Characterization of Ca^{2+} transport in rat renal brush-border membranes and its modulation by phosphatidic acid

Michael G. SOMERMEYER, Thomas C. KNAUSS, Joel M. WEINBERG and H. David HUMES*
Departments of Internal Medicine, Veterans Administration Medical Center and University of Michigan,
Ann Arbor, MI 48105, U.S.A.

(Received 6 December 1982/Accepted 21 March 1983)

The Ca^{2+} transport process by isolated renal brush-border membranes was characterized and the influence of the acidic phospholipid phosphatidic acid (PtdA) on this transport process was assessed. Ca^{2+} uptake by brush-border membranes exhibited saturation kinetics. It was inhibitable by a variety of multivalent cations, as well as by Ca^{2+} -entry inhibitors, including verapamil, Ruthenium Red and gentamicin. It was selective for Ca^{2+} compared with Mg^{2+} . This process was also electrophoretic since generation of K^+ and anion-diffusion potentials, negative inside the vesicle, increased Ca^{2+} uptake. Elevations in PtdA content of brush-border membranes by either exogenous addition or endogenous generation of PtdA by incubating brush-border membranes with MgATP^{2-} elevated the rate of Ca^{2+} uptake. This ATP effect could not be attributed to $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase or contaminating membrane fragments. PtdA also increased the magnitude and rate of Ca^{2+} efflux from brush-border membranes preloaded with Ca^{2+} . These modulations in uptake and efflux were not observed with phosphatidylcholine or phosphatidylinositol. In summary, these results are consistent with the presence of an electrophoretic uniport system for Ca^{2+} in renal brush-border membranes, and demonstrate that PtdA uniquely among phospholipids tested appears to facilitate transmembrane flux of Ca^{2+} across this membrane preparation.

Alterations in the metabolism of the acidic phospholipids, PtdA and PtdIns, occur in a variety of tissues simultaneously with hormone- and neurotransmitter-induced elevations in cellular Ca^{2+} influx (Michell *et al.*, 1977). Since these alterations are characterized by the breakdown of membrane PtdIns and formation of PtdA, PtdA has been suggested to directly promote Ca^{2+} translocation across the plasma membrane of the stimulated cell. In this regard, PtdA has been suggested to act as a Ca^{2+} ionophore in simple solvent systems (Tyson *et al.*, 1976; Putney *et al.*, 1980), multilamellar liposomes (Serhan *et al.*, 1981, 1982), sarcoplasmic-reticulum vesicles (Limas, 1980), nerve terminals (Harris *et al.*, 1981) and intact-cell and tissue preparations (Putney *et al.*, 1980; Salmon & Honeyman, 1980; Ohsako & Teguchi, 1981). This PtdIns/PtdA response may thus be a mechanism by

which a hormone promotes a rise in cytosolic $[\text{Ca}^{2+}]$ and utilizes Ca^{2+} as an intracellular second messenger.

Parathyroid hormone has been demonstrated to increase the content of PtdA in renal cortex and renal brush-border membranes (Farese *et al.*, 1980; Meltzer *et al.*, 1982; Hruska *et al.*, 1982). Although cyclic AMP has been considered to be the intracellular second messenger for parathyrin (parathyroid hormone) action on the renal tubular cell (Aurbuch & Chase, 1976), there is evidence to suggest that many parathyrin effects on the kidney are Ca^{2+} -dependent (Puschett, 1982; Rasmussen, 1981; McKeown, 1981; Amiel *et al.*, 1976). The present series of experiments was undertaken to characterize the Ca^{2+} -uptake process of isolated renal brush-border membranes and to determine whether PtdA could increase transmembrane Ca^{2+} flux across brush-border membranes, as it appears to in a variety of other membrane systems, thereby providing insight into a biochemical process by which parathyrin may produce an alteration in Ca^{2+} metabolism of the proximal renal tubule cell.

Abbreviations used: PtdA, phosphatidic acid; PtdIns, phosphatidylinositol; PtdCho, phosphatidylcholine.

* To whom reprint requests should be sent.

Methods

Membrane isolation

Brush-border membranes were prepared from homogenized renal cortex by a procedure identical with that described previously from our laboratory (Sastrasinh *et al.*, 1982a), except that for the precipitation step 10 mM-MgCl₂ was used instead of CaCl₂. Enzyme activities to assess purity of the brush-border-membrane preparation were also determined by methods described in detail elsewhere (Sastrasinh *et al.*, 1982a; Knauss *et al.*, 1983). The brush-border-membrane/homogenate average ratio for the brush-border-membrane marker enzyme alkaline phosphatase was 10.0, for the lysosomal marker enzyme *N*-acetyl- β -D-glucosaminidase was 0.4, for the endoplasmic-reticulum marker enzyme NADPH-cytochrome *c* reductase was 0.1, for the mitochondrial membrane marker enzyme cytochrome *c* oxidase was <0.05, the minimal detectable level by our assay, and for the basolateral membrane marker enzyme (Na⁺ + K⁺)-dependent ATPase was 2.0. These ratios are similar to previous reports and indicate substantial purity of the brush-border membrane fraction (Booth & Kenny, 1974; Kempson & Dousa, 1979; Sastrasinh *et al.*, 1982a; Knauss *et al.*, 1983). Protein concentrations were assessed by the method of Lowry *et al.* (1951).

Uptake studies

Brush-border membranes (approx. 200 μ g of protein) were added to an incubation buffer solution consisting of 200 mM-mannitol, 25 mM-Tris/Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4, and 2–4 μ Ci of ⁴⁵CaCl₂. Uptake studies were carried out at 25°C with a final incubation volume of 200 μ l and various total CaCl₂ concentrations. At various time intervals after the initiation of uptake, Ca²⁺ uptake was quenched by diluting the brush-border membrane preparation in 2 ml of ice-cold stop solution consisting of 200 mM-mannitol, 25 mM-Tris/Hepes, pH 7.4, and 1 mM-EGTA and immediately filtered using the Millipore filtration technique with 0.5 μ m cellulose acetate filters (Millipore Corp., Bedford, MA, U.S.A.). The filters were washed with 4 ml of ice-cold wash solution to remove extravesicular and outer-membrane-bound ⁴⁵Ca²⁺. The wash solution was identical with the stop solution. The dilution, filtration and washing steps took less than 12 s to complete. The filters containing the vesicles were placed in Aquasol (New England Nuclear, Boston, MA, U.S.A.) and the radioactivity measured using a Beckman scintillation counter.

To assess the modulation of ⁴⁵Ca²⁺ uptake by phospholipids, brush-border membranes were isolated and pre-incubated with 250 μ M-PtdA, -PtdIns and -PtdCho for 10 min at 25°C in the standard

incubation buffer. The suspensions were then centrifuged and the pellets washed and resuspended in standard buffer. ⁴⁵Ca²⁺ uptake was then measured in these treated vesicles.

The effect of ATP on Ca²⁺ uptake by brush-border membranes was assessed. Immediately after isolation, brush-border membranes were added to an incubation solution containing 200 mM-mannitol, 25 mM-Tris/Hepes, pH 7.4, 5 mM-MgCl₂, 5 mM-ATP and 0.1 mM-⁴⁵CaCl₂. ⁴⁵Ca²⁺ uptake was measured for various time intervals at 25°C.

Na⁺-dependent D-glucose uptake was assessed by the Millipore filtration technique. Uptake was initiated by adding 50 μ l of brush-border membrane suspension containing between 200 and 300 μ g of protein in 150 μ l of an uptake buffer consisting of 100 mM-mannitol, 50 μ M-NaCl, 5 mM-Tris/Hepes, pH 7.4, and 50 mM-[¹⁴C]glucose. Equilibrated uptake was determined after an incubation time of 90 min at 25°C. The uptake of D-glucose was terminated by adding 1 ml of ice-cold stop solution containing 100 mM-NaCl, 1 mM-phlorizin, a specific inhibitor of the D-glucose transport system, and 1 mM-Tris/Hepes, pH 7.4, and immediately poured on pre-wetted 0.45 μ m Millipore filters (HAWP). Brush-border membranes on the filters were washed twice with 4 ml of ice-cold rinse solution containing 154 mM-NaCl and 1 mM-Tris/Hepes, pH 7.4. The filters were dissolved in 10 ml of Aquasol and counted for radioactivity with a liquid-scintillation counter.

Ca²⁺ binding to brush-border membranes

Brush-border membranes (approx. 200 μ g of protein) were added to an assay solution consisting of 200 mM-mannitol, 25 mM-Tris/Hepes, pH 7.4, and various concentrations of ⁴⁵CaCl₂ with or without a 1000-fold excess of ⁴⁰CaCl₂. Inasmuch as ⁴⁵Ca²⁺ uptake by brush-border membranes approached a steady state after incubation for 60 min at 25°C, all binding studies were carried out at 60 min at 25°C. Incubation was abruptly stopped by diluting the brush-border membrane suspension 10-fold in 2 ml of ice-cold stop solution containing 200 mM-mannitol, 25 mM-Tris/Hepes, pH 7.4, and 1 mM-EGTA. Separation of membrane bound from the non-bound radioligand was accomplished by the Millipore filtration technique. Brush-border membranes on the filters were washed with 4 ml of ice-cold stop solution, and the radioactivity remaining on the filters was counted by liquid scintillation. Total radioactivity minus radioactivity obtained in the presence of a 1000-fold excess of unlabelled Ca²⁺ was used to define specific uptake of Ca²⁺ by brush-border membranes. Because brush-border membranes spontaneously reseal into vesicles, a portion of the radioligand associated with brush-

border membranes will be unbound and free in the intravesicular space. An estimate for this unbound $^{45}\text{Ca}^{2+}$ associated with brush-border membranes at equilibrium can be derived from multiplying the Ca^{2+} concentration of the incubation solution and the intravesicular volume. The intravesicular volume was estimated by measuring the equilibrated uptake of $[^{14}\text{C}]\text{glucose}$ (Aronson & Sacktor, 1975) using the methods described above. Specific binding of $^{45}\text{Ca}^{2+}$ to brush-border membranes was thus calculated by subtracting the $^{45}\text{Ca}^{2+}$ unbound and free within the intravesicular space from specific $^{45}\text{Ca}^{2+}$ uptake. Because the membrane-binding sites for Ca^{2+} are saturable, the percentage of total uptake representing the free intravesicular pool will increase as the Ca^{2+} concentration of the incubation medium rises. In this regard, for incubation Ca^{2+} concentrations of 5, 20, 50, 100, 200, 500, 1000 and 2000 μM , this percentage was measured and calculated to be 1.5, 3.7, 5.6, 6.6, 11.2, 15.8, 24.1 and 37.0% respectively. To measure total uptake of $^{45}\text{Ca}^{2+}$ at Ca^{2+} concentrations below 100 μM , 2 mM-nitrilotriacetic acid was added to the binding assay solution to attain accurate concentrations of Ca^{2+} in the micromolar range (Reed & Bygrave, 1975). The Ca^{2+} activity of the resulting solution was measured directly using a Ca^{2+} selective electrode (Radiometer model F2111Ca) as described in a previous report from our laboratory (Sastrasinh *et al.*, 1982b). Curvilinear Scatchard plots were analysed by the method of Priore & Rosenthal (1976).

Efflux studies

Brush-border membranes were pretreated with various phospholipids as described previously. The treated brush-border membranes were preloaded with $^{45}\text{Ca}^{2+}$ by incubation in the standard incubation buffer containing 0.1 mM- $^{45}\text{CaCl}_2$ at 4°C for 60 min. This temperature and this time interval were chosen after preliminary studies demonstrated that this procedure maximized $^{45}\text{Ca}^{2+}$ uptake but minimized the difference in $^{45}\text{Ca}^{2+}$ uptake between phospholipid-treated brush-border membranes and sham-control-treated brush-border membranes. Efflux of Ca^{2+} was initiated by diluting the brush-border membranes preloaded with $^{45}\text{Ca}^{2+}$ 10-fold into an incubation solution containing 200 mM-mannitol, 25 mM-Tris/Hepes, pH 7.4, and 1 mM-EGTA. At various time intervals, $^{45}\text{Ca}^{2+}$ efflux was immediately stopped by diluting the brush-border membranes in the standard EGTA-quench solution, followed by the usual filtration and washing steps.

$[\gamma\text{-}^{32}\text{P}]\text{ATP}$ labelling and measurement of brush-border-membrane phospholipids

Brush-border membranes were isolated and placed in the standard incubation buffer but also containing 5 mM- MgCl_2 and 5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. After

15 min of incubation, suspensions of brush-border membranes (approx. 4 mg of protein) were extracted, separated and measured by procedures described in detail in previous reports from our laboratory (Sastrasinh *et al.*, 1982a; Knauss *et al.*, 1983).

Reagents

$^{45}\text{CaCl}_2$, D- $[\text{U-}^{14}\text{C}]\text{-glucose}$ and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). All phospholipids, valinomycin, gentamicin sulphate and Ruthenium Red were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium orthovanadate was purchased from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.). Merck Silica Gel 60 plates were obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). Verapamil was a gift from Dr. Edward Kirstein, Knoll Pharmaceutical Co. (Wipanny, NJ, U.S.A.). Chloroform and methanol were 'HPLC' grade. All other chemicals and solvents were standard reagent grade.

Results

Binding studies

As shown in Fig. 1, Scatchard analysis of the specific binding curve of Ca^{2+} binding to internal membrane sites demonstrated a curvilinear relationship. Computer-derived best fits to this curve revealed two binding sites, a high-affinity site with a dissociation constant, K_d , of 13 μM and a low-affinity site with a K_d of 1.5 mM. Since normal free cytosolic $[\text{Ca}^{2+}]$ is less than 0.5 μM (Murphy *et al.*, 1980), a value substantially below the K_d for Ca^{2+}

Fig. 1. Scatchard analysis of Ca^{2+} binding to internal membrane sites of renal brush-border membranes. The two lines are computer-derived best fits to the curvilinear Scatchard plot. Each point is the mean of three separate determinations.

binding to interior membranes sites, virtually all the Ca^{2+} uptake by brush-border membranes determined with the EGTA-quench technique used in the present study is likely to be dissociated from interior binding sites under normal physiological conditions and, thereby, ultimately would represent trans-membrane flux of Ca^{2+} .

Uptake studies

Fig. 2 displays the initial velocity of Ca^{2+} uptake by brush-border membranes with increasing Ca^{2+} concentration, as defined by Ca^{2+} uptake in the first 20 s, which was the shortest time interval in which consistently reproducible uptakes were obtained. Because of a complex relationship between initial velocity and Ca^{2+} concentration at low Ca^{2+} concentrations, it was not possible to rigorously define the K_m values in this system, but, from graphical analysis of the substrate-velocity curve, the Ca^{2+} concentration at which the initial rate was half-maximal, $K_{0.5}$, was found to be 0.825 mM with a $V_{\max}$ of 2.7 nmol of Ca^{2+} /mg of protein per min.

To test if this transport process is electrophoretic, Ca^{2+} uptake by these vesicles was determined in the presence or absence of a K^+ diffusion potential. As can be seen in Fig. 3, the generation of a K^+ diffusion potential, negative inside, across the membrane significantly increased Ca^{2+} uptake compared with control conditions where no potential existed. The generation of a negative potential inside the membrane vesicle, therefore, increased the rate of Ca^{2+} uptake, suggesting that the translocation of Ca^{2+} across the membrane is electrophoretic.

Further support for the electrophoretic nature of this Ca^{2+} transport process was provided by

Fig. 2. Initial rates of Ca^{2+} uptake by brush-border membranes as a function of external Ca^{2+} concentration. Each point is mean $\pm$ S.E.M. of six experiments.

determining Ca^{2+} uptake in the presence of various anions of sodium salts, including SCN^- , NO_3^- and SO_4^{2-} . The relative abilities of these anions to

Fig. 3. Effect of K^+ diffusion potential, negative inside the vesicle, on Ca^{2+} uptake by brush-border membranes. An electrical gradient across the brush-border membranes was produced by imposing a K^+ concentration gradient with $\text{K}^+_{\text{inside}} ([\text{K}^+]_{\text{in}}) > \text{K}^+_{\text{outside}} ([\text{K}^+]_{\text{out}})$ in the presence of the K^+ ionophore valinomycin (O) (Henderson *et al.*, 1969). This condition was compared with the control situation in which no K^+ concentration gradient existed across the membrane ($[\text{K}^+]_{\text{in}} = [\text{K}^+]_{\text{out}}$; ●). Brush-border membranes were preloaded with K^+ by incubating the vesicles in 67 mM- K_2SO_4 /100 mM-mannitol/25 mM-Tris/Hepes, pH 7.4, for 15 min. The brush-border membranes were then diluted 10-fold either in an incubation medium containing 300 mM-mannitol, 25 mM-Tris/Hepes, pH 7.4, 0.5 mM- $^{45}\text{CaCl}_2$ and valinomycin (25 $\mu\text{g}/\text{ml}$) to establish a K^+ diffusion potential or in incubation medium identical with the preloading solution but containing 0.5 mM- $^{45}\text{CaCl}_2$ and valinomycin (25 $\mu\text{g}/\text{ml}$) as a control condition to clamp the membrane potential at 0 mV. Preliminary studies demonstrated a requirement for pre-equilibration of the brush-border membrane suspension with valinomycin to produce a maximal effect of K^+ diffusion potentials. Thus vesicles were washed, pelleted and resuspended in buffers containing valinomycin. Data are means $\pm$ S.E.M. for four experiments. For each time interval, differences between experimental and control values were statistically significant ($P < 0.01$, paired *t*-test, two-tailed).

Fig. 4. The effect of various anions on Ca^{2+} uptake by brush-border membranes

Brush-border membranes were isolated in 300 mM-mannitol/25 mM-Tris/Hepes, pH 7.4, and diluted 10-fold in incubation media containing 0.1 mM- $^{45}\text{CaCl}_2$ and 100 mM-NaSCN (●), 100 mM- NaNO_3 (△) or 100 mM- Na_2SO_4 (▲) to impose an anion concentration gradient across the vesicles ($[\text{anion}]_{\text{out}} > [\text{anion}]_{\text{in}}$). Each of these media also contained 25 mM-Tris/Hepes, pH 7.4, and for the buffers containing NaSCN and NaNO_3 100 mM-mannitol. $^{45}\text{Ca}^{2+}$ uptake was measured at various time intervals after initiating uptake. Each point is the mean of three separate experiments.

Fig. 5. Effects of various multivalent cations on Ca^{2+} uptake by brush-border membranes

Various concentrations of LaCl_3 , MnCl_2 , SrCl_2 , BaCl_2 , MgCl_2 and CaCl_2 were added to the incubation solution containing $20 \mu\text{M}$ - $^{45}\text{Ca}^{2+}$. Brush-border membranes were added to the incubation buffer and $^{45}\text{Ca}^{2+}$ uptakes were assessed after 6 min. Percentage control uptake is plotted as a function of increasing concentration of cation. Each point is the average of three separate experiments.

develop diffusion potentials, negative inside, are $\text{SCN}^- > \text{NO}_3^- > \text{SO}_4^{2-}$ (Gamble & Lehninger, 1973). As shown in Fig. 4, the action of these anions to increase Ca^{2+} uptake displayed a similar relationship, suggesting once more that Ca^{2+} flux across the vesicle membrane is electrophoretic.

To determine the specificity of the Ca^{2+} transport process, the effects of various cations on brush-border-membrane uptake of Ca^{2+} were assessed and are displayed in Fig. 5. Mg^{2+} and Ba^{2+} were least effective in competing with Ca^{2+} for vesicle uptake. Sr^{2+} and Mn^{2+} were slightly better, and La^{3+} was most effective. Of note is the selectivity of this uptake process for Ca^{2+} compared with Mg^{2+} , the other major bivalent cation in physiological fluids. Other classical inhibitors of Ca^{2+} transport were investigated in this system. Ruthenium Red, gentamicin and verapamil all inhibited Ca^{2+} uptake by brush-border membranes but the magnitude of inhibition was modest. Verapamil, at 0.1 and 1.0 mM, decreased Ca^{2+} uptake to 91 ± 5 and 79 ± 1 ($P < 0.001$) % of control values respectively; Ruthenium Red, at 10 and 100 μM , diminished Ca^{2+} uptake to 87 ± 5 ($P < 0.02$) and 66 ± 6 ($P < 0.001$) % of control values respectively; gentamicin, at 0.1 and

1.0 mM, reduced Ca^{2+} uptake to 79 ± 8 ($P < 0.05$) and 72 ± 6 ($P < 0.02$) of control values respectively. These results, therefore, characterize this Ca^{2+} transport process in renal brush-border membranes, as saturable, quite specific for Ca^{2+} and influenced by transmembrane potential.

Pretreatment of isolated brush-border membranes with 250 μM -PtdA doubled the brush-border-membrane content of PtdA from a control value of 614 to 1242 ng of lipid phosphorus/mg of protein ($n = 4$). This doubling of PtdA content of brush-border membranes was associated with a significant increase in Ca^{2+} uptake along the entire time course compared with control preparations (Fig. 6). The effect during the early time intervals was substantially smaller than at later time intervals. Pretreatment of brush-border membranes with the neutral phospholipid phosphatidylcholine, however, had no effect on Ca^{2+} uptake.

Efflux studies

Since these uptake values reflect both interior membrane binding and transport into the free intravesicular space but do not allow a clear

Fig. 6. Ca^{2+} uptake by brush-border membranes pretreated with PtdA (●), PtdCho (▲) or sham control (○). Incubation solution contained $0.1\text{ mM } ^{45}\text{CaCl}_2$. All values for PtdA pretreatment were significantly higher ($P < 0.05$) compared with controls except for the two earliest time intervals. No differences between PtdCho-treated vesicles and controls were demonstrated. Data are means $\pm$ S.E.M. for four separate experiments.

distinction between these two components, Ca^{2+} efflux studies were done to help better isolate the effect of PtdA on transmembrane transport. Ca^{2+} efflux was assessed in brush-border membranes pretreated with PtdA or sham controls and preloaded with $^{45}\text{Ca}^{2+}$ in an incubation medium containing $0.1\text{ mM } ^{45}\text{CaCl}_2$. The effect of PtdA to increase Ca^{2+} efflux over control values is depicted in Fig. 7. Since this increase was most apparent during the first 2 min of efflux, the effects of PtdA, PtdIns and PtdCho on the rate of efflux over this time interval was measured (Fig. 8). The increased rate of Ca^{2+} efflux from brush-border membranes preloaded with Ca^{2+} occurred only in vesicles pretreated with PtdA and not in vesicles pretreated with PtdIns or PtdCho.

Effect of ATP on Ca^{2+} uptake

Because 1,2-diacylglycerol kinase, which converts 1,2-diacylglycerol into PtdA in the presence of ATP, is a membrane-bound enzyme (Michell, 1975), an attempt to increase the PtdA content of brush-border membranes without exogenous administration of the phospholipid was made by incubating brush-border membranes with ATP. This incubation with ATP resulted in the synthesis of PtdA, as well as phosphatidylinositol monophosphate and phosphatidylinositol bisphosphate, within brush-border membranes as demonstrated by isotopic labelling of these phospholipids when $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was included in the incubation medium (Fig. 9). This increase in synthesis of these phospholipids was associated with a significant increase in Ca^{2+} uptake

Fig. 7. Ca^{2+} efflux out of vesicles pretreated with PtdA (●) or sham control (○) and preloaded with $^{45}\text{CaCl}_2$. Data are means $\pm$ S.E.M. for seven separate experiments.

Fig. 8. Effects of pretreatment of brush-border membranes with various phospholipids on the rate of Ca^{2+} efflux during the rapid initial 2 min phase of $^{45}\text{Ca}^{2+}$ release

PtdA was unique among the phospholipids tested in its action to increase significantly the rate of Ca^{2+} efflux.

by brush-border membranes compared with control conditions without ATP (Fig. 10). The effects during early time intervals, however, were substantially smaller than at later time intervals. However, if brush-border membranes were not used within 15 min of isolation, the ability of ATP to phosphorylate these lipids and to increase Ca^{2+} uptake was lost.

This increase in Ca^{2+} uptake appeared not to be due to a $(\text{Ca}^{2+} + \text{Mg}^{2+})$ -dependent ATPase, since the addition of the Ca^{2+} ionophore A23187 ($10\text{ }\mu\text{g/ml}$) at 20 and 60 min of incubation did not decrease the

Fig. 9. Tracings of ^{32}P autoradiographs of lipid extracts from brush-border membranes treated with 5 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the three separate solvent systems

Phospholipids were identified by co-chromatography with known pure standards. Only PtdA, phosphatidylinositol phosphate (PtdInsP) and phosphatidylinositol biphosphate (PtdInsP₂) were labeled by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Solvent systems A, B and C correspond respectively to A, C and D as described by Knauss *et al.* (1983).

Fig. 10. Effect of the addition of ATP to the incubation buffer on Ca^{2+} uptake by brush-border membranes

Ca^{2+} uptake was measured in an incubation medium containing 0.1 mM $^{45}\text{CaCl}_2$. Exposure of brush-border membranes to ATP significantly increased ($P < 0.05$) all uptake values except for the initial time interval compared with controls. The points to the right of the curves represent Ca^{2+} uptake values for both control and ATP-treated vesicles in the presence of A23187 (10 $\mu\text{g}/\text{ml}$) both at 20 and 60 min. Data are means $\pm$ S.E.M. for five experiments.

number of ATPases, did not diminish the effect of ATP to increase Ca^{2+} uptake by brush-border membranes (results not shown). Furthermore, this Ca^{2+} uptake was not due to mitochondrial contamination of the membrane preparation, since $^{45}\text{Ca}^{2+}$ uptake in control preparations was not inhibitable by addition of both oligomycin (5 $\mu\text{g}/\text{ml}$), an inhibitor of the mitochondrial ATPase, and antimycin (0.8 $\mu\text{g}/\text{ml}$), an inhibitor of the mitochondrial electron transport chain, to the incubation buffer (results not shown).

Discussion

The initial and probably rate-limiting step in trans-epithelial transport of Ca^{2+} across both intestinal and renal proximal epithelia occurs at the luminal brush-border membrane (Martin & DeLuca, 1969; Adams *et al.*, 1970). The characterization of this initial transport process is thus important in understanding the mechanism of directional Ca^{2+} transport across transporting epithelium. Furthermore, insight into the manner in which PtdA may modulate this Ca^{2+} transport step provides further understanding of a possible pathway by which hormones, in general, and parathyrin, in particular, may promote an increase in plasma-membrane permeability to Ca^{2+} .

A major difficulty in characterizing the Ca^{2+} transport process across isolated vesicular membrane systems is the existence of multiple Ca^{2+} pools associated with the membranes. In this regard, Ca^{2+} may bind both to external and internal membrane sites as well as being free within the intravesicular space. Since only the internal fraction is relevant to kinetic studies of Ca^{2+} transport, methods were employed in the present study to displace Ca^{2+} from external membrane binding sites. Similar to methods employed for the study of mitochondrial Ca^{2+} transport (Reed & Bygrave, 1975), Ca^{2+} transport in brush-border membranes was assessed by the use of $^{45}\text{Ca}^{2+}$, a rapid filtration step to separate the vesicles from the incubation media, a step to quench rapidly the transport process by using a stop solution containing 1 mM EGTA and a step to remove externally membrane-bound Ca^{2+} by washing brush-border membranes with solutions containing 1 mM-EGTA. The Ca^{2+} remaining with the brush-border membranes thereby represents Ca^{2+} that is either bound to internal membrane sites or is free in the intravesicular space. Attempts have been made to discriminate further between these two remaining Ca^{2+} pools (Gmaj *et al.*, 1979; Rasmussen *et al.*, 1979), since the internally bound pool has been suggested not to represent transmembrane cation transport as defined by movement of an ion from one aqueous compartment to another. In this regard, characterization of Ca^{2+} binding to internal mem-

amount of $^{45}\text{Ca}^{2+}$ associated with brush-border membranes and the incubation of brush-border membranes with vanadate (100 μM), an inhibitor of a

brane sites in the present study demonstrated that Ca^{2+} binds to at least two distinct sites with K_d values of $13\text{ }\mu\text{M}$ and 1.5 mM , values far in excess of the normal free cytosolic activity, which has been estimated to be less than $0.5\text{ }\mu\text{M}$ (Murphy *et al.*, 1980). Under normal physiological circumstances, therefore, Ca^{2+} that has moved from the vesicle exterior to interior membrane sites would quickly dissociate and thereby represent transmembrane flux of Ca^{2+} . Although the values of Ca^{2+} uptake by brush-border membranes reported in this present study represent two separate Ca^{2+} pools, the values appear to be reasonable measures of transmembrane Ca^{2+} transport across this vesicular membrane preparation.

This Ca^{2+} uptake process exhibited saturation kinetics with a $K_{0.5}$ of 0.875 mM and a V_{max} of $2.7\text{ nmol of Ca}^{2+}/\text{mg of protein per min}$ at 25°C . It was inhibitable with a variety of multivalent cations with $\text{La}^{3+} > \text{Mn}^{2+} \gg \text{Sr}^{2+} > \text{Ba}^{2+} \gg \text{Mg}^{2+}$. La^{3+} was the most effective at high concentrations; it was least effective at low concentrations. Furthermore, Mg^{2+} competed poorly with Ca^{2+} , so that this transport process was highly selective for Ca^{2+} compared with Mg^{2+} , the other major physiologically relevant bivalent cation.

A variety of Ca^{2+} transport inhibitors, including verapamil, Ruthenium Red and gentamicin, also inhibited Ca^{2+} uptake. Although the concentrations of these agents required to inhibit Ca^{2+} uptake by brush-border membranes are comparable with those that are effective in other membrane systems, the magnitude of their effects in brush-border membranes are small compared with the near complete inhibition some of these compounds have on other Ca^{2+} transport processes that have been shown to be inhibitable by these agents with high specificity, for example, the mitochondrial Ca^{2+} transporter for Ruthenium Red and the slow Ca^{2+} channel of myocardial cells for verapamil (Reed & Bygrave, 1974; Bayer *et al.*, 1975). The reason for this difference probably resides in the manner by which these compounds inhibit Ca^{2+} uptake by brush-border membranes. Rather than being specific inhibitors of this brush-border membrane transport process for Ca^{2+} , these agents displace Ca^{2+} from membrane binding sites (Reed & Bygrave, 1974; Lodhi *et al.*, 1976), resulting in a small but demonstrable inhibitory effect on Ca^{2+} uptake.

This Ca^{2+} transport process was electrophoretic. The generation of a K^+ diffusion potential, inside negative, by imposing a K^+ concentration gradient ($[\text{K}^+]_{\text{in}} > [\text{K}^+]_{\text{out}}$) in the presence of the electro-neutral K^+ ionophore significantly increased the rate of Ca^{2+} uptake by brush-border membranes. Further support for the electrophoretic nature of this transport process was demonstrated by the effects of SCN^- , NO_3^- , and SO_4^{2-} on Ca^{2+} uptake by

brush-border membranes. The rate of Ca^{2+} uptake by brush-border membranes in the presence of these anion gradients directly correlated with the anion's ability to generate a diffusion potential, inside negative, across the vesicular membrane. These results, therefore, characterize this Ca^{2+} transport process by isolated renal brush-border membranes as electrophoretic, inhibitable by multivalent cations and Ca^{2+} entry inhibitors, selective for Ca^{2+} compared with Mg^{2+} , and saturable. This characterization is very similar to the Ca^{2+} uptake process recently described for isolated brush-border membranes from rat intestine (Miller & Bronner, 1981).

The present experiments also demonstrate that elevations in PtdA content of brush-border membranes by either exogenous addition or endogenous generation of newly synthesized PtdA was associated with increases in Ca^{2+} uptake by brush-border membranes. Endogenous synthesis of PtdA was achieved by incubating isolated brush-border membranes in a solution containing ATP and Mg^{2+} . Since 1,2-diacylglycerol kinase, which converts 1,2-diacylglycerol into PtdA in the presence of ATP, is a membrane-bound enzyme (Michell, 1975), incubation of brush-border membranes with ATP results in the synthesis of PtdA. This process occurred in isolated brush-border membranes, as demonstrated by autoradiographs of phospholipid extracts of brush-border membranes incubated in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. This ATP-mediated increase in Ca^{2+} uptake may also have been due, at least in part, to an increase in membrane content of phosphatidylinositol phosphate, and phosphatidylinositol biphosphate, since the incorporation of ^{32}P into phosphatidylinositol phosphate and phosphatidylinositol biphosphate also occurred during this incubation and demonstrated that PtdIns kinase and phosphatidylinositol phosphate kinase are also membrane-bound enzymes of brush-border membranes.

At the concentrations of Ca^{2+} used in these experiments, the increase in brush-border membrane Ca^{2+} uptake after exogenous or endogenous increases in brush-border membrane PtdA content was predominantly due to internal membrane binding, thereby demonstrating the ability of PtdA to mediate Ca^{2+} transfer from membrane exterior to membrane interior sites. Since PtdA has an apparent stability constant of $1.6 \times 10^4\text{ M}^{-1}$ for Ca^{2+} (Abramson *et al.*, 1966), under normal physiological circumstances in which the cytosolic $[\text{Ca}^{2+}]$ is less than $0.5\text{ }\mu\text{M}$, Ca^{2+} associated with PtdA along the membrane interior would quickly dissociate. Thus the increase in Ca^{2+} uptake induced by PtdA in brush-border membranes is indicative of a transmembrane transport process. To demonstrate in a direct way the ability of PtdA to increase Ca^{2+} flux across brush-border membranes from one

aqueous compartment to another, $^{45}\text{Ca}^{2+}$ efflux studies were carried out in brush-border membranes preloaded with $^{45}\text{Ca}^{2+}$ and diluted into a solution containing EGTA. Since EGTA has an apparent association constant for Ca^{2+} of less than $2 \times 10^6 \text{ M}^{-1}$ (Bers, 1982), Ca^{2+} that has migrated from the vesicle interior to external binding sites will be chelated to EGTA present in large excess in the incubation solution and effectively removed from the membrane, so that under these conditions Ca^{2+} efflux most likely represents transmembrane transport of Ca^{2+} . In this regard, PtdA pretreatment of brush-border membranes increased both the magnitude and rate of Ca^{2+} transport across the preloaded vesicle compared with sham-control preparations. Furthermore, PtdA was unique among the phospholipids tested to increase Ca^{2+} efflux; PtdIns and PtdCho did not increase Ca^{2+} efflux above control levels.

These studies indicate an important influence of PtdA on Ca^{2+} uptake by renal brush-border membranes. The uptake studies demonstrate a significant effect of an increase in PtdA membrane content to augment the capacity for Ca^{2+} uptake by these vesicles, most likely by increasing membrane binding sites for Ca^{2+} . Initial rates for $^{45}\text{Ca}^{2+}$ uptake by brush-border membranes after exogenous or endogenous addition of PtdA were slightly, but not significantly, increased above control values, arguing against a calcium ionophoric role for PtdA. Only at later time intervals did $^{45}\text{Ca}^{2+}$ uptake by treated vesicles become significantly higher than control. A previous report has suggested that early uptake rates reflect primarily membrane binding events, whereas later rates assess transmembrane flux (Rasmussen *et al.*, 1979), so that the significantly greater $^{45}\text{Ca}^{2+}$ uptake rates in treated brush-border membranes compared with control preparations at later time intervals may still reflect an ionophoric capability for PtdA. In summary, these studies demonstrate that Ca^{2+} transport by brush-border membranes is saturable, highly specific for Ca^{2+} and is influenced substantially by transmembrane potential. They also suggest that PtdA may modulate Ca^{2+} transport in brush-border membranes, perhaps by facilitating transmembrane flux. Further studies are undoubtedly required to determine the precise role of PtdA in Ca^{2+} transport by renal brush-border membranes.

H. D. H. is an Established Investigator of the American Heart Association. J. M. W. is a Research Associate of the Veterans Administration. These studies were supported, in part, by the Veterans Administration Research Service, by Public Health Service Grant 1-R01-AM30879-01 and by a grant from the National Kidney Foundation of Michigan.

References

- Abramson, M. B., Katzman, R., Gregor, H. & Curci, R. (1966) *Biochemistry* **5**, 2207–2213
- Adams, T. H., Wong, R. G. & Norman, A. W. (1970) *J. Biol. Chem.* **245**, 4432–4442
- Amiel, C., Kuntziger, H., Couette, S., Coureau, C. & Bergounioux, N. (1976) *J. Clin. Invest.* **57**, 256–262
- Aronson, P. S. & Sacktor, B. (1975) *J. Biol. Chem.* **250**, 6032–6039
- Aurbuch, G. D. & Chase, L. R. (1976) *Handb. Physiol. Sect. 7: Endocrinol.* 353–380
- Bayer, R., Kalusche, D., Kaufman, R. & Mannhold, R. (1975) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **290**, 81–87
- Bers, D. M. (1982) *Am. J. Physiol.* **242**, C404–C408
- Booth, A. G. & Kenny, A. J. (1974) *Biochem. J.* **142**, 575–581
- Farese, R. V., Bidot-Lopez, P., Sabir, A., Smith, J. S., Schinbeckler, B. & Larsen, R. (1980) *J. Clin. Invest.* **65**, 1523–1526
- Gamble, J. G. & Lehninger, A. L. (1973) *J. Biol. Chem.* **248**, 610–618
- Gmaj, P., Murer, H. & Kinne, R. (1979) *Biochem. J.* **178**, 549–557
- Harris, R. A., Schmidt, J., Hitzemann, B. A. & Hitzemann, R. J. (1981) *Science* **212**, 1290–1291
- Henderson, P. J. F., McGivan, J. D. & Chappell, J. B. (1969) *Biochem. J.* **111**, 521–535
- Hruska, K., Khalifa, S. & Hammerman, M. (1982) *Clin. Res.* **30**, 451A
- Kempson, A. A. & Dousa, T. P. (1979) *Lyè Sci.* **24**, 881–888
- Knauss, T. C., Weinberg, J. M. & Humes, H. D. (1983) *Am. J. Physiol.* **244**, F535–F546
- Limas, C. J. (1980) *Biochem. Biophys. Res. Commun.* **94**, 541–546
- Lodhi, S., Weiner, N. & Schacht, J. (1976) *Biochem. Biophys. Acta* **426**, 781–795
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Martin, D. L. & DeLuca, H. F. (1969) *Arch. Biochem. Biophys.* **134**, 139–148
- McKeown, J. W. (1981) *Clin. Res.* **30**, 458A
- Meltzer, V., Weintreb, S., Bellorin-Font, E. & Hruska, K. A. (1982) *Biochim. Biophys. Acta* **712**, 258–267
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Michell, R. H., Jafferji, S. S. & Jones, L. M. (1977) *Adv. Exp. Med. Biol.* **85**, 447–464
- Miller, A., III and Bronner, F. (1981) *Biochem. J.* **196**, 391–401
- Murphy, E., Coll, K., Rich, T. L. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6600–6608
- Ohsako, S. & Teguchi, T. (1981) *J. Biol. Chem.* **256**, 10945–10948
- Priore, R. L. & Rosenthal, H. E. (1976) *Anal. Biochem.* **70**, 231–240
- Puschett, J. B. (1982) *Mineral Electrolyte Metab.* **7**, 281–284
- Putney, J. W. Jr., Weiss, S. J., Van De Walle, C. M. & Haddas, R. A. (1980) *Nature (London)* **284**, 345–347
- Rasmussen, H. (1981) *Calcium and cAMP as Synarchic*

- Messengers*, pp. 160–163, John Wiley and Sons, New York
- Rasmussen, H., Fontaine, O., Max, E. E. & Goodman, D. B. P. (1979) *J. Biol. Chem.* **254**, 2993–2999
- Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **140**, 143–155
- Reed, K. C. & Bygrave, F. L. (1975) *Anal. Biochem.* **67**, 44–54
- Salmon, D. M. & Honeyman, T. W. (1980) *Nature (London)* **284**, 344–345
- Sastrasinh, M., Knauss, T. C., Weinberg, J. M. & Humes, H. D. (1982a) *J. Pharmacol. Exp. Ther.* **222**, 350–358
- Sastrasinh, M., Weinberg, J. M. & Humes, H. D. (1982b) *Life Sci.* **30**, 2309–2315
- Serhan, C., Anderson, P., Goodman, E., Dunham, P. B. & Weissman, G. (1981) *J. Biol. Chem.* **256**, 2736–2741
- Serhan, C., Fridovich, J., Goetzl, E. J., Dunham, P. B. & Weissman, G. (1982) *J. Biol. Chem.* **257**, 4746–4752
- Tyson, C. A., Vande Zande, H. & Green, D. E. (1976) *J. Biol. Chem.* **251**, 1326–1332