Calcium Binding Fraction Detected from the Brush Border Membrane during Intestinal Calcium Absorption in the Rat

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Summary A fraction which had calcium binding activity was detected from the brush border membrane during intestinal calcium absorption in the rat. In situ duodenal loop experiment showed that $^{45}$Ca activity in mucosal homogenate, detergent-solubilized whole particulate fraction and detergent-solubilized brush border membrane was maximal at 30 min after the radioactive calcium solution was injected in the loop. The calcium radioactivities did not co-precipitate with anti-IMCal (integral membrane calcium binding protein) antibody. Gel permeation HPLC showed three calcium peaks from both solubilized whole particulate fraction and brush border membrane. In vitro calcium binding assay using the solubilized brush border membrane from the pylorus to proximal ileum showed the existence of specific binding sites with a dissociation constant of $2.19\pm0.27$ mM (mean$\pm$SE) while the number of binding sites was $3.4\pm0.4$ nmol/mg of protein. After in vitro binding, the solubilized brush border membrane was analyzed by gel filtration chromatography. Two calcium peaks, apparently 200 and 1 kDa, were separated and the former peak seemed to be the specific binding fraction but did not contain IMCal. This fraction may bind luminal calcium during intestinal calcium absorption.

Key Words calcium binding fraction, brush border membrane, intestinal calcium absorption, binding assay, IMCal, rat

Calcium is extremely important as a messenger of signal transduction and an element of bone mineralization, so that it has been extensively studied in that context. However, the mechanism of calcium absorption has not been well characterized despite the fact that no living organism can produce the element and the intestinal absorption of dietary calcium is essential for them. Currently, there

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are three possible mechanisms of calcium absorption: active transport, transport by a paracellular route, and transport by an endocytotic vesicular flow (1). Active transport, which is regulated by 1,25-dihydroxyvitamin D, involves three steps: entry across the brush border membrane, transport within the cytoplasm of the enterocyte, and extrusion across the basolateral membrane (2). The first step is not energy-dependent but requires saturable components on the brush border membrane (3). Intracellular transport is known to be facilitated by calbindin D (4). The extrusion step is an energy-dependent process which was first demonstrated by the everted gut sac method (5), and it was subsequently proven that calcium-dependent ATPase is responsible for this step (6). The paracellular diffusion process occurs in whole intestine when the luminal calcium concentration increases (7). The vesicular flow requires binding to the brush border membrane which then endocytosed in the cytoplasm to the basolateral membrane where the vesicle is exocytosed (8). Although both active transport and vesicular flow require calcium binding sites on the brush border membrane, no calcium channels or transporters have been reported (3).

To date, several brush border calcium-binding proteins have been reported. Brush border calmodulin (9) seems to be a microvillus core protein but it binds to a brush border membrane protein where it probably plays a regulatory role in calcium transport across the brush border (10). Calbindin D is mainly cytosoluble protein (11) although some of which is associated with the brush border membrane (12). IMCal (integral membrane calcium binding protein) is a vitamin D-dependent integral membrane protein but a direct relationship with calcium absorption has not been established (13, 14). The relationship between those proteins and brush border transport carrier(s) was not extensively investigated. We report here a calcium-binding fraction on the brush border membrane of the rat and relations with IMCal during intestinal calcium absorption.

**Experimental. Animals:** Male Wistar rats with body weights of 200–250 g were obtained from Saitama Experimental Animals (Saitama, Japan) and were fed a normal calcium diet with 1.2% Ca and 0.9% P (Oriental Yeast Co., Tokyo, Japan).

**In situ loop experiment:** Three to four animals were used in each experiment. After fasting overnight with water ad libitum, the duodenum was exposed by abdominal incision under the ether anesthesia and a loop was made by ligation immediately distal to Vater's papilla and 10 cm distal from there. For each rat, 0.75 ml of the calcium solution consisted of 20 mM CaCl₂, 120 mM NaCl, 4.9 mM KCl, and 370 kBq/ml ⁴⁵CaCl₂ in 10 mM Tris-HEPES, pH 7.0 was injected to the loop. Five, 30, or 120 min after the injection, rats were sacrificed by exsanguination from the abdominal aorta and the loops were removed. All of the following procedures were performed at 4°C. The duodenal loops were slit lengthwise in 10 ml of saline to collect luminal solution and the duodenal mucosa was scraped off with a glass slide. To obtain whole particulate fraction, the scraped mucosa was homogenized using a Polytron in 119 mM NaCl, 4.7 mM KCl, 0.1 mM phenylmethylsulfonyl

fluoride (PMSF), and 100 kU/ml aprotinin in 13 mM Tris-HCl, pH 7.4 and centrifuged at 140,000 × g for 60 min (15). The precipitate (whole particulate fraction) was solubilized in 1 ml/g of the solubilizing buffer consisting of 1.5% (v/v) Emulgen 109P (C12H25O(CH2CH2O)9H, Kao Co., Tokyo, Japan) and 0.1 mM PMSF in 13 mM Tris-HCl, pH 7.4 for 1 h then re-homogenized in a Potter-Elvehjem homogenizer and centrifuged at 10,000 × g for 30 min to obtain the supernatant as the detergent-solubilized whole particulate fraction. Emulgen 109P has an advantage compared with Triton X-100 because it does not have 280 nm absorbance, so that the solution can be measured by the spectrophotometer equipped with the HPLC system. To obtain brush border membrane, the magnesium precipitation method was employed (16). Briefly, the scraped mucosa was homogenized in a Waring Blender in 50 mM D-mannitol, 0.1 mM PMSF, and 100 kU/ml aprotinin in 2 mM Tris-HCl, pH 7.1, added solid MgCl2 to 20 mM and centrifuged at 6,000 × g for 10 min. The resultant supernatant was centrifuged at 27,000 × g for 30 min then the precipitate was homogenized in a Potter-Elvehjem homogenizer and added 10 mM MgSO4 to final concentration of 0.1 mM. This solution was again centrifuged at 6,000 × g for 10 min then 27,000 × g for 30 min. The precipitate (brush border membrane) was suspended in the solubilizing buffer, passed through a 27-gauge needle and solubilized as described above.

High-performance liquid chromatography (HPLC): Gel permeation chromatography was performed using a TSK Gel 3000SW column (7.5 mm × 30 cm, Tosoh, Tokyo, Japan) with TSK guard column (7.5 mm × 7.5 cm, Tosoh) in HLC-803D system (Tosoh). Elution buffer was 0.1% (v/v) Emulgen 109P and 0.3 M NaCl in 13 mM Tris-acetate, pH 7.3 and flow rate was 1 ml/min.

Immunoprecipitation against anti-IMCal antibody: IMCal (integral membrane calcium binding protein) was purified (13) and polyclonal antibody was raised in the rabbit. Two to four hundred microliters of the solubilized whole particulate fraction and the brush border membrane from the duodenal loop (0.4–4 mg protein) was added to 100 µl of anti-IMCal or control serum and incubated at 4°C for 16 h then 200 µl of Protein A was added and incubated at room temperature for 1 h. The solution was centrifuged at 2,000 × g for 10 min and washed three times, and the precipitate was solubilized in 1 N NaOH followed measurement of the radioactivity.

Calcium binding assay: Four rats were used in each experiment. The brush border membrane was prepared from four segments of the small intestine (15 cm from pylorus—duodenum and proximal jejunum, distal jejunum, proximal ileum, and distal ileum) and solubilized in 1.5% Emulgen 109P, 100 mM D-mannitol, and 100 mM NaCl in 10 mM Tris-HEPES, pH 7.5 after passing through a 27-gauge needle, then centrifuged at 100,000 × g for 30 min. Twenty microliters of the supernatant which contained 15–30 µg protein was spotted onto a nitrocellulose sheet (0.45 µm, Schleicher & Schuell, Dassel, Germany) using an Immunoblot microfiltration apparatus (Atto Corporation, Tokyo, Japan) under vacuum suction, then the spot was cut out and incubated in 1 ml of the incubation medium
(0.1% Emulgen 109P, 100 mM D-mannitol, and 100 mM NaCl in 10 mM Tris-HEPES, pH 7.5 with 0.1–15 mM CaCl₂ and 0.37–55.5 kBq ⁴⁵CaCl₂) at 4°C for 30 min. For the assay of nonspecific binding, 10 mM to 1.5 mM cold CaCl₂ was used. After the incubation time, the sheet was washed three times with 100 mM D-mannitol and 150 mM MgCl₂ in 10 mM Tris-HEPES, pH 7.5. The sheet was immersed in ACS-II (Amersham International, Amersham, U.K.) to solubilize and count the radioactivity and analyzed by a Scatchard analysis.

**Gel filtration:** Solubilized brush border membrane was incubated in the same condition as in the binding assay. After the incubation, the solution was dialyzed against the elution buffer (0.1% Emulgen 109P and 0.3 M NaCl in 13 mM Tris-HCl, pH 7.4) and applied on Bio-Gel A-0.5 m (Bio-Rad, Watford, U.K., 1.9 × 37.5 cm column).

**Western blot analysis:** Concentrated ⁴⁵Ca peak from Bio-Gel A-0.5 m was analyzed on 5–20% SDS-polyacrylamide gradient gel electrophoresis and transferred to a nitrocellulose sheet. Immunoblot was performed using rabbit anti-rat IMCa1 antibody diluted to 1 : 500, biotin conjugated goat anti-rabbit IgG and avidin conjugated horseradish peroxidase with 3,3′-diaminobenzidine as a substrate of color reaction (17).

**Other methods:** Activities of sucrase (EC 3.2.1.26), alkaline phosphatase (EC 3.1.3.1), succinate dehydrogenase (EC 1.3.99.1), Na,K-ATPase (EC 3.6.1.3), glucose-6-phosphatase (EC 3.1.3.9), and NADPH-cytochrome c reductase (EC 1.6.2.4) were determined by the methods of Dahlqvist (18), Kowarski and Schachter (19), Pennington (20), Scharschmidt et al. (21) with slight modification, Gierow and Jergil (22) and Williams and Kamin (23), respectively. Sucrase and alkaline phosphatase are marker enzymes for brush border, succinate dehydrogenase is for mitochondria, Na,K-ATPase is for basolateral membrane, and glucose-6-phosphatase and NADPH cytochrome c reductase are for microsomes. Protein concentration was determined by the method of Lowry et al. (24).

The protocol of this study was approved by the Animal Ethics Steering Committee of Nippon Medical School.

**Results and discussion.** Five, 30, and 120 min after the injection of the calcium solution into the in situ duodenal loop, ⁴⁵Ca activity was measured in plasma, mucosal homogenate, detergent-solubilized whole particulate fraction, or brush border membrane. Purity of the brush border membrane was confirmed by sucrase and alkaline phosphatase activities (Table 1A). In the mucosal homogenate, the detergent solubilized the whole particulate fraction and the detergent solubilized brush border membrane, the maximal ⁴⁵Ca activity was obtained at 30 min after the injection (Table 2). The percentage of the radioactivity in the brush border membrane for the radioactivity in the homogenate was maximal at 30 min, which suggests that the main radioactivity in the brush border membrane moved to the plasma and this radioactive calcium was substantially absorbed. However, the percentage of the radioactivity in the whole particulate fraction for the radioactivity in the homogenate showed the maximal value at 120 min, which suggests that
Table 1. Purity of the brush border membrane.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sucrase</th>
<th>Alkaline phosphatase</th>
<th>Succinate dehydrogenase</th>
<th>Na,K-ATPase</th>
<th>Glucose-6-phosphatase</th>
<th>NADPH cytochrome c reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10.54 (7)</td>
<td>8.76 (4)</td>
<td>0.049 (4)</td>
<td>0.55 (1)</td>
<td>2.27 (4)</td>
<td>ND</td>
</tr>
<tr>
<td>B</td>
<td>31.36 (8)</td>
<td>ND (8)</td>
<td>0.118 (8)</td>
<td>0.99 (8)</td>
<td>ND (8)</td>
<td>0.83 (8)</td>
</tr>
</tbody>
</table>

A shows in situ loop experiment and B shows the binding assay. Relative specific activities to homogenate are shown. Parentheses indicate number of experiments. ND, not determined.

Table 2. In situ loop experiment.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>n</th>
<th>Absorption (%)</th>
<th>Plasma (%)</th>
<th>Mucosal homogenate (%)</th>
<th>Solubilized brush border membrane (%)</th>
<th>Solubilized whole particulate fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4</td>
<td>44.96</td>
<td>1.80</td>
<td>3.70</td>
<td>0.011 (0.38)</td>
<td>—</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>71.71</td>
<td>6.07</td>
<td>7.74</td>
<td>0.034 (0.42)</td>
<td>—</td>
</tr>
<tr>
<td>120</td>
<td>4</td>
<td>98.13</td>
<td>2.88</td>
<td>3.91</td>
<td>0.012 (0.32)</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>31.85</td>
<td>1.76</td>
<td>5.33</td>
<td>—</td>
<td>2.20 (40.39)</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>76.19</td>
<td>5.52</td>
<td>5.98</td>
<td>—</td>
<td>2.42 (40.61)</td>
</tr>
<tr>
<td>120</td>
<td>3</td>
<td>98.95</td>
<td>3.08</td>
<td>2.77</td>
<td>—</td>
<td>1.64 (64.94)</td>
</tr>
</tbody>
</table>

$^{45}$Ca distribution after the injection of the calcium solution contained $^{45}$CaCl$_2$ into the duodenal loop. Absorption was calculated by subtracting remained luminal radioactivity from injected radioactivity. Values are expressed as the percentage of the radioactivity in each fraction for the injected radioactivity. Values in parentheses indicate the percentage of the radioactivity in each solubilized fraction for the radioactivity in the homogenate. $^1n=2$.

some of the radioactive calcium remained bound to intracellular membranes. Three calcium peaks were shown on a gel permeation HPLC from the detergent-solubilized brush border membrane, whose apparent molecular weights were estimated to be over 500, 200, and under 1 kDa. Figure 1 shows a representative elution pattern of the solubilized brush border membrane. The solubilized whole particulate fraction showed a similar pattern (data not shown). The radioactivity in each peak did not show a particular tendency to change with time after the injection.

Immunoprecipitation using anti-rat IMCal (integral membrane calcium binding protein) showed no radioactivity in the precipitate (data not shown). This result suggests that IMCal did not receive absorbed calcium directly.

In the in vitro calcium binding assay on the nitrocellulose sheet, specific binding was shown in the solubilized brush border membrane from upper three segments.
Fig. 1. A representative elution pattern of the solubilized brush border membrane after in situ loop experiment on gel permeation HPLC. Fifty microliters of solubilized brush border membrane was applied on TSK Gel 3000SW column (7.5 mm × 30 cm) and eluted with 0.1% Emulgen 109P and 0.3 M NaCl in 13 mM Tris-acetate, pH 7.3. Flow rate was 1 ml/min. Solid line indicates protein concentration measured by absorbance of 280 nm (arbitrary units). Dotted line indicates 45Ca activity (dpm). Arrows indicate molecular weight standards: bovine thyroglobulin, 670 kDa; bovine γ-globulin, 158 kDa; chicken ovalbumin, 44 kDa; horse myoglobin, 17 kDa; vitamin B12, 1.35 kDa (Bio-Rad).

but not from distal ileum (Table 3). The dissociation constant \( (K_d) \) was \( 2.19 ± 0.27 \) mM (mean ± SE) and the number of binding sites was \( 3.4 ± 0.4 \) nmol/mg of protein and no significant differences were observed among the three segments by one-way ANOVA \( (p < 0.05) \). The purity of the brush border membrane was approximately

<table>
<thead>
<tr>
<th>Position</th>
<th>( n )</th>
<th>Dissociation constant ( (K_d) ) (mM)</th>
<th>Number of binding sites (nmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 cm from pylorus</td>
<td>4</td>
<td>2.69 ± 0.23</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Distal jejunum</td>
<td>4</td>
<td>2.04 ± 0.61</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>Proximal ileum</td>
<td>4</td>
<td>1.86 ± 0.53</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>Distal ileum</td>
<td>3</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>12</td>
<td><strong>2.19 ± 0.27</strong></td>
<td><strong>3.4 ± 0.4</strong></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE. Distal ileum did not show specific binding. Significant differences were not detected among 15 cm from pylorus, distal jejunum, and proximal ileum by one-way ANOVA \( (p < 0.05) \).

31-fold greater than the homogenate, and contamination of the mitochondria, basolateral membrane, and microsome membrane were negligible (Table 1B).

After the in vitro calcium-binding experiment the solution contained $^{45}$Ca, and brush border membrane was applied on Bio-Gel A-0.5 m chromatography. Two separate $^{45}$Ca peaks were seen with apparent molecular weights were 200 and 1 kDa (Fig. 2). The 200-kDa peak disappeared when the binding experiment was performed in the nonspecific condition (100-fold concentration of cold calcium was added), suggesting that this peak may be a specific binding fraction. It is likely that 200-kDa HPLC peak from the in situ loop experiment and the 200-kDa peak from the solution after the in vitro binding experiment contained the same calcium binding fraction. The 1-kDa peak may be non-protein bound calcium. The first small peak in HPLC was not detected in this condition. Western blot analysis showed that the brush border membrane reacted against anti-IMCal antibody but the 200-kDa peak did not (Fig. 3), suggesting that the fraction may not contain IMCal which distributed on the brush border membrane.

![Fig. 2. A representative elution pattern of the solubilized brush border membrane after incubation with $^{45}$Ca on gel filtration chromatography. Approximately 4 ml of the sample (8 mg of protein) was applied on Bio-Gel A-0.5 m column (1.9 × 37.5 cm). Elution buffer was 0.1% Emulgen 109P and 0.3 m NaCl in 13 mM Tris-HCl, pH 7.4. Approximately 2.3 ml fraction was collected and protein (absorbance at 280 nm) and $^{45}$Ca activity (dpm) were measured. Solid line shows $^{45}$Ca activity and dotted line shows protein concentration. $V_o$ indicates the void volume and numbers indicate molecular weight standards (same as in Fig. 1).](image-url)
Fig. 3. Western blot analysis. Solubilized brush border membrane (lane 1, 44 μg of protein) and concentrated calcium peak (lane 2, 8.4 μg of protein) were applied on 5–20% SDS polyacrylamide gradient gel electrophoresis. After transfer to a nitrocellulose sheet, it was reacted with anti-IMCal antibody. The brush border membrane reacted but the calcium peak did not react with the antibody.

Those results suggest that the specific calcium binding fraction might bind calcium in the intestinal lumen and has a physiological importance since the order of magnitude of the $K_d$ was mM and the calcium concentration in the intestinal lumen should be the same order of magnitude (2).

The calcium entry step from the brush border was studied by several methods including in situ loop, everted gut sac, and brush border membrane vesicles (2). In this report, we used solubilized brush border membrane as a sample for the binding assay. This method may disrupt the membrane structure and eliminate the effects of membrane lipid, but it may detect the interaction between membrane proteins and calcium.

There are some reports of affinity of the brush border for calcium. In rats, Bronner and co-workers estimated $K_d$ and the number of binding sites using isolated brush border membrane vesicles and reported two classes of calcium binding sites in which the high-affinity site showed a $K_d$ of $1.6 \mu M$ and 0.8 nmol/mg protein of binding sites while the low-affinity site showed a $K_d$ of 3.6 mM and 33 nmol/mg protein of binding sites (25). Our results are consistent with the latter low-affinity site although they postulated that this site might be a membrane lipid. On the other hand, only high-affinity binding site was obtained from chick duodenum (26). Since their studies were performed using membrane vesicles, direct comparison
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with our method would be difficult, and no study could identify the calcium binding fraction.

There are several membrane-bound calcium binding proteins in the enterocyte (9, 11, 13) but calmodulin, calbindin D9K, and IMCal have μM order of magnitude of $K_d$, suggesting that they bind intracellular calcium and have different roles in calcium absorption from the calcium binding fraction we report. The time course experiment revealed that whole radioactivity in the brush border membrane was reduced, which suggests that the absolute amount of radioactivity in the peak might be absorbed, although the percentages of the radioactivity in three peaks did not change with time from the calcium injection. Calcium absorption is regulated mainly by 1,25-dihydroxyvitamin D but we did not search the effect of vitamin D on the binding fraction. Further purification and characterization of the fraction is needed to elucidate its role in calcium absorption.

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