Registry No. ATPase, 9000-83-3; Bz_2ATP, 105638-39-9; ATP, 56-65-5; L-Ser, 56-45-1.

References

Admon, A., & Hammes, G. G. (1987) *Biochemistry 26*, 3193–3197.

Baldwin, J. E., Bhatnagar, A. K., & Harper, R. W. (1970) *Chem. Commun.*, 659–661.

Breslow, R., Baldwin, S., Flechtner, T., Kalicky, P., Liu, S., & Washburn, W. (1973) *J. Am. Chem. Soc. 95*, 3251–3262.

Cremo, C. R., & Yount, R. G. (1987) *Biochemistry 26*, 7524–7534.

Grammer, J. C., Czarnecki, J. J., & Yount, R. G. (1985) *Biophys. J. 47*, 306a.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.

Hammer, J. A., III, Bowers, B., Paterson, B. M., & Korn, E. D. (1987) *J. Cell Biol. 105*, 913–925.

Johnson, N. D., Hunkapillar, M. W., & Hood, L. E. (1979) *Anal. Biochem. 100*, 335–338.

Karn, J., Brenner, S., & Barnett, L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4253–4257.

Leszyk, J., Collins, J. H., Leavis, P. C., & Tao, T. (1987) *Biochemistry 26*, 7042–7047.

Mahmood, R., & Yount, R. G. (1984) *J. Biol. Chem. 259*, 12956–12959.

Mahmood, R., Cremo, C., Nakamaye, K., & Yount, R. G. (1987) *J. Biol. Chem. 262*, 14479–14486.

Molina, M. I., Kropp, K. E., Gulick, J., & Robbins, J. (1987) *J. Biol. Chem. 262*, 6478–6488.

Nakamaye, K. L., & Yount, R. G. (1985) *J. Labelled Compd. Radiopharm. 22*, 607–613.

Nakamaye, K. L., Wells, J. A., Bridenbaugh, R. L., Okamoto, Y., & Yount, R. G. (1985) *Biochemistry 24*, 5226–5235.

Okamoto, Y., & Yount, R. G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1575–1579.

Okamoto, Y., Sekine, T., Grammer, J., & Yount, R. G. (1986) *Nature 324*, 78–80.

Rayment, I., & Winkelmann, D. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4378–4382.

Sperling, J., & Elad, D. (1971a) *J. Am. Chem. Soc. 93*, 967–971.

Sperling, J., & Elad, D. (1971b) *J. Am. Chem. Soc. 93*, 3839–3840.

Strehler, E. E., Strehler-Page, M., Perriard, J., Periasamy, M., & Nadel-Girard, B. (1986) *J. Mol. Biol. 90*, 291–317.

Warrick, H. M., De Lozanne, A., Leinwand, L. A., & Spudich, J. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9433–9437.

Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.

Wells, J. A., & Yount, R. G. (1982) *Methods Enzymol. 95*, 93–116.

Williams, N., & Coleman, P. (1982) *J. Biol. Chem. 257*, 2834–2841.

Williams, N., Ackerman, S. H., & Coleman, P. S. (1986) *Methods Enzymol. 126*, 667–682.

High Levels of Sodium–Calcium Exchange in Vascular Smooth Muscle Sarcolemmal Membrane Vesicles

Robert S. Slaughter,* Judith L. Shevell, John P. Felix, Maria L. Garcia, and Gregory J. Kaczorowski

Department of Membrane Biochemistry and Biophysics, Merck Institute for Therapeutic Research, P.O. Box 2000, Rahway, New Jersey 07065

Received August 15, 1988; Revised Manuscript Received January 27, 1989

ABSTRACT: Membrane vesicles which exhibit high levels of Na_i-dependent Ca^{2+} uptake have been prepared from either porcine or bovine aortic smooth muscle. These membranes are identified as being of sarcolemmal origin by enrichment of marker activities associated with the sarcolemma (e.g., binding of the ligands PN 200–110, iodocyanopindolol, and ouabain). The V_{max} of Na–Ca exchange in the two aortic sarcolemmal preparations [0.5–3.5 nmol s^{-1} (mg of protein)$^{-1}$] is significantly higher than that previously reported with membrane preparations derived from visceral and vascular smooth muscle and compares favorably with maximal values recorded in cardiac sarcolemmal membrane vesicles [5–20 $nmol^{-1}$ s^{-1} (mg of protein)$^{-1}$] under identical experimental conditions. The K_m of Ca^{2+} (15 ± 5 μM) and the K_m of Na^+ (15 ± 7 mM) are similar values as determined in heart. Aortic and cardiac Na–Ca exchange activities are equivalent in their sensitivity to inhibition by La^{3+} and two known classes of mechanism-based organic blockers of transport activity (i.e., amiloride analogues and bepridil-like agents). Both also display electrogenic behavior. However, Li^+, K^+, and choline all inhibit the smooth muscle transporter with markedly greater potency than found in heart, and intravesicular Ca^{2+} does not affect transport activity in smooth muscle membranes as it does in the cardiac system. When maximal transport velocities are compared, aortic membrane vesicles have 3–6-fold higher Na–Ca exchange than sarcolemmal Ca^{2+}-ATPase Ca^{2+} transporting capacities. The ratio of these activities and the specific activity of Na–Ca exchange in this tissue suggest that Na–Ca exchange is a major pathway for mediating sarcolemmal Ca^{2+} flux in vascular smooth muscle.

Tension development in vascular smooth muscle under conditions which implicate a Na–Ca exchange mechanism was originally reported by Bohr et al. (1958) and Reuter et al. (1973). Subsequent tissue-based contracture experiments proved equivocal because, while evidence continued to build in support of the presence of Na–Ca exchange (Ozaki & Urakawa, 1979, 1981), many experiments produced data inconsistent with a significant role for this process in controlling smooth muscle contractility (Mulvany et al., 1984; Mulvany, 1984; Aalkjaer & Mulvany, 1985). In addition, purified

* To whom correspondence should be addressed.

0006-2960/89/0428-3995$01.50/0

membrane vesicle preparations derived from other types of smooth muscle displayed transport levels that were quite low, especially in comparison with the sarcolemmal Ca^{2+}-ATPase (Grover et al., 1983; Morel & Godfraind, 1984). Many reviews (Brading & Lategan, 1985; Casteels et al., 1985; Daniel, 1985; Mulvany, 1985) have alluded to data obtained with these vesicle preparations to assess the level and significance of Na–Ca exchange in smooth muscle in general and, by implication, in vascular smooth muscle as well.

Recent reports of experiments with arterial rings have more consistently demonstrated modulation of tension development by a Na–Ca exchange mechanism (Blaustein et al., 1986; Johansson & Hellstrand, 1987; Ashida & Blaustein, 1987; Ashida et al., 1988). These data, together with Ca^{2+} flux measurements in cultured cells (Smith et al., 1987; Smith & Smith, 1987; Nabel et al., 1988) and in sarcolemmal membranes prepared from vascular smooth muscle (Matlib, 1988; Matlib & Reeves, 1988; Kahn et al., 1988), have unequivocally established the presence of Na–Ca exchange in this tissue. In addition, newly described membrane preparations derived from mesenteric artery (Matlib, 1988; Matlib & Reeves, 1988) and trachea (Slaughter et al., 1987a) have exhibited significant Na–Ca exchange activity. However, levels of transport in these preparations are still 10–50-fold lower than the activity of Na–Ca exchange that was inferred indirectly from measurements of Ca fluxes in aortic cells (Smith et al., 1987; Smith & Smith, 1987).

Na–Ca exchange has been implicated in the pathophysiology of essential hypertension (Blaustein, 1977). For this reason, it is important to establish the actual level of transport activity in sarcolemmal membranes from a vascular smooth muscle source. To this end, purified sarcolemmal membrane vesicles were prepared from either bovine or porcine aorta, and transport activity was characterized. Aortic Na–Ca exchange possesses many of the same properties as noted for the cardiac transport system. In addition, the specific activity of Na–Ca exchange in aortic vesicles is many times greater than found previously in other smooth muscle membrane preparations, including those prepared from vascular tissue. Moreover, this transport system possesses 3–6-fold greater maximal Ca^{2+} transport capacity than the sarcolemmal Ca^{2+}-ATPase, indicating that Na–Ca exchange is a major Ca^{2+} transport system in vascular smooth muscle. A preliminary report of this work has been presented in abstract form (Slaughter et al., 1987b).

Experimental Procedures

Preparation of Sarcolemmal Membranes. Thoracic aortas including the arch were obtained from either 20–30-kg pigs or 150-kg calves and placed in 3-(*N*-morpholino)propanesulfonic acid (MOPS)[1]-modified Tyrodes solution containing 155 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 1 g/L glucose, and 5 mM MOPS–Tris, pH 7.4. The procedure for preparing sarcolemmal membranes is similar to that previously described for porcine aortic tissue (Nishimura et al., 1986) but was further modified by using procedures described for trachea (Slaughter et al., 1987a) and heart (Slaughter et al., 1983). After the endothelium was scraped away, the medial smooth muscle layer was peeled away from the connective tissue. The muscle (400 g) was then minced, washed with an 8% sucrose solution (80 g/L sucrose and 20 mM MOPS–Tris, pH 7.4), and split into two parts, each of which was resuspended in 800 mL of 8% sucrose solution. The suspensions were homogenized 3 times with a Brinkmann Polytron (setting of 6) for 30 s, with 60-s intervals between homogenizations. The homogenate was filtered through one layer of cheesecloth, remaining tissue was resuspended in 8% sucrose solution, and the homogenization procedure was repeated until the filtrates began to clear (usually after three to four cycles). The filtered homogenate was subjected to centrifugation at 16000*g* for 20 min. The membranes remaining in the supernatant were then collected by centrifugation at 130000*g* for 60 min. This pellet was resuspended in an 8% sucrose solution, layered over equal volumes of 33% and 40% sucrose, and subjected to centrifugation at 160000*g* for 90 min. The membranes at the interfaces between 8 and 33% sucrose (F1) and between 33 and 40% sucrose (F2) were harvested, diluted 3-fold with 160 mM NaCl in 20 mM MOPS–Tris, pH 7.4, and collected by centrifugation at 160000*g* for 60 min. The two fractions were resuspended separately in NaCl solution, rapidly frozen in liquid nitrogen, and stored at −80 °C. Purified porcine cardiac sarcolemmal membrane vesicles were prepared as previously described (Slaughter et al., 1983). Membrane protein was determined by an amido-black dye binding assay (Schaffner & Weissmann, 1973), as modified by Newman et al. (1982), using bovine serum albumin as a standard.

Na–Ca Exchange Assay. To measure Na_i-dependent $^{45}Ca^{2+}$ uptake, purified sarcolemmal membrane vesicles were equilibrated with Na^+ by preincubation in 160 mM NaCl and 20 mM MOPS–Tris, pH 7.4, for 20 min at 37 °C. For Na_o-dependent $^{45}Ca^{2+}$ efflux studies, vesicles were preloaded with $^{45}Ca^{2+}$, either by preincubation at 0 °C for 4 h or by preloading using Na–Ca exchange-mediated Ca^{2+} uptake for 2 min, after which time the efflux reaction was initiated. All transport determinations were made at 37 °C. Other aspects of the Ca^{2+} uptake and efflux measurements were as previously described (Slaughter et al., 1988). However, addition of the ice-cold quench solution (1 mM EGTA, 160 mM KCl, and 20 mM MOPS–Tris, pH 7.4) in both uptake and efflux experiments was controlled by a timing device[2] which permitted data to be collected in a reproducible fashion at times as rapid as 250 ms.

ATP-Dependent Ca^{2+} Transport. ATP-dependent Ca^{2+} transport activity was measured by monitoring $^{45}Ca^{2+}$ uptake in sarcolemmal vesicles which had been preloaded with 160 mM KCl and 20 mM MOPS–Tris, pH 7.4, and pretreated with 50 μM EGTA for 6 h at 0 °C to remove contaminating intravesicular Ca^{2+}. Other conditions employed in this assay have previously been described (Barros & Kaczorowski, 1984). As in the Na–Ca exchange assay, this measurement was initiated by placing 2 μL of a membrane suspension on the side of a polystyrene 12 × 75 mm test tube and then mixing the vesicles into 200 μL of the reaction mixture. Subsequent procedures were identical with those employed for investigating the Na–Ca exchange reaction.

Evaluation of Plasma Membrane Marker Activities. Binding activities of iodocyanopindolol and ouabain were measured as previously described (Garcia et al., 1986). PN 200–110 binding was measured in membrane vesicles (20–40 μg) which were incubated with increasing concentrations of [^{3}H]PN 200–110 in a medium consisting of 10 μM $CaCl_2$, 10 μM $MgCl_2$, 0.2% bovine serum albumin, and 0.2% bacitracin

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[2] G. Kath and R. S. Slaughter (unpublished results).

Table I: Correlation of Na–Ca Exchange Activity with Sarcolemmal Membrane Markers in Aortic Membrane Fractions Isolated by Sucrose Density Gradient Centrifugation[a]

fraction	Na–Ca exchange act. [nmol·(mg of protein)$^{-1}$·s^{-1}]	PN 200–110 binding (pmol·mg of protein^{-1})	ouabain binding (pmol·mg of protein^{-1})	iodocyanopindolol binding (fmol·mg of protein^{-1})
applied sample	0.025	0.061	0.09	0.98
20/30% interface, F1	0.358	0.458	1.49	16.6
30/40% interface, F2	0.236	0.212	1.87	11.6
pellet	0.007	0.005	0.18	1.33

[a] Na–Ca exchange activity was determined at K_m Ca^{2+} concentration (15 μM). Binding of PN 200–110, ouabain, and iodocyanopindolol was performed by using 100 pM, 50 nM, and 74 pM ligand, respectively.

in 50 mM Tris-HCl, pH 7.4 (total reaction volume 2 mL). Incubations were carried out at room temperature until equilibrium was achieved (4 h). Nonspecific binding was determined in the presence of 1 μM nonradiolabeled PN 200–110. Samples were diluted with 4 mL of ice-cold 10 mM HEPES–Tris, pH 7.4, collected under reduced pressure by filtration onto GF/C glass fiber filters that had been presoaked in 0.3% poly(ethylenimine), and washed twice with the same solution.

Chemicals. [^{125}I]Iodocyanopindolol, [^{3}H]PN 200–110, and [^{3}H]ouabain were purchased from New England Nuclear, while $^{45}CaCl_2$ was bought from Amersham. The salts KCl, $LaCl_3$, and LiCl used in the inhibition experiments were all "puratonic grade" supplied by Alpha Products (Danvers, MA). Dichlorobenzamil was obtained from the Merck sample collection, while bepridil was a generous gift of McNeil Pharmaceuticals. All other reagents were supplied by commercial sources and were of the highest purity available.

Results

Membrane Characterization. To facilitate the study of Na–Ca exchange in vascular smooth muscle, sarcolemmal membranes have been purified from either porcine or bovine aortic smooth muscle strips. Membrane preparations with the highest transport activities are obtained from younger animals (20–30-kg pigs or 150-kg calves), when compared with preparations made from older, larger animals. The biochemical properties of transport activities present in tissues from both species are identical, and maximal levels of activity are found in each case in the same sucrose density gradient fraction (F1).

Several sarcolemmal membrane markers [i.e., binding of PN 200–110, ouabain, and iodocyanopindolol, representing interactions with L-type Ca^{2+} channels, (Na^+, K^+)-ATPase, and β-receptors, respectively] have been used to characterize membrane fractions resulting from sucrose density gradient centrifugation. Results of typical determinations made with a porcine aortic sarcolemmal membrane preparation are shown in Table I. The F1 membrane fraction is highest in sarcolemmal content as demonstrated by the binding profiles of PN 200–110 and iodocyanopindolol, while the F2 fraction has somewhat lower binding activities for both ligands. However, levels of ouabain binding are slightly higher in fraction F2. Purification levels of marker activities vary from 8- to 16-fold in F1 as compared with the applied sample, and there is little binding detectable in the pellet generated by the gradient. In F1, the maximal site density for PN 200–110 from a Scatchard analysis of ligand binding is 0.87 pmol/mg (data not shown), approximately half the level previously reported in purified porcine cardiac sarcolemmal vesicles (Garcia et al., 1986). This value is also much higher than levels reported for nitrendipine binding in various smooth muscle sarcolemmal membrane preparations (Luchowski et al., 1984), about twice

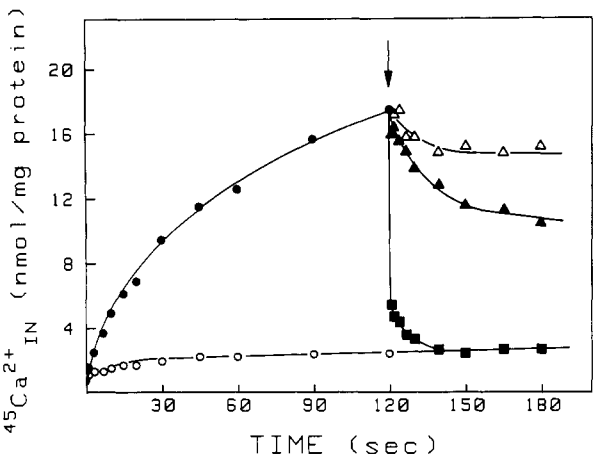

FIGURE 1: Na–Ca exchange in sarcolemmal membrane vesicles derived from bovine aortic smooth muscle. Aliquots (2 μL) of vesicles equilibrated in 160 mM NaCl were diluted 100-fold into either 160 mM KCl (●) or 160 mM NaCl (○) media containing 15 μM $^{45}CaCl_2$ at 37 °C. At the time indicated by the arrow, aliquots of the reaction mixture were diluted into 1 mL of either 160 mM KCl and 100 μM EGTA (△); 50 mM NaCl, 110 mM KCl, and 100 μM EGTA (▲); or 160 mM KCl, 2 μM A23187, and 100 μM EGTA (■) at 37 °C. All solutions were buffered with 20 mM MOPS–Tris, pH 7.4. Uptake and efflux reactions were terminated at indicated times as described under Experimental Procedures.

that found for dihydropyridine binding in both rat aortic membranes (0.50 pmol/mg; Wibo & Godfraind, 1984) and rat fundus plasma membranes (0.43 pmol/mg; Grover et al., 1984), and somewhat higher than the level of receptors present in rat myometrium plasma membranes (0.72 pmol/mg; Grover et al., 1984).

Na–Ca exchange activity, in general, parallels the distribution of the three ligands in the various membrane fractions, with a 12-fold increase in F1 over applied sample and very low activity in the pellet. In addition, the Ca^{2+} entry blocker diltiazem, which has been shown to be an effective inhibitor of mitochondrial Na–Ca exchange (Chiesi et al., 1987), has no effect on transport activity in these membranes at concentrations up to 100 μM, indicating that vesicles possessing Na–Ca exchange activity are not of intracellular origin. Since ligand binding profiles, coupled with the distribution of Na–Ca exchange activity, define F1 as the fraction most enriched in sarcolemmal membrane markers, and localize Na-dependent Ca^{2+} transport primarily to that fraction, F1 has been used for all subsequent characterization of the transporter. This preparation procedure, then, produces a highly purified membrane fraction suitable for study of many different vascular smooth muscle sarcolemmal systems.

Na–Ca Exchange Characteristics. Sarcolemmal membrane vesicles from aorta catalyze Na_i-dependent $^{45}Ca^{2+}$ uptake, well separated from nonmediated background accumulation of Ca^{2+}, as shown by the time course of Figure 1. Uptake into

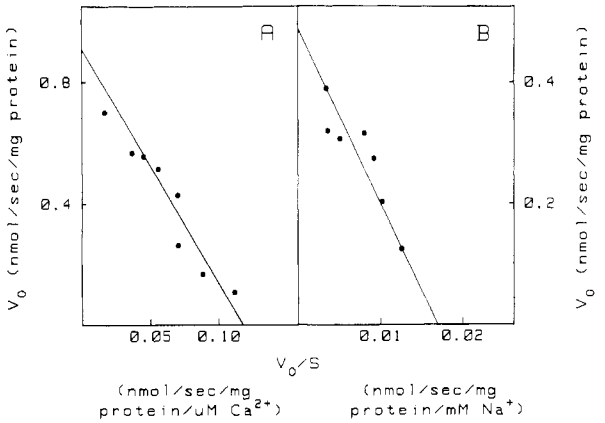

FIGURE 2: Kinetic analysis of Na–Ca exchange in sarcolemmal membrane vesicles derived from bovine aortic smooth muscle. (A) Na_i-dependent $^{45}Ca^{2+}$ uptake. Aliquots (2 μL) of membrane vesicles equilibrated in 160 mM NaCl were diluted 100-fold into 160 mM KCl media containing increasing concentrations of $^{45}CaCl_2$ at 37 °C. Reactions were terminated after 0.5 s as described under Experimental Procedures. Transport activity was determined from differences in $^{45}Ca^{2+}$ uptake with and without an outwardly directed Na^+ gradient present. Results are presented in an Eadie–Hofstee representation. (B) Na_o-dependent $^{45}Ca^{2+}$ efflux. Aliquots (2 μL) of membrane vesicles equilibrated in 160 mM NaCl were diluted 100-fold into 160 mM KCl media containing 75 μM $^{45}CaCl_2$ at 37 °C. At the end of a 2-min incubation period, samples were diluted into 1 mL of media containing different concentrations of NaCl. The osmolarity of the dilution medium was kept constant at 320 mOsm by balancing with KCl, and the medium was buffered with 20 mM MOPS–Tris and 100 μM EGTA, pH 7.4. The reaction was terminated after 2 s. Transport activity was determined from differences in Ca^{2+} efflux without and with Na^+ present in the dilution media. Results are presented in an Eadie–Hofstee representation.

Na^+-loaded vesicles from a K^+ medium at a K_m concentration of $^{45}Ca^{2+}$ (15 μM) is rapid, deviates from linearity after a few seconds, and nears equilibrium at 2 min. In the absence of a Na^+ gradient (i.e., with 160 mM NaCl present on both sides of the membrane), there is only a low background level of $^{45}Ca^{2+}$ accumulation. If vesicles are loaded with $^{45}Ca^{2+}$ via Na–Ca exchange and then exposed to the Ca^{2+} ionophore A23187, $^{45}Ca^{2+}$ is released to background binding levels within 3 s (Figure 1, arrow and squares). This finding confirms that $^{45}Ca^{2+}$, which had become associated with membranes during the transport reaction, is actually internalized into the vesicles. Reversibility of the Na–Ca exchange reaction is shown by experiments in which membranes are first loaded with $^{45}Ca^{2+}$ for 2 min via Na–Ca exchange and then diluted into a solution containing 50 mM NaCl, with 100 μM EGTA present to trap released $^{45}Ca^{2+}$ (Figure 1, arrow and closed triangles). Under these conditions, $^{45}Ca^{2+}$ is rapidly lost from vesicles. In control experiments where $^{45}Ca^{2+}$-loaded membranes are diluted into (K^+ plus EGTA)-containing medium (Figure 1, arrow and open triangles), only a small amount of vesicle-associated $^{45}Ca^{2+}$ is lost. These data indicate that vascular smooth muscle Na–Ca exchange is a bidirectional process, also a characteristic of the cardiac Na–Ca exchange reaction.

It is important to note that due to the curvature of the time course for uptake illustrated in Figure 1, measurements made after the first few seconds will grossly underestimate initial transport velocities. Consequently, K_m and V_{max} values calculated under noninitial velocity conditions could be inaccurate and misleading. To optimize the determination of these kinetic parameters in aortic sarcolemmal vesicles, Ca^{2+} uptake was measured at 0.5 s (Figure 2A) using a mixing-activated timing device.[2] The K_m of Ca^{2+} for influx as illustrated by the Eadie–Hofstee plot in Figure 2A is 8 μM. Average K_m values from eight different preparations are 15 ± 5 μM. Maximal transport velocities of these preparations range from 0.5 to 3.5 nmol s^{-1} (mg of protein)$^{-1}$, which are values from 10 to 100 times greater than earlier estimates made with purified membrane vesicle preparations from other smooth muscle sources. These previous reports of Na–Ca exchange activity determined kinetic parameters at time points as long as 60 s (Grover et al., 1983) or 3 min (Morel & Godfraind, 1984; Matlib et al., 1985), and, as discussed above, kinetic parameters are likely to be underestimated. Recent measurements made with preparations derived from trachea (Slaughter et al., 1987a) and mesenteric artery (Kahn et al., 1988) are of a greater magnitude than these previous determinations, but are still 3–10-fold lower in maximal activity than values reported in this study. Interestingly, when the maximal velocity of Na–Ca exchange in purified porcine cardiac sarcolemmal membrane vesicles is determined under these experimental conditions, resulting values [5–20 nmol s^{-1} (mg of protein)$^{-1}$] are only 5–10-fold higher than V_{max} values in aorta. Similarity in V_{max} values in these two preparations is even more apparent when it is considered that extravesicular K^+ is a much better inhibitor of the aortic than the cardiac transport reaction (see below). The K_m of Na^+ was determined at a 2-s time point in vesicles which had been preloaded with 70 μM $^{45}Ca^{2+}$ in 160 mM KCl and then diluted into solutions with 100 μM EGTA and total concentrations of KCl and NaCl present equal to 160 mM. The K_m of Na^+ from the Eadie–Hofstee plot of Figure 2B is 22 mM. Typical K_m values average 15 ± 7 mM in eight different membrane preparations. The K_m values for both Ca^{2+} and Na^+ are similar in magnitude to those previously determined for these substrates in the bovine (Slaughter et al., 1983) and porcine (Slaughter et al., 1988) cardiac transport reactions.

It is noteworthy that a large portion of Ca^{2+} released from internal stores of cultured rat aortic cells by angiotensin II can be removed from the cell by operation of Na–Ca exchange, which appears to be a major flux pathway in these cells (Smith et al., 1987; Smith & Smith, 1987; Nabel et al., 1988). By rough estimate of the proportion of plasma membrane protein comprising these cells, it has been speculated that Na–Ca exchange maximal velocities must be in the range of 240–600 nmol of Ca^{2+} transported min^{-1} (mg of plasma membrane protein)$^{-1}$. The most active sarcolemmal preparations from cow and pig that have been prepared in this study are over 200 nmol of Ca^{2+} transported min^{-1} (mg of protein)$^{-1}$. Thus, by two different procedures, similar estimates can be made for Na–Ca exchange activity in smooth muscle from aorta, despite differences in the species under investigation.

Comparison of Na–Ca Exchange and Sarcolemmal Ca^{2+}-ATPase Transport Activities. Ca^{2+}-ATPase is the only other plasmalemmal Ca^{2+} extrusion mechanism present in the cell. Because previous estimates of Na–Ca exchange activity in various types of smooth muscle have been quite low, it has been argued that Ca^{2+}-ATPase is the major, if not the only, Ca^{2+} efflux mechanism operative in these tissues. However, because of the high levels of Na–Ca exchange in the present study, a direct comparison of Na–Ca exchange and Ca^{2+}-ATPase transporting capacities is mandated in the same membrane preparation.

For the ATP-dependent Ca^{2+} transport assays, membranes have been pretreated with 100 μM EGTA for 6 h at 0 °C to remove endogenous Ca^{2+}, so that the Ca–Ca exchange mode of the Na–Ca exchange transporter would not contribute to non-ATP-mediated background accumulation of Ca^{2+}. Since membrane vesicles preloaded with 2 mM potassium oxalate do not display enhanced ATP-dependent Ca^{2+} uptake, a

characteristic of the sarcoplasmic reticulum, and there is no binding of the sarcoplasmic reticulum marker ryanodine to these membranes (P. K. S. Siegl, personal communication), this preparation does not appear to be significantly contaminated with membranes derived from that organelle, and ATP-dependent Ca^{2+} uptake must be due solely to sarcolemmal Ca^{2+}-ATPase action. ATP-dependent Ca^{2+} uptake has been measured at 1 min under initial velocity conditions, and transport activity determined in this fashion was compared with the activity of Na–Ca exchange in three different membrane preparations: two from porcine aorta and one from bovine aorta. The three Na–Ca exchange and the bovine Ca^{2+}-ATPase activities are all V_{max} determinations from Eadie–Hofstee analyses, while porcine ATP-driven Ca^{2+} transport measurements are made at saturating Ca^{2+} levels. This membrane preparation has been judged to be 40% right-side-out by monitoring binding of charybdotoxin, a peptidyl probe that only binds at the external face of Ca^{2+}-activated K^+ channels (Garcia et al., 1988a). Since externally applied ATP is only able to catalyze Ca^{2+} uptake into inside-out vesicles, the measured velocities for ATP-dependent Ca^{2+} uptake have been corrected for this sidedness ratio. In the three preparations, Na–Ca exchange activities, at 205, 24, and 73 nmol of Ca^{2+} transported min^{-1} (mg of protein)$^{-1}$, are 3–6-fold higher than the respective ATP-dependent Ca^{2+} uptake activities, 63, 8, and 12 nmol min^{-1} (mg of protein)$^{-1}$. The second set of values were obtained with vesicles isolated from older pigs, and while specific activities of both Na–Ca exchange and ATP-dependent Ca^{2+} uptake are reduced, the proportion still holds. Since these values for ATP-dependent Ca^{2+} uptake are at least as high as others previously reported in vascular smooth muscle (Wuytack et al., 1981; Grover et al., 1982; Sakai et al., 1983; Morel & Godfraind, 1984), the higher Na–Ca exchange activity identified in the present study argues that Na–Ca exchange is a significant mediator of Ca^{2+} flux in this tissue.

Modulation of Na–Ca Exchange Activity in Aorta. Two structurally different classes of Na–Ca exchange inhibitors that block the cardiac transport system have been described (Slaughter et al., 1988; Garcia et al., 1988b). These agents act as mechanism-based inhibitors, and their modes of action have been characterized according to a two ion binding site model describing carrier function (Reeves, 1985). Representative members of each of these inhibitor classes are also effective at blocking aortic Na–Ca exchange (Figure 3A). Amiloride analogues, which function as Na^+ mimics, have recently been characterized in the cardiac system as possessing a biphasic concentration dependence for inhibition (Slaughter et al., 1988). These agents interact preferentially at the B-site of the carrier, the site at which the third transported Na^+ presumably binds. At higher concentration, they occupy the A-site, which is thought to bind either Ca^{2+} or two Na^+. This pattern of behavior is also evident in aorta as shown by the inhibitory profile produced by 3,4-dichlorobenzamil, a terminal guanidino nitrogen substituted amiloride analogue (Figure 3A). Bepridil, a member of the other structural class of Na–Ca exchange inhibitors, causes partial inhibition of transport activity in heart under some conditions (Garcia et al., 1988b). This property is mechanism-based and appears related to the kinetics of carrier turnover. In aorta, bepridil's pattern of inhibition is similar to heart since this agent inhibits initial rates of Na_i-dependent Ca^{2+} uptake maximally by 50% when inhibitor is added at the external membrane surface. Importantly, the potencies of both classes of inhibitors are nearly identical in the two systems.

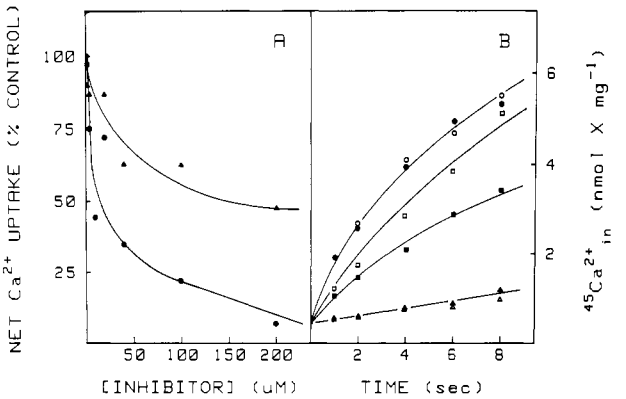

FIGURE 3: Modulation of Na–Ca exchange in sarcolemmal membrane vesicles derived from bovine aortic smooth muscle. (A) Effect of 3′,4′-dichlorobenzamil and bepridil. Membrane vesicles equilibrated with 160 mM NaCl and 20 mM MOPS–Tris, pH 7.4, were diluted 100-fold into 160 mM KCl buffered to pH 7.4 with 20 mM MOPS–Tris containing 15 μM $^{45}CaCl_2$ and increasing concentrations of either 3′,4′-dichlorobenzamil (●) or bepridil (▲) at 37 °C. Net Na^+-dependent uptake was determined after 0.5 s. Inhibition of $^{45}Ca^{2+}$ influx is represented as a function of inhibitor concentration relative to an untreated control. (B) Electrogenicity of Na–Ca exchange. Membrane vesicles equilibrated with 160 mM NaCl and 20 mM MOPS–Tris, pH 7.4, were diluted 100-fold into either 160 mM KCl (■, □), 320 mM sucrose (●, ○), or 160 mM NaCl (▲, △) containing 15 μM $^{45}CaCl_2$, in the absence (closed symbols) or presence (open symbols) of 2 μM valinomycin at 37 °C.

Another similarity with the cardiac transport system (Reeves & Sutko, 1980) is the electrogenic behavior of Na–Ca exchange in aortic smooth muscle. This property is demonstrated by the ability of K^+ and valinomycin to stimulate transport activity through a charge compensation mechanism. As shown in Figure 3B, valinomycin stimulates initial rates of Na_i-dependent Ca^{2+} uptake approximately 1.5-fold. Stimulation in the presence of valinomycin is dependent on the presence of extravesicular K^+, because the ionophore has no effect on basal rates of Na–Ca exchange when external sucrose is substituted for K^+. Stimulation of transport activity caused by an ionophore-induced internally positive membrane potential is consistent with a stoichiometry of more than two Na^+ transported for each Ca^{2+} during carrier turnover. Another interpretation involves movement of charged portions of the transport protein in response to the imposed membrane potential. However, since the stoichiometry of the cardiac transport reaction has been firmly established at 3 Na^+/1 Ca^{2+} (Reeves & Hale, 1984), the most likely explanation for this behavior is that the aortic Na–Ca exchange reaction has a similar stoichiometry. Also noteworthy is the fact that the rate of the Na–Ca exchange reaction is faster in sucrose than in K^+ media (Figure 3B). Such a phenomenon has previously been observed with the cardiac Na–Ca exchange reaction operating in both uptake and efflux modes, and has been modeled as inhibition of transport due to K^+ competing at the Ca^{2+} binding site of the carrier (Slaughter et al., 1988).

Not only is external K^+ inhibitory to the aortic transport reaction but so also are external Li^+ and choline when increasing concentrations of these ions are substituted for sucrose under conditions where the osmolarity of the solution is maintained at 320 mOsm. Interestingly, as shown in Figure 4A, these ions are clearly much more potent inhibitors of smooth muscle Na–Ca exchange than of the cardiac transport system. When these data are reanalyzed in the form of a Hill plot, the IC_{50}'s and Hill coefficients, respectively, for these ions in aorta are as follows: K^+, 54 mM, 0.97; Li^+, 28 mM, 0.90; choline, 32 mM, 0.78. In heart, they are the following: K^+, 630 mM, 1.00; Li^+, 195 mM, 1.07; choline, 670 mM, 0.67.

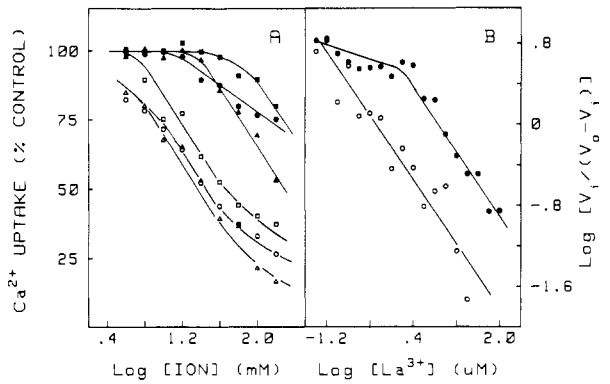

FIGURE 4: Effect of ions on Na–Ca exchange activity. (A) Monovalent cations. Aliquots (2 μL) of either cardiac sarcolemmal (closed symbols) or bovine aortic sarcolemmal membrane vesicles (open symbols) equilibrated in 160 mM NaCl and 20 mM MOPS–Tris, pH 7.4, were diluted 100-fold into solution with different concentrations of KCl (■, □), choline chloride (●, ○), or LiCl (▲, △) containing 15 μM $^{45}CaCl_2$ at 37 °C. The osmolarity of the dilution media was kept constant by balancing the solutions with sucrose, and uptake was determined at 0.5 s. Data are presented relative to a control which was determined by diluting vesicles into 320 mM sucrose. (B) Inhibition of bovine aortic Na–Ca exchange by $LaCl_3$. Aliquots (2 μL) of vesicles equilibrated in 160 mM NaCl and 20 mM MOPS–Tris, pH 7.4, were diluted 100-fold into either 320 mM sucrose (●) or 160 mM KCl (○) media containing 15 μM $^{45}CaCl_2$ in the absence or presence of increasing concentrations of $LaCl_3$ at 37 °C. Uptake was determined at 0.5 s. Data are presented in the form of a Hill plot.

Hill coefficients of ca. 1 would suggest an interaction directly at the level of the exchange protein. Differences in IC_{50} values of 7- to over 20-fold suggest that the aortic transport reaction is much more sensitive to modulation by ions than the cardiac muscle system. These data imply that monovalent cations may regulate Na–Ca exchange in vascular smooth muscle and that perhaps relative Na^+ and K^+ concentrations have some bearing on movement of Ca^{2+} in this tissue. Inhibition of Na–Ca exchange by monovalent cations in both vascular smooth muscle and cardiac sarcolemmal vesicles contrasts markedly with the putative requirement for low concentrations of alkali metal ions to stimulate carrier turnover in intact myocytes (Gadsby et al., 1988) and squid giant axon (Allen & Baker, 1986).

It is well-known that La^{3+} inhibits Na–Ca exchange activity in heart (Reeves, 1985). Aortic smooth muscle Na–Ca exchange is inhibited by La^{3+} with an identical concentration dependence as found for the cardiac transport system. In both cases, La^{3+} is 10-fold more potent in the presence of 160 mM KCl than with 320 mM sucrose present (Figure 4B), but Hill coefficients are always ca. 1, suggesting an interaction at only one site on the carrier. This pattern of more effective La^{3+} inhibition in KCl than in sucrose medium is opposite of that observed by Smith et al. (1987) for the effects of various divalent cations on Ca^{2+} efflux in aortic cells in culture. In this system, divalent ions were always found to be more inhibitory in sucrose than in KCl, as would be expected if K^+ and divalent ions compete for a common site(s). However, the ability of K^+ to potentiate inhibition by La^{3+} in the vesicle systems would suggest that K^+ is not acting at the same site.

In cardiac sarcolemmal membranes, raised internal Ca^{2+} levels result in stimulation of Na–Ca exchange activity (Reeves & Poronnik, 1987). This phenomenon is thought to be due to a nonspecific Ca^{2+} interaction and not to binding of Ca^{2+} at an additional carrier site on the inner surface of the membrane (Durkin & Reeves, 1988), such as that which has been described for the squid giant axon (Baker & Dipolo, 1984) and barnacle muscle (Nelson & Blaustein, 1981) transport systems. Nonetheless, EGTA treatment removes internal Ca^{2+} and markedly lowers Na–Ca exchange activity in cardiac vesicles. When aortic sarcolemmal vesicles are pretreated with EGTA, this procedure has no effect on Na_i-dependent Ca^{2+} uptake, while when the same protocol is used, the activity of cardiac Na–Ca exchange falls to 30% of its original level. This insensitivity of Na–Ca exchange activity to EGTA has also been observed in trachea (Slaughter et al., 1987a), which implies that both vascular and visceral smooth muscle transport reactions differ from the cardiac system in this respect.

Discussion

The results presented in this paper describe the existence of high levels of Na–Ca exchange activity in purified sarcolemmal membrane vesicles derived from either porcine or bovine aortic smooth muscle. This finding has important implications and counters the prevailing consensus, as expressed in recent reviews (see the introduction), that Na–Ca exchange is only a minor component of vascular smooth muscle sarcolemmal Ca^{2+} flux. The specific activity of Na–Ca exchange determined in the present study is over 100 times higher than previously reported values from other types of smooth muscle (Grover et al., 1983; Wibo & Godfraind, 1984) on which this consensus is in large part based. High levels of Na–Ca exchange are in agreement with recent work on Ca^{2+} transport in cultured rat arterial smooth muscle cells which demonstrates that a major portion of Ca^{2+} efflux from these cells is Na-dependent (Smith et al., 1987; Smith & Smith, 1987; Nabel et al., 1988). The findings that Ca^{2+} efflux from aortic cells (Smith et al., 1987) and from aortic rings (Ashida & Blaustein, 1987) is 70–90% dependent on the presence of external Na^+ imply that the Ca^{2+}-ATPase would account for no more Ca^{2+} efflux than the remaining percentage. Thus, the observation reported in this study that the maximal Na^+-dependent Ca^{2+} transport capacity is 3–6 times that of ATP-dependent Ca^{2+} transport in the same sarcolemmal membrane preparation correlates well with Ca^{2+} efflux data obtained from intact systems. The high level of Na–Ca exchange found in purified aortic sarcolemmal membranes, coupled with the correlation between vesicle, cellular, and tissue data, suggests that Na–Ca exchange must play a significant role in mediating Ca^{2+} fluxes in vascular smooth muscle.

Na–Ca exchange in vascular smooth muscle displays many properties in common with the corresponding cardiac transport system, although some differences which may be significant in terms of carrier regulation are evident. Thus, aortic and cardiac Na–Ca exchange systems exhibit similar K_m values for Na^+ and Ca^{2+}, are both electrogenic and bidirectional, and have similar sensitivities to two classes of mechanism-based organic inhibitors and the inorganic inhibitor La^{3+}. On the other hand, the vascular smooth muscle system is far more sensitive to inhibition by monovalent metal ions and is completely insensitive to removal of endogenous Ca^{2+} by EGTA.

The elevated levels of Na–Ca exchange activity [30–210 nmol of Ca^{2+} transported min^{-1} (mg of protein)$^{-1}$] described in this study depend on obtaining highly purified sarcolemmal membranes from aortic smooth muscle and on measuring true initial velocities of transmembrane Ca^{2+} flux. The high density of receptors for the dihydropyridine antagonist PN 200–110 in fraction F1 indicates that this membrane preparation has increased levels of sarcolemmal markers over those reported in various vesicle preparations from vascular (Wibo & Godfraind, 1984) and from other smooth muscle types (Grover et al., 1984). While the purification procedures used here do not differ greatly from those previously reported, use of greater amounts of starting material can result in lower proportional

losses and may be more protective of Na–Ca exchange activity. It should be noted that membranes derived from rat myometrium, in which the initial demonstration of Na–Ca exchange in a smooth muscle tissue was made (Grover et al., 1983), were reported to be 80–90% sealed vesicles and were able to support the formation of a 1000-fold gradient of Ca^{2+} (i.e., measured as ATP-dependent Ca^{2+} uptake; Daniel, 1985). Therefore, it seems unlikely that differences in Ca^{2+} transport measurements could be due to variations in membrane integrity among different preparations. Earlier reports of smooth muscle Na–Ca exchange activity (Godfraind et al., 1983; Morel & Godfraind, 1984; Matlib et al., 1985) were based on measurements taken at time points well past the region of transport linearity which would greatly underestimate maximal transport capacities. V_{max} determinations in this report were all made under initial velocity conditions (0.5 s) which should give a more accurate measurement of Na–Ca exchange activity. Because the earlier studies may not have been conducted under optimal conditions, the actual levels of Na–Ca exchange activity in these tissues may have been underestimated.

Potent inhibition of aortic Na–Ca exchange by K^+, Li^+, and choline indicates that data obtained from tissue studies in which external Na^+ is replaced by these ions (i.e., Na^+ withdrawal experiments) must be interpreted with caution. While there are differences in experimental design between tissue contracture and isolated vesicle flux measurements, demonstration of significant Na–Ca exchange in smooth muscle membranes must be taken into account in interpreting ex vivo tissue experiments. The comparable activities found in the present studies and in whole cell experiments (Smith et al., 1987; Smith & Smith, 1987; Nabel et al., 1988) indicate that membrane vesicles are a suitable preparation for studying Na–Ca exchange in vascular smooth muscle.

A role for Na–Ca exchange in the development of essential hypertension has been proposed (Blaustein, 1977). However, it remains to be proven whether all types of peripheral vascular tissue display high levels of Na–Ca exchange activity. Aortic smooth muscle, as expected for a tissue which has large amounts of this transport activity, is more likely to contract in response to Na depletion protocols and cardiac glycoside block of the Na pump (Mulvany, 1984) than is peripheral smooth muscle. Nevertheless, substantial Na–Ca exchange activity has been demonstrated in bovine (Kahn et al., 1988) and dog (Matlib, 1988) mesenteric artery membranes and in smooth muscle cells from bovine tail artery (Blaustein et al., 1986). In addition, contractions consistent with the operation of this transport reaction in various peripheral vascular tissues have been demonstrated (Ozaki & Urakawa, 1979; Johansson & Hellstrand, 1987; Ashida & Blaustein, 1987). Thus, some types of peripheral smooth muscle may ultimately prove to have significant Na–Ca exchange activity in isolated sarcolemmal membrane preparations.

The high activity of Na–Ca exchange in one type of vascular smooth muscle strongly suggests that this system plays a significant role in mediating trans-sarcolemmal Ca^{2+} flux and in controlling intracellular Ca^{2+} levels. However, development of a specific inhibitor is needed to confirm the physiological role of this transport system in whole tissue. In pathophysiological conditions of elevated internal Na^+ (i.e., essential hypertension), a specific inhibitor would block influx of Ca^{2+} through this transport reaction and allow Ca^{2+}-ATPase to clear Ca^{2+} from the cell and promote muscle relaxation.

Acknowledgments

We thank V. F. King for excellent technical assistance during development of the aortic sarcolemmal membrane vesicle preparation and Mary Coward for help in the preparation of the manuscript.

Registry No. ATPase, 9000-83-3; Na, 7440-23-5; Ca, 7440-70-2; La, 7439-91-0; K, 7440-09-7; 3′,4′-dichlorobenzamil, 1166-01-4; bepridil, 64706-54-3.

References

Aalkjaer, C., & Mulvany, M. J. (1985) *J. Physiol. (London) 362*, 215–231.

Allen, T. J. A., & Baker, P. F. (1986) *J. Physiol. (London) 378*, 53–76.

Ashida, T., & Blaustein, M. P. (1987) *J. Physiol. (London) 392*, 617–635.

Ashida, T., Schaeffer, J., Goldman, W. F., Wade, J. B., & Blaustein, M. P. (1988) *Circ. Res. 62*, 854–863.

Baker, P. F., & Dipolo, R. (1984) *Curr. Top. Membr. Transp. 22*, 195–247.

Barros, F., & Kaczorowski, G. J. (1984) *J. Biol. Chem. 259*, 9404–9410.

Blaustein, M. P. (1977) *Am. J. Physiol. 232 (3)*, C165–C173.

Blaustein, M. P., Ashida, T., Goldman, W. F., Mer, W. G., & Hamlyn, J. N. (1986) *Ann. N.Y. Acad. Sci. 488*, 199–216.

Bohr, D. F., Brodie, D., & Cheu, D. (1958) *Circulation 17*, 796–799.

Brading, A. F., & Lategan, T. W. (1985) *J. Hypertens. 3*, 109–116.

Casteels, R., Raeymaekers, L., Droogmans, L., & Wuytack, F. (1985) *J. Cardiovasc. Pharmacol., Suppl. 3*, S103–S110.

Chiesi, M., Rogg, H., Eichenberger, K., Gazzotti, P., & Carafoli, E. (1987) *Biochem. Pharmacol. 36*, 2735–2740.

Daniel, E. E. (1985) *Experientia 41*, 905–913.

Durkin, J. T., & Reeves, J. P. (1988) *J. Physiol. (London) 407*, 134P.

Gadsby, D. G., Nakao, M., Noda, M., & Shepherd, R. N. (1988) *J. Physiol. (London) 407*, 135P.

Garcia, M. L., King, V. F., Siegl, P. K. S., Reuben, J. P., & Kaczorowski, G. J. (1986) *J. Biol. Chem. 261*, 8146–8157.

Garcia, M. L., Vazquez, J., Reuben, J. P., & Kaczorowski, G. J. (1988a) *Physiologist 31*, A88.

Garcia, M. L., Slaughter, R. S., King, V. F., & Kaczorowski, G. J. (1988b) *Biochemistry 27*, 2410–2415.

Grover, A. K., Kwan, C. Y., & Daniel, E. E. (1982) *Am. J. Physiol. 242*, C278–C282.

Grover, A. K., Kwan, C. Y., Rangachari, P. K., & Daniel, E. E. (1983) *Am. J. Physiol. 244*, C158–C165.

Grover, A. K., Kwan, C. Y., Luchowski, E., Daniel, E. E., & Triggle, D. J. (1984) *J. Biol. Chem. 259*, 2223–2226.

Johansson, B., & Hellstrand, P. (1987) *J. Cardiovasc. Pharmacol. 10*, 575–581.

Kahn, A. N., Allen, J. C., & Shelat, H. (1988) *Am. J. Physiol. 254*, C441–C449.

Luchowski, E. M., Yousif, F., Triggle, D. J., Maurer, S. C., Sarmiento, J. G., & Janis, R. A. (1984) *J. Pharmacol. Exp. Ther. 230*, 607–613.

Matlib, M. A. (1988) *Am. J. Physiol. 255*, 1323–1330.

Matlib, M. A., & Reeves, J. P. (1987) *Biochim. Biophys. Acta 904*, 145.

Matlib, M. A., Schwartz, A., & Yamori, Y. (1985) *Am. J. Physiol. 249*, C166–C172.

Morel, N., & Godfraind, T. (1984) *Biochem. J. 218*, 421–427.

Mulvany, M. J. (1984) *J. Cardiovasc. Pharmacol. 6*, 582–587.

Mulvany, M. (1985) *J. Hypertens. 3*, 429–436.

Mulvany, M. J., Aalkjaer, C., & Peterson, T. T. (1984) *Circ. Res. 54*, 740–749.

Nabel, E. G., Berk, B. C., Brock, T. A., & Smith, T. W. (1988) *Circ. Res. 62*, 486-493.
Nelson, M. T., & Blaustein, M. P. (1981) *Nature 289*, 314-316.
Newman, M. J., Foster, D. L., Wilson, T. H., & Kaback, H. R. (1982) *J. Biol. Chem. 256*, 11804-11808.
Nishimura, J., Kanaide, H., & Nakamura, M. (1986) *J. Pharmacol. Exp. Ther. 236*, 789-793.
Ozaki, H., & Urakawa, N. (1979) *Naunyn-Schmiedeberg's Arch. Pharmacol. 309*, 171-178.
Ozaki, H., & Urakawa, N. (1981) *Pfluegers Arch. 390*, 107-112.
Reeves, J. P. (1985) *Curr. Top. Membr. Transp. 25*, 77-127.
Reeves, J. P., & Sutko, J. L. (1980) *Science 208*, 1461-1464.
Reeves, J. P., & Hale, C. C. (1984) *J. Biol. Chem. 259*, 7733-7734.
Reeves, J. P., & Poronnik, P. (1987) *Am. J. Physiol. 252*, C17-C23.
Reuter, H., Blaustein, M. P., & Haeusler, G. (1973) *Philos. Trans. R. Soc. London B 265*, 87-94.
Sakai, V., Grover, A. K., Fox, J. E. T., & Daniel, E. E. (1983) *Can. J. Physiol. Pharmacol. 61*, 699-704.
Schaffner, W., & Weissmann, C. (1973) *Anal. Biochem. 56*, 502-514.
Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183-3190.
Slaughter, R. S., Welton, A. F., & Morgan, D. W. (1987a) *Biochim. Biophys. Acta 904*, 92-104.
Slaughter, R. S., Garcia, M. L., King, V. F., & Kaczorowski, G. J. (1987b) *Biophys. J. 51*, 386a.
Slaughter, R. S., Garcia, M. L., Cragoe, E. J., Jr., Reeves, J. P., & Kaczorowski, G. J. (1988) *Biochemistry 27*, 2403-2409.
Smith, J. B., & Smith, L. (1987) *J. Biol. Chem. 262*, 17455-17460.
Smith, J. B., Cragoe, E. J., & Smith, L. (1987) *J. Biol. Chem. 262*, 11988-11994.
Wibo, M., & Godfraind, T. (1984) *Arch. Int. Pharmacodyn. Ther. 270*, 333-334.
Wuytack, F., De Schutter, G., & Casteels, R. (1981) *Biochem. J. 198*, 215-271.

Isolation and Characterization of the N-Terminal 23-Kilodalton Fragment of Myosin Subfragment 1†

Andras Muhlrad*
Cardiovascular Research Institute, University of California, San Francisco, California 94143, and Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, Jerusalem 91010, Israel

Received August 4, 1988; Revised Manuscript Received December 2, 1988

ABSTRACT: The 23-kDa N-terminal tryptic fragment was isolated from the heavy chain of rabbit skeletal myosin subfragment 1 (S-1). The heavy-chain fragments were dissociated by guanidine hydrochloride following limited trypsinolysis, and the 23-kDa fragment was isolated by gel filtration and ion-exchange chromatography. Finally, the fragment was renatured by removing the denaturants. The CD spectrum of the renatured fragment shows the presence of ordered structure. The tryptophan fluorescence emission spectrum of the fragment is considerably shifted to the red upon adding guanidine hydrochloride which indicates that the tryptophans are located in relatively hydrophobic environments. The two 23-kDa tryptophans, unlike the rest of the S-1 tryptophans, are fully accessible to acrylamide as indicated by fluorescence quenching. The isolated 23-kDa fragment cosediments with F-actin in the ultracentrifuge and significantly increases the light scattering of actin in solution which indicates actin binding. The binding is rather tight (K_d = 0.1 μM) and ionic strength dependent (decreasing with increasing ionic strength). ATP, pyrophosphate, and ADP dissociate the 23-kDa-actin complex with decreasing effectiveness. The isolated 23-kDa fragment does not have ATPase activity; however, it inhibits the actin-activated ATPase activity of S-1 by competing presumably with S-1 for binding sites on actin. F-Actin binds to the 23-kDa fragment immobilized on the nitrocellulose membrane. The fragment was further cleaved, and one of the resulting peptides, containing the 130-204 stretch of residues, was found to bind actin on the nitrocellulose membrane, indicating that this region of the 23-kDa fragment participates in forming an actin binding site.

Myosin subfragment 1 (S-1),[1] the head segment of myosin, contains separate actin and nucleotide binding sites of the molecule. These sites are involved in ATPase activity and actin-myosin interactions which are believed to have central significance in the molecular mechanism of muscle contraction. In order to understand the mechanism of these contraction events and the communication between the actin and nucleotide binding sites, it is necessary to define the S-1 structure.

The structure of S-1 heavy chain has been thoroughly studied by limited proteolytic digestion. Balint et al. (1978) found that the chain consists of three trypsin-resistant fragments (23, 50, and 20 kDa) aligned from the N-terminus in

† This research was supported by Grant 00241/85 from the US-Israel Binational Research Foundation, by Grant HL-16683, and by a grant from the Muscular Dystrophy Association.

* Address correspondence to this author at the Department of Oral Biology, Hebrew University-Hadassah School of Dental Medicine, Jerusalem 91010, Israel.

[1] Abbreviations: S-1, chymotryptic subfragment 1; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone; PMSF, phenylmethanesulfonyl fluoride; ϵ-ATP, 1,N^6-ethenoadenosine 5′-triphosphate; PP_i, pyrophosphate; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kDa, kilodalton(s); NCS, *N*-chlorosuccinimide; DTE, dithioerythritol; DTT, dithiothreitol; BSA, bovine serum albumin; MeOH, methanol; TMR, tetramethylrhodamine iodoacetamide; Trp, tryptophan.

0006-2960/89/0428-4002$01.50/0