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Ca^{2+} Dependency of Na^+ Transport by Rabbit Renal Brush Border Membrane

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Summary. Intracellular Ca^{2+} has been suggested to play an important role in the regulation of epithelial Na^+ transport. Previous studies showed that preincubation of toad urinary bladder, a tight epithelium, in Ca^{2+}-free medium enhanced Na^+ uptake by the subsequently isolated apical membrane vesicles, suggesting the downregulation of Na^+ entry across the apical membrane by intracellular Ca^{2+}. In the present study, we have examined the effect of Ca^{2+}-free preincubation on apical membrane Na^+ transport in a leaky epithelium, i.e., brush border membrane (BBM) of rabbit renal proximal tubule. In contrast to toad urinary bladder, it was found that BBM vesicles derived from proximal tubules incubated in 1 mM Ca^{2+} medium exhibited higher Na^+ uptake than those derived from proximal tubules incubated in Ca^{2+}-free EGTA medium. Such effect of Ca^{2+} in the preincubation medium was temperature dependent and could not be replaced by another divalent cation, Ba^{2+} (1 mM). Ca^{2+} in the preincubation medium did not affect Na^+-dependent BBM glucose uptake, and its effect on BBM Na^+ uptake was pH gradient dependent and amiloride (10^{-3} M) sensitive, suggesting the involvement of Na^+/H^+ antiport system. Addition of verapamil (10^{-4} M) to 1 mM Ca^{2+} preincubation medium abolished while ionomycin (10^{-6} M) potentiated the effect of Ca^{2+} to increase BBM Na^+ uptake, suggesting that the effect of Ca^{2+} in the preincubation medium is likely to be mediated by Ca^{2+}-dependent cellular pathways and not due to a direct effect of extracellular Ca^{2+} on BBM. Neither the proximal tubule content of cAMP nor the inhibitory effect of 8,bromo-cAMP (0.1 mM) on BBM Na^+ uptake was affected by the presence of Ca^{2+} in the preincubation medium, suggesting that Ca^{2+} in the preincubation medium did not increase BBM Na^+ uptake by removing the inhibitory effect of cAMP. Addition of calmodulin inhibitor, trifluoperazine (10^{-4} M) to 1 mM Ca^{2+} preincubation medium did not prevent the increase in BBM Na^+ uptake. The effect of Ca^{2+} was, however, abolished when protein kinase C in the proximal tubule was downregulated by prolonged (24 hr) incubation with phorbol 12-myristate 13-acetate (10^{-6} M). In summary, these results show the Ca^{2+} dependency of Na^+ transport by renal BBM, possibly through stimulation of Na^+/H^+ exchanger by protein kinase C.

Key Words renal brush border membrane · Na^+/H^+ exchange · intracellular calcium · cAMP · calmodulin · protein kinase C

Introduction

In Na^+ transporting epithelia, the apical Na^+ entry is usually the rate-limiting step of the transepithelial Na^+ transport and is subject to wide range of variation. In order to prevent sharp changes in cellular Na^+ activity, Na^+ entry across the apical membrane is normally under the control of a variety of hormones and intracellular regulatory processes [22].

While it remains not fully understood how the apical membrane Na^+ transport activity is regulated, intracellular Ca^{2+} has been suggested to play an important role [31]. An increase in intracellular Na^+ activity, resulting from either an increased apical Na^+ uptake or a decreased basolateral pump activity, may raise intracellular Ca^{2+} through Na^+/Ca^+ exchange mechanism and reduce apical membrane Na^+ permeability [4, 25, 31]. In tight epithelia, such as frog skin, toad urinary bladder and renal distal tubules, removal of contraluminal Na^+ reduces apical Na^+ entry in a Ca^{2+}-dependent manner [11], and an increase in intracellular Ca^{2+} induced by quinidine or Ca^{2+} ionophore reduces apical Na^+ entry [24, 30]. Further support for the notion that increased levels of intracellular Ca^{2+} reduce apical membrane Na^+ permeability was provided by studies with isolated membrane vesicles. With toad urinary bladder, Chase et al. found that the Na^+ uptake by the isolated apical membrane vesicles was inhibited by either direct addition of Ca^{2+} [6] or by maneuvers which raise intracellular Ca^{2+} levels prior to the isolation of the apical membrane [5]. By incubating toad urinary bladder in Ca^{2+}-free medium, Garty et al. also reported an activation of Na^+ channels in the subsequently isolated apical membrane vesicles [9, 10], consistent with the contention that intracellular Ca^{2+} downregulates apical membrane Na^+ transport.

In leaky epithelium, such as renal proximal tubule, similar interactions between intracellular Ca^{2+}

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Table 1. Enrichment of enzyme markers in BBM vesicles isolated from proximal tubules preincubated in Ca^{2+}-free (I) or 1 mM Ca^{2+} (II) KRB medium for 30 min, and from proximal tubules incubated in culture medium for 24 hr (III)

	Alkaline phosphatase	Na,K-ATPase	Cytochrome *c* oxidase	Glucose-6-phosphatase
I ($n = 4$)	8.2 ± 0.3	0.85 ± 0.03	0.32 ± 0.04	0.61 ± 0.08
II ($n = 5$)	8.7 ± 0.5	0.90 ± 0.07	0.29 ± 0.03	0.70 ± 0.11
III ($n = 3$)	9.5 ± 0.8	0.83 ± 0.02	0.29 ± 0.06	0.67 ± 0.09

and Na^+ transport have also been proposed. Raising intracellular Ca^{2+} levels by means of lowering peritubular Na^+ concentrations, increasing external Ca^{2+} concentrations, or addition of quinidine or Ca^{2+} ionophore, was shown to inhibit Na^+ transport by proximal tubules [8, 17]. The mechanism whereby intracellular Ca^{2+} inhibits Na^+ transport remains unclear, however. It is not certain if intracellular Ca^{2+} also exerts inhibitory effect on Na^+ transport across the luminal brush border membrane (BBM) in renal proximal tubule. With the difference in apical membrane Na^+ transport mechanism between tight and leaky epithelia, i.e., Na^+-permselective channels for the former and carrier-mediated processes for the latter, observations made in toad urinary bladder may not necessarily be applicable to renal proximal tubule. In fact, in the in vitro isolated perfused rabbit renal proximal tubules, McKinney et al. reported that a reduction of luminal Ca^{2+} concentration rather suppressed the fluid transport rate [19]. With the isolated rabbit renal BBM vesicles, Aronson et al. found that direct addition of Ca^{2+} did not affect the Na^+ uptake by these vesicles [1].

In the present study, we have examined the effect of preincubating rabbit renal proximal tubules in either 1 mM Ca^+ or nominally Ca^{2+}-free solution on the subsequently isolated BBM Na^+ uptake. It was found that preincubation of proximal tubules in Ca^{2+}-free medium rather led to a decrease in BBM Na^+ uptake.

Materials and Methods

Materials

$^{22}Na^+$ (carrier free) was purchased from New England Nuclear (Boston, MA), verapamil hydrochloride from Knoll Pharmaceutical (Whippany, NJ), EGTA from Fluka (Switzerland); all other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Methods

Experimental Animals

New Zealand white rabbits weighing 1.5–2.5 kg were used in these studies. The animals were maintained on an ad libitum diet of standard rabbit chow with free access to tap water for drinking.

Preparation of BBM Vesicles

Effect of Ca^{2+} was examined by preparing BBM vesicles from suspension of pretreated proximal tubules. Proximal tubule suspension was prepared by the method described previously [7, 20]. In brief, after rabbits were killed by cervical dislocation under light anesthesia (nembutol 5 mg/kg i.v.), kidneys were rapidly excised and the renal cortex was removed, minced in small pieces and homogenized with five strokes in a Dounce homogenizer (B pestle). The homogenate was filtered through 250 and 83 μm pore size nylon mesh (Nytex, Tetco, Elmsdorf, NY). The proximal tubules retained on the 83-μm mesh screen were removed, washed three times and suspended in modified Krebs-Ringer bicarbonate (KRB) buffer with the following composition (mM): NaCl 120, $NaHCO_3$ 25, KCl 5.0, $MgSO_4$ 1.2, $NaHPO_4$ 1.2, L-lactate 3, L-alanine 3, Na-acetate 3, glucose 5, containing either 1 mM $CaCl_2$ or 0.5 mM ethylene glycol bis-(-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and pre-equilibrated with 95% O_2/5% CO_2 (vol/vol) at 25°C (pH 7.4). Proximal tubules were incubated in this medium in a metabolic shaker (40 cycles/min) at 25°C for 30 min. In order to avoid BBM exposure to Ca^{2+} during isolation procedure, proximal tubules were washed three times in ice-cold Ca-free KRB at the end of the incubation and BBM vesicles were isolated by $MgCl_2$ precipitation method [3]. Table 1 shows the enrichment of enzyme markers in BBM as compared to proximal tubules. In general, there was a significant increase in BBM enzyme marker alkaline phosphatase [16], while other enzyme markers such as Na,K-ATPase for basolateral membrane [21], cytochrome *c* oxidase for mitochondria [29] and glucose-6-phosphatase for endoplasmic reticulum [14] were reduced. There was no difference between BBM vesicles obtained from proximal tubules incubated under different conditions.

Measurement of BBM Na^+ and Glucose Uptake

Transport experiments were performed at room temperature by a Millipore rapid filtration procedure [2]. BBM vesicles were prepared and suspended in a medium comprised of 300 mM mannitol, 10 mM $MgSO_4$ and 10 mM Tris-16 mM HEPES, pH 7.5. $^{22}Na^+$ uptake was measured in the presence of an outward H^+ gradient induced by preincubation of vesicles for 2 hr at room temperature in a medium comprised of 273 mM mannitol, 10 mM $MgSO_4$, 9 mM Tris, 14 mM HEPES and 30 mM MES, pH 6.0. Uptake was initiated by incubation of vesicles with a medium comprised of 1 mM $^{22}NaCl$ (2.5 μCi/ml), 286 mM mannitol, 2 mM $MgSO_4$, 13 mM Tris, 15 mM HEPES, 6 mM MES, pH 7.5, and was stopped by an ice-cold isosmotic solution. The rate of Na^+ uptake was calculated from the accumulated $^{22}Na^+$ and expressed in moles per milligram protein per unit time. BBM ^{14}C-

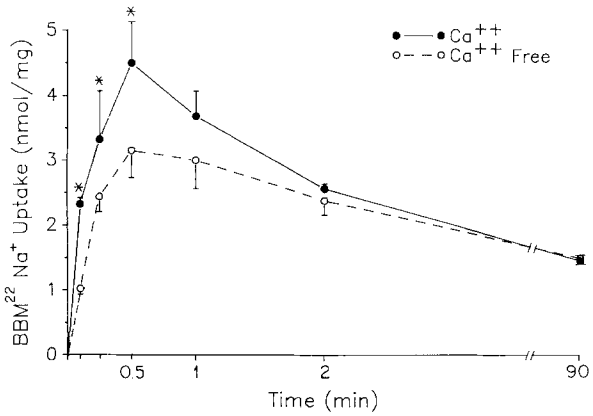

Fig. 1. Effect of Ca^{2+} on BBM Na^+ uptake. The initial Na^+ uptake was higher in BBM vesicles isolated from proximal tubules preincubated in medium containing 1 mM Ca^{2+} (●—●) as compared to BBM vesicles isolated from proximal tubules preincubated in nominally Ca^{2+} free (0.5 mM EGTA) medium (○---○). (Mean ± SE, $n = 4$, $^*P < 0.05$)

glucose uptake was initiated by incubation of vesicles with a medium comprised of 100 mM NaCl, 100 mM mannitol, 5 mM HEPES-Tris, pH 7.5, and 0.1 mM [U-^{14}C]-glucose (2.5 μCi/ml). Sodium-dependent glucose uptake was determined from the difference of accumulated [U-^{14}C-]-glucose in the presence or absence of sodium in the uptake solution. Protein concentration was assayed using Coomassie Brilliant blue G250 with bovine serum albumin as the reference protein [23]. All measurements were carried out in triplicate with freshly prepared membranes.

Intracellular Calcium (Ca_i) Measurements

Ca_i of proximal tubular cells was measured in a suspension of proximal tubules with the cell permeant fluorescent calcium indicator fura 2/AM. Proximal tubules were incubated with fura 2/AM (5 μM) in nominally calcium-free KRB at 25°C for 30 min. After repeated medium wash, proximal tubules suspended in 1.5 ml of calcium-free KRB were placed on an inverted stage fluorescence microscope (Diavert, Leitz, Rockleigh, NJ) in a custom-made chamber. Fluorescence measurements were made with a computer-assisted microscopic spectrofluorometer (MPVSP, Leitz). For each measurement the excitation wavelength was altered between 350 and 380 nm by movement of the respective filter (UVF 350 and 380, Melles Griot, Irvine, CA) placed in the excitation pathway. The emission was collected at 500 nm. The viewing field was focused though a 40× objective lens (UVFL-40, Olympus) and the boundaries were defined by an adjustable rectangular diaphragm. In general, not more than five tubule segments were present in the viewing field. Autofluorescence was determined with comparable number of tubules without fura 2/AM loading and was subtracted from the fluorescence measurements made at respective excitation wavelengths. The ratio of 350 to 380 thus obtained (R) was used for calculation of Ca_i according to the equation [13]

$$Ca_i = K_d \cdot (R - R_{min}/R_{max} - R) \cdot (Sf2/Sb2)$$

where K_d is 224 nM; R_{min} and R_{max} are R obtained in calcium free and 1 mM calcium solution, respectively; $Sf2$ and $Sb2$ are 380 nm measurements in calcium-free and 1 mM calcium solution, respectively. Ca_i was measured at 1, 3 and 5 min after the addition of calcium or test agents, and the mean of the three measurements was obtained for each period.

Table 2. Effect of preincubation time[a]

	Preincubation time	
	1 min	30 min
	(nmol/mg/5 sec)	
Ca^{2+}-free	1.47 ± 0.26	1.32 ± 0.09
1 mM Ca^{2+}	1.98 ± 0.08[b]	1.82 ± 0.33[b]

[a] The difference in Na^+ uptake by BBM vesicles isolated from proximal tubules preincubated in 1 mM Ca^{2+} or Ca^{2+}-free medium was already present with preincubation time as short as 1 min. (Mean ± SE; $n = 3$; [b]$P < 0.05$ *vs.* Ca^{2+}-free.)

cAMP Assays

After incubation in medium containing either 1 mM Ca^{2+} or 0.5 mM EGTA, proximal tubules were separated from the incubation medium and were extracted with 5% trichloroacetic acid. To determine total cAMP, neutralized extract and incubation medium from each sample were pooled and eluted from AG 1-X2 columns (recovery of [^{3}H]cAMP was 80–90%). The purified samples were dried by lyophilization and resuspended in 0.05 M sodium acetate buffer, pH 6.2. Aliquots were analyzed for their cAMP content with a commercial radioimmunoassay kit (New England Nuclear, Boston, MA). Protein content was measured in separate samples.

Statistical Analysis

Data are presented as mean ± SE and compared with Student t tests for paired and unpaired data as appropriate.

Results and Discussion

The initial Na^+ uptake by BBM vesicles derived from proximal tubules preincubated in 1 mM Ca^{2+} medium for 30 min at 25°C was increased as compared to those derived from proximal tubules incubated in Ca^{2+}-free EGTA medium (2.28 ± 0.1 *vs.* 1.54 ± 0.08 nmol/mg/5 sec, $n = 14$, $P < 0.02$). BBM Na^+ uptake as the function of time and the effect of Ca^{2+} in proximal tubule preincubation medium are shown in Fig. 1. Such effect was found to be temperature dependent so that the difference in BBM Na^+ uptake was reduced when proximal tubules were preincubated at either 4 or 37°C as compared to 25°C (Fig. 2). Alteration in BBM Na^+ uptake took place within short period of time so that the difference in BBM Na^+ uptake could be seen with proximal tubule preincubation time as short as 1 min (Table 2). Such effect on BBM Na^+ uptake

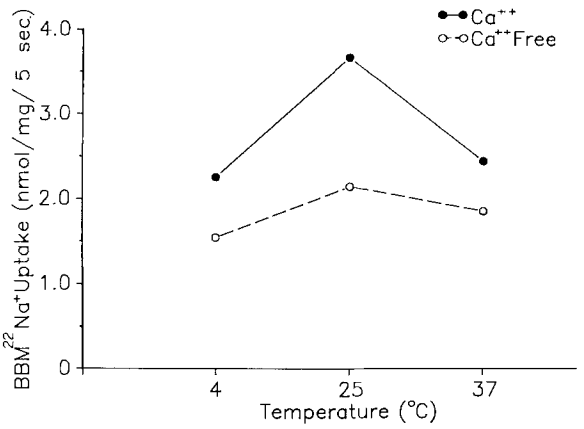

Fig. 2. Effect of preincubation temperature. The difference between the initial Na^+ uptake by BBM vesicles isolated from proximal tubules preincubated in 1 mM Ca^{2+} (●—●) and Ca^{2+}-free (O---O) medium was reduced at preincubation temperature of either 4 or 37°C as compared to 25°C. (Result from a representative study is shown)

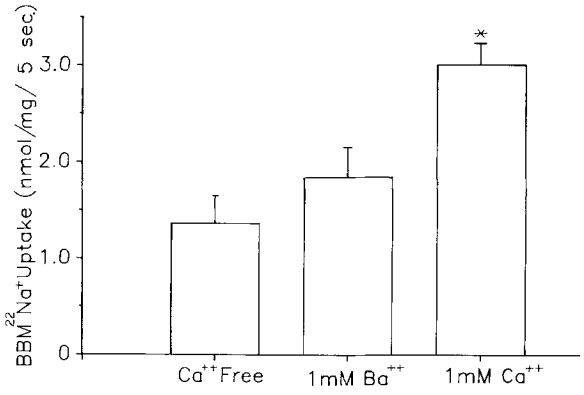

Fig. 3. Effects of Ba^{2+} and Ca^{2+} on BBM Na^+ uptake. Replacement of Ca^{2+} in the proximal tubule preincubation medium by another divalent cation, Ba^{2+} (1 mM), failed to increase the initial Na^+ uptake by the subsequently isolated BBM vesicles. (Mean ± SE, $n = 3$, $^*P < 0.05$ *vs.* Ca^{2+}-free)

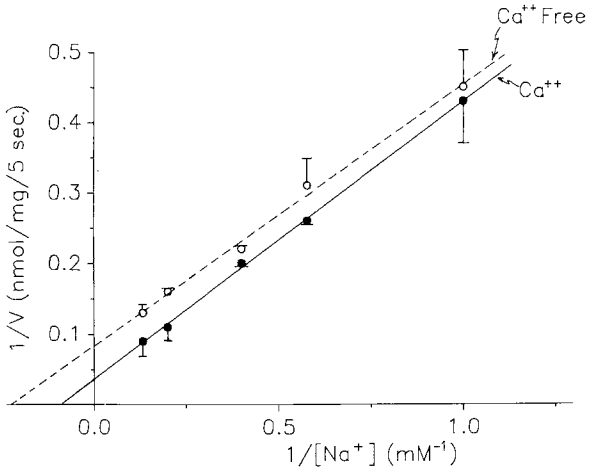

Fig. 4. Lineweaver-Burk plot of Ca^{2+} effect on BBM Na^+ uptake. BBM Na^+ uptake was measured with Na^+ concentration in the uptake medium varied from 1 to 7.5 mM. BBM vesicles isolated from proximal tubules preincubated in 1 mM Ca^{2+} medium (●—●) showed an increase in V_{max} and a decrease in K_m for Na^+ as compared to vesicles isolated from proximal tubules preincubated in Ca^{2+}-free medium (O---O) (*see* text). (Mean ± SE, $n = 3$)

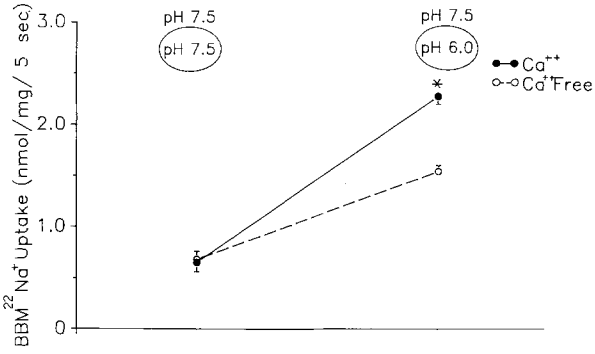

Fig. 5. Effect of proton gradient across BBM vesicles. The difference between initial Na^+ uptake by BBM vesicles isolated from proximal tubules preincubated in 1 mM Ca^{2+} (●—●) and Ca^{2+}-free (O---O) medium was observed only when BBM vesicles were imposed with an outwardly directed proton gradient. (Mean ± SE, $n = 3$, $^*P < 0.05$)

could not be reproduced when Ca^{2+} in the proximal tubule preincubation medium was replaced by another divalent cation, barium (Ba^{2+}, 1 mM) (Fig. 3). Kinetic analyses showed that the higher Na^+ uptake by BBM vesicles isolated from proximal tubules preincubated in 1 mM Ca^{2+} medium was associated with an increase in maximum velocity (V_{max}) (31.4 ± 1.4 *vs.* 11.8 ± 1.4 pmol/mg/5 sec., $n = 3$, $P < 0.02$) and a decrease in affinity (K_m) for Na^+ (12.6 ± 1.7 *vs.* 4.2 ± 0.9 mM, $n = 3$, $P < 0.01$) (Fig. 4). Since the K_m value for Na^+ is much lower than the extracellular Na^+ concentration, the effect of Ca^{2+} on K_m is probably of less physiological significance. These results thus suggest that the presence of Ca^{2+} in the preincubation medium is able to induce changes in BBM either directly or indirectly and enhance its Na^+ transport activity.

The effect of Ca^{2+} on Na^+ uptake was observed only when BBM vesicles were imposed with an outwardly directed proton gradient (Fig. 5). Addition of amiloride (10^{-3} M) to BBM vesicles abolished the effect of Ca^{2+} so that the amiloride-insensitive BBM Na^+ uptake was not different between the two groups (Fig. 6). Furthermore, the presence or absence of Ca^{2+} in proximal tubule preincubation medium did not affect Na^+-dependent glucose uptake

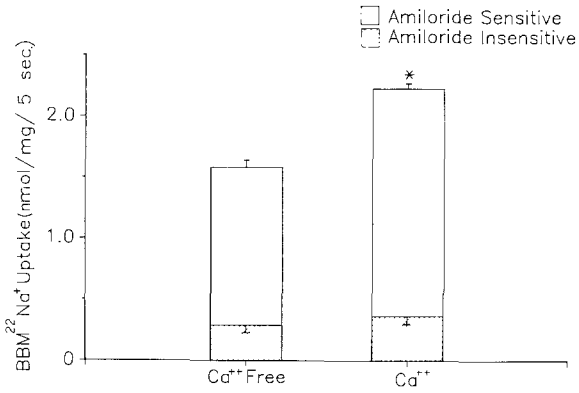

Fig. 6. Effect of Ca^{2+} on amiloride-sensitive and insensitive BBM Na^+ uptake. The higher Na^+ uptake by BBM vesicles isolated from Ca^{2+}-preincubated proximal tubules was mainly due to an increase in amiloride-sensitive Na^+ uptake (open bars). The amiloride-insensitive Na^+ uptake (shaded bars) was not different between the two groups. (Mean ± SE, $n = 4$, $^*P < 0.05$)

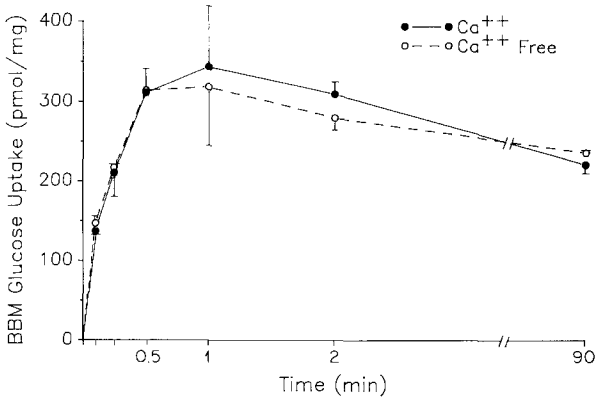

Fig. 7. Effect of Ca^{2+} on BBM Na^+-dependent glucose uptake. Presence (●—●) or absence (○---○) of Ca^{2+} in proximal tubule preincubation medium did not affect the Na^+-dependent glucose uptake by the subsequently isolated BBM vesicles. (Mean ± SE, $n = 3$)

by the subsequently isolated BBM vesicles (Fig. 7). Taken together, these results suggest that the effect of Ca^{2+} on BBM Na^+ transport involves mainly Na^+/H^+ antiport system.

Since all BBM vesicles were not exposed to Ca^{2+} during the isolation procedure, the difference in Na^+ uptake observed in these studies is unlikely to be due to a direct effect of extracellular Ca^{2+} on BBM. However, the possibility that a direct effect of extracellular Ca^{2+} on BBM during proximal tubule preincubation which persisted during the isolation procedure cannot be completely excluded. In order to differentiate the effects from extra- and intracellular Ca^{2+}, proximal tubules were incubated in 1 mM Ca^{2+} medium where intracellular Ca^{2+} levels were either raised by adding Ca^{2+} ionophore, ionomycin (5 μM), or lowered by adding Ca^{2+} channel blocker, verapamil (10^{-4} M), to the incubation medium. As is shown in Fig. 8, addition of ionomycin potentiated while addition of verapamil abolished the effect of Ca^{2+} to increase BBM Na^+ uptake. To further ascertain the role of intracellular Ca^{2+}, changes in intracellular Ca^{2+} concentration were monitored with cell-permeant calcium indicator, fura 2/AM. Basal intracellular Ca^{2+} concentration of the proximal tubules suspended in Ca^{2+}-free medium averaged 73 ± 19 nM ($n = 5$). This was increased to 156 ± 32 nM ($n = 5$, $P < 0.05$) upon addition of 1 mM Ca^{2+} to the medium. Further addition of ionomycin (5 μM) caused a rapid increase in intracellular Ca^{2+} (>1 μM) within 30 sec and followed by incomplete recovery (468 ± 132 nM, $n = 5$, $P < 0.05$). In separate sets of samples, addition of verapamil (10^{-4} M) to proximal tubules suspended in Ca^{2+}-free medium did not significantly alter the intracellular Ca^{2+} concentration (from 82 ± 20 to 89 ± 21 nM, $n = 5$). Addition of 1 mM Ca^{2+} in the presence of verapamil also did not affect the intracellular Ca^{2+} concentration significantly (93 ± 23 nM, $n = 5$). These results thus indicate that BBM Na^+ transport property can be altered by changes in intracellular Ca^{2+} levels and suggest that the effect of Ca^{2+}in the preincubation medium is likely to be mediated through Ca^{2+}-dependent cellular pathways rather than a direct effect of extracellular Ca^{2+}on BBM.

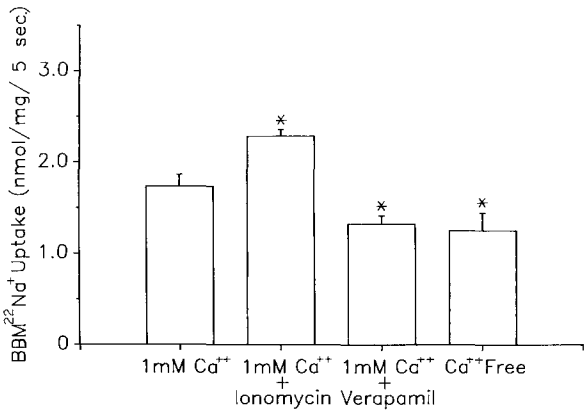

Fig. 8. Effects of Ca^{2+}, ionomycin and verapamil on BBM Na^+ uptake. The higher initial Na^+ uptake by BBM vesicles isolated from proximal tubules preincubated in 1 mM Ca^{2+} medium was potentiated by ionomycin (5 μM) and was abolished by verapamil (10^{-4} M) added in the preincubation medium. (Mean ± SE, $n = 4$, $^*P < 0.05$ *vs.* 1 mM Ca^{2+})

Several of the Ca^{2+}-dependent cellular pathways such as Ca^{2+}-cytoskeleton interactions and activation of phospholipases or protein kinases have been implicated to modulate Na^+/H^+ ex-

Table 3. Proximal tubule content of cAMP and the inhibitory effect of cAMP on BBM Na^+ uptake[a]

	Ca^{2+}-free	1 mM Ca^{2+}	1 mM Ca^{2+} + 8,bromo-cAMP
cAMP content (pmol/mg protein)	3.08 ± 0.15	3.28 ± 0.04	
BBM Na^+ uptake (nmol/mg/5 sec)	0.95 ± 0.12[b]	1.51 ± 0.07	1.03 ± 0.11[b]

[a] Cellular content of cAMP was not different in proximal tubules incubated for 30 min in either 1 mM Ca^{2+} or Ca^{2+}-free medium. Addition of 8,bromo-cAMP (10^{-4} M) to 1 mM Ca^{2+} preincubation medium was able to inhibit Na^+ uptake by the subsequently isolated BBM vesicles. (Mean ± SE; $n = 4$ each; [b]$P < 0.05$ *vs.* 1 mM Ca^{2+}.)

Table 4. Effects of Ca^{2+} and trifluoperazine (TFP)[a]

	Ca^{2+}-free	1 mM Ca^{2+}	1 mM Ca^{2+} TFP
BBM Na^+ uptake (nmol/mg/5 sec)	1.05 ± 0.08	1.6 ± 0.12[b]	1.69 ± 0.12[b]

[a] Addition of TFP (10^{-4} M) to 1 mM ca^{2+} preincubation medium did not prevent the effect of Ca^{2+} to increase BBM Na^+ uptake. (Mean ± SE; $n = 3$; [b]$P < 0.05$ *vs.* Ca^{2+}-free.)

change activity [12, 18]. It is therefore possible that the effect of Ca^{2+} on BBM Na^+/H^+ exchange observed in the present study may be mediated by these Ca^{2+}-dependent cellular pathways. In view of the prevailing evidence that renal BBM Na^+/H^+ exchanger is under the influence of different protein kinase systems [15, 26–28], we have attempted in the present study to examine the roles of (i) cAMP-dependent protein kinase, (ii) calmodulin-dependent protein kinase and (iii) protein kinase C.

Kahn and Weinman et al. have recently shown that cAMP-dependent protein kinase-induced protein phosphorylation inhibits renal BBM Na^+/H^+ exchanger [17, 26]. It is thus conceivable that Ca^{2+} may enhance BBM Na^+/H^+ exchange by removing the inhibitory effect of cAMP-dependent protein kinase through either reducing intracellular cAMP level or prohibiting the effect of cAMP. To test such possibility, we have examined the effects of Ca^{2+} on both the proximal tubule cAMP content and the inhibitory effect of exogenous cAMP on BBM Na^+/H^+ exchange. As is shown in Table 3, incubation in either 1 mM Ca^{2+} or Ca^{2+}-free EGTA medium at 25°C for 30 min did not alter cAMP content in the proximal tubular cells. On the other hand, 8,bromo-cAMP (0.1 mM), when added to the proximal tubule preincubation medium containing 1 mM Ca^{2+}, was able to cause significant reduction in Na^+ uptake by the subsequently isolated BBM vesicles (Table 3). Thus preincubation of proximal tubules in the presence of 1 mM Ca^{2+} did not affect either the endogenous cAMP level or the inhibitory effect of cAMP on BBM Na^+ uptake. These results therefore suggest that the enhancement of BBM Na^+/H^+ exchange by Ca^{2+} is unlikely to occur as the result of removal of the inhibitory influence from cAMP.

To test the possible role of calmodulin-dependent protein kinase, calmodulin inhibitor, trifluoperazine (TFP, 10^{-4} M), was added to proximal tubules incubated in 1 mM Ca^{2+} medium. As is shown in Table 4, addition of TFP did not prevent the effect of Ca^{2+} to increase BBM Na^+ uptake. Thus it seems also unlikely that the effect of Ca^{2+} on BBM Na^+ uptake is mediated through activation of calmodulin-dependent protein kinase. The recent report by Weinman et al. [27] that renal BBM Na^+/H^+ exchanger is rather inhibited by calmodulin-dependent protein kinase-induced protein phosphorylation lends further support to such notion.

Finally, in contrast to cAMP and calmodulin-dependent protein kinases, Weinman et al. have shown that protein phosphorylation mediated by protein kinase C stimulates renal BBM Na^+/H^+ exchanger [27, 28]. It is therefore possible that Ca^{2+} may enhance renal BBM Na^+/H^+ exchange through activation of protein kinase C. To testify to such possibility, the effect of Ca^{2+} was examined in proximal tubules where protein kinase C was downregulated by prolonged incubation with phorbol 12-myristate 13-acetate (PMA). Proximal tubules were incubated in culture medium (DMEM + HAMS) with or without PMA (10^{-6} M) at 37°C under 95% O_2/5% CO_2 for 24 hr. After this 24 hr incubation, the effect of Ca^{2+} was tested by resuspending and incubating proximal tubules in modified KRB medium containing either 1 mM Ca^{2+} or 0.5 mM EGTA at

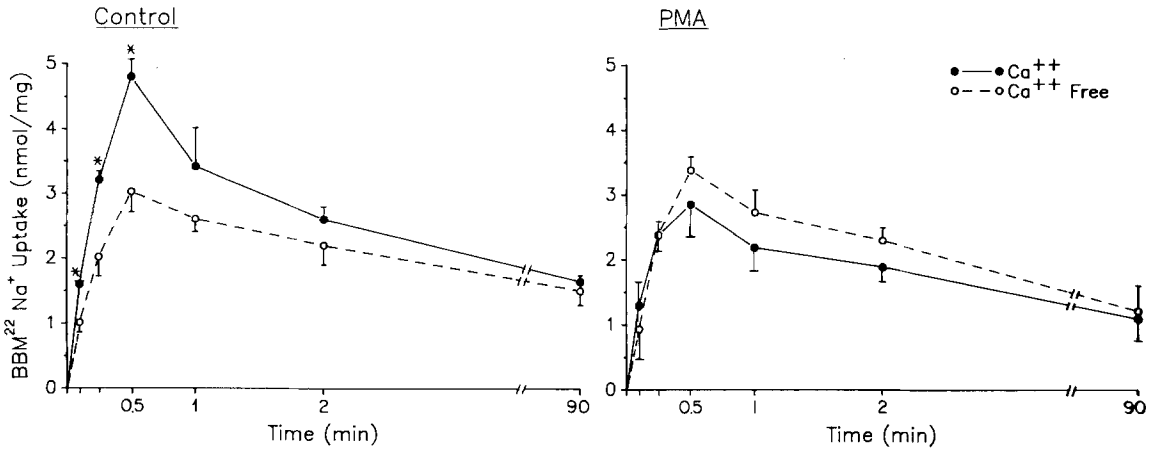

Fig. 9. Effect of phorbol 12-myristate 13-acetate (PMA). The effect of Ca^{2+} to increase BBM Na^+ uptake persisted in proximal tubules after 24 hr incubation in culture medium without PMA (left panel). Such effect of Ca^{2+} was, however, abolished when proximal tubules were incubated for 24 hr in culture medium containing PMA (10^{-6} M) (right panel). (Mean ± SE, $n = 4$, $^*P < 0.05$)

25°C for 30 min prior to the isolation of BBM vesicles. At the end of 24 hr incubation, the majority of the tubular cells remained intact as inferred from more than 95% cell exclusion of trypan blue; the intactness of BBM vesicles thus isolated is also supported from the similarity in enzyme enrichment (Table 1) and in the pattern of Na^+ uptake (Fig. 9). As is shown in Figure 9, the effect of Ca^{2+} to increase BBM Na^+ uptake was preserved in proximal tubules after 24 hr incubation without PMA. In contrast, 24 hr incubation of proximal tubules with PMA abolished the effect of Ca^{2+} to increase Na^+ uptake by the subsequently isolated BBM vesicles (Fig. 9). These results thus suggest that activation of protein kinase C may be involved in the enhancement of BBM Na^+/H^+ exchange by Ca^{2+}.

In summary, results of the present study show the Ca^{2+} dependency of renal BBM Na^+ transport possibly through stimulation of BBM Na^+/H^+ exchanger by protein kinase C. The enhancement of BBM Na^+/H^+ exchange by Ca^{2+} found in the present study thus differs from the previously reported downregulatory effect of Ca^{2+} on Na^+ channels in the apical membrane of toad urinary bladder [5, 9, 10]. While this is likely to be related to the different apical Na^+ transport mechanisms between tight and leaky epithelium, it is not certain if such difference represents a generalized feature for other tight and leaky epithelia as well. The enhancement of BBM Na^+/H^+ exchange by Ca^{2+} and ionomycin found in the present study also appears contradictory to the prevailed evidence that increased intracellular Ca^{2+} levels suppress proximal tubule Na^+ transport [8, 17]. Although the fact that the stimulatory effect of Ca^{2+} is lessened by preincubation at 37°C as compared to 25°C may minimize its significance under physiological condition, this still does not explain the inhibitory effect of Ca^{2+} on proximal tubule Na^+ transport. If one assumes that the effect of Ca^{2+} found in the present study with isolated BBM vesicles applies to BBM in the intact proximal tubule, it may be speculated that the inhibitory effect of intracellular Ca^{2+} on transtubular Na^+ transport in intact proximal tubule may result from the effect of Ca^{2+} on Na^+ transport at steps other than that across the BBM, e.g., Na^+ exclusion by Na,K-ATPase across the basolateral membrane. Further studies are required to clarify these issues.

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