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An Effect of Ca^{2+} on the Intrinsic Cl^--conductance of Rat Kidney Cortex Brush Border Membrane Vesicles

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Abstract. Brush-border membrane vesicles (BBMV) were prepared from superficial rat renal cortex by a divalent^{2+}-precipitation technique using either $CaCl_2$ or $MgCl_2$. The dependence of the initial [^{14}C]-D-glucose (or [^{3}H]-L-proline) uptake rate and the extent of the overshoot of D-glucose or L-proline uphill accumulation from solutions containing 100 mM Na^+ salt, was found to be dependent upon the precipitating divalent cation. With Mg^{2+} precipitation the initial uptake and overshoot accumulation of either D-glucose or L-proline were enhanced compared to BBMV prepared by Ca^{2+} precipitation. When the anion composition of the media was varied (uptake in Cl^- media in comparison to gluconate$^-$-containing media) it was found that the Cl^--dependent component of the initial uptake was markedly depressed with Ca^{2+}-prepared BBMV (104.99 ± 33.31 *vs.* 13.83 ± 1.44 pmoles/sec/mg protein for Mg^{2+} and Ca^{2+} prepared vesicles respectively). When Ca^{2+} was loaded into Mg^{2+} prepared BBMV using a freeze-thaw technique, it was found that the magnitude and Cl^- enhancement of D-glucose transport was reduced in a dose-dependent manner. Neomycin, an inhibitor of phospholipase C, had no effect on the reduction of D-glucose uptake by Ca^{2+} in Mg^{2+} prepared vesicles.

In contrast, phosphatase inhibitors such as vanadate and fluoride were able to partially reverse the Ca^{2+} inhibition of D-glucose uptake and restore the enhancement due to Cl^- media. In addition, inhibitors of protein phosphatase 2B, deltamethrin (50 nM) and trifluoperazine (10 μM), caused partial reversal of Ca^{2+}-dependent inhibition of D-glucose uptake.

Direct measurement of changes in the bi-ionic (Cl^- *vs.* gluconate$^-$) transmembrane electrical potential differences using the cyanine dye, 3,3′-dipropylthiodicarbocyanine iodide $DiSC_3$-(5) confirmed that Cl^- conductance was reduced in Ca^{2+}-prepared vesicles.

We conclude that a Cl^- conductance coexists with Na^+ cotransport in rat renal BBMV and this may be subject to negative regulation by Ca^{2+} via stimulation of protein phosphatase (PP2B).

Key words: Brush border — Membrane vesicle — Cl^- conductance — Protein phosphatase — Na^+/glucose cotransport

Introduction

In brush border membrane vesicles (BBMV) prepared from rat kidney superficial cortex by a Mg^{2+} precipitation method it is apparent that Cl^- conductance coexists with Na^+ cotransport and this may function to regulate the activity of electrogenic transport systems at this membrane (Brown, King & Simmons, 1993). The physiological relevance of the intrinsic permeabilities of isolated BBMV to both cations and anions has been questioned since exact correlation of such permeabilities with those seen in intact tubules has been difficult to draw. This may result from dynamic regulation of such conductances; intact tubule studies are predominately made in the absence of regulatory factors; for instance a proximal tubule brush-border Cl^- conductance has been shown to be subject to regulation by protein kinase A and C using parathyroid hormone as a physiological regulator in this tubule segment (Lipkowitz & Abramson, 1989, 1992; Suzuki et al., 1991). An additional factor may concern the method by which BBMV are prepared.

In the divalent cation precipitation method either Mg^{2+} or Ca^{2+} ions may be used in the differential precipitation of cytosolic membranes and basolateral mem-

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branes *vs.* brush border membranes. It has been well documented that significant differences in the transport properties of such vesicles exist; thus Biber et al. (1981) noted that the extent of D-glucose transport in Ca^{2+}-prepared vesicles was depressed compared to Mg^{2+}-prepared vesicles. The question whether this results from the direct regulation of the transporter or is due to the change in the driving force for transport (or its dissipation) is still unresolved (Lin et al., 1989). Sabolic & Burkhardt (1984) have shown that both the K^+ and H^+ conductances in Ca^{2+}-prepared rat renal vesicles are increased whilst Na^+/H^+ exchange is reduced.

The intrinsic Cl^--conductance of rat renal BBMV may be important in the regulation of electrogenic solute transport at this membrane. We have assessed whether a reduction in Cl^- conductance may contribute to the reported differences between Ca^{2+} and Mg^{2+}-prepared vesicles. Two experimental protocols were used; (i) an indirect measurement of Cl^- conductance utilizing Cl^--gradient enhancement of electrogenic transport of D-glucose or L-proline into BBMV and (ii) by direct measurement of changes in the bi-ionic transmembrane potential differences using the cyanine dye 3,3′-dipropylthiodicarbocyanine iodide ($DiSC_3$-(5)). Evidence is presented for the potential role of a Ca^{2+}-activated phosphatase in the regulation of kidney BBMV Cl^--conductance.

Materials and Methods

Preparation of Rat Renal Brush Border Membrane Vesicles

Renal cortical brush border membrane vesicles were prepared by a double cation precipitation procedure as described by Biber et al (1981). Kidneys from 3 male Wistar rats (220–250 g body weight) were removed and decapsulated before cutting thin slices (1–2 mm thick) of superficial cortex. The tissue was suspended 10% (w/v) in buffer containing (in mM): 100 mannitol, 5 EGTA, 0.2 PMSF, 10 Hepes/Tris pH 7.4 and homogenized (setting 5 for 2 min) with a Kinematica homogenizer. The homogenate volume was adjusted to 40 ml with water, divided into 2 equal volumes for comparison of Mg^{2+} *vs.* Ca^{2+} prepared vesicles and either 2.0 M $MgCl_2$ or 2.0 M $CaCl_2$ added to give a final concentration of 12 mM. This suspension was centrifuged at 2,400 × *g* for 15 min to remove large cellular debris and cation aggregated material. Brush border membrane vesicles remaining in the supernatant were collected by centrifuging at 30,900 × *g* for 30 min. The pellet was resuspended in 20 ml buffer solution containing (in mM): 50 mannitol, 2.5 EGTA, 0.1 PMSF, 5.0 Hepes/Tris pH 7.4 and the cation precipitation step repeated. The final brush border membrane vesicle fraction was resuspended in 400–500 μl of a solution containing 300 mM mannitol and 10 mM Hepes/Tris, pH 7.4. Vesicles (containing 8–10 mg/ml protein) were stored in liquid nitrogen until use. All steps of the procedure were carried out at 4°C.

Protein Assay and Marker Enzyme Analysis

Protein determination was carried out by the method of Bradford (1976) using bovine serum albumin as the standard. Alkaline phosphatase and leucine aminopeptidase were used as marker enzymes for brush border membrane vesicles. Alkaline phosphatase activity was enriched 18.01 ± 1.05 and 18.20 ± 1.14-fold (SE $n = 5$) over tissue homogenates for Mg^{2+} and Ca^{2+} prepared vesicles respectively while leucine aminopeptidase activity was enriched 14.11 ± 1.57 and 15.59 ± 1.79-fold (SE n = 5) over tissue homogenates for Mg^{2+} and Ca^{2+} prepared vesicles respectively.

Transport Studies

The uptake of D-glucose into rat renal brush border membrane vesicles was measured at room temperature (20°C) using a rapid filtration stop technique as described by Berner et al. (1976). Brush border membrane vesicles (containing 40–50 μg protein/time point) were pipetted into a 1 ml tube. Reactions were started by the addition of 750 μl incubation medium which contained (in mM): 100 NaCl, 100 mannitol, 10 Hepes/Tris pH 7.4 and 0.1 [^{14}C]-D-glucose (about 40,000 c.p.m.). The reaction mix was taken up into the tip of a 1-ml electronic pipette (Biohit Proline, Helsinki, Finland). At appropriate time points 60 μl volumes of reaction mix were dispensed directly onto nitrocellulose filters (0.45 μm pore size) under vacuum. Filters were washed, 3 × 3 ml ice-cold stop solution containing (in mM): 150 KCl, 10 Hepes/Tris pH 7.4 and 0.1 phloridzin. The filters were then removed and processed for liquid scintillation counting. Zero time points were determined by dispensing 60 μl volumes of incubation medium onto the filters and washing with 3 × 3 ml stop solution. Measurements of initial rates of transport were performed. Over a period of 10 sec, uptake was directly proportional to time.

The procedure for the measurement of [^{2,3}H]-L-proline transport activity was similar to that for D-glucose. The incubation medium contained (in mM): 100 NaCl, 100 mannitol, 10 Hepes/Tris pH 7.4 and 0.1 [^{2,3}H]-L-proline (about 30,000 c.p.m.). The stop solution contained; .150 KCl, 10 Hepes/Tris pH 7.4.

Uptake experiments using freshly prepared vesicles or vesicles stored overnight in liquid nitrogen showed little difference in substrate transport activity (*data not shown*).

Intravesicular Loading of Brush Border Membrane Vesicles with Ca^{2+}

Using a freeze-thaw technique, the intravesicular composition of brush border membrane vesicles can be manipulated to incorporate a range of macromolecules (Donowitz et al., 1987). The loading of Ca^{2+} into Mg^{2+}-prepared vesicles was carried out by the method of Lin et al. (1989) using the freeze-thaw technique (Donowitz et al., 1987). Mg^{2+}-prepared brush border membrane vesicles (100 μl) were pelleted by centrifugation at 30,900 × *g* for 10 min. The supernatant was discarded and the pellet resuspended in 100 μl of media containing (in mM): 10 EGTA, 10 Hepes/Tris pH 7.4, and either 0 (control), 6.60, 9.50, 9.96, 10.00 and 11.00 mM concentrations of $CaCl_2$ to achieve final free Ca^{2+} concentrations of 0 (control) 0.1, 1.0, 10, 100, 1000 μM respectively. The osmolarity of the Ca^{2+}/EGTA buffer was maintained at 310 mosmol using mannitol. Free Ca^{2+} concentrations were calculated using the EQCAL computer program (Backman, L. 1988 Biosoft, Cambridge, UK). After resusupension, the samples were frozen in liquid nitrogen and subsequently allowed to thaw at 37°C for 30 min. After a further 30-min incubation at 20°C the transport of either D-glucose or L-proline into the vesicles was measured.

Measurements of Fluorescence Quenching

Calibration of the fluorescence response with imposed K^+-valinomycin diffusion potentials and measurements of the bi-ionic membrane po-

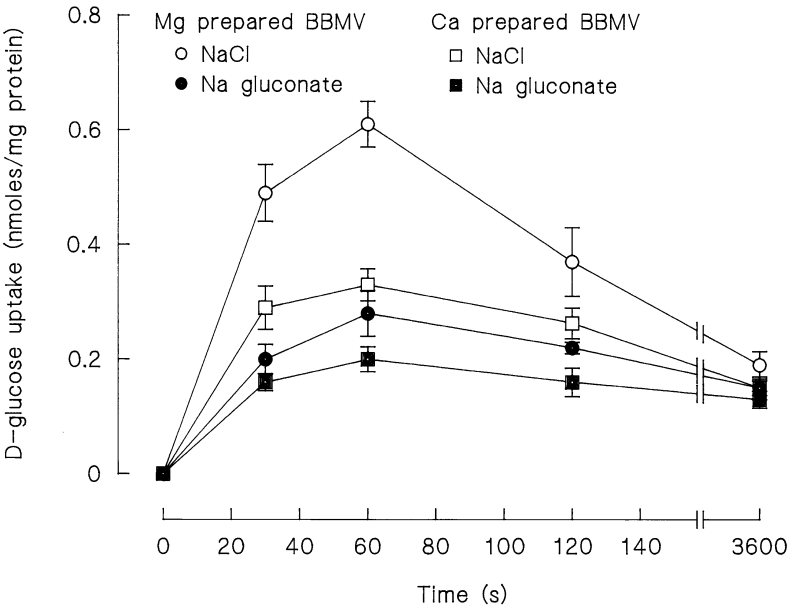

Fig. 1. Time course (0–60 min) of the anion-dependent transport of D-glucose into Mg^{2+}- or Ca^{2+}-prepared rat kidney BBMV. Vesicles were prepared and D-glucose transport activity measured as described in Materials and Methods. The intravesicular composition was (in mM: 300 mannitol, 10 Hepes/Tris pH 7.4. BBMV were incubated for different time periods in extravesicular uptake media that contained (in mM): 100 mannitol, 10 Hepes/Tris pH 7.4, 0.1 [^{14}C]-D-glucose and either 100 NaCl or 100 Na gluconate. The transport of D-glucose into Mg^{2+} (○,●) or Ca^{2+} (□,■) prepared vesicles in the presence of NaCl (○,□) or Na gluconate (●,■) respectively is shown. Values are the mean ± SE $n = 4$ separate preparations where individual points were performed in triplicate.

tential difference between Mg^{2+}- and Ca^{2+}-prepared vesicles were carried out as described by the methods of Gunther, et al. (1984) and Cassano et al. (1988), using the voltage-dependent fluorescence of the cyanine dye $DiSC_3$-(5). BBMV were prepared either by a Mg^{2+} or Ca^{2+} precipitation procedure and left overnight at 4°C, in loading buffer (*see* Table legend for details). In a plastic cuvette the following reagents were added, 1960 μl external solution, 10 μl 0.6 mM dye and 10 μl 0.89 mM valinomycin, to achieve the final concentrations shown in the Table legend). The fluorescence was stabilized at 90 arbitrary units and the reaction started by the addition of 10 μl BBMV (80 μg protein) to the cuvette. The fluorescence of the cyanine dye was measured spectrofluorometrically ($\lambda_{ex\ 620nm}$ $\lambda_{em\ 670nm}$) for 2 min.

Statistics

Tests of significance of difference between mean values were made using ANOVA (one-way analysis of variance) with a BonFerroni method for multiple comparison *t* tests between data pairs; *n* is the number of separate preparations used.

Results

Na^+-dependent Transport of D-glucose in Mg^{2+} or Ca^{2+} Prepared Rat Renal Brush Border Membrane Vesicles

To overcome the possible contamination of the vesicle population with endosomal Cl^--channels (Schmid, Burckhardt & Gogelein, 1989), brush border membrane Cl^--conductance was measured indirectly using the electrogenic low affinity, high capacity Na^+/glucose transporter (SGLT2) expressed in the superficial rat renal cortex (Kanai et al., 1994, Lee et al., 1994).

The time course of D-glucose transport into Mg^{2+} or Ca^{2+} prepared vesicles is shown in Fig 1. In the presence of an inwardly directed 100 mM-NaCl gradient, there was a rapid accumulation of D-glucose into the Mg^{2+} prepared vesicles. The initial rate of uptake (Table 1) was nearly 8 times higher than the initial rate of D-glucose uptake into Ca^{2+}-prepared vesicles. The accumulation of D-glucose into both Mg^{2+} and Ca^{2+}-prepared vesicles continued up to 60 sec (Fig 1). After this point the concentration of D-glucose inside the vesicles began to fall. The overshoot accumulation of D-glucose into Ca^{2+}-prepared vesicles was significantly lower (1.88 ± 0.28-fold SE $n = 4$ $P < 0.01$) than the overshoot in Mg^{2+}-prepared vesicles (0.61 ± 0.04 *vs.* 0.33 ± 0.02 pmoles/mg protein, for Mg^{2+} and Ca^{2+} BBMV respectively.

Replacing the external Cl^--ion gradient with the membrane impermeant gluconate$^-$-ion resulted in a reduction of Na^+-dependent D-glucose transport into Mg^{2+}-prepared vesicles (Fig 1). In contrast in Ca^{2+}-prepared vesicles, replacement of Cl^- by gluconate did not markedly reduce initial D-glucose uptake nor accumulation at 60 secs. The intravesicular concentrations at equilibrium were essentially identical for all conditions. The equilibrium volume of the vesicles was 1.57 ± 0.10 μl/mg protein.

The initial rate of transport of D-glucose into Mg^{2+}-prepared vesicles was unaffected in the presence of an external 100 mM NaSCN driving force (Table 1) compared to NaCl. In contrast, in Ca^{2+}-prepared vesicles the initial race of D-glucose uptake in the presence of an external 100 mM NaSCN gradient was similar to that observed in Mg^{2+}-prepared vesicles. Thus, the acceleration of D-glucose uptake into BBMV observed with Cl^--containing media compared to gluconate$^-$ media in Mg^{2+}-prepared vesicles is consistent with the existence of a Cl^- conductance. This observation is in agreement with the NPPB-sensitive Cl^- conductance, previously demonstrated on these vesicles (Brown et al., 1993).

Table 1. Initial rates of D-glucose transport into rat kidney BBMV in the presence of different membrane permeant anions

Vesicles	Anion	D-glucose uptake (pmoles/sec/mg protein)
Mg^{2+} prepared	Cl^-	104.99 ± 33.31
	$Gluconate^-$	25.36 ± 3.92[a]
	SCN^-	125.91 ± 11.38[b]
Ca^{2+} prepared	Cl^-	13.83 ± 1.44[a]
	$Gluconate^-$	15.15 ± 1.14[a,c]
	SCN^-	114.96 ± 5.81[ns]

The preparation of rat kidney BBMV and the measurement of the transport of D-glucose are described in Materials and Methods. Mg^{2+}- or Ca^{2+}-prepared vesicles were made from the same divided homogenate. The intravesicular media contained (in mM): 300 mannitol, 10 Hepes/Tris pH 7.4. The initial rate of D-glucose uptake was carried out in reaction media containing (in mM): 100 mannitol, 10 Hepes/Tris pH 7.4, 0.1 [^{14}C]-D-glucose and either 100 NaCl or 100 Na gluconate or 100 NaSCN. Values are the mean ± SE ($n = 3$), [a], significantly different from Cl^- media Mg^{2+}-prepared vesicles $P < 0.05$, [b], not significantly different, [c], not significantly different from equivalent media, Cl^-, $gluconate^-$, SCN^- Mg^{2+} *vs.* Ca^{2+} prepared vesicles.

These workers noted a 55% reduction in Cl^--dependent D-glucose uptake, in the presence of the channel blocker, NPPB (500 μM); Cl^--driven D-glucose transport was insensitive to the channel blocker DIDS (Brown et al., 1993).

The abolition of the Cl^--acceleration in Ca^{2+}-prepared vesicles and the restoration of D-glucose uptake in SCN^- media, without change in the equilibrium volume, indicates that the relative Cl^- permeability of Ca^{2+} prepared BBMV is reduced.

L-proline Transport in Mg^{2+} or Ca^{2+} Prepared Rat Renal Brush Border Membrane Vesicles

The electrogenic transport of the amino acid L-proline (Chesney et al., 1991) was investigated as an additional intravesicular monitor of the BBMV membrane potential.

The initial rate of L-proline uptake into Mg^{2+}-prepared vesicles incubated in the presence of 100 mM NaCl gradient was 23.73 ± 6.19 pmoles/sec/mg protein (Fig 2). This was 3 times higher than the initial rate of L-proline transport into Ca^{2+} prepared vesicles (7.72 ± 2.45 pmoles/sec/mg protein $P < 0.05$ paired data) (Fig. 2). Mg^{2+}-prepared vesicles showed maximal overshoot accumulation of substrate within 2 min in NaCl media (0.94 ± 0.13 nmoles/mg protein), whereas the peak of the overshoot for Ca^{2+}-prepared vesicles in NaCl media (0.20 ± 0.03 nmoles/mg protein) was significantly lower (4.65 ± 0.40-fold SE $n = 4$ $P < 0.05$) (Fig 2).

There was no difference in the initial rates of L-proline transport into either Mg^{2+}- or Ca^{2+}-prepared vesicles (2.82 ± 1.14 pmoles/sec/mg protein and 2.83 ± 0.10 pmoles/sec/mg protein $P < 0.05$) respectively when the external media contained gluconate-ions (Fig. 2). Thus, a reduction in Cl^--conductance, in BBMV prepared by Ca^{2+} precipitation, is observed with an alternative electrogenic solute transport system.

Effect of Ca^{2+} Concentration on D-glucose Transport

Mg^{2+}-prepared vesicles were manipulated to contain variable concentrations of intravesicular free Ca^{2+} ((0, control), 0.1–1000 μM) with EGTA buffers using a freeze-thaw technique (*see* Materials and Methods). Figure 3A shows that D-glucose transport with an inwardly directed NaCl gradient was reduced as the concentration of Ca^{2+} increased ($P < 0.001$ ANOVA). In contrast, the transport of D-glucose in Na gluconate media was not significantly effected as the concentration of Ca^{2+} increased ($P > 0.05$ ANOVA) (Fig. 3*A*). In the presence of 0.1, 1.0, 10.0, 100.0, and 1000 μM Ca^{2+} Cl^--driven D-glucose transport was reduced by 62, 75, 82, 87 and 100% respectively ($P < 0.01$ ANOVA) (Fig. 3*B*). Furthermore, 1.0 μM Ca^{2+} loaded into Mg^{2+}-prepared vesicles also reduced Cl^--dependent L-proline transport (Fig. 4*B*). Thus, it is likely that the intrinsic Cl^--conductance of rat BBMV is responsive to concentrations of Ca^{2+} within a physiological range; it also implies that the mechanism by which Ca^{2+} acts is intrinsic to the brush border membrane.

Phospholipase C Inhibitor, Neomycin, on D-glucose and L-proline Transport in Ca^{2+}-loaded BBMV

Rat kidney brush border membrane vesicles have been shown to contain a Ca^{2+}-activated phospholipase C (Schwertz, Kreisberg & Venkatachalam, 1984). To test whether a reduction in Cl^- conductance in Ca^{2+} loaded BBMV was attributable to Ca^{2+}-activated phospholipase C activity, the effect of phospholipase C inhibitor, neomycin, was studied. Mg^{2+}-prepared vesicles were preincubated for 1 hr at 20°C in the presence of 0.5 mM neomycin. Ca^{2+} (1.0 μM) was loaded into the vesicles by freeze-thaw (*see above*) and the Cl^--dependent acceleration of transport of either D-glucose (Fig. 4*A*) or L-proline (Fig. 4*B*) measured. Vesicles pretreated with neomycin showed no increase in the Cl^--driven component in D-glucose or L-proline transport, over Ca^{2+}-loaded controls. The small reduction in D-glucose transport activity into Mg^{2+}-prepared vesicles in Cl^- media ($P < 0.01$) and Ca^{2+}-loaded vesicles in Cl^- media ($P > 0.05$) may be due to a direct inhibitory action of neomycin on the transporter (Lin et al., 1989). Neomycin had no affect on L-proline transport. It can be concluded that the reduction in Cl^--conductance caused by Ca^{2+} is not associated with the action of Ca^{2+}-dependent phospholipase C activity.

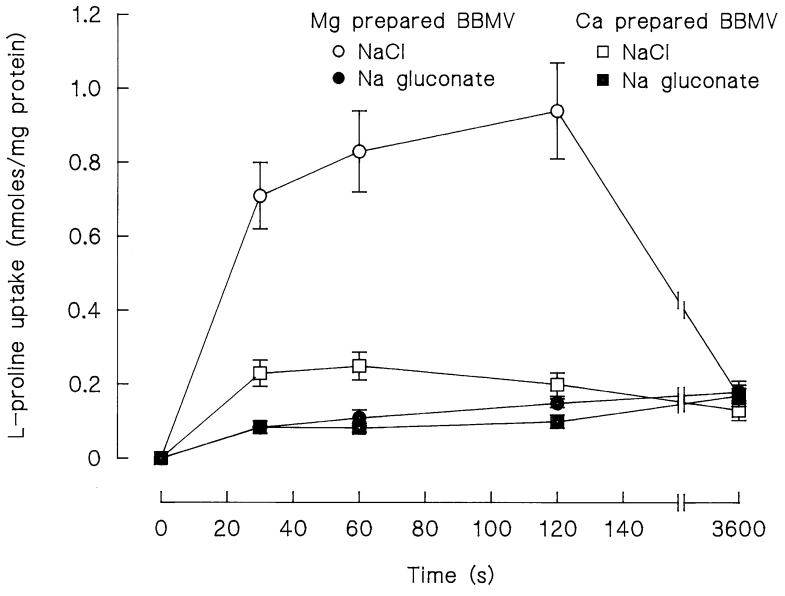

Fig. 2. Time course (0–60 min) of the transport of L-proline into Mg^{2+}- or Ca^{2+}-prepared rat kidney BBMV. Vesicles were prepared and L-proline transport activity measured as described in Materials and Methods. The intravesicular composition was (in mM): 300 mannitol, 10 Hepes/Tris pH 7.4. BBMV were incubated for different time periods in reaction media that contained; 100 mannitol, 10 Hepes/Tris pH 7.4, 0.1 [^{2,3}H]-L-proline and either 100 NaCl or 100 Na gluconate. The transport of L-proline into Mg^{2+} (○,●) or Ca^{2+} (□,■) prepared vesicles in the presence of NaCl (○,□) or Na gluconate (●,■) respectively is shown. Values are the mean ± SE n = 4 separate preparations.

Protein Phosphatase Inhibitors on D-glucose Transport in Ca^{2+}-loaded BBMV

The role of a Ca^{2+}-activated phosphatase in the regulation of BBMV Cl^- conductance was investigated using the protein phosphatase inhibitors, levamisole, Na orthovanadate and NaF (Van Belle, 1972; Swarup, Cohen & Garbers 1982; Damuni, 1990). Mg^{2+}-prepared BBMV were preincubated for 1 hr at 20°C with 0.5 mM concentrations of the various inhibitors. Ca^{2+} (1.0 μM) was then loaded into the BBMV via the freeze-thaw technique and the transport of D-glucose measured in NaCl and Na gluconate media. These agents had only minor effects on D-glucose transport in control vesicles (Fig. 5*A*) ($P > 0.05$ for all treatments *vs.* control). Incubation of Mg^{2+}-prepared vesicles with Ca^{2+} again significantly reduced the Cl^--dependent acceleration of D-glucose uptake (Fig. 5B) ($P < 0.01$). In comparison to control Ca^{2+}-loaded vesicles, the presence of Na orthovanadate or NaF resulted in a significant increase in the Cl^--dependent acceleration of D-glucose transport (3.5-fold and 6.0-fold over Ca^{2+} controls respectively ($P < 0.05$). Levamisole had no significant effect on reversing the action of Ca^{2+}.

Synthetic type II pyrethroid insecticides are potent inhibitors of isolated bovine brain protein phosphatase 2B (calcineurin) (Enan & Matsumura, 1992). The role of Ca^{2+}-activated protein phosphatase 2B in the regulation of a renal brush border Cl^- conductance was investigated using the type II pyrethroid, deltamethrin. Mg^{2+} prepared BBMV were preincubated for 1–2 hr, on ice, with 50 nM-deltamethrin or 50 nM of its nonactive analogue, permethrin, prior to loading them with 1.0 μM Ca^{2+} using the freeze-thaw technique. The transport of D-glucose into the vesicles in the presence of either 100 mM NaCl or 100 mM Na gluconate was then measured. The results are shown in Fig. 6*A* and *B*. Preincubation of Mg^{2+} prepared BBMV with 50 nM deltamethrin resulted in a significant increase (2.7-fold) in Cl^--driven D-glucose transport activity (Fig. 6*B*) compared to Ca^{2+} loaded BBMV (0.92 ± 0.09 *vs.* 0.34 ± 0.05 nmoles/min/mg protein respectively ($P < 0.05$)). BBMV pretreated with permethrin (negative control) showed a decrease in the rate of Cl^--driven D-glucose uptake to those treated with deltamethrin ($P > 0.05$) and a similar rate of Cl^--driven D-glucose uptake to Ca^{2+}-loaded BBMV (0.51 ± 0.09 *vs.* 0.34 ± 0.05 nmoles/min/mg protein respectively ($P > 0.05$)).

Addition of the phenothiazine drug, 10 μm-trifluoperazine, also known to inhibit protein phosphatase 2B (Ingebritsen & Cohen, 1983), to Mg^{2+} vesicles prior to the addition of Ca^{2+} (1.0 μM) resulted in a 2.5-fold increase of Cl^--driven D-glucose uptake (Fig. 6*B*) (0.87 ± 0.08 *vs.* 0.34 ± 0.05 nmoles/min/mg protein for vesicles pretreated with trifluoperazine prior to loading with Ca^{2+} and Ca^{2+} loaded vesicles respectively $P < 0.05$). Deltamethrin, permethrin and trifluoperazine had only minor effects on D-glucose transport in control vesicles (Fig. 6*A*) ($P > 0.05$ for all treatments *vs.* control).

These data suggest that an endogenous Ca^{2+}-activated phosphatase (probably protein phosphatase 2B (also calcineurin) Cohen (1989), Shenolikar & Nairn (1991), Klee, Draetta & Hubbard (1988)) reduces the activity of a Cl^--conductance pathway in the rat kidney BBMV.

In rat brain, PP 2B has been shown to regulate the activity of other protein phosphatases (Mulkey et al., 1994). To test whether PP 2B was activating a second phosphatase in renal BBMV, the action of the protein phosphatase inhibitor, calyculin A (Suganuma et al., 1990), was investigated. The addition of Calyculin A

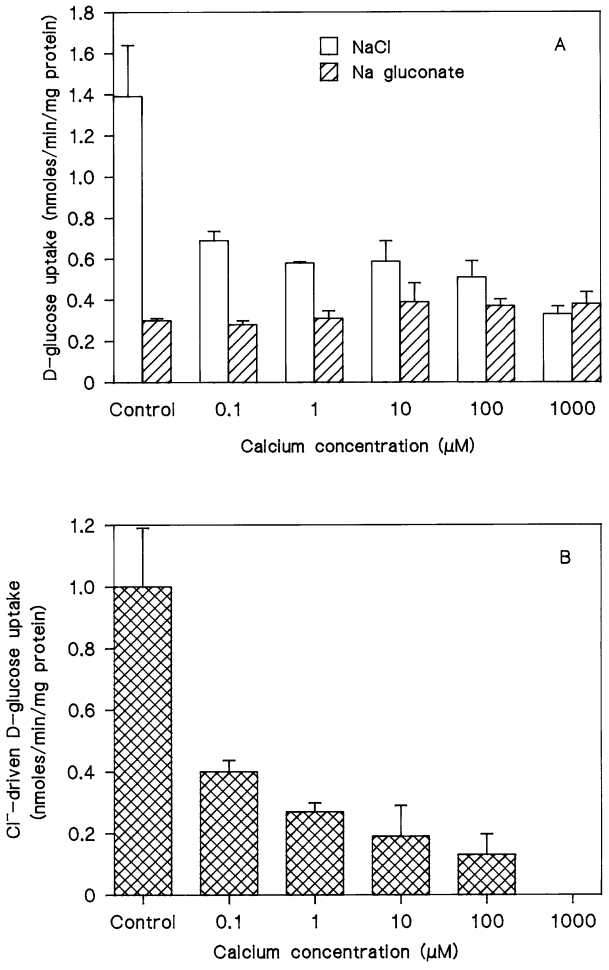

Fig. 3. Effect of Ca^{2+} concentration on D-glucose transport into rat kidney BBMV. (*A*) Rat kidney BBMV were prepared by a Mg^{2+} precipitation procedure and located with Ca^{2+} buffers using a freeze-thaw technique as described in Materials and Methods. The intravesicular composition of the vesicles was (in mM): 290 mannitol, 10 Hepes/Tris pH 7.4, 10 EGTA and Ca^{2+} added to achieve final 'free' Ca^{2+} concentrations of 0.1–1000 μM. D-glucose transport was measured at 20°C in reaction media that contained (in mM): 90 mannitol, 10 Hepes/Tris pH 7.4, 0.1 [^{14}C]-D-glucose, 10 EGTA, Ca^{2+} added to achieve final 'free' Ca^{2+} concentrations of 0.1–1000 μM and either 100 NaCl (open bars) or 100 Na gluconate (striped bars). D-glucose uptake was measured at 60 sec. (*B*) Cl^- enhancement of D-glucose uptake expressed as the difference between Cl^- and gluconate$^-$ media. Values are the mean ± SE $n = 3$.

(2 nM) to Mg^{2+} prepared BBMV, prior to loading them with Ca^{2+} did not result in an increase in the Cl^--driven component of D-glucose transport (0.47 nmoles/min/mg protein *vs.* 0.48 nmoles/min/mg protein for BBMV loaded with Ca^{2+} and those pretreated with 2 nM calyculin A ($n = 3$ $P > 0.05$)). Since Cl^--driven D-glucose uptake was not increased in the presence of calyculin A, it is likely that the Ca^{2+}-dependent phosphatase interacts via direct dephosphorylation of the renal Cl^- conductance rather than acting in a phosphatase cascade system.

Ca^{2+}-activated Protein Kinase C Activity

Amino acid transport into rat kidney cortex brush border membrane vesicles has been shown to be regulated by Ca^{2+}-dependent protein kinase C activity (Zelikovic & Przekwas, 1993, 1995). To test whether Na^+/glucose transport activity was affected by Ca^{2+}-dependent PKC activity, the action of the protein kinase C inhibitor peptide [19–36] (RFARKGALRQKNVHEVKN) (House & Kemp, 1987) was investigated. The results for a pooled preparation from 2 animals showed that the addition of PKC pseudosubstrate [19–36] (5, 20 and 100 μM) to Mg^{2+}-prepared BBMV (1 hr on ice), prior to loading them with Ca^{2+} (1.0 μM, using the freeze-thaw technique) did not result in an increase in the Cl^--driven component of D-glucose transport (0.46 nmoles/min/mg protein *vs.* 0.46, 0.67 and 0.46 nmoles/min/mg protein for BBMV loaded with Ca^{2+} and those pretreated with 5, 20 and 100 μM PKC [19–36] peptide, respectively. PKC inhibitor peptide had no effect on D-glucose transport activity in Mg^{2+} prepared vesicles (*not shown*). In an identical experiment, the transport activity of L-proline into Ca^{2+} loaded BBMV in the absence or presence of 5, 20 or 100 μM PKC inhibitor peptide [19–36], was measured. As predicted, in the absence of peptide Ca^{2+}-loaded vesicles showed nearly 4-fold reduction in Cl^--driven L-proline transport. Vesicles pretreated with the peptide prior to the addition of Ca^{2+} showed no stimulation in Cl^--driven L-proline transport activity, 0.63 nmoles/min/mg protein *vs.* 0.75, 0.66 and 0.93 nmoles/min/mg protein for Ca^{2+} BBMV and vesicles pretreated with peptide respectively.

Since ATP was absent from the assay system, it is unlikely PKC derived phosphate from an alternative source, thus its role in phosphorylation inactivation of SGLT2 or the L-proline transporter under these conditions was not demonstrated. It can therefore be concluded that the addition of Ca^{2+} to BBMV affects the relative activity of a Cl^- conductance on the brush border membrane rather than indirect inactivation of the electrogenic cotransporter(s).

Bi-ionic Potential Measurements in Mg^{2+} or Ca^{2+} Prepared Rat Renal Brush-border Membrane Vesicles

Electrical potential differences across the membrane of Mg^{2+} or Ca^{2+}-prepared brush border vesicles, were measured using a direct approach, using the voltage-dependent fluorescence of the cyanine dye $DiSC_3$-(5). A calibration of fluorescence response with imposed K^+-valinomycin diffusion potentials for a typical experiment, (representative of 3 such experiments) are shown in Table 2. Inside-positive membrane potentials were generated by diluting Mg^{2+} or Ca^{2+} prepared vesicles, equilibrated in 10 mM K gluconate buffer, in external cuvette buffer to obtain an extravesicular concentration

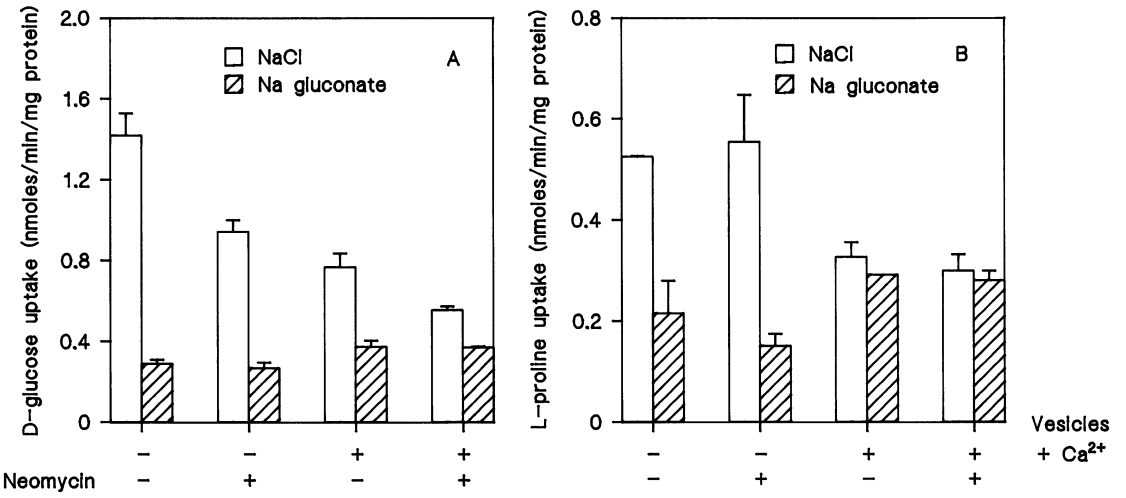

Fig. 4. Effect of neomycin on D-glucose (*A*) and L-proline (*B*) transport into rat kidney BBMV by Mg^{2+} precipitation. Rat kidney BBMV were prepared and D-glucose and L-proline transport activity measured as described in Materials and Methods. BBMV were preincubated with 0.5 mM-neomycin, at 20°C, prior to freeze-thaw loading with Ca^{2+} buffer to 1.0 μM. The transport of D-glucose or L-proline was measured in reaction media that contained (in mM): 90 mannitol, 10 Hepes/Tris pH 7.4, 10 EGTA, Ca^{2+} added to achieve a final free Ca^{2+} concentration of 1.0 μM and either 0.1 [^{14}C]-D-glucose or 0.1 [^{2,3}H]-L-proline, in the presence of 100 NaCl (open bars) or 100 Na gluconate (striped bars). Values are the mean ± SE $n = 3$.

of 100 mM K gluconate. Baseline fluorescence was recorded when there were no ion gradients i.e., $K_i = K_o$ and $E_m = 0$ mV. Table 2 shows that for Mg^{2+}-prepared vesicles, in the presence of 100 mM K gluconate, fluorescence (F) increased by 6 units before valinomycin and 9.34 units after valinomycin. In the presence of valinomycin, $E_m = E_k = RT/F \log_{10} 100/10 = 58$ mV. Thus, in the absence of valinomycin the increase in F (6 units) corresponds to a membrane potential of +37.25 mV (with respect to extravesicular medium). For 3 separate experiments, each fluorescence quench recorded in triplicate, the mean potential difference was +45.30 ± 3.28 (SE) mV.

For Ca^{2+}-prepared vesicles (isolated from the same homogenate), dye quenching was increased in K^+-gradient conditions compared to Mg^{2+}-prepared vesicles. Using the calibration from Ca^{2+} vesicles with a K^+-gradient in the presence of valinomycin the mean p.d. minus valinomycin was +39.04 ± 3.88 (SE) mV which was not significantly different from the membrane potential calculated for Mg^{2+} prepared vesicles ($P > 0.05$ $n = 3$ separate experiments).

For studies involving the measurement of the bi-ionic diffusion potential (Cl^- *vs.* gluconate$^-$) Mg^{2+} or Ca^{2+} BBMV were pre-equilibrated overnight at 4°C, in buffer containing (in mM): 10 K gluconate, 90 NMDG gluconate, 100 mannitol and 10 Hepes/Tris pH 7.4.

A Cl^- *vs.* gluconate$^-$ diffusion potential was generated by adding vesicles to cuvette buffer containing (in mM): 90 KCl, 10 K gluconate, 100 mannitol, 10 Hepes/Tris pH 7.4. Note that in these conditions an inward K^+ gradient also exists. With Mg^{2+}-prepared vesicles fluorescence was now seen to decrease. The Cl^--diffusion p.d. must predominate even in the presence of the inward K^+ gradient. This also holds even at the concentration of valinomycin used (Table 2) i.e., $P_{Cl} > P_K$. Compared to Mg^{2+} prepared vesicles, Ca^{2+}-prepared vesicles (from the same homogenate) showed a significant, 2.88 ± 0.83-fold lowering of membrane p.d. −23.31 ± 7.61 mV *vs.* −48.36 ± 9.15 mV for Ca^{2+} and Mg^{2+}-prepared vesicles respectively (SE $P < 0.05$ $n = 3$ separate preparations). It can be concluded that differences in membrane potential exist between Mg^{2+} and Ca^{2+}-prepared vesicles; these changes are consistent with a Ca^{2+}-induced reduction of Cl^- conductance.

Discussion

The question of the existence and significance of a Cl^- conductance in the apical membrane of the renal proximal tubule is still the subject of controversy. In the present study, two independent methods were used to study rat renal BBMV Cl^- conductance; (i) an indirect approach utilizing the electrogenicity of Na^+-dependent transport of either D-glucose (Kanai et al., 1994) or L-proline (Chesney et al., 1991) and (ii) a direct measurement of membrane-diffusion potentials, using the voltage-dependent fluorescence-quenching of the dye, $DiSC_3$-(5). For rat BBMV, voltage-sensitive $^{36}Cl^-$ uptakes have also provided evidence for a Cl^- conductance; there is therefore agreement between data obtained using these independent measurements for a Cl^- conductance (Lipkowitz & Abramson, 1989; Lipkowitz et al., 1992).

Both Mg^{2+} and Ca^{2+} have been routinely used to prepare BBMV by differential precipitation of microsomes from homogenates of small intestine and from

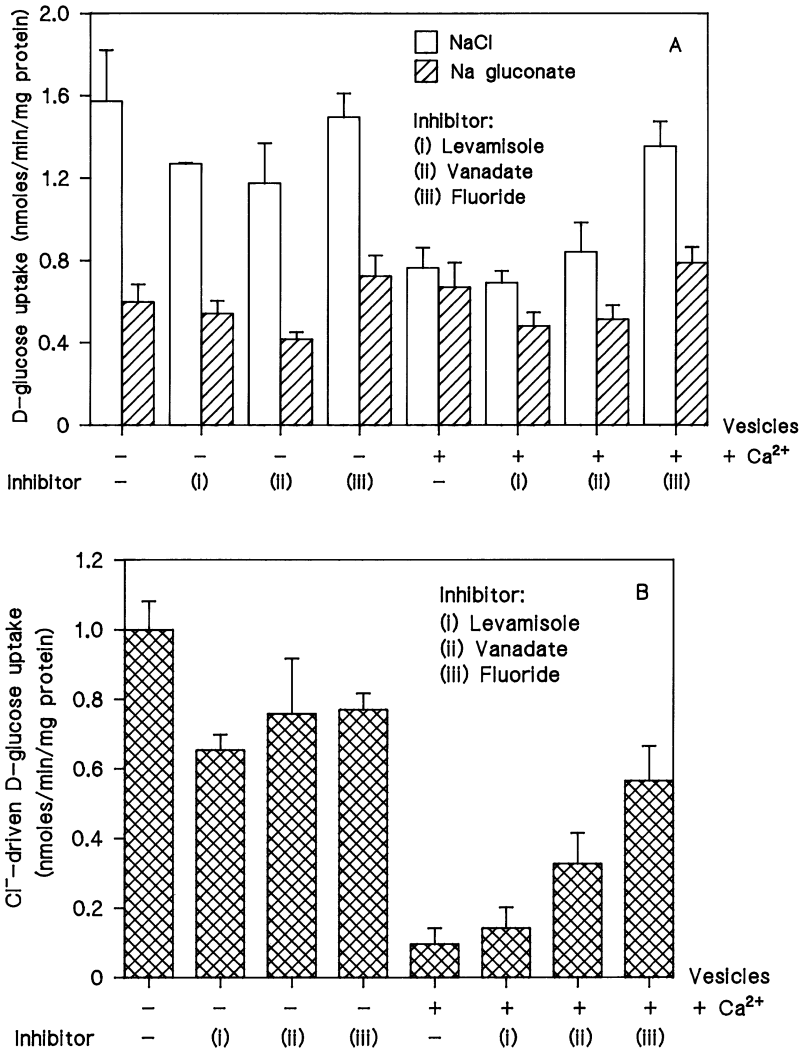

Fig. 5. Effect of protein phosphatase inhibitors on D-glucose transport into rat kidney BBMV prepared by Mg^{2+} precipitation. (*A*) Vesicles were preincubated with 0.5 mM concentrations of either (i) levamisole, (ii) Na orthovanadate or (iii) NaF for 1 hr at 20°C, prior to freeze-thaw loading with Ca^{2+} buffer to 1.0 μM. The transport of D-glucose was measured at 20°C in reaction media that contained (in mM): 90 mannitol, 10 Hepes/Tris pH 7.4, 0.1 [^{14}C]-D-glucose 10 EGTA, Ca^{2+} added to achieve a final 'free' Ca^{2+} of 1.0 μM and either 100 NaCl (open bars) or 100 Na gluconate (striped bars). Values are the mean ± SE $n = 3$. (*B*) Cl^--enhancement of D-glucose uptake expressed as the difference between Cl^- and gluconate$^-$ values. Other details as for 5 (*A*).

renal cortex (Hopfer et al., 1973; Evers et al., 1978; Biber et al., 1981). Differences between the properties of Mg^{2+}- and Ca^{2+}-precipitated vesicles have been noted (*see* Biber et al., 1981). The Na^+-dependent transport of taurine or b-alanine into rabbit intestinal BBMV has also been shown to be affected by the precipitating divalent cation (Miyamoto et al., 1990*a,b*). These workers observed a reduction in Na^+-coupled substrate transport activity when vesicles were prepared by Ca^{2+} precipitation. In addition they found the effect of Ca^{2+} on the transport activity to be time and dose-dependent. In our present studies, rat kidney brush border membrane vesicles prepared in parallel by Ca^{2+} precipitation exhibited a noticeable reduction in the initial rate and overshoot accumulation of D-glucose or L-proline compared to vesicles isolated in the presence of Mg^{2+} when substrate uptake was energized with an external NaCl gradient in agreement with Biber et al. (1981). When the Cl^--ion gradient was replaced with the membrane impermeant gluconate ion, there was no significant difference in the overshoot accumulation of either substrate into Mg^{2+}- or Ca^{2+}-prepared vesicles. The initial rate of D-glucose transport into Ca^{2+}-prepared vesicles was approximately 8 times lower than that into Mg^{2+}-prepared vesicles. Whereas in the presence of the membrane permeant anion (e.g., SCN^-) the initial rates of D-glucose uptake into Mg^{2+}- or Ca^{2+}-prepared vesicles were similar. The similar behavior of both D-glucose and L-proline transport is consistent with a common action via the vesicular membrane potential. The ability of SCN^- to increase substrate transport is consistent with permeation via a lipophilic route. If permeability was due entirely to permeation via Cl^- channels, a reduction in Cl^- permeability should have been paralleled by a similar reduction in SCN^- permeability. This was not the case. It has previously been shown that Cl^--conductance in Mg^{2+} prepared rat kidney vesicles is inhibited by the Cl^--channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB 0.5 mM) and insensitive to the stilbene 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS 0.5 mM) (Brown et

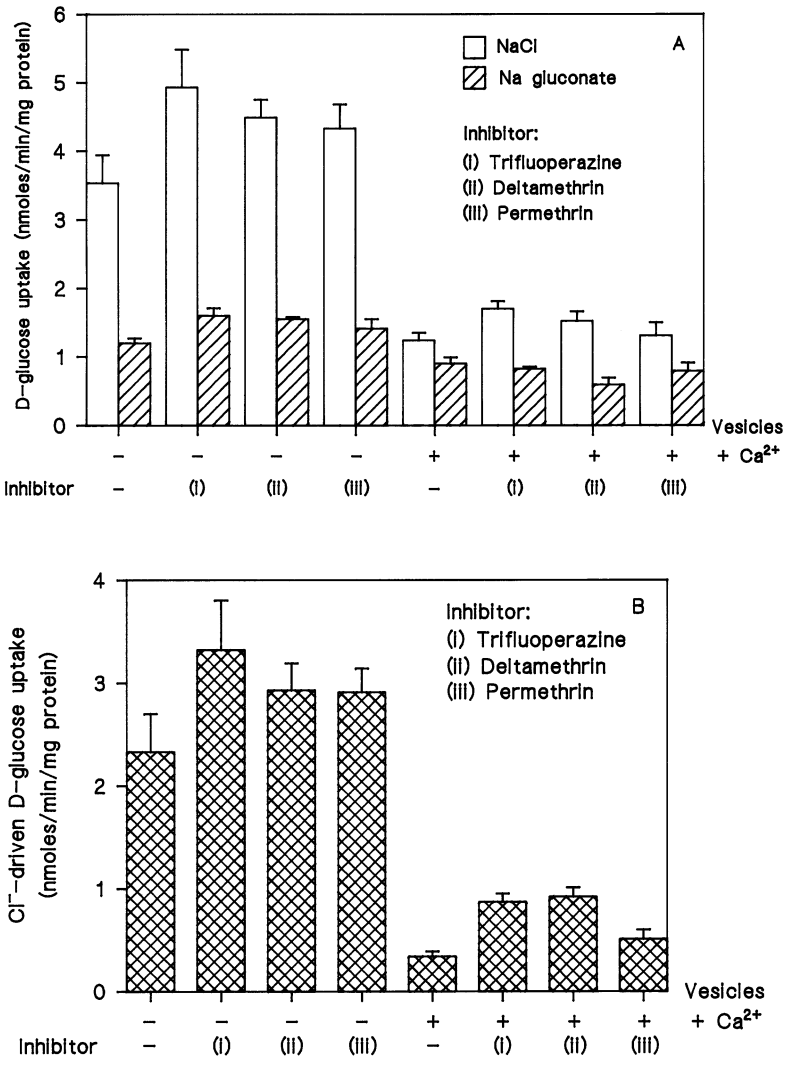

Fig. 6. Effect of protein phosphatase inhibitors on D-glucose transport into rat kidney BBMV prepared by Mg^{2+} precipitation. (*A*) Vesicles were preincubated with either (i) 10 μM trifluoperazine, (ii) 50 nM deltamethrin or (iii) 50 nM permethrin for 1–2 hr, on ice, prior to freeze-thaw loading with Ca^{2+} buffer to 1.0 μM. The transport of D-glucose was measured at 20°C in reaction media that contained (in mM): 90 mannitol, 10 Hepes/Tris pH 7.4, 0.1 [^{14}C]-D-glucose 10 EGTA, Ca^{2+} added to achieve a final 'free' Ca^{2+} of 1.0 μM and either 100 NaCl (open bars) or 100 Na gluconate (striped bars). Values are the mean ± SE $n = 3$. Vesicles pretreated with trifluoperazine were protected from light. Reactions involving deltamethrin and permethrin were carried out in glass tubes pretreated with 1% (w/v) polyethylene glycol 8,000, as described by the manufacturers. (*B*) Cl^- enhancement of D-glucose uptake expressed as the difference between Cl^- and gluconate$^-$ values. Other details as for 6 (*A*).

al., 1993). Therefore, we conclude that a Cl^- conductance coexists with electrogenic Na^+-substrate cotransport in proximal tubule brush border and that the presence of Ca^{2+} during the membrane isolation procedure causes a reduction in this intrinsic Cl^- conductance.

We have confirmed these observations by direct measurements of the bi-ionic membrane potential (Cl^- *vs.* gluconate$^-$) between Mg^{2+}- and Ca^{2+}-prepared vesicles using the voltage sensitive dye, $DiSC_3$-(5).

Rat kidney Cl^- conductance was shown to be dependent on the Ca^{2+} concentration. Mg^{2+}-prepared vesicles loaded with increasing concentrations of Ca^{2+} (0.1–1000 μM), using a freeze-thaw technique, resulted in a dose dependent reduction in the Cl^--driven transport of D-glucose. Inhibition of transport was observed at physiological concentrations of Ca^{2+}. That Cl^--driven D-glucose transport can be downregulated in Mg^{2+}-prepared vesicles loaded with Ca^{2+} indicates that the mechanism by which Ca^{2+} acts is intrinsic to the renal brush border membrane.

It has been reported that the reduction in Na^+-coupled substrate transport activity into Ca^{2+}-prepared vesicles is the result of increased membrane permeability to Na^+ (Lin et al., 1989, Miyamoto et al., 1990*a*,*b*). This is thought to be caused by Ca^{2+}-activated phospholipase C activity. Lin et al. (1989) showed that D-glucose transport activity was recoverable in Mg^{2+}-prepared vesicles pretreated with the phospholipase C inhibitor, neomycin, prior to loading them with Ca^{2+}. In our study, the preincubation of Mg^{2+}-prepared vesicles with neomycin prior to loading them with Ca^{2+} had little or no effect on restoring the Cl^--driven D-glucose or L-proline transport into the vesicles. This implies that in rat kidney BBMV the Ca^{2+} reduced Cl^--driven D-glucose or L-proline transport, is not a result of increased Na^+ permeability caused by Ca^{2+}-activated phospholipase C activity.

Cl^- channels are regulated by phosphorylation-dephosphorylation reactions (Anderson & Welsh, 1989; La et al., 1991, Tabcharani & Hanrahan, 1991; Finn et al., 1992, Becq et al., 1993). Here we provide evidence

Table 2. Membrane potential measurement of Cl^- conductance into Mg^{2+}- or Ca^{2+}-prepared BBMV

Condition ± valinomycin	Preparation of vesicles			
	Mg		Ca	
	ΔF	E_m	ΔF	E_m
(a) 10 k_i/100K_o (−)	6.00	37.25	9.16	29.79
(+)	9.34	58.00	17.83	58.00
(b) 10K_i/90Cl_o (−)	−8.83	−54.81	−8.46	−27.51
(+)	−9.00	−55.87	−5.59	−18.18

BBMV were prepared either by a Mg^{2+}- or Ca^{2+}-precipitation procedure from the same homogenate and membrane potential measurements made as described in Materials and Methods. The intravesicular composition of the vesicles was (in mM); 10 K gluconate, 90 NMDG gluconate, 100 mannitol, 10 Hepes/Tris pH 7.4. The reaction was started by adding BBMV (80 μg protein) to incubation media containing (in mM): (*a*), 100 K gluconate, 100 mannitol, 3 μM-$DiSC_3$-(5) dye, 10 Hepes/Tris pH 7.4; (*b*), 90 KCl, 10 NMDG gluconate, 100 mannitol, 10 Hepes/Tris pH 7.4 and 3 μM-$DiSC_3$-(5). All reactions were performed ± 4.5 μM-valinomycin. Data shown represent the mean values of a typical experiment, (representative of 3 such experiments), with each fluorescence measurement performed in triplicate. *F*, fluorescence (arbitrary units), E_m, membrane potential, (mV).

for the potential role of a Ca^{2+}-activated phosphatase in the dephosphorylation of renal brush border Cl^- conductance. The addition of the protein phosphatase inhibitors, Na orthovanadate, (Swarup et al., 1982), or Na fluoride (Damuni, 1990), to Mg^{2+} prepared vesicles prior to the addition of Ca^{2+}, caused partial restoration of the Cl^--driven transport of D-glucose. That 50 nM of the type II pyrethroid insecticide, deltamethrin (Enan & Matsumura, 1992) or 10 μM of the phenothiazine drug, trifluoperazine (Ingebritsen & Cohen, 1983), were able to restore Cl^--driven D-glucose uptake indicates the possible role of the Ca^{2+}-dependent protein phosphatase 2B (PP 2B) in the regulation of kidney Cl^--conductance. Addition of the protein phosphatase 1 inhibitor, calyculin A (Suganuma et al., 1990), did not cause any enhancement in the Cl^--driven component of D-glucose uptake. This suggests that protein phosphatase 1 (PP 1) is not involved in the regulation of renal Cl^- conductance.

Recent studies have shown that renal amino acid transport is regulated via Ca^{2+}-dependent protein kinases (Zelikovic & Przekwas, 1993, 1995). These workers have shown that endogenous as well exogenous PKC is responsible for the phosphorylation inactivation of L-proline transport in rat kidney cortex BBMV. The uptake of L-proline into these vesicles in the presence of 50 μM ATP and Ca^{2+}, was significantly reduced compared to L-proline uptake in control media. L-proline transport activity was recoverable by inhibiting PKC activity using the specific PKC inhibitor peptide [19–31]. To obviate the possibility that Na/glucose cotransport (via SGLT2) or Na/L-proline cotransport might undergo similar phosphorylation inactivation and thus account for the reduced Cl^--driven D-glucose uptake in the presence of Ca^{2+}, we investigated the effect of PKC pseudosubstrate [19–36] (RFARKGALRQKNVHEVKN) (House & Kemp, 1987) on Cl^--driven D-glucose or L-proline uptake. The addition of peptide, to BBMV loaded with Ca^{2+}, did not cause any enhancement in the Cl^--driven component of either D-glucose or L-proline uptake. Coupled to the absence of ATP in our vesicle preparation it is unlikely that Ca^{2+}-dependent PKC activity is involved in the action of calcium reported here.

In rat primary cultured proximal convoluted tubule cells patch clamp studies have shown a multiplicity of Cl^--channels subject to cAMP and Ca^{2+}-dependent regulation (Darvish, Winaver & Dagan, 1994). A Ca^{2+}-activated Cl^- conductance cannot be the major Cl^--conductance present in BBMV, rather, the net effect of Ca^{2+} concentration is to progressively reduce Cl^- conductance. The relative contribution of individual Cl^- channels to the ensemble conductance remains to be elucidated.

In pig jejunal BBMV where a Cl^- conductance in the brush border of the enterocyte would contribute to intestinal secretion, Forsyth & Gabriel (1989) have demonstrated that in imidazolium-prepared vesicles with a low intrinsic Cl^- conductance incorporation of ATP, AsO_4^{3-} and F^- to stabilize the presence of phosphoproteins, a cAMP-mediated increase in Cl^- conductance could be observed. The nature of the pathways maintaining a phosphorylated (open) state of the Cl^- conductance in rat renal BBMV remain to be elucidated. It is likely that this is subject to cAMP-dependent regulation (Lipkowitz & Abramson, 1989; Suzuki et al., 1991). For rat BBMV, Cl^- conductance may be increased by pretreatment of tissue homogenates with cholera toxin or parathyroid hormone (G_S) and decreased by pertussis toxin (G_i) (Lipkowitz et al., 1992).

The physiological relevance of a transcellular route for Cl^- transport in the early proximal tubule (and hence of Cl^- conductance) has been shown in micropuncture studies which show that 40–50% of total Cl^- reabsorption is via a regulated transcellular route independent of organic acid exchange (Wong, Berry & Cogan, 1995).

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References

Anderson, M.P., Welsh, M.J. 1989. An endogenous calcium-dependent phosphatase regulates airway epithelial chloride channels. *J. Clin. Res.* **37:**917*A* (*Abstr.*)

Becq, F., Fanjul, M., Merten, M., Figarelle, C., Hollane, E., Gola, M. 1993. Possible regulation of CFTR-chloride channel by membrane bound phosphatases in pancreatic duct cells. *FEBS Lett.* **327:**337–342

Berner, W., Kinne, R., Murer, H. 1976. Phosphate transport into brush-border membrane vesicles isolated from the rat small intestine. *Biochem. J.* **160:**467–474

Biber, J., Stieger, B., Hasse, W., Murer, H. 1981. A high yield preparation for rat kidney brush-border membranes: different behaviour of lysosomal markers. *Biochim. Biophys. Acta.* **647:**169–176

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of dye binding. *Anal. Biochem.* **72:**248–254

Brown, C.D.A., King, N., Simmons, N.L. 1993. Co-expression of an anion conductance pathway with Na^+-glucose cotransport in rat renal brush-border membrane vesicles. *Eur. J. Physiol.* **423:**406–410

Cassano, G., Maffia, M., Vilella, S., Storelli, C. 1988. Effects of membrane potential on Na cotransport in eel intestinal brush-border membrane vesicles: studies with a fluorescent dye. *J. Membrane Biol.* **101:**225–236

Chesney, R.W., Zelikovic, I., Budreau. A., Randle, D. 1991. Chloride and membrane potential dependence of sodium ion-proline symport. *J. Am. Soc. Nephrol.* **2:**885–893

Cohen, P. 1989. The structure and regulation of protein phosphatases. *Ann. Rev. Biochem.* **58:**453–508

Damuni, Z. 1990. Inactivation of bovine kidney cytosolic protamine kinase by the catalytic subunit of protein phosphatase 2A. *Biochem. Biophys. Res. Commun.* **166:**449–456

Darvish, N., Winaver, J., Dagan, D. 1994. Diverse modulations of chloride channels in renal proximal tubules. *Am. J. Physiol.* **267:**F716–724

Donowitz, M., Emmer, E., McCullen, J., Reinlib, L., Cohen, M., Rood, R.P., Madara, J., Sharp, G.W.G., Murer, H., Malmstron, K. 1987. Freeze-thaw and high voltage discharge allow macromolecule uptake into ileal brush-border vesicles. *Am. J. Physiol.* **252:**G723–G735

Enan, E., Matsumura, F. 1992. Specific inhibition of calcineurin by type II synthetic pyrethroid insecticides. *Biochem. Pharmacol.* **111:**1777–1784

Evers, C., Haase, W., Murer, H., Kinne, R. 1978. Properties of brush border vesicles isolated from kidney cortex by calcium precipitation. *Membr. Biochem.* **1:**203–219

Finn, A.L., Gaido, M.L., Dillard, M., Brautigan, D.L. 1992. Regulation of an epithelial chloride channel by direct phosphorylation and dephosphorylation. *Am. J. Physiol.* **263:**C172–C175

Forsyth, G.W., Gabriel, S.E. 1989. Activation of chloride conductance in pig jejunal brush border vesicles. *J. Membr and Biol.* **107:**137–144

Gunther, R.D., Schell, R.E., Wright, E.M. 1984. Ion permeability of rabbit intestinal brush border membrane vesicles. *J. Membr and Biol.* **78:**119–127

Hopfer, U., Nelson, K., Perrotto, J., Isselbacher, K.J. 1973. Glucose transport in isolated brush border membrane from rat small intestine. *J. Biol. Chem.* **218:**25–32

House, C., Kemp, B.E. 1987. Protein kinase C contains a pseudosubstrate prototype in its regulatory domain. *Science* **238:**1726–1728

Ingebritsen, T.S., Cohen, P. 1983. The protein phosphatases involved in cellular regulation. *Eur. J. Biochem.* **132:**255–261

Kanai, Y., Lee, W-S., You, G., Brown, D., Hediger, M.A. 1994. The human kidney, low affinity Na^+/glucose cotransporter SGLT2: Delineation of the major renal reabsorptive mechanism for D-glucose. *J. Clin. Invest.* **93:**397–404

Klee, C.B., Draetta, G.F., Hubbard, M.J. 1988. Calcineurin. *Adv. Enzymol.* **61:**149–200

La, B-Q., Carosi, S.L., Valentich, J., Shenolikar, S., Sansom, S.C. 1991. Regulation of epithelial chloride channels by protein phosphatase. *Am. J. Physiol.* **260:**C1217–C1223

Lee, W-S, Kanai, Y., Wells, R.G., Hediger, M.A. 1994. The high affinity Na^+/glucose cotransporter re-evaluation of function and distribution of expression. *J. Biol. Chem.* **269:**12032–12039

Lin, J-T, Xu, Z-J, Lovelace, C., Windhager, E.E., Heinz, E. 1989. Effect of Ca on Na-D-glucose cotransport across isolated renal brush-border membranes. *Am. J. Physiol.* **257:**F126–F136

Lipkowitz, M.S., Abramson, R.G. 1989. Modulation of the ionic permeability of renal cortical brush-border membranes by cAMP. *Am. J. Physiol.* **257:**F769–F776

Lipkowitz, M.S., London, R.D., Beck, J.C., Abramson, R.G. 1992. Hormonal regulation of rat renal proximal tubule brush-border membrane ionic permeability. *Am. J. Physiol.* **263:**F144–F151

Miyamoto, Y., Kulanthaivel, P., Ganapathy, V., Whitford, G.M., Leibach, F.H. 1990*a*. Calcium-induced inhibition of taurine transport in brush-border membrane vesicles from rabbit small intesting. *Biochim. Biophys. Acta.* **1030:**189–194

Miyamoto, Y., Nakamura, H., Hoshi, T., Ganapathy, V., Leibach, F.H. 1990*b*. Uphill, transport of B-alanine in intestinal brush-border membrane vesicles. *Am. J. Physiol.* **259:**G372–G379

Mulkey, R.M., Endo, S., Shenolikar, S., Malenka, R.C. 1994. Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* **369:**486–488

Sabolic, I., Burckhardt, G. 1984. Effect of the preparation method on Na^+-H^+ exchange and ion permeabilities in rat renal brush-border membranes. *Biochim. Biophys. Acta.* **772:**140–148

Schmid, A., Burckhardt, G., Gogelein, H. 1989. Single chloride channels in endosomal vesicle populations from rat kidney cortex. *J. Membrane Biol.* **111:**265–275

Schwertz, D.W., Kreisberg, J.I., Venkatachalam, M.A. 1984. Effects of aminoglycosides on proximal tubule brush-border membrane phosphatidylinositol-specific phospholipase C. *J. Pharmacol. Exp. Ther.* **231:**48–55

Shenolikar, S. Nairn, A.C. 1991. Protein phosphatase: Recent progress. *In*: Adv. Sec. Mess. Phosphopr. Res. P. Greengard and G.A. Robinson, editors. vol 23, pp. 1–122. Raven Press, New York

Suganuma, M., Fujiki, H., Furuya-Suguri, H., Yoshizawa, S., Yasumoto, S., Kato, Y., Fusetani, N., Sugimura, T. 1990. Calyculin A, an inhibitor of protein phosphatases, a potent tumour promoter on CD-1 mouse skin. *Cancer Res.* **50:**3521–3525

Suzuki, M., Morita, T., Hanaoka, K., Kawaguchi, Y., Sakai, O. 1991. A Cl channel activated by parathyroid hormone in rabbit renal proximal tubule cells. *J. Clin. Invest.* **88:**735–742

Swarup, G., Cohen, S., Garbers, D.L. 1982. Inhibition of membrane phosphotyrosyl-protein phosphatase activity by vanadate. *Biochem. Biophys. Res. Commun.* **107:**1104–1109

Tabcharani, J.A., Chang, X-B., Riordan, J.R., Hanrahan, J.W. 1991. Phosphorylation-regulated Cl^- channel in CHO cells stably expressing the cystic fibrosis gene. *Nature* **352:**628–631

Van Belle, H. 1972. Kinetics and inhibition of alkaline phosphatases from canine tissue. *Biochim. Biophys. Acta.* **289:**158–168

Wong, K.R., Berry, C.A., Cogan, M.G. 1995. Chloride transport in the rat S1 proximal tubule. *Am. J. Physiol.* **268:**F723–F729

Zelikovic, I., Przekwas, J. 1993. The role of protein phosphorylation in renal amino acid transport. *Pediatr. Nephrol.* **7:**621–629

Zelikovic, I., Przekwas, J. 1995. Ca^{2+}-dependent protein kinases modulate proline transport across the renal brush-border membrane. *Am. J. Physiol.* **268:**F155–162