Na^+/P_i co-transport alters rapidly cytoskeletal protein polymerization dynamics in opossum kidney cells

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We studied with biochemical and immunofluorescent techniques the interactions between the actin microfilament and tubulin microtubule cytoskeleton and Na^+/P_i co-transport in opossum kidney cells, a line with proximal tubular characteristics. On brief (5 min) incubation of the cells with a low (0.1 mM) concentration of P_i, a rapid F-actin depolymerization takes place, which fails to occur in cells incubated under similar conditions with 1 mM P_i. The disassembly of actin microfilaments could be quantitatively expressed as a 33% increase in the ratio of monomeric G-actin to polymerized F-actin (G/F-actin ratio from 0.80 ± 0.03 to 1.06 ± 0.06, $n = 28$, $P < 0.01$), owing to a significant decrease in the latter. Under these conditions microfilaments were also markedly destabilized, as shown by their diminished resistance to graded cytochalasin B concentrations. In addition, incubation of opossum kidney cells with low P_i concentrations (0.1 mM) resulted within 5 min in a substantial depolymerization of microtubules, shown by immunofluorescence microscopy and measured as a $70.9 \pm 6.9\%$ ($n = 11$, $P < 0.01$) decrement by immunoblot analysis. These changes, which occur only when extracellular P_i concentrations are kept low, seem to be related to a significant increase within 5 min in the rate of cellular P_i uptake by 25.5% under these conditions. The shifts in the dynamic equilibria between monomeric and polymerized actin and tubulin in response to cellular P_i uptake were transient, being fully reversible within 30 min. Moreover, the effect of P_i seemed to be specific because inhibition of its uptake by phosphonoformic acid blunted microtubular disassembly markedly. In contrast, measurement of P_i uptake in the presence of agents known to stabilize cytoskeletal structures showed a substantial decrease with phallacidin, which stabilizes microfilaments, whereas the microtubule stabilizer taxol had no apparent effect. These results indicate that acute alterations in the polymerization dynamics and stability of both microfilaments and microtubules are involved in the modulation of Na^+/P_i co-transport and suggest important cytoskeletal participation in proximal tubular transport functions.

INTRODUCTION

Microfilaments and microtubules are involved in diverse cellular functions including secretion, exocytosis and endocytosis, intracellular transport, membrane trafficking and the organization of various organelles [1–6]. Both cytoskeletal constituents exist in dynamic equilibria between their polymerized and monomeric basic components (actin and tubulin), and the ratios between the respective monomers and polymeric forms are influenced by a variety of stimuli, including ions, amino acids, hormones and growth factors [7–12]. In turn, the activities of various transport proteins [13], as well as channels for ions [14–17] or water [18,19], seem to depend on the extent of actin and tubulin polymerization, indicating the participation of cytoskeletal mechanisms in the modulation of transmembrane transport processes too (for review see [4]).

The role of cytoskeletal participation in the regulation of the Na^+/P_i co-transporting systems that reside on the cellular membrane remains unclear. Inorganic phosphate balance is essential for the maintenance of normal cellular functions and relies heavily on renal P_i reabsorption, which is mainly a proximal tubular function. Reabsorption of P_i by renal tubular epithelia is performed by at least two Na^+/P_i co-transporter types, which are located in the apical membrane [20]. The activity of the co-transport seems to be regulated mainly through changes of the system's V_{max}, consistent with alterations in the number of co-transporter units contained in the luminal membrane as well as with the existence of recycling mechanisms capable of transferring them to and from a subapical cytoplasmic location to dock them apically [21–23]. Furthermore, because phosphorylation pathways seem to be involved in cytoskeletal component polymerization, it is conceivable that increases in cellular P_i uptake affect cytoskeletal protein polymerization dynamics [16,24–27].

To explore these questions we investigated in a cell line with a sufficiently active and well defined Na^+/P_i symporter system (opossum kidney cells) whether Na^+/P_i co-transport induces alterations in cytoskeletal protein polymerization dynamics. Using the appropriate biochemical techniques we determined the cellular actin and tubulin polymerization state on Na^+/P_i co-transport up-regulation and examined morphologically the relative stability of microfilaments, as well as the polymerization state of microtubules by fluorescence microscopy. In addition, to explore whether the state of cytoskeletal protein polymerization affects Na^+/P_i co-transport we measured P_i uptake in the presence of agents known to stabilize cytoskeletal structures. The results indicate that in opossum kidney cells both microfilament and microtubule polymerization dynamics are altered with P_i uptake, whereas in turn the stabilization of cytoskeletal components affects cellular Na^+/P_i co-transport.

MATERIALS AND METHODS

Materials

Culture media, Dulbecco's modified Eagle's medium (DMEM)/Ham's F12, trypsin and EDTA solutions were from Biochrom

Abbreviations used: CB, cytochalasin B; DMEM, Dulbecco's modified Eagle's medium; PFA, phosphonoformic acid.

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KG (Berlin, Germany), and fetal calf serum was purchased from Flow Laboratories (Irvine, Scotland, U.K.). BSA, DNase I, DNA, taxol, phosphonoformic acid (PFA), phallacidin, cytochalasin B (CB) and the monoclonal antibody against β-tubulin (for immunofluorescence staining) were obtained from Sigma (St. Louis, MO, U.S.A.). Monoclonal antibody against β-tubulin used in Western-blotting experiments and the ECL Western-blotting kit were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). Type IV collagen was from Gibco BRL (Life Technologies, Inc.). Rhodamine–phalloidin and fluorescein-labelled goat anti-mouse IgG were from Molecular Probes, Inc. (Eugene, OR, U.S.A.). Actin from rabbit muscle and tubulin from pig brain were our own preparations. All other chemicals were obtained from usual commercial sources at the purest grade available.

Cell culture

Opossum kidney cells were from the American Type Culture Collection and were studied between passages 40 and 50. The cells were maintained in a humidified atmosphere of 5% CO_2 in air in 75 cm^2 flasks, or 35 mm plastic dishes, at 37 °C and fed twice weekly with 1:1 (v/v) DMEM/Ham's F12 medium, which contained 1 mM P_i supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 20 mM $NaHCO_3$, 22 mM Hepes, 50 i.u./ml penicillin and 50 mg/ml streptomycin. Subcultivation was performed with Ca- and Mg-free PBS containing 0.25% trypsin and 0.2% (w/v) EDTA. Cells were initially cultured for 48 h with complete medium as described above; 15–20 h before the experiments the medium was changed to serum-free medium, containing 0.1% BSA. All experiments described below were performed while cells were still in their exponential growth phase.

P_i uptake experiments

Cells were first washed twice with uptake solution (150 mM NaCl, 1.8 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM Hepes, pH 7.4), then 2 ml of the above solution containing 1 mM P_i (control) or 0.1 mM P_i (low P_i) was added to the cells and the preincubations were performed for 5 min unless otherwise specified. In experiments in which P_i uptake was actually measured, incubations were performed for 5 min after the addition of radioactively labelled 0.1 mM K_2HPO_4 (1 μCi/ml $^{32}PO_4$, specific radioactivity 1000 mCi/mmol). P_i uptake was stopped by rapidly washing the cells three times with 2 ml of ice-cold stop solution, the composition of which was identical with the uptake medium except that NaCl was replaced equimolarly with choline chloride. Cells were solubilized with 0.4 ml of 0.5% Triton X-100 and the radioactivity was counted in 100 μl aliquots by liquid scintillation. Separate aliquots were taken for protein measurement. In distinct experiments before P_i uptake measurements, which were performed for 5–30 min as described above, phallacidin or taxol (final concentrations 1 μM) were added as aliquots direct to the growth medium and preincubations were performed at 37 °C for 60 min. PFA (final concentration 10 mM), dissolved in uptake solution containing 0.1 mM P_i, was added to the cells and incubations were performed for 10 min, followed by measurement of P_i uptake as described above.

Cellular monomeric (G) and filamentous (F) actin levels

Monomeric (G) and total actin was measured in cells that had been grown in culture medium containing 1 mM P_i and incubated for 5–30 min in uptake solution containing 1 mM P_i (control cells) or 0.1 mM P_i (low P_i), by using the G-actin-dependent DNase I inhibition assay [28] with minor modifications [9]: 5×10^6 cells, washed twice with stop solution, were treated with 300 μl of lysis buffer containing 10 mM K_2HPO_4, 100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 0.2 mM dithiothreitol, 0.5% Triton X-100, 1 M sucrose, pH 7.0. For determination of the G-actin content, 10 μl of the lysate was added to the assay mixture containing 10 μl of DNase I solution (0.1 mg/ml DNase I in 50 mM Tris/HCl, 10 mM PMSF, 0.5 mM $CaCl_2$, pH 7.5) and 1 ml of DNA solution (40 μg/ml DNA in 100 mM Tris/HCl, 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.5). The DNase I activity was monitored continuously at 260 nm with a Perkin-Elmer Lambda 15 double-beam spectrophotometer. Actin in the sample was measured by reference to a standard curve for the inhibition of DNase I activity, prepared with rabbit muscle G-actin isolated as previously described [29]. A linear relationship was observed over the range of 25–70% inhibition of DNase I activity. To measure total actin, aliquots of the lysed cell suspension were diluted 2–3 times with lysis buffer and then incubated for 10 min with an equal volume of guanidine/HCl buffer (1.5 M guanidine/HCl, 1 M sodium acetate, 1 mM $CaCl_2$, 1 mM ATP, 20 mM Tris/HCl, pH 7.5) to depolymerize F-actin to monomeric G-actin. The cellular F-actin content was calculated from the difference between total and G-actin. Both the G-actin and F-actin contents in opossum kidney cells were related to the total protein content. Protein concentrations were measured with the Bio-Rad protein determination kit (Bio-Rad Laboratories, Palo Alto, CA, U.S.A.), using BSA as standard.

Total cellular tubulin and microtubule polymer determinations

Total cellular tubulin and microtubule polymer amounts were determined in cells pretreated exactly as described for the actin measurements, by using a modification of the immunoblot analysis method described previously [30]. Briefly, to determine total tubulin content, cells were rinsed with microtubule stabilizing buffer (0.1 M Pipes, 1 mM EGTA, 1 mM $MgSO_4$, 10 μg/ml leupeptin, 5 μg/ml PMSF, 2 M glycerol, pH 6.75), followed by solubilization for 5 min at 37 °C in lysis buffer [25 mM Tris/HCl, 0.4 M NaCl, 1% (v/v) Nonidet P-40, 0.5% SDS, pH 7.4]. For the measurements of microtubule polymer amounts, cells were extracted twice for 15 min with microtubule stabilizing buffer containing 0.1% Triton X-100. The remaining 'Triton insoluble cytoskeletal fraction', which included polymerized tubulin, was solubilized in lysis buffer. Equal amounts of protein (5 or 10 μg) were subjected to SDS gel electrophoresis and the resulting protein bands were transferred to nitrocellulose membranes by using an LKB electroblot apparatus (LKB, Bromma, Sweden). Nitrocellulose blots were incubated with monoclonal mouse anti-β-tubulin antibody followed by incubation with the appropriate labelled second antibody, by using the ECL Western-blotting kit. Nitrocellulose blots were exposed to Kodak X-Omat AR film for various lengths of time. Band intensities were measured by PC-based Image Analysis (Image Analysis Inc., Ontario, Canada). The tubulin content of the sample was calculated by reference to a standard curve of pig brain tubulin, purified as previously described [31]. In additional experiments, tubulin determinations were performed in cells incubated for 10 min in low (0.1 mM) P_i concentrations in the presence of 10 mM PFA.

Fluorescence microscopy experiments

For morphological observations by fluorescence, opossum kidney cells were cultured onto type IV collagen-covered glass

slides, to ensure cellular attachment and orientation conditions analogous to those obtained for renal tubular epithelia *in situ*. Direct fluorescence microscopy of F-actin was performed by rhodamine–phalloidin staining. Cultured opossum kidney cells, preincubated for 5 min in uptake solution containing 1 mM or 0.1 mM P_i, were incubated for 10 min with 10 or 5 μM CB dissolved in culture medium. Cell fixation and direct fluorescence staining of microfilaments were performed as previously described [6]. Indirect immunofluorescence microscopy of microtubules was performed after cell incubation for 5–30 min under the conditions described above. In additional experiments opossum kidney cells were incubated for 10 min in 0.1 mM P_i in the presence of 10 mM PFA. Cell fixation and immunological labelling was performed with monoclonal anti-β-tubulin antibody, as described previously [6]. All specimens were examined with a Leitz Dialux 2 OEB microscope (Wetzlar, Germany), equipped with epifluorescent illumination. Micrographs were photographed with a 35 mm (C-35AD-4) camera and Kodak P3200 black and white films.

Statistical methods

Results are expressed as means $\pm$ S.E.M., and data obtained from different cell preparations were compared by an unpaired Student's *t*-test, *P* values less than 0.05 being considered significant. In the P_i uptake experiments in the presence of cytoskeletal stabilizing agents, comparisons of slopes between linear regression equations were performed by analysis of covariance by the method of Sokal and Rohlf [32].

RESULTS

Effects of ambient P_i on Na^+/P_i co-transport by opossum kidney cells, on the dynamic equilibrium between monomeric (G) and filamentous (F) actin and on the stability of microfilaments

In cells grown in 1 mM P_i-containing DMEM/F12 culture medium, the ratio of monomeric (G) to filamentous (F) actin, i.e. the G/F-actin ratio, was 0.80 ± 0.03 ($n = 28$) and remained unchanged when cells were further incubated for 30 min in uptake solution also containing 1 mM P_i. As shown in Table 1, incubation for 5 min at low P_i (0.1 mM) induced a significant increase by 33 % of the G/F-actin ratio (1.06 ± 0.06, $n = 28$, $P < 0.01$), consistent with a decrease in the proportion of filamentous actin. In line with this, the level of polymerized actin decreased significantly whereas the total actin content was not appreciably changed (Table 1).

The increase in the G/F-actin ratio in opossum kidney cells, initiated by their exposure to low-P_i medium, was a very rapid but transient event, because it occurred within 5 min and approx. 30 min later the ratio was restored to control levels, without significant changes in the total actin content (results not shown).

Table 1 Effect of low-P_i incubation on the polymerization of actin microfilaments

Opossum kidney cells were exposed for 5 min to 1 mM (control) P_i or 0.1 mM (low) P_i. Measurement of monomeric (G) and total cellular actin was performed by the DNase I assay to determine the G/F-actin ratios, the ratio of the F-actin content to total cellular protein and the ratio of the total actin content to total cellular protein. Significance levels: *, $P < 0.05$; **, $P < 0.01$, $n = 28$).

P_i concentration (mM)	G/F-actin ratio	F-actin/protein (μg/mg)	Total actin/protein (μg/mg)
1	0.80 ± 0.03	40.0 ± 2.20	71.0 ± 3.40
0.1	1.06 ± 0.06**	34.1 ± 1.60*	70.2 ± 2.80

Table 2 Effect of PFA on the uptake of P_i and the polymerization state of microtubules of opossum kidney cells

Microtubule polymer amounts were measured in the absence of PFA ($n = 11$) or in cells incubated for 10 min with 10 mM PFA ($n = 6$, $P < 0.01$). The effect of 10 min incubation of the cells with 10 mM PFA on the uptake of phosphate by opossum kidney cells is shown ($n = 6$, $P < 0.01$). Significance levels: **, $P < 0.01$.

Treatment	Polymerized tubulin (%)	$[^{32}P]P_i$ uptake (pmol/min per mg of protein)
1 mM P_i	100	1.314 ± 0.392 ($n = 3$)
0.1 mM P_i	29.1 ± 6.9** ($n = 11$)	2.993 ± 0.235 ($n = 6$)
PFA + 0.1 mM P_i	99.2 ± 8.0 ($n = 6$)	0.969 ± 0.059** ($n = 6$)

P_i uptake under the experimental conditions used was significantly increased by 25.5 % when cells were preincubated for 5 min in uptake solution containing 0.1 mM P_i, compared with the control cells preincubated similarly with uptake solution containing 1 mM P_i (7.74 ± 0.20 compared with 9.71 ± 0.63 pmol/5 min per mg, $n = 6$, $P < 0.01$). Moreover, in the presence of 10 mM PFA, an inhibitor of P_i uptake by blockade of Na^+/P_i co-transport [33], P_i uptake was significantly decreased by 64 %, as shown in Table 2.

Microfilament organization and stability studied by direct fluorescence microscopy [34,35] under control conditions (1 mM P_i) remained unaffected by exposure of the cells to 10 μM CB (Figure 1b). Incubation with low P_i caused the focal appearance of blotchy fluorescence in opossum kidney cells exposed to the same CB concentration, but with the majority of actin filaments continuing to be regularly distributed (Figure 1b′). The decrease in microfilament stability became clearer after exposure to 5 μM CB: under these conditions, and before the lowering of ambient P_i, only few of the actin filaments had undergone depolymerization, whereas their overall network seemed largely intact (Figure 1c). In contrast, in cells incubated in low P_i and then incubated with CB (5 μM), an almost complete disappearance of the characteristic microfilamentous network was observed (Figure 1c′). These findings indicate that exposure of opossum kidney cells to low P_i renders actin filaments less resistant to the action of CB, suggesting a marked destabilization of actin microfilaments under these experimental conditions.

Effect of low-P_i incubations on the equilibrium between monomeric and polymeric tubulin and microtubular morphology

Quantitative immunoblot analysis of the proportions of polymerized and total tubulin in cells exposed for 5 min to 0.1 mM P_i uptake solution revealed a significant depolymerization of tubulin (Figure 2, lanes 1 and 2) by 70.9 ± 6.9 % (Table 2, $n = 11$, $P < 0.01$). This finding clearly demonstrates that with tubulin, too, a dramatic shift takes place in the dynamic equilibrium between its monomeric and polymeric forms.

To examine the specificity of P_i flux on the depolymerization of microtubules we further studied the effect of PFA. In line with previously published data, P_i uptake by opossum kidney cells decreased within 10 min by 64 % in the presence of 10 mM PFA (Table 2). Predictably, the decrease in polymerized tubulin content was virtually abolished when cells were incubated for 10 min in the presence of 10 mM PFA (Figure 3f), and 99.2 ± 8 % of microtubules remained polymerized in the presence of the P_i uptake inhibitor (Table 2).

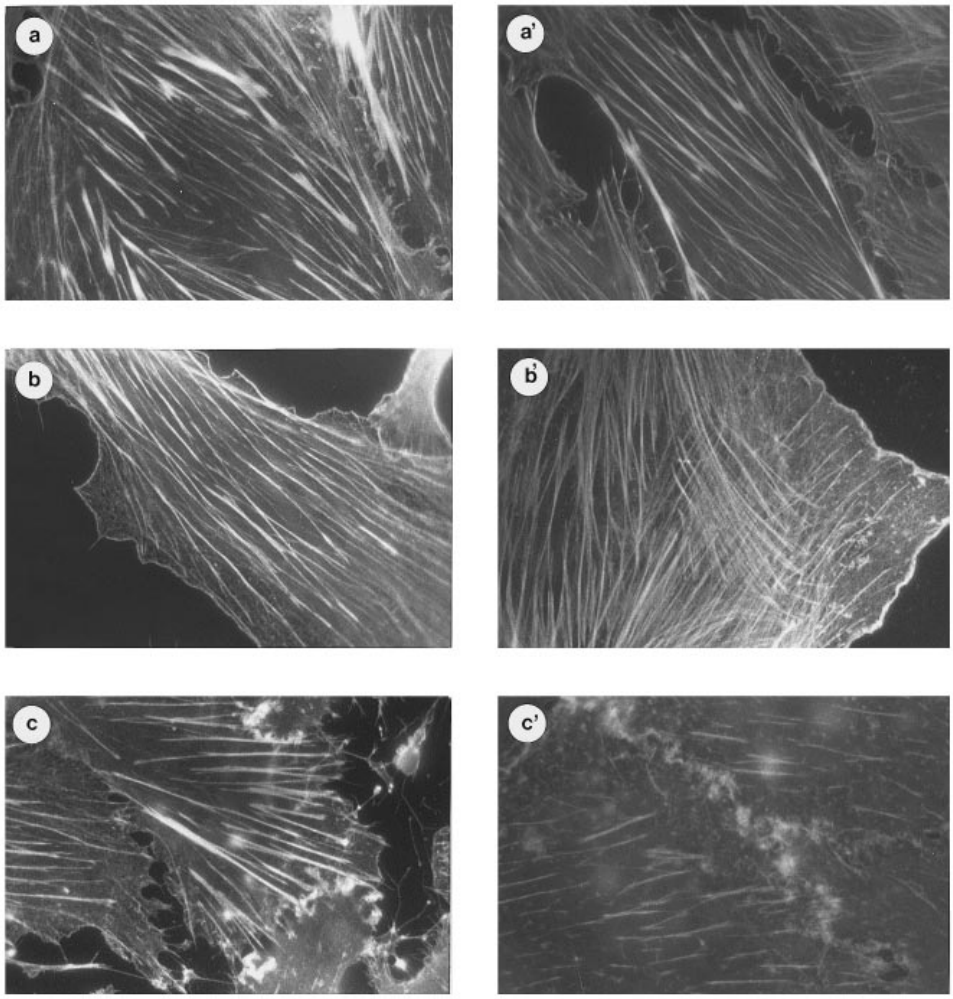

Figure 1 Effect of low-P_i incubation on the stability of microfilaments

Rhodamine–phalloidin staining of microfilaments in opossum kidney cells incubated with 1 mM P_i (control; **a**, **b**, **c**) and with 0.1 mM P_i (low; **a′**, **b′**, **c′**). Actin filament stability was examined after incubation for 10 min with 5 and 1 μM concentrations of CB. (**a**, **a′**) CB-untreated cells; (**b**, **b′**) effect of 1 μM CB; (**c**, **c′**) effect of 5 μM CB. (Magnification $\times$ 1000.)

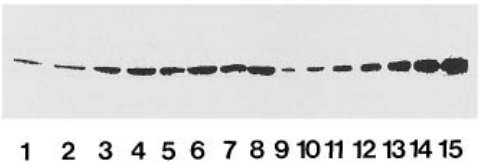

Figure 2 Effect of low-P_i incubation on the polymerization state of microtubules

Quantitative Western-blot analysis (representative of a total of 11 experiments) depicting polymeric tubulin amounts (lanes 1–4) and the corresponding total tubulin contents (lanes 5–8), after 5 min preincubations of opossum kidney cells in uptake medium containing either 1 or 0.1 mM P_i. Lanes 1 and 2, polymer amounts, in 5 and 10 μg cellular protein aliquots respectively, after cell preincubation in 0.1 mM (low) P_i. Lanes 3 and 4, polymer amounts, in 5 and 10 μg cellular protein aliquots respectively, in control cells preincubated in 1 mM (control) P_i. Lanes 5 and 6, total tubulin content, in 5 and 10 μg cellular protein aliquots respectively, after cell preincubation in 0.1 mM P_i. Lanes 7 and 8, total tubulin content, in 5 and 10 μg cellular protein aliquots respectively, in control cells preincubated in 1 mM P_i. Lanes 9–15, standard curve of isolated pure pig brain tubulin (0.15–0.75 μg).

Incubation of opossum kidney cells in low-P_i uptake medium was characterized morphologically by marked disassembly of the microtubular network, as demonstrated by the indirect immunofluorescence microscopy depicted in Figure 3. Within 5 min of the exposure to low ambient concentrations of P_i, there was an almost complete disappearance of microtubules with persistence of only scant polymeric tubulin structures (Figure 3b), which persisted for up to 10 min (Figure 3c). The extensive depolymerization of microtubules on exposure to low P_i was, however, also a transient event. Partial rearrangement of the microtubular network became evident within 15 min (Figure 3d), and after 30 min of cell incubation with 0.1 mM P_i the microtubules were fully reorganized (Figure 3e). Moreover, as shown in Figure 3f, tubulin depolymerization was abolished in cells exposed for 10 min to low P_i in the presence of 10 mM PFA.

P_i uptake experiments in the presence of cytoskeletal stabilizing agents

P_i uptake by opossum kidney cells over different time periods (5, 10, 15, 20 and 30 min) of 0.1 mM P_i incubation was determined

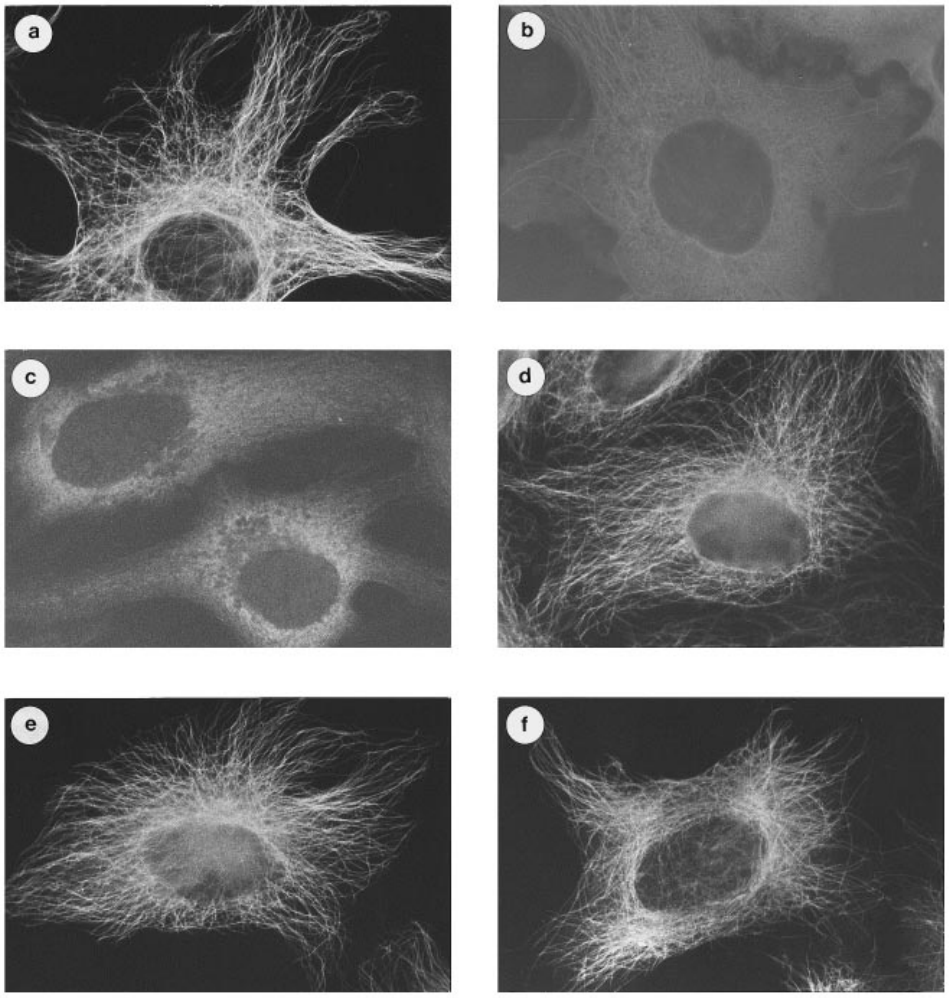

Figure 3 Immunofluorescence staining of microtubules in opossum kidney cells exposed to low-P_i uptake medium, and the effect of PFA

Time course of microtubule disintegration in response to incubation in 0.1 mM P_i. (**a**) Untreated cells; (**b**) cells preincubated with 0.1 mM P_i for 5 min; (**c**) for 10 min; (**d**) for 15 min; (**e**) for 30 min. (**f**) Effect of 10 mM PFA in cells incubated with 0.1 mM P_i for 10 min. (Magnification × 1000.)

after 60 min preincubations with the microfilament stabilizer phallacidin (Figure 4A) and the microtubule stabilizer taxol (Figure 4B). In control experiments P_i uptake was measured in cells in which preincubation was with media free of phallacidin and taxol. P_i uptake was clearly decreased in cells preincubated for 60 min with 1 μM phallacidin, compared with untreated opossum kidney cells (Figure 4A), as shown by a significant difference in the slopes of the respective linear regressions. On the other hand, in cells treated for 60 min with 1 μM taxol, P_i uptake remained unaffected (Figure 4B).

DISCUSSION

It has long been known that even short-term incubation of cultured cells in low-P_i media augments the rate of their phosphate uptake through Na^+-coupled phosphate co-transport [22,36]. In the present experiments we showed that such a change in P_i uptake was detectable already after a 5 min preincubation of opossum kidney cells in uptake medium containing 0.1 mM P_i, i.e. under conditions traditionally used by most investigators for the assay of P_i uptake by intact tubular cells or brush-border membranes [21,37–39]. This widely employed method does not, however, permit a distinction between true membrane transport activity and P_i incorporation into cellular metabolites, or a combination of the two. Because identically conducted preincubations with an uptake medium containing 1 mM P_i instead (but otherwise of the same composition) did not have this effect, it seems reasonable to conclude that the augmentation in Na^+/P_i co-transport observed was an event related to low extracellular P_i concentration. This phenomenon therefore seems consistent with the previously recognized short-term up-regulation of Na^+/P_i co-transport brought about by low concentrations of this anion in the incubation medium of phosphate-transporting cells [22,23,36].

The observed depolymerization phenomena for both the actin–microfilament and the tubulin–microtubule systems were, however, transient: reassembly of both cytoskeletal systems seemed complete within 30 min, despite the continuous cell exposure to low ambient P_i. We consider this reversibility important because it supports the notion that we are not simply describing toxic effects on cytoskeletal structures, but rather a physiologically relevant phenomenon. Finally the demonstration

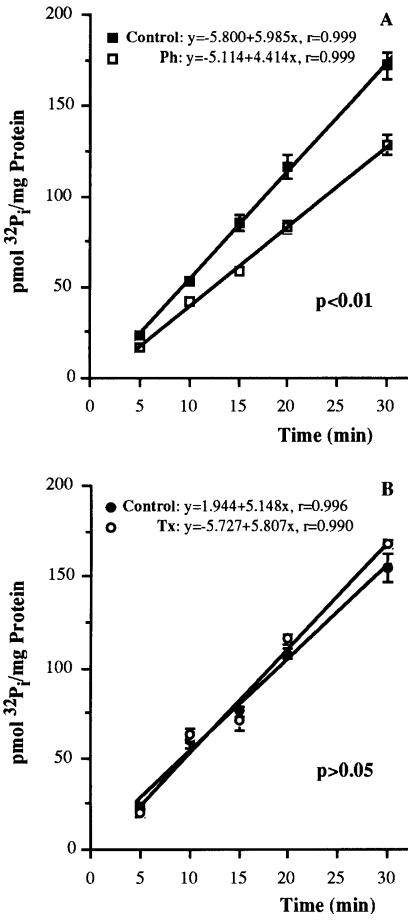

Figure 4 Effect of phallacidin and taxol on the time course of P_i uptake by opossum kidney cells

Time course of P_i transport in cells preincubated for 60 min with (**A**) 1 μM phallacidin (Ph) (□), (B), 1 μM taxol (Tx) (○) or with the vehicles of the respective cytoskeletal stabilizing agents (■, ●).The slopes of regressions when compared with an analysis of covariance revealed significant differences in the presence of phallacidin ($P < 0.01$). Individual data points represent the means for three separate experiments performed in duplicate.

that microtubule disassembly is prevented by PFA indicates that the observed change in cytoskeletal protein polymerization dynamics is a relatively specific phenomenon, and one dependent on P_i uptake. [Disassembly of actin microfilaments during short-term low-P_i incubations was not appreciably influenced by the presence of PFA in the medium. The significance of this finding remains uncertain, however, because it could reflect the considerably less pronounced changes observed with the actin cytoskeleton (33%), compared with those seen with the microtubules (70.9%).] The cytoskeleton stabilizing effect of PFA in cells exposed to low P_i indicates that the transient depolymerization of actin and tubulin that we observed is related primarily to augmentation in the rates of P_i transport and its up-regulation and not to decreased intracellular levels of P_i, which if anything would have been expected to become even lower in the presence of PFA. Additionally the time frame of these effects would be too short because a decrease in intracellular P_i of opossum kidney cells exposed to P_i-free media cannot be detected before 60 min from the beginning of such incubations [40]. It is additionally conceivable that the observed cytoskeletal alterations are related to cell volume changes brought about by the augmentations in Na^+-coupled P_i transport [41].

Trafficking of a variety of channels and transporters between intracellular pools and the plasma membrane seems to be an important mechanism that rapidly regulates the transport capacity of this membrane for many valuable substrates [42,43], ions [44–46] and even water [47,48]. Therefore it is possible that similar recycling mechanisms exist for the Na^+/P_i symporters [49], especially in the time frame of their short-term up-regulation as under the experimental conditions employed in the present study. According to this notion, up-regulation of Na^+/P_i co-transport after short-term exposure to lowered extracellular P_i concentrations would cause the number of symporters in the apical membrane to increase, implying the existence of a trafficking mechanism that shuttles symporter units between the membrane and a submembranous compartment harbouring them in reserve. It is conceivable that the rapid dissolution of the submembranous actin network, brought about by partial disassembly of microfilaments, as well as the simultaneously occurring transient depolymerization of microtubules, are related to such co-transporter traffic. Indirect experimental evidence consistent with the above suggestion is provided by our results for P_i uptake by opossum kidney cells exposed to cytoskeleton-stabilizing agents. Thus when opossum kidney cells were preincubated with phallacidin, which stabilizes the microfilamentous network, P_i uptake was clearly inhibited, consistent with a blockade of the symporter's transport towards the apical membrane. In contrast, cell incubation with taxol did not affect appreciably P_i uptake, which is compatible with the view that stabilization of immobilized microtubules promotes and/or prolongs the residence of functional co-transporters in the apical membrane. The Na^+/P_i symporter trafficking concept is further supported by previous work that documented (1) alterations in Na^+/P_i co-transport in parallel with changes in the abundance of Na^+/P_i symporters as determined by Western blot [45,50] or by the number of their binding sites available to specific inhibitors (PFA) [51]; (2) the occurrence of these responses without parallel changes in the Na^+/P_i co-transporter's mRNA expression [45] and even in the face of its arrested synthesis [52]; (3) the existence of functional Na^+/P_i co-transporters in renal cortical endosomes [49,50]; (4) smaller decreases in P_i uptake in response to parathyroid hormone when endocytosis is generally prevented by hypertonic cell exposure [53].

Our findings are compatible with recently published data [54] on the role of the cytoskeleton in the adaptive response of Na^+/P_i co-transport to low P_i. That report dealt, however, with relatively long-term (5 h) effects of anti-cytoskeletal agents on P_i uptake when it showed that CB did not affect P_i uptake. Because CB promptly induces microfilament depolymerization and destabilization, the failure to document changes in P_i uptake after relatively prolonged exposure of opossum kidney cells to the drug is not inconsistent with our suggestion that apical insertion of Na^+/P_i co-transporters is facilitated by early destabilization of the subapical microfilamentous network. However, the reported decrease in the adaptive co-transporter response to low P_i in the presence of microtubular depolymerizing agents [54] also has to be interpreted, taking into consideration that it represents a 5 h effect of these drugs. This interval cannot be compared with our experimental conditions, under which we were able to demonstrate the occurrence of a very rapid and transient depolymerization of microtubules.

In summary our results demonstrate that in opossum kidney cells a brief incubation in low-P_i uptake medium induces a rapid and significant, but transient, depolymerization of microfilaments and microtubules, possibly related to the phenomenon of short-

term up-regulation of Na^+/P_i co-transport. Because the cellular uptake of P_i is also influenced by agents that alter cytoskeletal stability, we suggest that cytoskeletal elements participate in Na^+/P_i co-transport modulation.

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