Cell-matrix interactions modulate transepithelial phosphate transport in P\(_1\)-deprived OK cells

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Barac-Nieto M, Weinman EJ, Spitzer A. Cell-matrix interactions modulate transepithelial phosphate transport in P\(_1\)-deprived OK cells. Am J Physiol Cell Physiol 293: C1272–C1277, 2007. First published July 25, 2007; doi:10.1152/ajpcell.00051.2006.—In opossum kidney (OK) cells as well as in kidney proximal tubules, P\(_1\) deprivation increases apical (A) and basolateral (B) Na\(^+\)-dependent P\(_1\) cell influxes. In OK cells’ monolayers in contrast to proximal tubules, there is no increase in transepithelial P\(_1\) transport. This limitation may be due to altered cell-matrix interactions. A and B cell 32P\(_i\), uptakes and transepithelial 32P\(_i\) and [14C]mannitol fluxes were measured in OK cells grown on uncoated or on Matrigel-coated filter inserts. Cells were exposed overnight to solution of either low (0.25 mM) or high (2.5 mM) Pi. When grown on Matrigel, immunofluorescence of apical (A) cell membranes were exposed (2).

The absence of transepithelial Pi transport in vitro may be the consequence of large paracellular fluxes that may overwhelm and mask any increase in transcellular P\(_i\) transport. Indeed, we found that large (60–80%) of the P\(_i\) fluxes of mannitol occur across monolayers of OK cells grown on uncoated filters (2).

We have also shown that in OK cells grown on uncoated filters, transepithelial P\(_i\) fluxes and the intracellular P\(_i\) pool were reduced in response to severe P\(_1\) deprivation, despite a decrease in paracellular fluxes and increased influx of P\(_i\) across A and B cell membranes (1, 2). Effective permeability for cell P\(_i\) efflux across B or A cell membranes and the distribution of intracellular P\(_i\) among inorganic and organophosphates fractions were unaltered (2). These findings suggest that in severely P\(_1\)-deprived OK cells grown on uncoated filters, P\(_i\) enters cell compartments where it is not available for transepithelial transport. Cell compartments depend on the structure of the cytoskeleton (14), which may be influenced by cell-matrix interactions through kinases and proteins linking the cytoskeleton to the cell membrane (NHERF1-PDZK1-MAP17-Ezrin) (4, 5, 7). Our findings on P\(_1\)-depleted (P\(_i\),d) cells stimulated our interest to study transepithelial P\(_i\) transport in OK cells grown on a matrix more akin to the natural basement membrane.

METHODS

Monolayers of OK cells were grown to confluence in a 5% CO\(_2\)-95% air atmosphere at 37°C, on uncoated or Matrigel-coated Cyclospore (polyethylene terephthalate, PET) filter (0.45-μm pore diameter) inserts. Inserts were placed in the wells of plates containing 2 ml DMEM-Ham F12 10% FBS in the compartment (B) to which the basolateral cell membranes were exposed. Two milliliters of the same medium were placed in the insert compartment (A) to which the apical cell membranes were exposed (2).

The cells were exposed 16 h to FBS-free Ham F12 solutions containing either low (0.25 mM) or high (2.5 mM) P\(_i\). The cells were then exposed to 2 ml FBS-free Ham-F12 containing 0.25 mM 32P\(_i\), and [14C]mannitol in the A or the B compartments and to 2 ml FBS-free Ham F12 containing 0.25 mM unlabeled P\(_i\), and 0.25 mM mannitol in the opposite compartment. Aliquots (25 μl) were sampled from the latter compartment (B or A, respectively) after 5 or 15, 30, and 60 min to determine transepithelial 32P\(_i\) and [14C]mannitol fluxes, in triplicate wells.

Uptakes of 32P\(_i\), from either the A or the B compartments into the OK cell monolayers were measured after 5 min exposure to 0.25 mM 32P\(_i\). Uptake was stopped by aspiration and by three rinsings of the two compartments with ice-cold 100 mM Mg gluconate. The cells were lysed in 2 ml 0.5% Triton X100 in 0.2 N KOH in a plate shaker for 1 h. Aliquots of the lysate were used for Liquid Scintillation counting in 10 ml Aquasol (2). Proteins were measured using the

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Bio-Rad Coomassie Blue reagent (2) and γ-globulin in 0.5% Triton 0.2 N KOH as standard.

Fluorescence microscopy. OK cells were grown to confluency on filter inserts uncoated or coated with Matrigel (1:10 in DMEM-F12), exposed for 16 h to 0.25 or 2.5 mM Pi in DMEM-F12, fixed for 20 min with 3% paraformaldehyde in PBS supplemented with Ca²⁺- Mg²⁺, and quenched for 10 min with 20 mM glycerine before staining. After being blocked with 10% goat serum in PBS, cells were incubated overnight with COOH-terminal anti-NaPi4 (an isoform of the sodium-phosphate cotransporter) chicken primary antibody and the next day rinsed and incubated with Alexa 488 Fluor-conjugated secondary rabbit antibody (Molecular Probes, Eugene, OR) against chicken IgG/IgY. Samples were mounted in 90% glycerol (Merck, Darmstadt, Germany) and 10% PBS containing 2.5% 1,4-diazabicyclo[2.2.2]octane (Sigma). Imaging was done with a confocal microscope (LSM 410, C. Zeiss, Thornwood, NY) with ×63 and ×40 oil immersion objectives.

The OK cell line was kindly provided by Drs. Biber and Murer (Zurich, Switzerland). Cell culture supplies, including the PET filter inserts, were obtained from BD, Falcon (Lincoln Park, NJ), or Gibco-BRL (Grand Island, NY). Radioisotopes were purchased from DuPont New England Nuclear (Boston, MA). Chemicals were procured from Sigma (St. Louis, MO) or Baker (Phillipsburg, NJ). The NaPi4 antibody was kindly provided by Dr. Moshe Levy (Denver, CO).

Data were analyzed using paired or unpaired t-tests. A P < 0.05 was considered statistically significant. Values are presented as means ± SE of at least three experiments using in each, triplicate determinations for any given experimental condition.

RESULTS

Effects of Pi depletion. In cells grown on Matrigel (Fig. 1A), exposure to 0.25 mM instead of 2.5 mM Pi, in the culture medium (Pi depletion) increased transepithelial unidirectional Pi fluxes (A to B by 4.6-fold, P < 0.001 and B to A by 3.5-fold, P < 0.05). Net transepithelial Pi transport was from A to B and increased 10-fold (P < 0.001) with Pi depletion: A to B by 10-fold, B to A nearly 4-fold (both P < 0.05). The net transepithelial Pi flux was from A to B and was enhanced 8-fold (P < 0.05) (Table 1).

In cells grown on uncoated filters (Fig. 2A), transepithelial Pi fluxes decreased with Pi depletion: the transepithelial A to B flux 0.61 times, (P < 0.001); the B to A flux 0.62 times (P < 0.001). The net transepithelial Pi transport (3 nmol·mg⁻¹·h⁻¹) was only a fraction (0.14) of the unidirectional fluxes and did not significantly change with Pi depletion (P > 0.3, Table 1). The B to A [¹⁴C]mannitol flux (Fig. 2B) did not significantly exceed the A to B flux. These fluxes decreased by a factor of 0.7 (P < 0.01 and P < 0.001, respectively) with Pi depletion. The net transepithelial mannitol flux was from B to A and decreased insignificantly (P > 0.1) with Pi depletion (Table 1).

The estimated unidirectional transcellular Pi fluxes (Table 1) were not significantly altered (P > 0.3) by Pi depletion. The net transcellular Pi flux from A to B decreased by half, but the change was not significant (P > 0.4).

Effects of cell matrix interactions. When compared with cells grown on uncoated filters, Pi-repleted cells (Pi,r) grown on Matrigel (Table 1) had 1.4-fold (P < 0.05) higher transepithelial A to B and B to A Pi fluxes and lower (P < 0.05) paracellular A to B and B to A [¹⁴C]mannitol fluxes. Transcellular unidirectional Pi fluxes were 3.1-fold (A to B, P < 0.05) and 25 fold (B to A, P < 0.05) higher on Matrigel than on uncoated filters (Table 1). However, the net transepithelial Pi, net transcellular Pi, and net paracellular mannitol fluxes in Pi,r cells grown on Matrigel were not significantly different from those observed in Pi,r cells grown on uncoated filters (all P > 0.1) (Table 1).

The largest effects of Matrigel were seen in Pi,d OK cells: Pi,d cells grown on Matrigel-coated compared with uncoated filters had higher fluxes of both Pi and mannitol (Table 1). This was true for the transepithelial unidirectional [⁵²P]Pi flux from A to B, which was 10.6-fold higher (P < 0.001); for the transepithelial Pi flux from B to A, which was 7.7-fold higher (P <
influx into cells. B–C, basolateral influx. *Estimated unidirectional transcellular Pi fluxes (Table 1) were smaller percentages of the respective transepithelial Pi fluxes in cells grown on Matrigel than in those grown on uncoated filters. Consequently, the estimated transcellular Pi fluxes represented a larger percentage of the transepithelial Pi fluxes in cells grown on Matrigel than in those grown on uncoated filters (Table 1).

**Table 1.** Pi fluxes in Pi-replete and Pi-depleted opossum kidney cell confluent monolayers grown on uncoated or on Matrigel-coated porous membrane filters

<table>
<thead>
<tr>
<th></th>
<th>Pi, Replete Cells</th>
<th>Pi, Depleted Cells</th>
<th>Depletion Effect</th>
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<tbody>
<tr>
<td></td>
<td>Matrigel</td>
<td>Uncouated</td>
<td>Mg/Un</td>
</tr>
<tr>
<td>TE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-B</td>
<td>30±4</td>
<td>21±1.4</td>
<td>1.4*</td>
</tr>
<tr>
<td>B-A</td>
<td>25±3</td>
<td>18±0.8</td>
<td>1.4*</td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-B</td>
<td>10.0±0.2</td>
<td>14.5±0.8</td>
<td>0.7‡</td>
</tr>
<tr>
<td>B-A</td>
<td>12.5±1.0</td>
<td>17±1.5</td>
<td>0.7*</td>
</tr>
<tr>
<td>TC</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A-B</td>
<td>20.0±5</td>
<td>6.5±1.4</td>
<td>3.1*</td>
</tr>
<tr>
<td>B-A</td>
<td>12.5±5</td>
<td>0.5±0.8</td>
<td>25*</td>
</tr>
<tr>
<td>TCnet A-B</td>
<td>5.0±1.8</td>
<td>3±0.6</td>
<td>1.7</td>
</tr>
<tr>
<td>PCnet B-A</td>
<td>2.5±1.2</td>
<td>3±1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>TCnet A-B</td>
<td>7.5±2.0</td>
<td>6±1.8</td>
<td>1.3</td>
</tr>
<tr>
<td>A-C</td>
<td>20±4</td>
<td>6.5±1.4</td>
<td>3.1*</td>
</tr>
<tr>
<td>B-C</td>
<td>25±2</td>
<td>7.5±1.0</td>
<td>3.3*</td>
</tr>
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Values are means ± SE (in mmol·mg⁻¹·h⁻¹); for each n = 9–15 wells. Pi, replete cells were exposed overnight to 2.5 mM Pi, Pi-depleted cells were exposed overnight to 0.25 mM Pi. Fluxes of 0.25 mM ³²Pi, and [¹⁴C]mannitol across the monolayer or into the cells were measured. TE, transepithelial (Pi); PC, paracellular (mannitol); TC, transcellular (Pi); A-B, apical to basolateral; B-A, basolateral to apical; Mg/Un: flux in cells on Matrigel/flux in cells on uncoated filters. Depletion effect: flux in Pi-depleted cells/flux in Pi-replete cells. TE and TC net = A-B flux – B-A flux; PC net = B-A flux – A-B flux. A-C, apical influx into cells. B-C, basolateral influx. *P < 0.05, †P < 0.01 comparing flux in Pi,d cells with the respective estimated unidirectional rates in Pi,r cells.

Fig. 2. Cumulative Pi (A) and mannitol (B) fluxes in Pi,d and Pi,r OK cells grown on uncoated porous supports. Fluxes of 0.25 mM ³²Pi (A) and [¹⁴C]mannitol (B) were measured at 5 or 15, 30, and 60 min. Fluxes increased linearly with time. Transepithelial fluxes in the apical (A) to basolateral (B) or B to A directions were measured in contiguous wells of the same culture plate (Fig. 1). Net fluxes were calculated from the difference between these two unidirectional fluxes. Letter r and d refer to fluxes measured in Pi-replete or Pi-deprived OK cells, respectively, as described in METHODS. Measurements were done in triplicate wells. Data points presented are means ± SE of the averages measured in at least three different cell cultures plates. *P < 0.05 comparing flux in Pi,d cells with the same flux measured in Pi,r cells.
transcellular A to B Pi fluxes (Table 1). Consequently, in all these conditions, the apical uptake rates set the pace of the transcellular A to B Pi fluxes. By contrast, in P_{i,d} cells grown on uncoated filters, the A to C Pi uptake rate was 4-fold higher than the estimated transcellular A to B Pi flux (Table 1). In all conditions, basolateral (B to C) Pi uptake into cells was larger than the estimated respective transcellular B to A Pi fluxes (Table 1). Thus the rate of transcellular B to A Pi flux is not set by the basolateral Pi uptake.

Immunofluorescence. Confocal imaging at ×63 exhibited (Fig. 3, XY plane at center of each image) abundance of cells labeled by indirect NaPi4 immunofluorescence with a patchy distribution on the cell surface, in P_{i,d} cells grown on Matrigel but not on those grown on uncoated filters (Fig. 3). Orthogonal sections in the XZ plane (Fig. 3, top insets) and in the YZ plane (Fig. 3, right inset) confirmed the apical localization of the immunofluorescence in the Matrigel grown Pi_{i}-deprived cells and its scarcity in cells grown on uncoated filters. Conventional fluorescence imaging at ×40 revealed that indirect NaPi4 immunofluorescence in P_{i,d} OK cells was 2.5-fold stronger (P < 0.01) in cells grown on Matrigel than in those grown on uncoated filters (see Fig. 3) In turn, P_{i,d} cells exhibited stronger fluorescence intensity than P_{i}, cells grown on uncoated or on Matrigel-coated filters, which hardly exhibited any immunofluorescence. The enhanced expression of NaPi4 in P_{i,d} cells grown on Matrigel than in those grown on uncoated filters is congruent with the higher A to C and A to B Pi fluxes observed in these cells.

In summary, in OK cells grown on Matrigel, Pi_{i} depletion greatly increased unidirectional transepithelial, transcellular Pi_{i}, and paracellular fluxes and markedly enhanced net transepithelial and transcellular apical to basolateral Pi_{i} transport despite an absolute increase in net paracellular flux in the opposite direction. In cells grown on uncoated filters, Pi_{i} depletion increased cell Pi_{i} uptake, but decreased unidirectional transepithelial, transcellular Pi_{i}, and paracellular fluxes similarly in both directions. Net rates of transepithelial, transcellular, and paracellular Pi_{i} transport were not altered by Pi_{i} depletion.

Cell-matrix (Matrigel) interactions increased unidirectional and net transepithelial and transcellular Pi_{i} fluxes, as well as paracellular mannitol fluxes, in P_{i,d} OK cells. In P_{i,d} OK cells, matrix interactions reduced unidirectional Pi_{i} transepithelial and paracellular mannitol fluxes but did not significantly alter net transepithelial, transcellular Pi_{i}, or paracellular fluxes.

**DISCUSSION**

The major new finding of this study was the large increase in net transepithelial Pi_{i} flux from A to B compartments observed in P_{i,d} OK cells monolayers grown on Matrigel. This net increase was mainly due to a fivefold increase in the influx of Pi_{i} across the apical cell membrane and to entry of all Pi_{i} crossing the apical membrane into the transcellular Pi_{i} transport pool, such that the apical influx rate did not differ from the estimated transcellular A to B Pi_{i} flux rate. The large increment in apical Pi_{i} influx was associated with enhanced expression of NaPi4 cotransporters at the apical cell surface and was sufficient to result in a net increment in transepithelial Pi_{i} transport despite increases in net paracellular flux and unidirectional Pi_{i} flux in the opposite direction.

These findings differ from those in cells grown on uncoated filters (2). Under these conditions, the apical Pi_{i} uptake also increased with Pi_{i} depletion (10), but the change was smaller (2 vs. 5-fold) compared with that in cells growing on Matrigel. Since interactions between cell integrins and matrix can alter the cytoskeleton (7) and interactions between the cytoskeleton and the cell membrane are involved in the upregulation of Pi_{i} transport in response to Pi_{i} depletion (4), it is not surprising that the magnitude of the upregulatory response was found to be dependent on cell-matrix interactions. The higher Pi_{i} fluxes
observed in Pi,d OK cells grown on Matrigel correspond with the enhanced expression of NaPi4 cotransporters observed by indirect immunofluorescence confocal microscopy (Fig. 3).

In Pi,d cells grown on uncoated filters only a fraction (0.25) of Pi entering across the apical cell membrane reached the Pi transport pool and appeared on the B side. The restriction was not at the basolateral cell membrane. Indeed, the unidirectional basolateral Pi efflux (C to B) rate, estimated from the net transcellular Pi transport rate plus the measured basolateral (B to C) Pi uptake rate, was found to exceed by sixfold the magnitude of the transcellular A to B Pi flux. Thus basolateral Pi efflux could not have limited the access of Pi transported across the apical cell membrane to the B compartment. It is more likely that in Pi,d cells grown on uncoated filters, most Pi transported across the apical membrane reaches a Pi pool that is unavailable for transcellular transport, perhaps a subapical pool or another intracellular pool, from which it can backflow to the A compartment or enter other Pi pools. Apparently, changes in cell matrix interactions led to changes in the compartmentalization of the intracellular Pi pools possibly through altered cytoskeletal and membrane-cytoskeletal interactions (7, 14).

Cell-matrix (Matrigel) interactions also led to changes in paracellular (mannitol) fluxes in Pi,r and Pi,d cells. This is consistent with their influence of these interactions on the permeability of tight junctions (13). The effect of cell-matrix interactions on paracellular fluxes was found to depend on the Pi status of the cells. Paracellular fluxes were lower in Pi,r cells (0.7×) but higher (3.5×) in Pi,d cells, when grown on Matrigel compared with uncoated filters. This suggests that signaling initiated by cell-matrix interactions is altered by Pi depletion or repletion. However, the fraction of the transcellular Pi flux that could be accounted for by paracellular flux in the same direction was smaller (for example, A to B flux, 0.33 vs. 0.68) in cells grown on Matrigel than in cells grown on uncoated filters, independently of their Pi status. This was due to the concomitant increases in transcellular Pi fluxes observed in cells grown on Matrigel (≥12×), which were particularly large in Pi,d cells (≥35×). That means that changes in transcellular Pi transport have a larger influence on transepithelial Pi transport in cells grown on Matrigel than in those grown on uncoated filters.

The interaction of cells with Matrigel was associated with larger basolateral Pi influx (B to C) than observed in cells grown on uncoated filters. This suggests that Matrigel-cell interactions promote the expression or the activity of yet to be identified Pi transporters at the B membrane, particularly in Pi,d cells. In all cases, however, the basolateral Pi uptake rates exceeded the transcellular B to A Pi fluxes, indicating that this transcellular flux was not limited by basolateral Pi uptake.

Indeed except for Pi,d cells on uncoated filters, the transcellular B to A Pi flux was found to nearly equal the magnitude of the apical Pi backflux [estimated as the difference between the measured apical Pi uptake (A to C) rate and the net transcellular Pi flux]. This suggests that in these cells the pace of transcellular B to A Pi flux was set by the rate of Pi efflux across the apical cell membrane. However, in Pi,d cells grown on uncoated filters, the transcellular B to A Pi flux was lower than the estimated apical Pi efflux suggesting that, in this condition, there is entry of basolateral Pi into pools that do not participate in transcellular Pi transport. Such trapping of Pi in pools that do not exchange with the Pi transport pool reflects intracellular Pi compartmentalization, dependent on the nature of cell-matrix interactions in Pi,d cells. That Pi entering via A or B membranes go into different OK cell compartments was already suggested by the finding that adaptive upregulation of Pi transport in response to Pi depletion only occurs when the depletion is at the apical compartment (10).

Because the B to C Pi uptake rate was always larger than the transcellular B to A Pi flux, it is likely that a large fraction of the basolateral Pi uptake recycles across the basolateral membrane. Indeed, we have shown exchange of basolateral for intracellular Pi and transstimulation of basolateral Pi efflux from OK cells by Pi in the basolateral compartment (3).

Exchange of basolateral HPO4=D= for cell H2PO4=D= results in net Pi transport across the basolateral membrane if the exchange ratio is not 1. In all conditions described here, there was net transfer of Pi to the basolateral compartment, and the estimated basolateral Pi efflux exceeded the basolateral Pi uptake into the cells. The estimated basolateral Pi exchange ratios in Pi,d and Pi,r cells were 1.7 and 1.3 on Matrigel and 1.2 and 1.7 on uncoated filters, respectively. A high exchange ratio may be necessary, but is not sufficient, for net apical to basolateral transepithelial Pi transport, because in Pi,r replete cells grown on uncoated filters, the ratio was high but net transepithelial Pi transport was the lowest.

A potentially significant caveat is that, in our discussion, paracellular Pi fluxes have been equated with those of mannitol. This implies that Pi and mannitol have the same permeance across the paracellular pathway. Since size and charge differ between these molecules, the assumption may be inaccurate. If mannitol fluxes overestimate paracellular Pi fluxes, then transcellular fluxes may have been underestimated. However, in most conditions studied (except for Pi,d cells on uncoated filters) the transcellular A to B fluxes were found to closely match the magnitude of the Pi influxes measured across the apical cell membranes. This widely accepted characteristic of renal transepithelial Pi transport (6) lends support to our postulate that paracellular permeability to Pi and mannitol are similar. Since there is no reason to believe that paracellular Pi and mannitol permeabilities only differ in Pi,d cells grown on uncoated filters, we surmise that in cells grown on Matrigel and in Pi,r cells grown on uncoated filters, but not in Pi,d cells grown on uncoated filters, transcellular Pi fluxes are limited by apical membrane fluxes rather than by intracellular compartmentalization of Pi. Furthermore, in no condition basolateral membrane fluxes were found to limit tranellular Pi fluxes.

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