*Biochimica et Biophysica Acta*, 981 (1989) 185–192
Elsevier



BBA 74421

# The $Na^+/P_i$-cotransporter of OK cells: reaction and tentative identification with *N*-acetylimidazole

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(Received 13 October 1988)
(Revised manuscript received 17 January 1989)

Key words: Phosphate transport; Sodium ion cotransport; Proximal tubule; *N*-Acetylimidazole; (OK cell)

**Using an established renal epithelial cell line (OK cells) the effect of the amino-acid side-chain modifying reagent *N*-acetylimidazole (NAI) upon the sodium-dependent transport of phosphate ($P_i$) was investigated. After an incubation with 10 mM NAI for 20 min, cellular $Na^+/P_i$ uptake was inhibited by 70%. The presence of 5 mM $P_i$ protected this transport function from being affected by NAI by 80 to 100%. Since the presence of sulfate was unable to protect the $Na^+/P_i$ transport inactivation by NAI and since the presence of $P_i$ did not affect NAI inhibition of other transport systems, it is suggested that NAI interacts with the $P_i$ transporter directly. The protective effect of $P_i$ was used as a criterion to identify $P_i$-protectable [$^3$H]NAI labelling of OK cell plasma membrane proteins. $P_i$ protection was observed in four molecular mass regions: 31, 53, 104 and 176 kDa. Since the incorporation of [$^3$H]NAI into these proteins was also affected by parathyroid hormone at $10^{-10}$ M, it is concluded that the identified proteins represent possible candidates for the renal $Na^+/P_i$ cotransporter.**

## Introduction

The sodium-dependent transport system for phosphate, which is localized in the luminal membrane of the proximal tubular cell, plays a key role in the renal reabsorption of inorganic phosphate. The importance of this transport system in the homeostatic control of phosphate becomes evident from numerous observations which have shown that this transport system underlies a regulatory control of a variety of hormonal and non-hormonal factors [1–3].

Despite the fact that this transport system has been functionally well-characterized, its molecular identity is still unkown. Recently, Beliveau et al. have reported a functional molecular mass of 234 kDa, which was obtained by radiation-inactivation studies on renal brush-border membrane vesicles [4].

So far, only a few studies have been reported on the chemical modification of this transport system. Recently, phenylglyoxal has been used to inhibit renal $Na^+/P_i$ cotransport successfully, indicating the involvement of functionally important arginine residues [5,6]. Here, we report the effect of the group-specific (tyrosine and/or lysine) reagent, NAI, on the sodium-dependent transport of phosphate in an established renal epithelial cell line (OK cells). Since the inhibition of the phosphate transport by NAI could be protected by the presence of phosphate, the use of tritiated NAI allowed the identification of four molecular mass regions corresponding to candidates for the renal $Na^+$-dependent $P_i$ cotransporter.

## Methods and Materials

*Cell culture.* Opossum kidney (OK) cells were kept in culture (passage number between 80 and 100) in a 1:1 mixture of Ham's F12 and DMEM medium (buffered with 20 mM Hepes/22 mM bicarbonate/10% $CO_2$) as described in Refs. 7 and 8. For experimental purposes, trypsinized cells were plated in 35 mm or 220 × 220 mm petri dishes (Nunc) and grown to confluency for 4–5 days. If necessary, the cells were treated with $10^{-10}$ M

Abbreviations: NAI, *N*-acetylimidazole, DMEM, Dulbecco's modified Eagles' medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PTH, parathyroid hormone.

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parathyroid hormone (bPTH 1–34, Bachem, Switzerland) for 3 h [7] prior to the NAI treatment.

*Treatment of OK cells with NAI and cellular transport measurements.* Confluent OK cell monolayers were washed twice with the buffer used for the subsequent NAI incubation. Incubation with NAI (made up immediately before its use) was performed at 25°C in 1 ml of either 300 mM mannitol/5 mM Hepes-KOH (pH 7.4) or 100 mM NaCl/100 mM mannitol/5 mM Hepes-KOH (pH 7.4). After incubation, the monolayers were washed two times with transport buffer (in mM): NaCl 128/KCl 4.73/$CaCl_2$ 1.25/$MgSO_4$ 1.25/Hepes 5 (pH 7.4) with NaOH). 1 ml of transport buffer containing 0.1 mM of the solute (1 $\mu$Ci) was then added and transport was measured at 25°C for the time given in Table III. The uptake medium was aspirated off and the monolayers were washed four times with ice-cold Tris-buffered (10 mM) (pH 7.4) saline (150 mM). An aliquot of cells solubilized in 0.5% Triton X-100 was used for scintillation counting or for the determination of the protein. Sodium-independent transport rates were determined in the presence of *N*-methylglucamine.

In order to study the effect of NAI on the $P_i$ transport in sodium-equilibrated OK cells, cells were pretreated with 100 $\mu$M ouabain for 4 h at 37°C in the transport buffer (see above), which contained in addition 1 mM phosphate and 5 mM D-glucose. This treatment results in a complete equilibration of both sodium and potassium ions as determined by flame photometry (Montrose, M., unpublished data). After this ouabain treatment, the cells were treated with NAI (8 mM, 20 min) in 130 mM NaCl/40 mM mannitol/5 mM Hepes-KOH (pH 7.4) and $P_i$ transport was determined in the presence of 130 mM NaCl or equimolar amounts of *N*-methylglucamine for 12 min at 25°C.

*Synthesis of [$^3$H]NAI.* 50 $\mu$mol (5 mCi) of [$^3$H]acetic anhydride (New England Nuclear, NET-108H) were added to 3 ml benzene containing 0.15 ml tetrahydrofuran. After addition of 50 $\mu$mol of water, the hydrolysis of acetic anhydride was performed at 25°C overnight. 100 $\mu$mol (16.2 mg) of carbonyldiimidazole (Fluka, Switzerland) were added and the reaction was performed under constant stirring for 3 h at 25°C [9]. The product was filtered through glass wool and the solvent was evaporated. The residue was redissolved in dry benzene and distributed into aliquots which were dried and used the same day.

By thin-layer chromatography (hexane/ methyl acetoacetate, 2 : 1, v/v; Silica gel 60/Merck) the following $R_f$ values were obtained: 0.22 for carbonyldiimidazole and 0.08 for the product, which was identical to that obtained for NAI (Fluka).

The product inhibited the uptake of $P_i$ into OK cells in an identical way to non-radioactive NAI: at 8 mM [$^3$H]NAI, 80% inhibition of $P_i$ uptake was observed which could be almost completely protected by 5 mM $P_i$ (data not shown).

*[$^3$H]NAI labelling.* Confluent OK cell monolayers (35 mm dishes) were washed twice with NaCl buffer (100 mM NaCl/100 mM mannitol/5 mM Hepes-KOH (pH 7.4)) or with NaCl buffer containing 5 mM $NaH_2PO_4$. The cells were then incubate at 25°C with 8 mM [$^3$H]NAI (approx. 1.6 mCi/mg protein) in either 1 ml of the NaCl buffer or the NaCl/$P_i$ buffer for 15 min. The incubation was stopped by two washes with 300 mM mannitol/5 mM Hepes-KOH (pH 7.4) and one wash with 5 mM Hepes-KOH (pH 7.4). Cells were then scraped off, homogenized in the latter buffer using a 23 g syringe and mixed with the carrier (unlabelled) cells (see below).

*Partial purification of OK cell plasma membranes.* Confluent OK cells, grown in 220 × 220 mm plates were washed once with cold 150 mM NaCl/5 mM Tris-HCl (pH 7.2) and once with 5 mM Hepes-KOH (pH 7.2). Cells were then scraped off into an aliquot of the latter buffer and homogenized by syringing six times through a 18 g needle. After the addition of the labelled cell homogenate to this carrier, cell homogenate, cell debris and nuclei were removed by centrifugation (5 min at $279 \times g_{max}$, 4°C). The supernatant was centrifuged at 4°C and $48\,000 \times g_{max}$ for 30 min and the pellet was resuspended in 1 ml of 5 mM Hepes-KOH/0.5 mM EDTA (pH 7.2). The membranous solution was layered onto a discontinuous-sucrose gradient, which was composed of four layers of sucrose: 40; 32.5; 29 and 10 (%, w/w) prepared in 5 mM Hepes-KOH/0.5 mM EDTA (pH 7.2). The gradient was run for 90 min at 33 000 rpm ($140\,000 \times g_{av}$) (Beckmann, TST 41) and three fractions were recovered: fraction 1 from the 10/29% interphase; fraction 2 from the 29/32.5% interphase and fraction 3 from the 32.5/40% interphase.

*SDS-gel electrophoresis.* Membrane fractions as recovered from the sucrose gradient were precipitated in methanol/chloroform as described in Ref. 10. By a gel-electrophoretic analysis (silver staining) of the organic phase obtained by this precipitation technique, no evidence for a selective extraction of membrane proteins was obtained. The precipitated proteins were solubilized in SDS (2%) and separated by SDS-polyacrylamide gel electrophoresis in 12 × 0.5 cm glass tubes [11]. The final gels were cut into 2 mm slices which were treated with 0.3 ml of Protosol (Dupont) for 2 h prior to liquid-scintillation counting. Molecular mass markers were purchased from Bio-Rad.

*Miscellaneous techniques.* The activities of the $Na^+/K^+$-ATPase (EC 3.6.1.3) and succinate–cytochrome-*c* oxidoreductase (EC 1.3.99.1) were assayed as described in Ref. 12. Dipeptidyl peptidase IV (EC 3.4.14.5) and γ-glutamyl transferase (EC 2.3.2.2) were determined according to Refs. 13 and 14. Protein was

determined according to Ref. 15 using the Bio-Rad standard. The rate of hydrolysis of NAI and its reactivity towards L-tyrosine in the various buffers used were determined by measuring the decrease in optical absorbance at 275 nm [16].

## Results

OK cells were treated with NAI in isotonic buffers consisting of either 300 mM mannitol (mannitol buffer) or 100 mM NaCl/100 mM mannitol (NaCl/mannitol buffer). The effect of NAI (depending on its concentration and on the incubation time) with respect to the activity of the sodium-dependent transport of phosphate ($P_i$) is shown in Fig. 1. When incubated in the NaCl/mannitol buffer, $Na^+/P_i$ cotransport was maximally inhibited by 70% (by 10 mM NAI, Fig. 1A). In the mannitol buffer, the same NAI concentration inhibited $Na^+/P_i$ cotransport by only 20%. Maximal inhibition by 10 mM NAI was achieved within 30 min of incubation in the NaCl/mannitol buffer (Fig. 1B). Importantly, the uptake of $P_i$ in the presence of *N*-methylglucamine, which represents about 2% of total $P_i$ uptake (see Ref. 7), was not changed under the conditions used in Fig. 1 (data not shown).

Under conditions of maximal inhibition, NAI effects a 2-fold decrease in the maximal velocity ($V_{max}$) ($15 \pm 2$ versus $7.5 \pm 0.5$ nmol $P_i$/mg per min). Under all conditions used, the apparent $K_m$ for $P_i$ remained unchanged ($132 \pm 17$ versus $123 \pm 10$ M).

In order to exclude the possibility that the inhibition of $Na^+/P_i$ transport is secondary to the effects of NAI on driving forces (e.g., sodium gradient), OK cells were – prior to the NAI treatment – incubated for 4 h with 100 $\mu$M ouabain at 37°C in the transport buffer. Such

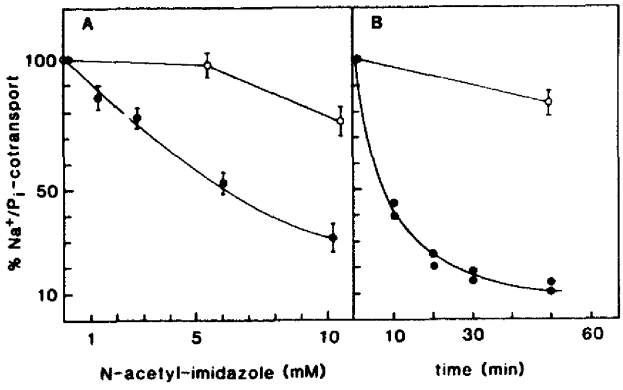


Fig. 1. Inhibition of $Na^+/P_i$ cotransport by NAI. OK cells were incubated with various concentrations of NAI (A) for 20 min or with 10 mM NAI for various times (B). The incubations wre performed in either 300 mM mannitol/5 mM Hepes-KOH (pH 7.4) (○) or in 100 mM NaCl/100 mM mannitol/5 mM Hepes-KOH (pH 7.4) (●). 100% uptake represents $8.7 \pm 0.1$ nmol $P_i$/mg per 6 min. The data in (A) represent the means ± S.D. of three independent experiments. In (B) two independent experiments are given.

TABLE I

*Effect of NAI on $Na^+/P_i$ uptake in sodium-equilibrated OK cells*

OK cells were treated with 100 uM ouabain prior to incubation with 10 mM NAI for 15 min. Total $P_i$ uptake has been corrected by the $P_i$ uptake in the presence of equimolar amounts of *N*-methylglucamine, which was 0.17 nmol $P_i$/mg per 12 min. The results are the means ± S.D. of three independent experiments.

| | Net $Na^+$-dependent $P_i$ uptake | |
|---|---|---|
| | nmol $P_i$/mg per 12 min | inhibition (%) |
| Control cells | $1.28 \pm 0.08$ | |
| NAI-treated | $0.71 \pm 0.04$ | $44 \pm 3$ |
| Controls + 5 mM $P_i$ | $1.23 \pm 0.10$ | |
| NAI plus 5 mM $P_i$ | $1.01 \pm 0.17$ | $18 \pm 4$ * |

* Unpaired Student's *t*-test; $P < 0.01$, compared to the absence of $P_i$.

a treatment resulted in the complete equilibration of both sodium and potassium ions as determined by flame photometry (Montrose, M., unpublished data). As illustrated in Table I, $Na^+/P_i$ transport into equilibrated cells was inhibited by 44% using 10 mM NAI. This indicates that the major effect of NAI, as observed in intact cells, is not due to a dissipation of the driving force (sodium gradient), but rather is due to a direct interaction of NAI with the $Na^+/P_i$ transport system.

To investigate the effect of NAI on other sodium-dependent transport processes (see Ref. 17), OK cells were treated with 10 mM NAI for 15 min in either the mannitol or the NaCl/mannitol buffer. As indicated in Table II, the $Na^+$-dependent transports of L-glutamic acid, L-proline and methyl-$\alpha$-glucopyranoside were also – though less than $P_i$-transport – inhibited by NAI after an incubation in the NaCl/mannitol buffer, but were not significantly inhibited after an incubation in the mannitol buffer. Independently of the buffer used,

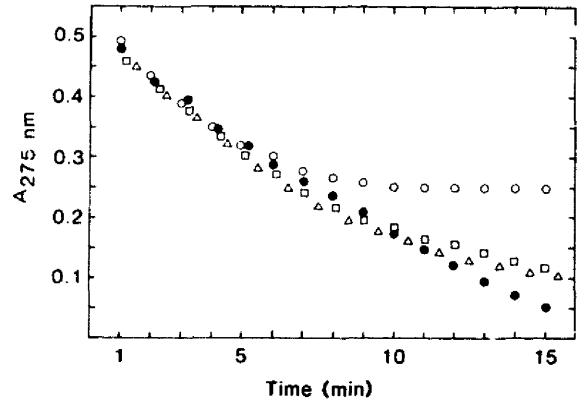


Fig. 2. Reaction of NAI with L-tyrosine. At zero time, L-tyrosine (up to 2 mM) was added to 5 mM NAI and the reaction was followed at 25°C at 275 nm against NAI as reference. The reaction rates were determined in the mannitol buffer (○), the NaCl/mannitol buffer (●), NaCl/mannitol plus 10 mM $Na_2HPO_4$ (△) or NaCl/mannitol plus 10 mM $Na_2SO_4$ (□).

TABLE II

*NAI inhibition of various $Na^+$-dependent transports of OK cells*

All solutions were used at a final extracellular concentration of 0.1 mM. Transport measurements were performed for $x$ min. OK cells were incubated with 10 mM NAI for 15 min in the buffer indicated prior to the transport experiments. All data are presented as the means ± S.D. of three experiments. Student's unpaired $t$-test: $P$ values refer to the absence of sodium.

| Solute and conditions | Net $Na^+$-dependent transport (pmol/mg per $x$ min) | | |
|---|---|---|---|
| | control | NAI-treated | inhibition (%) |
| Phosphate ($x = 6$) | | | |
| mannitol buffer [a] | 9500 ± 220 | 8368 ± 880 | 12 ± 9 [c] |
| NaCl/mannitol [b] | 9600 ± 160 | 3200 ± 100 | 67 ± 2 |
| | | | ($P < 0.01$) |
| L-Glutamic acid ($x = 6$) | | | |
| mannitol buffer | 6500 ± 640 | 5474 ± 160 | 16 ± 9 [c] |
| NaCl/mannitol | 6660 ± 400 | 4200 ± 240 | 37 ± 5 |
| | | | ($P < 0.02$) |
| L-Proline ($x = 15$) | | | |
| mannitol buffer | 5340 ± 300 | 4840 ± 80 | 8 ± 2 [c] |
| NaCl/mannitol | 6600 ± 340 | 4660 ± 280 | 30 ± 5 |
| | | | ($P < 0.01$) |
| Methyl-α-glucopyranoside ($x = 15$) | | | |
| mannitol buffer | 3200 ± 400 | 3336 ± 200 | 0 ± 14 [c] |
| NaCl/mannitol | 2960 ± 200 | 1780 ± 100 | 40 ± 5 |
| | | | ($P < 0.01$) |
| L-Alanine ($x = 3$) | | | |
| mannitol buffer | 14900 ± 720 | 15168 ± 520 | 0 ± 6 [c] |
| NaCl/mannitol | 15160 ± 1160 | 14780 ± 100 | 3 ± 7 |
| | | | ($P > 0.2$) |

[a] 300 mM mannitol/5 mM Hepes-KOH (pH 7.4).
[b] 100 mM NaCl/100 mM mannitol/5 mM Hepes-KOH (pH 7.4).
[c] Not significantly different from zero.

$Na^+$-dependent transport of L-alanine was not inhibited by NAI.

Whether NAI inhibits the $Na^+/P_i$ transport by modifying the $Na^+/P_i$ cotransporter directly (possibly via a tyrosine and/or lysine residue) was tested by using $P_i$ as a protective agent. The data presented in Table III demonstrate that, in the presence of 5 mM $P_i$, the inhibition of the $Na^+/P_i$ transport was almost completely prevented, suggesting that NAI might inhibit the $Na^+/P_i$ transport by interacting directly with the transport protein(s). As illustrated in Table I, the presence of $P_i$ during the incubation of sodium-equilibrated cells with NAI, also significantly reduced the inhibition of the $Na^+/P_i$-cotransport rate.

Whether this protective effect of $P_i$ was due to a direct interaction of $P_i$ with the $Na^+/P_i$ transporter or due to a nonspecific interaction of phosphate with the reaction of NAI with the plasma membrane was answered in the following ways. First, to decide if the $P_i$ anion exhibits a general anion effect on the NAI inhibition, $P_i$ was replaced equimolarly by sulfate. As the data given in Table III indicate, the presence of 5 mM $Na_2SO_4$ did not prevent the inhibitory effect of NAI upon the $Na^+/P_i$ cotransport, excluding the possibility for an indirect and general anion effect on the NAI reaction. Secondly, the effect of the presence of $P_i$ upon the NAI inhibition of other $Na^+$-dependent transport rates was tested. As indicated in Table III, inhibition of the $Na^+$/L-glutamic-acid transport by NAI was not

TABLE III

*Effect of $P_i$ and sulfate on $Na^+$-dependent transport of $P_i$ and L-glutamic acid*

The data are the means ± S.D. of three independent experiments. Student's $t$-test was used. n.d., not determined.

| Conditions | $Na^+$-dependent uptake (%) of [b] | |
|---|---|---|
| | phosphate | L-glutamic acid |
| Mannitol [a] | 92 ± 10 | 85 ± 10 |
| NaCl/mannitol [a] | 51 ± 5 [c] | 66 ± 2 [c] |
| NaCl/mannitol + 5 mM $P_i$ | 91 ± 9 [d] | 58 ± 2 [c] |
| NaCl/mannitol + 5 mM $SO_4^{2-}$ | 43 ± 6 [c] | n.d. |

[a] See Table II and Methods and Materials for composition.
[b] The percentages of uptake were calculated using controls, which were treated the same way, but in the absence of NAI. The control uptakes were (in nmol $P_i$/mg per 6 min): 9.3 ± 0.7 for mannitol; 9.1 ± 0.7 for NaCl; 6.6 ± 0.5 for NaCl/$P_i$ and 9.6 ± 0.6 for NaCl/$SO_4^{2-}$.
[c] Not significantly different from zero.
[d] $P < 0.01$ as compared to the absence of $P_i$.

TABLE IV

*Partial purification of OK cell plasma membranes by discontinuous gradient centrifugation*

The data (in mU/mg) were obtained from five independent experiments and are presented as the means ± S.D.

| | $Na^+/K^+$-ATPase | γ-Glutamyl-transferase | Dipeptidyl peptidase IV | Succinate-cyto-chrome-c oxidase | Protein recovery (%) |
|---|---|---|---|---|---|
| Homogenate | 41 ± 7 | 23 ± 7 | 196 ± 44 | 25 ± 1 | 100 |
| Fraction 1 | 93 ± 30 | 93 ± 9 | 977 ± 59 | 4 ± 1 | 4.6 ± 0.5 |
| Fraction 2 | 375 ± 52 | 123 ± 27 | 728 ± 59 | 23 ± 1 | 2.8 ± 0.2 |
| Fraction 3 | 99 ± 41 | 14 ± 5 | 69 ± 27 | 317 ± 9 | 9.0 ± 1.7 |

protected by the presence of 5 mM $P_i$. In fact, the presence of 5 mM L-glutamic acid did not prevent inhibition of the $Na^+/P_i$ cotransport by NAI (data not shown). Thus, the above two controls suggest that $P_i$ indeed might protect the $Na^+/P_i$ cotransporter from being modified by NAI. Furthermore, the data suggest that the site modified by NAI is directly involved in the binding and/or translocation of phosphate.

In order to further test for a possible indirect interaction of $P_i$ with the chemical reactivity of NAI, the rate of the reaction of NAI with L-tyrosine in the various buffers used was measured. As illustrated in Fig. 2, the reactivities of NAI with L-tyrosine in NaCl/mannitol buffer containing either 5 mM $P_i$ or sulfate did not differ and were very similar to the reaction rate in the NaCl/mannitol buffer. Therefore, it is suggested that the protective effect of $P_i$ as described above is a result of a direct interaction of $P_i$ with a membrane protein involved in the $P_i$ transport process. In the mannitol buffer, however, the reactivity of NAI with L-tyrosine is greatly impaired (see Fig. 2). This effect of a high concentration of mannitol on the NAI reactivity towards L-tyrosine could explain the smaller effect of NAI when incubated in the mannitol buffer.

Since the above-presented results strongly indicate that NAI directly modifies the $Na^+/P_i$ cotransporter of the OK cells and that this inhibition can be protected in the presence of $P_i$, $^3$H-labelled NAI has been used to trace for plasma membrane proteins, eventually showing $P_i$-protectable NAI incorporation. A crude membranous fraction was obtained by discontinuous-sucrose-gradient centrifugation (see Materials and Methods). As plasma membrane markers, the activities of the $Na^+/K^+$-ATPase, γ-glutamyl-transferase (γ-GTP) and dipeptidyl peptidase IV (DPP IV) were measured. Three membrane fractions were recovered, which were characterized as follows (Table IV): fraction 1 was characterized by an enrichment of the γ-GTP (4 ×) and DPP IV (5 ×) but only by a 2-fold enrichment of the $Na^+/K^+$-ATPase. In fraction 2, the enrichment factors of γ-GTP and DPP IV were similar to those in fraction 1, but $Na^+/K^+$-ATPase was enriched 9-fold. It is concluded that fractions 1 and 2 are enriched in plasma membranes of the OK cells but that a clear distinction

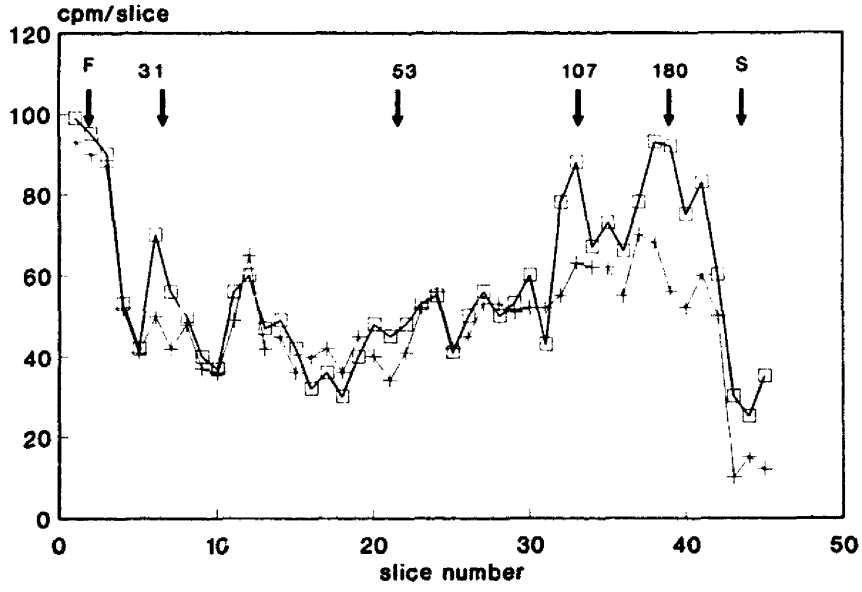


Fig. 3. Effect of $P_i$ on [$^3$H]NAI labelling of OK cell plasma membranes. OK cells were treated with 8 mM [$^3$H]NAI in the absence (□) or presence (+) of 5 mM $P_i$ in the NaCl/mannitol buffer and 100 μg of fraction 1 were applied onto a 10% gel. Apparent molecular masses are given in $10^{-3}$ kDa. S: start; F: front. These data represent one typical experiment out of three, which are summarized in Table V.

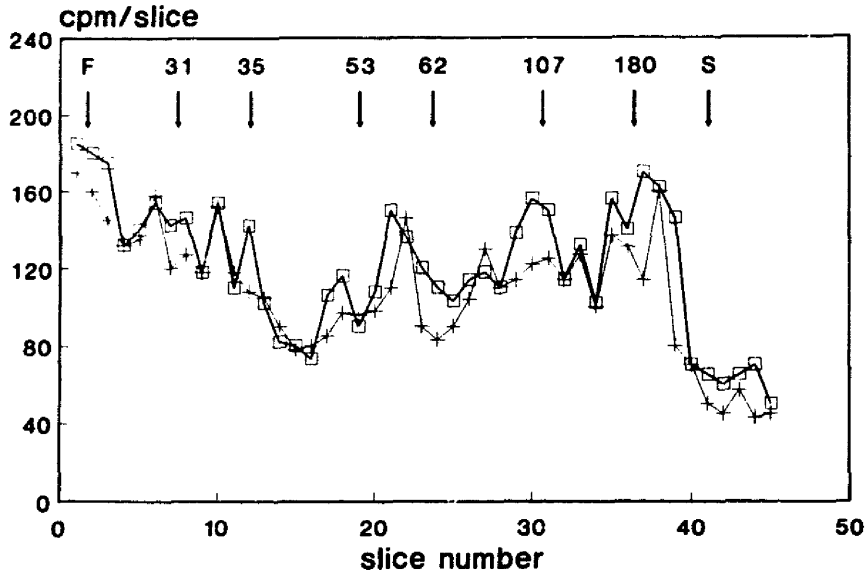


Fig. 1. Effect of PTH on [$^3$H]NAI labelling of OK cell plasma membranes. Control cells or PTH-treated cells ($10^{-10}$ M, 4 h) were labelled with 8 mM [$^3$H]NAI for 15 min. 100 $\mu$g of the corresponding fractions 1 were analyzed with a 10% gel. □, control cells; +, PTH-treated cells. One typical experiment out of two is shown.

between apical and basolateral membranes can not be made. Based on the activity of the $Na^+/K^+$-ATPase, fraction 1 was enriched 4-times with respect to apical membranes compared to fraction 2. In contrast to fractions 1 and 2, fraction 3 was highly enriched (12 ×) in the mitochondrial marker, succinate–cytochrome-*c* oxidoreductase. Importantly, only a very low activity of this mitochondrial marker could be detected in fraction 1 (7-times lower than in the homogenate), suggesting a minor contamination of fraction 1 with mitochondrial particles.

The labelling patterns shown in the following were all derived from membranes recovered from fraction 1. In fraction 2, the results of the $^3$H-labelling experiments were similar to those obtained from fraction 1, whereas, in fraction 3, no differences of the [$^3$H]NAI labelling pattern due to the presence or absence of $P_i$ (as shown in Figs. 3 and 4) were observed (data not shown).

A typical [$^3$H]NAI labelling pattern of fraction 1 is shown in Fig. 3. As also illustrated, the presence of 5 mM $P_i$ during the labelling procedure decreased the incorporation of [$^3$H]NAI in the molecular mass regions of 31 ± 3; 53 ± 3; 107 ± 7 and 176 ± 12 kDa. The protective effect of $P_i$ upon the [$^3$H]NAI incorporation into these four regions of fraction 1 has been quantitated and is summarized in Table V. In all four regions a similar degree of protection by $P_i$ could be observed.

To confirm the physiological relevance of these four $P_i$-protectable regions, we have looked at the effect of parathyroid hormone (PTH) on [$^3$H]NAI incorporation into the proteins of fraction 1. When treated with $10^{-10}$ M PTH for 3–4 h, $Na^+/P_i$ cotransport of OK cells is specifically inhibited by approx. 70% [7,18,19]. Indirect experimental evidence suggests that the PTH-induced inhibition of the $Na^+/P_i$ uptake is due to a reduction in the number of transport sites residing in the apical membrane [20]. Thus, less [$^3$H]NAI incorporation into the protein(s) in question of the plasma membrane of PTH-treated OK cells would be expected. The labelling patterns of fractions 1 of control and PTH-treated cells

TABLE V

*Protective effect of $P_i$ on the [$^3$H]NAI incorporation into OK cell plasma membrane proteins*

| $M_r$ (×$10^{-3}$) | Protection (%) [a] | |
|---|---|---|
| | single observations [b] | mean ± S.D. |
| 176 ± 12 | 15 | |
| | 30 | 22 ± 7 |
| | 21 | |
| 104 ± 7 | 15 | |
| | 17 | |
| | 48 | 37 ± 25 |
| | 68 | |
| 53 ± 3 | 23 | |
| | 20 | 20 ± 3 |
| | 17 | |
| 31 ± 3 | 23 | |
| | 33 | 28 ± 5 |
| | 27 | |

[a] Quantitation was performed by cutting out and weighing the peak regions which showed reproducible protection by $P_i$.

[b] Single observations were derived from totally independent experiment swith respect to [$^3$H]NAI synthesis and cell culture (passage number).

are shown in Fig. 4 and demonstrate reduced [$^3$H]NAI incorporation into the same molecular mass regions as observed by the $P_i$-protection experiments (Fig. 3). In addition, PTH affected the incorporation of [$^3$H]NAI also into proteins of 35 and 62 kDa. It is important to note that the plasma membranes obtained from the PTH-treated cells were enriched to the same degree with respect to the marker enzymes tested as the membranes obtained from the control cells. Therefore, the labelling patterns of control and PTH-treated cells are directly comparable.

## Discussion

This work demonstrates that the $Na^+/P_i$ cotransport activity present in OK cells can be inhibited by NAI in a phosphate-protectable way and that this substrate protection can be used to identify proteins of the plasma membrane of the OK cell, representing possible candidates for the $Na^+/P_i$ cotransport system. These data were obtained with an established epithelial cell line which originally was derived from an opossum kidney [21]. As described recently [7,18,19,20], the OK cell line represents a suitable cellular model system for the investigation of renal proximal tubular $Na^+/P_i$ cotransport. In the course of this study, we have also used brush-border membrane vesicles isolated from rat kidney cortex. When treated with NAI up to 30 mM, $Na^+/P_i$ cotransport into these vesicles was also inhibited by 80%. Furthermore, this inhibition could also be protected (approx. 25%) by the presence of $P_i$ (data not shown), indicating similarities of the $Na^+/P_i$ cotransport systems of the OK cells and rat renal brush-border vesicles.

NAI is considered to be rather specific in acetylating phenolic hydroxyls, but sulfhydryl groups and lysine amino groups also might react under the conditions used [16]. From this work, which was performed on intact OK cells, it could however not be distinguished between these different side-groups. However, in brush-border vesicles isolated from rat kidney cortex, the NAI inhibition of $Na^+/P_i$ cotransport could not be released by a wash in dithiothreitol, suggesting that sulfhydryl groups are not involved.

To achieve maximal inhibition, a rather high concentration of NAI had to be used. Nevertheless, the following observations suggest that the NAI treatment did not impair the functional intactness of the cells. (i) Sodium-independent uptake of $P_i$ (in the presence of *N*-methylglucamine) was not affected by NAI (data not shown). (ii) NAI-treated cells exhibited the same $Na^+$-dependent uptake of L-alanine as control cells, which is evidence for an intact gradient of sodium in NAI-treated cells. This is also supported by the results obtained with sodium-equilibrated cells (Table I), which indicate that NAI did not inhibit $P_i$ uptake via a nonspecific alteration of the driving force ($Na^+$ gradient) but rather inhibits $P_i$ uptake via a direct interaction of NAI with the transport system.

The successful use of similar side-chain-specific reagents has been demonstrated many times by applying the protection criteria. Thus, one would have to expect protection of either NaCl, $P_i$ or both, since the cotransport system is characterized by a 2 $Na^+$/1 $P_i$ stoichiometry (for a review, see Ref. 4). However, as the results demonstrate, the presence of sodium alone did not prevent inhibition of the $Na^+/P_i$ transport by NAI, nor of the other transport systems tested. These data might be in contrast to the recently described reaction of NAI with the intestinal $Na^+$-dependent transporters of D-glucose and L-proline [22,23]. Since however an approx. 100-fold lower concentration of NAI has been used during the studies using extracted intestinal brush-border membranes, the noncovalent interaction of sodium with the binding site(s) might be favored compared to the high NAI concentrations used in the present study. In the presence of $P_i$, protection of the transport activity from being inactivated by NAI could be achieved (see Table III). This protective effect was in the order of 80–100% and, based on the following criteria, it is suggested to be a specific protection of the $Na^+$ cotransport system. (i) The reaction rate of NAI with L-tyrosine in the presence of $P_i$ did not differ compared to the absence of $P_i$ (Fig. 3). (ii) Replacement of $P_i$ with equimolar amounts of sulfate did not protect NAI inhibition of the $Na^+/P_i$ transport. (iii) The presence of $P_i$ did not affect NAI inhibition of other transport systems (e.g., L-glutamic acid transport), nor did L-glutamic acid protect the $Na^+/P_i$ cotransporter.

Recently, evidence for the involvement of (a) arginine residue(s) in the transport of $Na^+/P_i$ has been obtained by the use of phenylglyoxal [5,6]. As further demonstrated, inhibition of $Na^+/P_i$ transport into renal brush-border vesicles by phenylglyoxal could be protected by $P_i$ as well [6]. Studies performed with OK cells also suggested the involvement of arginine residues in the $Na^+/P_i$ cotransport of these cells, since at 2 mM, phenylglyoxal $P_i$ uptake was inhibited by 70% (data not shown).

Based on the above conclusions, radioactive NAI has been synthesized and used to identify proteins of isolated plasma membranes of OK cells which show $P_i$-protectable NAI-labelling. As demonstrated in Figs. 3 and 4, four regions could be identified in which the NAI incorporation was reproducibly reduced by the presence of $P_i$ (Table V). Quantitative correlation of the protective effect of $P_i$ upon the transport rate on the one hand and upon the NAI incorporation on the other hand is, however, not possible, since by one-dimensional gel electrophoresis incomplete separation of the proteins is obtained.

Fortunately, OK cells offer the possibility to test for a physiological significance of the findings obtained with the [$^3$H]NAI-labelling experiments. As shown previously, $Na^+/P_i$ transport of OK cells can be inhibited by near-physiological concentrations of PTH [7,18,19]. Importantly, at $10^{-10}$ M PTH (which has been used throughout this study), $Na^+/P_i$ cotransport is inhibited highly specifically. At higher concentrations, however, PTH ($10^{-8}$ M) also effects the inhibition of the $Na^+$–$H^+$ exchanger (Ref. 24 and Helmle, C., unpublished data). Evidence was obtained that the PTH-induced inhibition of $Na^+/P_i$ transport might be due to a decrease in the number of transport sites at the cell surface [20]. Thus, if the proteins identified in Fig. 3 represent proteins involved in the $Na^+/P_i$ cotransport process, reduced labelling of NAI into these proteins should also be observed after PTH treatment. As illustrated in Fig. 4, PTH indeed effects a decreased NAI labelling of the same proteins as identified by the $P_i$-protection experiments and, in addition, effects a reduced labelling of two other proteins.

In summary, we conclude that the renal $Na^+/P_i$ cotransporter is inhibited by NAI via a modification of a yet-unknown amino side-chain (possibly tyrosine and/or lysine), which is located near or at the binding/translocation site of $P_i$. Furthermore, four possible candidates for the renal $Na^+/P_i$ cotransporter of the OK cells could be identified (31, 53, 107 and 176 kDa proteins). At present, no precise apparent molecular mass of the transport system or its subunits can be given, since it is currently not known whether, after gel electrophoresis, we are dealing with single and discrete proteins or rather with several aggregation states of one or several proteins.

## Acknowledgments

We gratefully acknowledge the help of Dr. B. Bernet (Dept. of Organic Chemistry, University Zurich) in synthesizing the radioactive NAI. This work was financially supported by the Swiss National Fonds (Grant Nos. 3881.185 and 3.949.0.87).

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