Characterization of calcium binding to brush-border membranes from rat duodenum

Alexander MILLER, III, Shu-Tung LI and Felix BRONNER

Department of Oral Biology, University of Connecticut Health Center, Farmington, CT 06032, U.S.A.

(Received 12 July 1982/Accepted 20 August 1982)

The Ca^{2+} -binding properties of isolated brush-border membranes at physiological ionic strength and pH were examined by rapid Millipore filtration. A comprehensive analysis of the binding data suggested the presence of two types of Ca^{2+} -binding sites. The high-affinity sites, $K_a = (6.3 \pm 3.3) \times 10^5 \text{ M}^{-1}$ (mean $\pm$ s.e.m.), bound $0.8 \pm 0.1 \text{ nmol}$ of Ca^{2+} /mg of protein and the low-affinity sites, $K_a = (2.8 \pm 0.3) \times 10^2 \text{ M}^{-1}$, bound $33 \pm 3.5 \text{ nmol}$ of Ca^{2+} /mg of protein. The high-affinity site exhibited a selectivity for Ca^{2+} , since high concentrations of competing bivalent cations were required to inhibit Ca^{2+} binding. The relative effectiveness of the competing cations (1 and 10 mM) for the high-affinity site was $\text{Mn}^{2+} \approx \text{Sr}^{2+} > \text{Ba}^{2+} > \text{Mg}^{2+}$. Data from the pH studies, treatment of the membranes with carbodi-imide and extraction of phospholipids with aqueous acetone and NH_3 provided evidence that the low-affinity sites were primarily phospholipids and the high-affinity sites were either phosphoprotein or protein with associated phospholipid. Two possible roles for the high-affinity binding sites are suggested. Either high-affinity Ca^{2+} binding is involved with specific enzyme activities or Ca^{2+} transport across the luminal membrane occurs via a Ca^{2+} channel which contains a high-affinity Ca^{2+} -specific binding site that may regulate the intracellular Ca^{2+} concentration and gating of the channel.

Ca^{2+} uptake by brush-border vesicles prepared from rat duodenum consists of two components: binding and/or incorporation into membrane constituents and transport into an osmotically sensitive space (Miller & Bronner, 1981). Preliminary data indicated that the energy-independent Ca^{2+} -binding component consisted of at least two types of binding sites, high- and low-affinity. Similar cation-binding characteristics have been reported for various membrane systems; rat liver plasma membranes (Schlatz & Marinetti, 1972), cardiac sarcolemma (Williamson *et al.*, 1975), sarcoplasmic reticulum (Chevallier & Butow, 1971), renal membranes (Palmer & Posey, 1970), brain plasma membranes (Hemminki, 1974) and adipocyte plasma membranes (McDonald *et al.*, 1976).

The presence of a small number of high-affinity sites in the intestinal brush-border membrane with fairly high binding affinity (Miller & Bronner, 1981) is interesting and generally suggestive of membranes that are involved in the active regulation of intracellular Ca^{2+} . In the intestinal epithelial cell, the low intracellular Ca^{2+} concentration ($< 1 \mu\text{M}$) is maintained and/or regulated by the permeability of the membrane, the binding of the cation to available binding sites and by an ATP-dependent Ca^{2+} -pump

mechanism as well as a $\text{Ca}^{2+}/\text{Na}^+$ exchange system (Hildmann *et al.*, 1980).

Since binding may be considered the first step in the transport process, this investigation was initiated in an attempt to characterize further the Ca^{2+} -binding sites of the brush-border membrane with respect to possible structure, function and location.

Materials and methods

Male Sprague–Dawley rats (100–120 g), obtained from ARS, Madison, WI, U.S.A., were fed *ad libitum* on a low- Ca^{2+} (0.06%) low-phosphorus (0.2%) semi-synthetic diet (Bronner & Freund, 1975) for several days before being killed. $^{45}\text{CaCl}_2$ (sp. radioactivity 13.6–26 mCi/mg) and the Ca^{2+} ionophore A23187 were purchased from New England Nuclear, Boston, MA, U.S.A., and Calbiochem–Behring Corp., La Jolla, CA, U.S.A., respectively. Carbodi-imide [1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride] was obtained from Story Chemical Corp., Muskegon, WI, U.S.A. Ruthenium Red (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used without further purification, since spectral analysis indicated a major peak at 535–540 nm and an insignificant peak at 730–740 nm (Luft, 1971). All other chemicals and

reagents were purchased from standard sources and were analytical-reagent grade. Stock solutions of CaCl_2 and BaCl_2 were prepared from their corresponding carbonates.

Preparation of brush-border membranes

Animals were killed by cervical dislocation and brush-border membranes and vesicles were isolated as described previously (Miller & Bronner, 1981). Sucrase (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26) specific activity of the membrane vesicles was enriched more than 20-fold compared with the initial homogenate. No intact mitochondria or mitochondrial fragments were identified by electron microscopy and 2,4-dinitrophenol and NaN_3 had no significant effect on vesicular Ca^{2+} uptake. The vesicles were loaded with either 100 mM-D-mannitol/10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/Tris buffer, pH 7.5–8.0, or the same medium also containing 100 mM-NaCl as described by Miller & Bronner (1981). NaCl (100 mM) was included in the incubation medium to maintain physiological ionic strength and to minimize Donnan effects (McDonald *et al.*, 1976). The final protein concentration of the membrane preparations, determined by the method of Lowry *et al.* (1951), was generally 3–5 mg/ml.

Ca^{2+} -binding assay

The binding of Ca^{2+} to brush-border vesicles was determined by rapid Millipore filtration (Miller & Bronner, 1981). The incubation medium (total volume 70 μl) contained 100 mM-D-mannitol, 10 mM-Hepes/Tris, 100 mM-NaCl, pH 7.5–8.0, $^{45}\text{CaCl}_2$ (3×10^5 – 5×10^5 c.p.m.), the desired concentrations of non-radioactive CaCl_2 and membrane protein (30–60 μg). Further details are given in the Figure legends, Millipore filters (type HA; 0.45 μm pore size; diameter 25 mm) were presoaked in 0.25 M-KCl before use (Palmer & Posey, 1970). When Ca^{2+} uptake (binding plus transport) reached equilibrium, vesicles were osmotically lysed with cold water, filtered with a model FH 225 V filtration unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.), and the filters were washed twice with 100 mM-D-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer (pH 7.5). Further washings did not significantly decrease the amount of radioactivity retained by the membranes. The filters were then allowed to air-dry, 3–4 ml of Formula-963 scintillation fluid (New England Nuclear) was added and the radioactivity retained by the membranes was measured in a Nuclear-Chicago liquid-scintillation spectrometer (Unilux II-A).

Binding values were expressed as nmol of Ca^{2+} equivalent per mg of membrane protein after appropriate corrections for non-specific radioactivity retained by the filters, 0.1–0.4% depending on the Ca^{2+} concentration.

Analysis of Ca^{2+} binding

Ca^{2+} binding was determined as a function of the Ca^{2+} concentration at 25°C and pH 7.5–8.0. The Ca^{2+} -binding data were fitted to a two-term mass-action equation under the assumption that each site bound Ca^{2+} independently. The curve-fitting algorithm consists of a least-squares computational method (Li & Katz, 1977) that simultaneously converged to the best values of n_1 and n_2 (the numbers of high- and low-affinity sites respectively) and the respective affinity constants (K_a). The initial parameters used for curve fitting were estimated from a Scatchard (1949) plot.

1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide treatment

Vesicles were isolated in 100 mM-D-mannitol/10 mM-Hepes/Tris buffer (pH 7.5), lysed in ice-cold water and treated with carbodi-imide (1–2 mg) for approx. 20 min at room temperature with occasional mixing. Additional carbodi-imide was added and the incubation continued for another 20 min. The controls, without reagent, were also treated as above. The control and treated preparations were then washed twice with cold water and resuspended in 100 mM-D-mannitol/10 mM-Hepes/Tris buffer (pH 7.5) for subsequent binding assays as described above.

Organic phosphate determination

The organic phosphate content of brush-border vesicles was determined essentially as described by Hurst (1964) after acid hydrolysis (6 M-HCl) in sealed tubes at 120°C for 48 h. A Technicon autoanalyser (Technicon Corp., Tarrytown, NY, U.S.A.) was used for all assays and the organic phosphate content was expressed as nmol of P/mg of membrane protein.

Atomic absorption analysis of endogenous Ca^{2+}

Samples of medium, isolated plasma membranes and appropriate standards were treated with 12% (w/v) HClO_4 containing 10 mM- SrCl_2 , and denatured protein was removed by centrifugation. Ca^{2+} concentrations were determined with the aid of a Varian AA575 atomic absorption spectrophotometer, by using a nitrous oxide/acetylene flame and the resonance line of 4227 nm for Ca^{2+} . After injection of 50 μl samples into the burner via a capillary holder, absorbance was measured as peak height integrated over 1 s.

Results

Effects of protein concentration and pH on Ca^{2+} binding

Preliminary experiments, conducted in the presence of 100 mM-NaCl, indicated that Ca^{2+} binding

Fig. 1. Time course of Ca^{2+} binding by brush-border membrane vesicles

Ca^{2+} binding, at (a) 3.6 mM- Ca^{2+} and (b) 0.0092 mM- Ca^{2+} , was determined in 100 mM-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer (pH 7.5). The vesicles were prepared in 100 mM-mannitol/10 mM-Hepes/Tris buffer (pH 7.5).

to brush-border membranes was linear with increasing concentrations of membrane protein, from 10 μg to at least 90 μg of protein per assay tube. Ca^{2+} binding reached equilibrium by approx. 60 min at high cation concentrations (Fig. 1a) and by 5 min at low concentrations (Fig. 1b), and remained relatively constant up to 2 h, indicating no significant change in the structural integrity of the membranes. The kinetic data agree well with those reported by Wilson & Lawson (1980) for chick intestinal brush-border membranes. A continual increase in Ca^{2+} binding was observed between pH 6.5 and pH 9.0 when the medium contained 3.6 mM- Ca^{2+} (Fig. 2a), whereas a definite peak at pH 8.0 was apparent at 0.0092 mM- Ca^{2+} (Fig. 2b). Similar pH profiles have been reported for Ca^{2+} binding to adipocyte plasma membranes (McDonald *et al.*, 1976) and rat liver plasma membranes (Schlatz & Marinetti, 1972). Therefore all subsequent binding assays were performed at pH 7.5–8.0 with 30–60 μg of membrane protein and terminated at equilibrium as indicated in the Materials and methods section.

Scatchard-plot analyses

A typical Ca^{2+} -binding curve and the corresponding Scatchard plot of Ca^{2+} binding to brush-border

Fig. 2. Effect of pH on Ca^{2+} binding to brush-border membranes

Over the pH range 6.5–9.5, 100 mM-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer was used; between pH 4.0 and 6.0 100 mM-mannitol/100 mM-NaCl/10 mM-Mes/Tris was used. Binding assays with (a) 3.6 mM- Ca^{2+} and (b) 0.0092 mM- Ca^{2+} were terminated at 60 and 15 min respectively.

membranes are shown in Fig. 3. The biphasic nature of the Scatchard plot indicated the presence of at least two types of binding sites. The high-affinity sites, $K_a = (6.3 \pm 3.3) \times 10^5 \text{ M}^{-1}$ (mean $\pm$ S.E.M.), bound $0.8 \pm 0.1 \text{ nmol}$ of Ca^{2+} /mg of protein, whereas the low-affinity sites, $K_a = (2.8 \pm 0.3) \times 10^2 \text{ M}^{-1}$, accommodated $33 \pm 3.5 \text{ nmol}$ of Ca^{2+} /mg of protein. The data were reproducible with different membrane preparations; however, the calculated affinity constants were higher than those reported earlier (Miller & Bronner, 1981). In the present

study, results obtained from analyses of endogenous Ca^{2+} in the medium (approx. $5 \mu\text{M}$) and the use of a greater number of Ca^{2+} concentrations between 10 mM and $1 \mu\text{M}$ permitted a more accurate computer-assisted analysis of the binding data. Ca^{2+} associated with the membranes after isolation (10 nmol/mg of protein) was assumed to be non-exchangeable (McDonald *et al.*, 1976).

Effect of inhibitors on Ca^{2+} binding

The effect of Ruthenium Red ($100 \mu\text{M}$) and La^{3+} ($100 \mu\text{M}$) on Ca^{2+} binding to brush-border membranes is shown in Table 1. Both cations inhibited high- and low-affinity Ca^{2+} binding. However, La^{3+}

Fig. 3. Ca^{2+} -binding curve and Scatchard plot of concentration-dependent Ca^{2+} binding by brush-border membranes

Membranes were isolated in $100 \text{ mM-D-mannitol/10 mM-Hepes/Tris}$ buffer (pH 7.5). The binding assay medium contained the same buffer with 100 mM-NaCl , approx. $50 \mu\text{g}$ of membrane protein and the indicated concentrations of Ca^{2+} . When Ca^{2+} uptake reached equilibrium, each assay tube was diluted 50-fold with ice-cold water, filtered, washed twice more with buffer containing 100 mM-NaCl , and the radioactivity retained by the membranes was assessed.

appeared to be more effective than Ruthenium Red on the high-affinity binding sites. If the latter sites were located on the inner membrane surface and inaccessible to La^{3+} and Ruthenium Red during Ca^{2+} uptake by intact vesicles, displacement of Ca^{2+} from these internal binding sites might have occurred after lysis by osmotic shock. It is generally recognized that La^{3+} interacts strongly with Ca^{2+} -binding sites in biological systems and displaces membrane-bound Ca^{2+} . Therefore, the data indicate that La^{3+} and Ruthenium Red may have been bound to the same high-affinity site as Ca^{2+} , in addition to being bound to a large number of low-affinity sites for which Ca^{2+} is not a strong competitor.

Effect of pH on Ca^{2+} binding

The effect of pH on Ca^{2+} binding was examined further in an attempt to assess the nature of the anionic groups in the membrane reacting with Ca^{2+} . Ca^{2+} binding to the high-affinity binding sites was decreased by approx. 50% between pH 7.5 and 5.5 as well as between pH 5.5 and 4.0 (Table 2). At

Table 2. Effect of pH on Ca^{2+} binding to brush-border membranes

For pH 7.5, membranes were prepared in $100 \text{ mM-mannitol/10 mM-Hepes/Tris}$ buffer (pH 7.5) and the assay medium contained the same buffer plus 100 mM-NaCl and the indicated Ca^{2+} concentrations. For pH 5.5 and 4.0, the assay medium contained 100 mM-mannitol , 100 mM-NaCl , 10 mM-Mes (4-morpholine-ethanesulphonic acid)/Tris. Data are the means for two membrane preparations, each with quadruplicate determinations; s.e.m. values are shown in parentheses.

$[\text{Ca}^{2+}]$ (M)	Ca^{2+} bound (nmol/mg of protein)		
	pH 7.5	pH 5.5	pH 4.0
9.4×10^{-6}	0.34 (0.03)	0.17 (0.02)	0.07 (0.02)
1.4×10^{-5}	0.58 (0.05)	0.25 (0.04)	0.10 (0.02)
2.7×10^{-5}	0.89 (0.12)	0.38 (0.04)	0.16 (0.02)
8.6×10^{-4}	8.7 (0.7)	2.1 (0.2)	1.2 (0.1)
2.1×10^{-3}	15.0 (1.0)	2.3 (0.4)	2.1 (0.3)

Table 1. Effect of Ruthenium Red and La^{3+} on Ca^{2+} binding to brush-border membranes

The assay medium contained 100 mM-mannitol , 100 mM-NaCl , 10 mM-Hepes/Tris buffer (pH 7.5) and either $100 \mu\text{M}$ -Ruthenium Red or $100 \mu\text{M}$ - LaCl_3 or no addition (control). Binding was assessed as explained in the text. Data are means of quadruplicate determinations, with the s.e.m. values in parentheses.

$[\text{Ca}^{2+}]$ (M)	Ca^{2+} bound (nmol/mg of protein)			
	Control	Ruthenium Red	Control	La^{3+}
9.4×10^{-6}	0.49 (0.1)	0.37 (0.1)	0.58 (0.04)	0.28 (0.02)
1.4×10^{-5}	0.66 (0.04)	0.49 (0.03)	0.95 (0.03)	0.41 (0.02)
2.7×10^{-5}	1.1 (0.03)	0.76 (0.03)	1.4 (0.05)	0.49 (0.09)
8.6×10^{-4}	5.1 (2.4)	1.3 (0.2)	7.6 (0.07)	3.0 (0.3)
2.1×10^{-3}	—	—	18.6 (4.0)	3.9 (0.9)

Table 3. *Effect of 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide treatment on Ca²⁺ binding to brush-border membranes*

Vesicles, isolated in 100 mM-mannitol/10 mM-Hepes/Tris buffer (pH 7.5), were lysed in cold water and treated with 1–2 mg of carbodi-imide for 20 min at room temperature. Additional reagent was added and the incubation continued for 20 min. The controls (no carbodi-imide) were also treated as above. The control and treated preparations were washed twice with cold water and resuspended in 100 mM-mannitol/10 mM-Hepes/Tris buffer (pH 7.5) for subsequent binding assays. Data are the averages of quadruplicate determinations, with S.E.M. values shown in parentheses.

[Ca ²⁺] (M)	Ca ²⁺ bound (nmol/mg of protein)	
	Control	Carbodi-imide-treated
1.4 × 10 ⁻⁵	0.39 (0.04)	0.31 (0.02)
2.7 × 10 ⁻⁵	0.52 (0.02)	0.44 (0.02)
8.6 × 10 ⁻⁴	4.2 (0.4)	2.8 (0.09)
2.1 × 10 ⁻³	9.3 (1.6)	3.1 (0.3)

relatively high Ca²⁺ concentrations (2 mM) the decrease in binding was more pronounced, 4–7-fold, between pH 7.5 and 5.5, and either no or only a small effect was observed between pH 5.5 and 4.0. Since the pK values of carboxy and phosphate groups are in the range 4–5 and 6–7 respectively, the data are consistent with the presence of both groups in the high-affinity sites and indicate that the low-affinity sites contain primarily phosphate groups.

Carbodi-imide treatment and Ca²⁺ binding

The effect of carbodi-imide treatment on Ca²⁺ binding to brush-border membranes is shown in Table 3. The experiment was performed in the absence of nucleophilic agents other than water; therefore the rate of the reaction was slow and non-specific. Under these experimental conditions, carbodi-imide would be expected to modify both carboxy and phosphate groups. Decreased Ca²⁺ binding was noted at the high-affinity and, to a greater degree, the low-affinity sites after treatment with reagent, again indicating the involvement of carboxy and phosphate groups respectively.

Phospholipid extraction and Ca²⁺ binding

Extraction of phospholipids, which constitute approx. 30% of the total lipid in the microvillus membrane (Forstner *et al.*, 1968), with aqueous acetone and NH₃ (Fleischer *et al.*, 1967) resulted in a marked decrease in Ca²⁺ binding to membrane preparations at high Ca²⁺ concentrations (Table 4). The effect of solvent extraction on binding to the high-affinity sites was less pronounced. Although no

Table 4. *Effect of solvent extraction on Ca²⁺ binding to brush-border membranes*

Brush-border vesicles were extracted with 90% acetone, 10% water and 0.01% NH₃ on ice for 10 min (Fleischer *et al.*, 1967). The suspension was centrifuged at 24 000g for 10 min, washed twice with 100 mM-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer (pH 7.5) and resuspended in the same buffer for subsequent binding assays. Data are the averages of quadruplicate determinations, with S.E.M. values in parentheses.

[Ca ²⁺] (M)	Ca ²⁺ bound (nmol/mg of protein)	
	Control	Solvent-treated
9.4 × 10 ⁻⁶	0.32 (0.06)	0.22 (0.06)
1.4 × 10 ⁻⁵	0.51 (0.04)	0.31 (0.02)
2.7 × 10 ⁻⁵	0.78 (0.06)	0.43 (0.02)
8.6 × 10 ⁻⁴	6.5 (0.5)	2.2 (0.2)
2.1 × 10 ⁻³	14.9 (2.6)	1.5 (0.3)

significant difference in protein concentration was observed between the control and extracted membrane preparations, the extraction method may have removed other minor membrane constituents capable of binding Ca²⁺.

Effect of competing bivalent cations on Ca²⁺ binding

A comparison of the inhibitory effects of selected bivalent cations on Ca²⁺ binding to brush-border membranes was conducted by using vesicles that were isolated in 100 mM-D-mannitol/10 mM-Hepes/Tris buffer (pH 7.5) and pre-equilibrated with 100 mM-NaCl. Extravesicular Mn²⁺ (1 mM) inhibited Ca²⁺ (0.027 mM) binding more effectively (*P* ≤ 0.05) than did 1 mM-Sr²⁺, -Ba²⁺ or -Mg²⁺ (Fig. 4a). Since a similar trend of inhibition was observed when the concentration of competing cations was increased to 10 mM, the relative effectiveness of the bivalent cations appears to be Mn²⁺ ≈ Sr²⁺ > Ba²⁺ > Mg²⁺. A similar inhibitory pattern was evident when the incubation medium contained 0.86 mM-Ca²⁺ (Fig. 4b); however, at a competing cation concentration of 10 mM, the differences in inhibition were not considered statistically significant (*P* > 0.05). Since Ba²⁺ had an effect similar to that of Sr²⁺ and Mg²⁺, the data were not plotted, for the sake of clarity. The lack of inhibition of Ca²⁺ binding to the high-affinity sites in the presence of low concentrations of competing bivalent cations (< 0.1 mM) indicates that the high-affinity binding sites are Ca²⁺-specific.

Effect of membrane potential on Ca²⁺ binding

The effect of altering the electrochemical potential difference across the brush-border membrane on Ca²⁺ binding is shown in Fig. 5. Replacement of Cl⁻ by either SCN⁻ or NO₃⁻ increased Ca²⁺ binding

Fig. 4. Effect of competing bivalent cations on Ca^{2+} binding to brush-border membranes

Brush-border vesicles were pre-equilibrated in 100 mM-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer, pH 7.5 (Miller & Bronner, 1981). After loading samples (10 μl) of the vesicle preparation were added to the assay medium (final volume 70 μl), containing the above buffer with (a) 0.027 mM- Ca^{2+} or (b) 0.86 mM- Ca^{2+} and 0.01, 0.1, 1.0 or 10 mM- MgCl_2 (●), BaCl_2 (Δ), SrCl_2 (○), or MnCl_2 (▲). Uptakes were terminated at 15 or 60 min. Error bars have been omitted for clarity except for the controls (□), with no competing bivalent cations.

significantly. Decreased binding was observed in the presence of the less-permeant anion gluconate. The data indicate that Ca^{2+} binding to the high-affinity sites was potential-sensitive; Miller & Bronner (1981) previously demonstrated considerable bind-

Fig. 5. Effect of anion replacement on Ca^{2+} (0.0079 mM) binding by brush-border membranes

Membranes were prepared in 100 mM-mannitol/10 mM-Hepes/Tris buffer (pH 7.5). Gradients (100 mM) of NaCl (●), NaSCN (Δ), NaNO_3 (▲) and sodium gluconate (○) were directed from the extravesicular medium to the intravesicular space. At the indicated times, the vesicles were lysed with ice-cold water, filtered, washed twice with the above buffer containing 100 mM-NaCl and assessed for radioactivity bound to the membranes.

ing of Ca^{2+} to the vesicle interior under conditions that increased negativity inside.

Effect of extravesicular bivalent cations on Ca^{2+} efflux

Brush-border vesicles were pre-equilibrated with 100 mM-D-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer (pH 7.5) and loaded with 0.96 mM- $^{45}\text{Ca}^{2+}$ for 90–120 min at 25°C. Ca^{2+} efflux was initiated by adding 10 μl of the vesicles to 240 μl of the following reaction media: 100 mM-D-mannitol, 100 mM-NaCl, 10 mM-Hepes/Tris buffer (pH 7.5), buffer with 10 mM unlabelled Ca^{2+} , Sr^{2+} or Mg^{2+} , and buffer with either 0.1 mM-EGTA or 0.1 mM-EGTA plus 15 μM -ionophore A23187. ^{45}Ca in the extravesicular medium owing to carry-over was 0.038 mM. As shown in Fig. 6, $^{45}\text{Ca}^{2+}$ taken up by the vesicles does not appear to exchange appreciably with externally added unlabelled Ca^{2+} , whereas $^{45}\text{Ca}^{2+}$ release was quite rapid when the extravesicular medium contained EGTA plus ionophore A23187. Since the ionophore would not be expected to remove bound Ca^{2+} , the data are consistent with a transmembrane

Fig. 6. Effects of bivalent cations, EGTA and ionophore A23187 on Ca^{2+} release from brush-border vesicles. Vesicles isolated in 100 mM-D-mannitol/10 mM-Hepes/Tris buffer (pH 7.5) were pre-equilibrated with the same medium containing 100 mM-NaCl (Miller & Bronner, 1981) and loaded with $0.96 \text{ mM-}^{45}\text{Ca}^{2+}$ for 90–120 min at 25°C with shaking. The loaded vesicles were then diluted 25-fold into the following media: (○) 100 mM-D-mannitol/100 mM-NaCl/10 mM-Hepes/Tris buffer (pH 7.5); buffer with either (▲) 10 mM- Ca^{2+} , (△) 10 mM- Sr^{2+} , (■) 10 mM- Mg^{2+} , (□) 0.1 mM-EGTA or (◇) 0.1 mM-EGTA plus $15 \mu\text{M}$ -ionophore A23187. (●), Undiluted control; 0.01 ml samples of the loaded vesicles were placed on the membrane filters, filtered and washed three times with the stop-and-wash solution, which contained 100 mM-D-mannitol, 5 mM-EGTA and 10 mM-Hepes/Tris (pH 7.5).

process as previously reported (Miller & Bronner, 1981).

Essentially the same amount of $^{45}\text{Ca}^{2+}$ was released in the presence of either 10 mM- Ca^{2+} or 0.1 mM-EGTA, which would be expected to remove only reversibly bound Ca^{2+} from the external membrane surface. The additional loss of radioactivity in the presence of unlabelled Ca^{2+} , compared with the diluted control, is probably due to an exchange with the externally bound radiolabelled cation. The effect of 10 mM- Sr^{2+} in the extravesicular medium at 15 min was similar to that of Ca^{2+} and EGTA; Mg^{2+} did not affect $^{45}\text{Ca}^{2+}$ release.

Discussion

Two classes of energy-independent Ca^{2+} -binding sites that differed significantly in their affinity for Ca^{2+} were demonstrated in brush-border vesicles isolated from rat duodenum. The high-affinity sites bound $0.8 \pm 0.1 \text{ nmol}$ of Ca^{2+} /mg of protein and had

an affinity for Ca^{2+} which was more than 2000 times that for the low-affinity sites, which bound $33 \pm 3.5 \text{ nmol}$ of Ca^{2+} /mg of protein; $K_m = 1.6 \mu\text{M}$ and $3571 \mu\text{M}$ respectively. The presence of two classes of binding sites is generally characteristic of plasma membranes from a variety of cellular types. The apparent binding constant for the low-affinity sites, $K_a = 2.8 \times 10^2 \text{ M}^{-1}$, is similar to reported values for phosphoserine and phospholipid (Hendrickson & Fullington, 1965). Assuming that some sites are not accessible for binding, the number of low-affinity binding sites, $33 \pm 3.5 \text{ nmol/mg}$ of protein, correlated reasonably well with the amount of organic phosphorus determined, $46 \pm 12 \text{ nmol/mg}$ of membrane protein. The binding constant for the high-affinity site, $10^5\text{--}10^6 \text{ M}^{-1}$, is similar in magnitude to those obtained for Ca^{2+} -binding proteins such as calmodulin (Cheung, 1980), brush-border CaBP_x (Miller *et al.*, 1979) and IMCal (Kowarski & Schachter, 1980), which is discussed below.

Data from the pH studies (Table 2), carbodi-imide treatment (Table 3) and solvent extraction (Table 4) correlate well with the inhibition of Ca^{2+} uptake or binding by Ruthenium Red, Na^+ , K^+ and tetracaine (Miller & Bronner, 1981) and substantiate the initial observation that the low-affinity binding sites are primarily phospholipid and that the high-affinity sites consist mainly of protein, phosphoprotein or protein with associated phospholipid. The indication that phospholipid is a possible constituent of the high-affinity binding site is not surprising, since there is substantial evidence that phospholipid molecules are associated with and determine the enzymic activity of membrane-bound protein (Warren *et al.*, 1974; Hesketh *et al.*, 1976; Bennett *et al.*, 1978). The presence of a small number of high-affinity sites specific for Ca^{2+} suggests that they may be associated with the regulation of membrane-bound enzymes or may involve a specific carrier molecule implicated in Ca^{2+} translocation across the brush-border membrane.

Phosphorylation of proteins is generally considered a regulatory step for a number of physiological and metabolic processes. However, the significance of protein or membrane phosphorylation as far as regulation of membrane function is concerned is far from resolved (Lever, 1980; Hosey & Tao, 1977). A Ca^{2+} -stimulated ATPase in brush-border and basolateral membranes of rat duodenum with high-affinity sites for Ca^{2+} ions was reported by Ghijsen & van Os (1979). The K_m values for the high-affinity sites were 0.5 and $1.1 \mu\text{M}$ for basolateral and brush-border membranes respectively. The latter value is similar to that ($K_m = 1.6 \mu\text{M}$) calculated for the high-affinity site reported in the present study. Although the later reports by Ghijsen *et al.* (1980) and De Jonge *et al.* (1981) associate Ca^{2+} -stimulated ATPase activity

exclusively with the basolateral membrane, Toury & Toury (1979) reported a stimulation of Ca^{2+} uptake into brush-border vesicles isolated from rat jejunum and ileum that were preloaded with ATP. Wilson & Lawson (1981) reported the presence of a phosphorylated vitamin D-responsive protein, mol.wt. 84000, in chick intestinal brush-border membrane. Treatment of the protein with hydroxylamine did not release phosphate, which indicated that the reaction product was not an acyl phosphate, typically found in ATPases.

A Ca^{2+} -binding complex, designated IMCal, which contains a Ca^{2+} -binding protein of high affinity, a Ca^{2+} -dependent ATPase and *p*-nitrophenyl phosphatase activity, was identified by Kowarski & Schachter (1980) in rat intestinal-mucosal homogenate. Whether any of the above proteins is analogous to the high-affinity binding protein reported in the present study remains to be determined. In any case, our results correlate well with those of Wilson & Lawson (1980), who reported a Ca^{2+} -specific high-affinity binding site in the chick intestinal brush-border membrane with an association constant for Ca^{2+} of $5 \times 10^5 \text{ M}^{-1}$. The latter authors also showed that the number of high-affinity sites was increased in the presence of vitamin D or by pretreatment of D-deficient birds with 1,25-dihydroxycholecalciferol. Preliminary Scatchard analyses of Ca^{2+} -binding data obtained for brush-border vesicles isolated from vitamin D-replete or vitamin D-deficient rats indicated a decrease of at least 20% in the total number of sites available for binding in the vitamin D-deficient membranes (A. Miller & F. Bronner, unpublished work). However, vitamin D did not significantly affect the Ca^{2+} -binding affinity of the different vesicle preparations, which is in close agreement with results reported by Wilson & Lawson (1980) for the chick.

The binding of Ca^{2+} by brush-border membrane vesicles was dependent on the ionic strength and H^+ concentration of the medium. The observed decrease in binding when the pH of the extravesicular medium was below 7.5 (Table 2 and Fig. 2) suggests that H^+ ions compete with Ca^{2+} for the same membrane binding sites. Increased H^+ concentrations block ionic channels (Hille, 1968; Woodhull, 1973) and the H^+ ion is bound much more strongly than Ca^{2+} . Consistent with the involvement of phosphate groups, Ca^{2+} bound to the low-affinity sites increased linearly as the external pH was raised from 6.5 to 8.0 (Fig. 2a) and continued to increase up to pH 9.0, probably because masked phosphate and carboxy groups became accessible for binding (Li *et al.*, 1975; Li & Katz, 1976). The dissociation constant for organic phosphate is in the range 10^{-7} – 10^{-8} M . Ca^{2+} binding to the high-affinity sites reached a maximum at approx. pH 8.0 (Fig. 2b).

Between pH 4.5 and 8.0 the involvement of carboxy groups, with ionization constant of 10^{-4} – 10^{-6} M , and phosphate is indicated.

With the possible exception of rat liver mitochondria (Reed & Bygrave, 1974), La^{3+} displaces membrane-bound Ca^{2+} but does not appear to be transported across cellular membranes to any great extent (Laszlo *et al.*, 1952; Langer & Frank, 1972; Sarkadi *et al.*, 1977; Reeves & Sutko, 1979). If it is assumed that La^{3+} is not transported across the brush-border membrane, then a large number of the low-affinity binding sites are most probably located on the external surface of the membrane. Since Ca^{2+} and La^{3+} bind to ionized oxygen groups electrostatically and La^{3+} does so with greater affinity because of a higher charge density and similar ionic radius (Williams, 1970), the Ca^{2+} -binding sites are probably acidic oxygen atoms, phosphate and carboxylate groups, as indicated previously.

Although the passive influx of Ca^{2+} into the intestinal epithelial cell is compensated by a Ca^{2+} -extrusion mechanism in the basolateral membrane, the brush-border membrane, in addition to acting as a permeability barrier, may be more directly involved in the regulation of the intracellular Ca^{2+} concentration. The kinetic data for the high-affinity binding site, assumed to be located on the inner membrane surface, indicate that half-maximal binding occurred in 30–60 s (Fig. 1b). If the cell required a fairly rapid Ca^{2+} uptake, this physiological requirement could be satisfied by either a specific channel or a carrier system. Countertransport stimulation of Ca^{2+} uptake and/or release constitutes strong evidence for a carrier function; however, under our experimental conditions, $^{45}\text{Ca}^{2+}$ taken up by brush-border vesicles apparently does not exchange significantly with unlabelled Ca^{2+} in the extravesicular medium (Fig. 6). Significant changes in net Ca^{2+} uptake and efflux in response to chemical gradients of the cation across the membrane would suggest substantial Ca^{2+} movement through a channel. However, the study of net Ca^{2+} movement across the brush-border membrane, as well as the determination of the diffusion or transport rates, remains difficult at present and may be of limited value because of the large amount of membrane-bound Ca^{2+} observed during uptake (Miller & Bronner, 1981). De Jonge *et al.* (1981) calculated that $3.3 \times 10^6 \text{ Ca}^{2+}$ ions/s can be transported out of each duodenal cell via the Ca^{2+} -ATPase pump located in the basolateral membrane. Assuming steady-state conditions, a similar rate for Ca^{2+} entry may be too fast for known carriers and could indicate the presence of a Ca^{2+} channel. The concept that Ca^{2+} transport across the brush-border membrane may be facilitated by a Ca^{2+} -specific protein channel has previously been suggested by Norman *et al.* (1982), Rasmussen *et al.* (1982) and Schachter &

Kowarski (1982). However, how the channel is controlled or regulated remains an open question.

Although speculative, an attractive model for Ca^{2+} uptake by brush-border membranes would be a Ca^{2+} channel with a high-affinity binding site located within the membrane field. This potential-sensitive Ca^{2+} -specific binding site, which is in either the free or the bound state, may regulate the flux and gating of the channel. Since the intestinal epithelial cell maintains a large electrochemical gradient for Ca^{2+} between the extracellular environment and the cytosolic interior, $>1\text{ mM}$ and $<1\text{ }\mu\text{M}$ respectively, the calculated affinity constant for Ca^{2+} , $K_a = 10^5$ – 10^6 M^{-1} , seems very reasonable for sites interacting with intracellular Ca^{2+} . In any case, it should be recognized that intracellular organelles such as mitochondria, endoplasmic reticulum and Ca^{2+} -binding proteins may also be involved in the regulation of intracellular Ca^{2+} concentrations as well as participating in transcellular Ca^{2+} transport. As emphasized above, high-affinity sites specific for Ca^{2+} may be associated with the regulation of membrane-bound enzymes. Therefore the functional role of the high-affinity site in Ca^{2+} uptake by brush-border membranes remains to be established, and further investigation will require the isolation of the protein in question and appropriate reconstitution studies.

This study was supported by NSF grant no. PCM 78-23366 and the University of Connecticut Research Foundation. Special appreciation goes to Dr. A. Scarpa, Johnson Research Foundation, University of Pennsylvania, for analysing the medium and brush-border membranes for Ca^{2+} content.

References

- Bennett, J. P., McGill, K. A. & Warren, G. B. (1978) *Nature (London)* **274**, 823–825
- Bronner, F. & Freund, T. (1975) *Am. J. Physiol.* **229**, 689–694
- Cheung, W. Y. (1980) *Science* **207**, 19–27
- Chevallier, J. & Butow, R. A. (1971) *Biochemistry* **10**, 2733–2737
- De Jonge, H. R., Ghijsen, W. E. J. M. & van Os, C. H. (1981) *Biochim. Biophys. Acta* **647**, 140–149
- Fleischer, S., Fleischer, B. & Stoeckenius, W. (1967) *J. Cell Biol.* **32**, 193–208
- Forstner, G. G., Tanaka, K. & Isselbacher, K. H. (1968) *Biochem. J.* **109**, 51–59
- Ghijsen, W. E. J. M. & van Os, C. H. (1979) *Nature (London)* **279**, 802–803
- Ghijsen, W. E. J. M., De Jonge, M. D. & van Os, C. H. (1980) *Biochim. Biophys. Acta* **599**, 538–551
- Hemminki, K. (1974) *Biochim. Biophys. Acta* **363**, 202–210
- Hendrickson, H. S. & Fullington, J. G. (1965) *Biochemistry* **4**, 1599–1605
- Hesketh, T. R., Smith, G. A., Houslay, M. D., McGill, K. A., Birdsall, N. J. M., Metcalfe, J. C. & Warren, G. B. (1976) *Biochemistry* **15**, 4145–4151
- Hildmann, B., Schmidt, A., Storelli, C. & Mürer, H. (1980) *Proc. Int. Union Physiol. Sci.* **14**, 472
- Hille, B. (1968) *J. Gen. Physiol.* **51**, 221–236
- Hosey, M. M. & Tao, M. (1977) *Curr. Top. Membr. Transp.* **9**, 233–320
- Hurst, R. D. (1964) *Can. J. Biochem.* **42**, 287–292
- Kowarski, S. & Schachter, D. (1980) *J. Biol. Chem.* **255**, 10834–10840
- Langer, G. A. & Frank, J. S. (1972) *J. Cell Biol.* **54**, 441–455
- Laszlo, D., Ekstein, D. M., Lewin, R. & Stern, K. G. (1952) *J. Natl. Cancer Inst.* **13**, 559–571
- Lever, J. E. (1980) *CRC Crit. Rev. Biochem.* **7**, 187–246
- Li, S.-T. & Katz, E. P. (1976) *Biopolymers* **15**, 1439–1460
- Li, S.-T. & Katz, E. P. (1977) *Calcif. Tissue Res.* **22**, 275–284
- Li, S.-T., Golub, E. & Katz, E. P. (1975) *J. Mol. Biol.* **98**, 835–839
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Luft, J. H. (1971) *Anat. Rec.* **171**, 347–368
- McDonald, J. M., Bruns, D. E. & Jarett, L. (1976) *J. Biol. Chem.* **251**, 5345–5351
- Miller, A., III & Bronner, F. (1981) *Biochem. J.* **196**, 391–401
- Miller, A., III, Ueng, T.-H. & Bronner, F. (1979) *FEBS Lett.* **103**, 319–322
- Norman, A. W., Putkey, J. A. & Nemere, I. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 78–83
- Palmer, R. F. & Posey, V. A. (1970) *J. Gen. Physiol.* **55**, 89–103
- Rasmussen, H., Matsumoto, T., Fontaine, O. & Goodman, D. B. P. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 72–77
- Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **138**, 239–252
- Reeves, J. P. & Sutko, J. L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 590–594
- Sarkadi, B., Szász, I., Gerlocozy, A. & Gárdos, G. (1977) *Biochim. Biophys. Acta* **464**, 92–107
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Schachter, D. & Kowarski, S. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 84–87
- Schlitz, L. & Marinetti, G. V. (1972) *Biochim. Biophys. Acta* **290**, 70–83
- Toury, C. & Toury, R. (1979) *C. R. Hebd. Seances Acad. Sci. Ser. D* **289**, 109–112
- Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974) *Biochemistry* **13**, 5501–5507
- Williams, R. J. P. (1970) *Q. Rev. Chem. Soc.* **24**, 331–365
- Williamson, J. R., Woodrow, M. L. & Scarpa, A. (1975) *Recent Adv. Stud. Card. Struct. Metab.* **5**, 61–71
- Wilson, P. W. & Lawson, D. E. M. (1980) *Pflügers Arch.* **389**, 69–74
- Wilson, P. W. & Lawson, D. E. M. (1981) *Nature (London)* **289**, 600–602
- Woodhull, A. M. (1973) *J. Gen. Physiol.* **61**, 687–708