chem. 258, 3183–3190
33 Bodansky, O. and Schwartz, M.K. (1963) J. Biol. Chem. 238, 3420–3427
34 Vandermeers, A., Vandermeers-Piret, M.C., Rathé, J., Katzner, R., Delfruge, A. and Chrostophe, J. (1977) Eur. J. Biochem. 81, 379–386
35 Pederson, T.C., Buege, J.A. and Aust, S.D. (1973) J. Biol. Chem. 248, 7134–7141
36 Cooperstein, S.J. and Lazarow, A. (1951) J. Biol. Chem. 189, 665–670
37 Schaffner, W. and Weissmann, C. (1973) Anal. Biochem. 56, 502–514
38 Newman, M.J., Foster, D.L., Wilson, T.H. and Kaback, H.R. (1982) J. Biol. Chem. 256, 11804–11808
39 Reeves, J.P. and Sutko, J.L. (1983) J. Biol. Chem. 258, 3178–3182
40 Nicolls, D. and Akerman, K. (1982) Biochim. Biophys. Acta 683, 57–88

41 Philipson, K.D. and Nishimoto, A.Y. (1982) Am. J. Physiol. 243, C191–C195
42 Reeves, J.P., Bailey, C.A. and Hale, C.C. (1986) J. Biol. Chem. 261, 4948–4955
43 Kuwayama, H. and Kanazawa, T. (1982) J. Biochem. 91, 1419–1426
44 Reeves, J.P. and Hale, C.C. (1984) J. Biol. Chem. 259, 7733–7734
45 Reeves, J.P. (1985) in Pathobiology of Cardiovascular Injury (Stone, H.L. and Weglicki, W.B., eds.), pp. 232–244, Martinus Nijhoff, Boston
46 Eisner, D.A. and Lederer, W.J. (1985) Am. J. Physiol. 248, C189–C202
47 Mullins, L.J. (1979) Am. J. Physiol. 236, C103–C110