Sodium-dependent transport of phosphate in neuronal and related cells

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Abstract

Sodium-dependent phosphate entry into neuronal cells was demonstrated in synaptic plasma membrane vesicles and synaptosomes prepared from rat brains, in PC12 cells and in primary culture of pituitary cells. The extent of the sodium-dependent phosphate transport in the synaptic plasma membrane preparation, at $[Na]_{out} = 110$ mM and $[P_i]_{out} = 0.1$ mM, varied between 0.28 to 1.02 nmol phosphate/mg membrane protein/min. In pituitary cells the value was only about 0.05 nmol P_i/mg protein/min. In PC12 cells the activity increased from 0.0085 to 0.26 nmol P_i/mg protein/min in the transit from undifferentiated to differentiated cells. The dependence of phosphate on sodium concentrations fits a model in which two sodium ions are required to transfer the phosphate into the cells with a $K_{[Na]0.5}$ of 43 mM. The K_m for the phosphate transport in the synaptic plasma membrane preparations was between 0.1 and 0.45 mM. It is concluded that sodium-driven active transport of phosphate is a ubiquitous activity in various types of neuronal cells.

Keywords: Phosphate homeostasis; Plasma membrane vesicle; Synaptosome; PC12 cell; (Brain)

1. Introduction

Phosphate is a substrate and/or an effector of several cytosolic key enzymes of the living cell. Phosphate level in the cells is determined by the balance between the rate of ATP (and to a lesser extent other high energy phosphate compounds) production and utilization in the cell and by the balance of phosphate fluxes between the intracellular and extracellular compartments through the cell membrane. The balance between influx and efflux of phosphate is determined by the extracellular phosphate concentration, by the transport proteins that are involved in phosphate passage through the membranes and by the driving forces that act on the phosphate (for review see Ref. [1]).

In higher organisms, the phosphate concentration in the extracellular fluid is kept within a narrow range that is species specific [2]. In humans the normal plasma phosphate concentration is 1.1 mM $\pm$ 0.2 mM while in other mammals the phosphate concentration in the extracellular fluid is up to threefold higher [2]. In rat liver, cytosolic phosphate concentration is approximately 1 mM whereas the extracellular concentration is about 2 mM [3]. In some aglomerular fishes the phosphate concentration in the plasma can be as high as 6 mM [4]. Since the resting membrane potential can be in some excitable cells in the vicinity of -90 mV, the equilibrium intracellular phosphate concentration should be between 30-times to 1000-times lower, i.e., if only the monovalent or only the divalent phosphate anion, respectively, can pass through the membrane. The fact is that phosphate concentration in the cytosol of cells is in the same

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order of magnitude as the concentration in the extracellular fluid. This implies that some type of active transport of phosphate into the cells must be operating in the plasma membrane. One of these well characterized phosphate active transport into cells is the sodium-driven phosphate transport that was characterized in renal and intestinal epithelial luminal membranes and is responsible for the active reabsorption of phosphate in these organs [1]. In addition, these transport systems were described also in non-epithelial cells such as liver [5,6], osteoblasts [7], RBC [8] and heart muscle cells [9].

Phosphate transport was scarcely studied in neuronal and related cells. It was first demonstrated it squid and frog neurons [10–12] and partially characterized in vagus nerve [13]. Miyamoto et al [14] found that mRNA encoding the Na^+-dependent P_i cotransporter (NPT-1) is expressed in the kidney cortex, the liver and the brain but not in other tissues. Ni et al [15] identified a brain specific sodium-dependent phosphate cotransporter (BNPI) that is expressed by specific neuron populations in the CNS. Recently, Glinn et al [16] characterized Na^+-dependent P_i uptake in rat fetal cortical neurons and showed that over 90% of saturable phosphate uptake is dependent on sodium. The aim of this study was to show the presence of sodium-dependent phosphate transport in subcellular preparations of neuronal cells and in tissue cultures of related cells and characterize its kinetic parameters and sensitivity to transport inhibitors.

2. Materials and methods

2.1. Preparation of synaptic plasma membrane vesicles

Synaptic plasma membrane vesicles were prepared from rat brains as described by Kanner et al. with minor modifications [17]. Sabra rats were killed by cervical dislocation. Brains excluding the cerebellum were homogenized ten volumes of 0.32 M mannitol, 1 mM EDTA solution, using a Wheaton all glass homogenizer B (three strokes). The homogenate was centrifuged at $3000 \times g$ for 10 min (Sigma 3K20 centrifuge, Germany), the supernatant was collected and centrifuged again for 15 min at $27\,000 \times g$ resulting in a crude mitochondrial pellet. This pellet was homogenized (three strokes) in a minimal volume of 5 mM Tris-HCl, 1 mM K-EDTA (pH 7.4) buffer and subsequently the volume was adjusted to 40 ml using the same buffer. After stirring for 45 min, the suspension was centrifuged for 20 min at $27\,000 \times g$. The resulted pellet contained mainly membranes from the synaptic plasma membrane. The pellet was suspended in 0.3 M sucrose, 20 mM Tris-HCl (pH 7.4), and homogenized in a glass Teflon homogenizer. Aliquots were frozen in liquid nitrogen and kept at -70°C. Under these conditions, the transport ability of the vesicles was stable for at least two months.

2.2. Preparation of pinched-off presynaptic nerve terminals (synaptosomes)

Synaptosomes were prepared as described by Krueger et al. [18] with minor modification as described previously [19]. Freshly prepared synaptosomes were used in all transport experiments.

2.3. Preparation of primary cells and tissue culture

Anterior pituitary primary cells were obtained by enzymatic dispersion of the anterior pituitary glands of male rats using 0.1% trypsin, 0.06% collagenase (type II), 0.05% hyaluronidase (type IV), 200 units DNase (type I), 0.25% bovine serum albumin (BSA) and 2.5 mM kanamycin sulfate, as described by Ben Tabou et al. [20]. The resulting somatotrophs were plated in 24-well culture dishes (Nunc Multidish) and stored in an incubator (37°C, 5–6% CO_2). Two h later Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum were added to the culture dish. The cells were kept in the incubator for 1–9 days before the experiment. Pheochromocytoma cell line, PC12, were grown in culture plates in DMEM containing 7.5% fetal bovine serum, 7.5% horse serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. The cultures were grown in incubators (37°C, 5% CO_2). The medium was replaced twice a week. Once a week the cultures were split 1:6. Prior to the experiment the cells were grown in 24-well tissue-culture dishes (Nunc Multidish) coated by a solution containing polylysin 10 μg/ml and collagen 10 μg/ml (1:1). Differentiation of PC12 cells was achieved by the addition of NGF 50 ng/ml, isolated from submaxillary glands by liq-

uid chromatography and purified by HPLC [21], to the cell culture daily for 7 days.

2.4. $^{32}P_i$ transport to synaptic plasma membrane vesicles

Transport assays were performed by a rapid filtration technique. The plasma membrane synaptic vesicles were thawed at room temperature and suspended in a loading solution (5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol and 20 mM Tris-HCl (pH 7.4)). The suspension was incubated in 37°C for 15 min and then centrifuged for 20 min at $27\,000 \times g$. The pellet was suspended in the loading solution, and kept at 37°C for the duration of the experiment. The reactions were initiated by the addition of 50 μl of membrane vesicle suspension to 450 μl of the incubation medium containing 0.02 μCi $^{32}P_i$ (1Ci/mmol) and 0.1 mM unlabeled P_i, and incubation at 37°C. After the desired time, the reaction was stopped by washing the vesicles three times with 2 ml of an ice-cold stop-solution containing 4 mM $CaCl_2$, 5.4 mM KCl, 130 mM NaCl, 30 mM Tris-HCl (pH 7.4), 0.32 M sucrose, and polyethylimine 0.1%. The vesicles were collected on 0.6-μm cellulose nitrate filters (Schleicher and Schuell, Germany) by suction. A liquid scintillation counter was used to determine the amount of radioactive P_i remaining on the filter, with 'quicksafe' scintillation fluid (Zinsser Analytic, Germany). Non-specific binding of $^{32}P_i$ to the membranes and filter was determined by measuring the radioactivity in tubes in which the stop-solution was added to the reaction mixture prior to the addition of the vesicles.

2.5. $^{32}P_i$ transport to synaptosomes

Freshly prepared synaptosomes were suspended in an incubation medium containing 5 mM NaCl, 125 mM choline-Cl, 0.1 mM $CaCl_2$, 0.1 mM KH_2PO_4 and 20 mM Tris-Hepes (pH 7.4). The synaptosome suspension was kept at 37°C for the duration of the experiment. The transport assays were performed by a rapid filtration technique as described above for the synaptic membrane vesicles. the synaptosomes were collected on 1 μm GF/B filters (Whatman, England) by suction. The amount of radioactive P_i remaining on the filters was determined as described above for synaptic plasma membrane vesicles.

2.6. $^{32}P_i$ transport to PC12 and pituitary cells

P_i uptake was performed on cells grown in 24-well tissue-cultured dishes, prepared as described above. $^{32}P_i$ uptake was initiated by the addition of 200 μl of a solution containing: 5 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol, 18 mM Tris-HCl (pH 7.4), 0.1 mM KH_2PO_4, 100 mM NaCl, 5 mM choline-Cl and 0.02 μCi $^{32}P_i$ (1Ci/mmol) to the tissue-cultured wells. Sodium independent transport was determined by the addition of the same buffer in which high sodium concentration was replaced by high choline, i.e. 100 mM choline-Cl and 5 mM NaCl. The uptake was terminated at the desired time following incubation at room temperature, by suction of the radioactive medium and rinsing of the wells, three times, with an ice-cold stop-solution containing 4 mM $CaCl_2$, 5.4 mM KCl, 130 mM NaCl, 30 mM Tris-HCl (pH 7.4) and 0.32 M sucrose. The cells were dissolved by the addition of 1 ml 0.5 N NaOH. The solution was transferred to scintillation vials to which 'quicksafe' scintillation fluid was added, and the radioactivity determined. Protein was determined according to the Bradford Bio-Rad method using BSA as standard [22]. Since NaOH is known to interfere in the Bradford assay, in samples in which NaOH was present in significant amounts (more than 0.1 N) the BSA standard curve was generated in the presence of equal amount of NaOH.

3. Results

3.1. Sodium-dependent phosphate transport in synaptic membrane vesicles

Sodium-dependent phosphate intake by synaptic plasma membrane vesicles was studied at 1 min after the addition of the radioactive element into the solution. This time interval was chosen because it represented rate of phosphate uptake well before the equilibrium distribution of the labeled compound between the external solution and the cytosol of cells (data not shown). The results of the experiments where the external phosphate concentration was kept at 0.1 mM, and the sodium concentration in the medium varied between zero and 120 mM, are shown in Fig. 1. The curve in the figure was obtained by a best to a Hill expression of the type $V = V(\mathrm{Na})_{\max}([\mathrm{Na}]^n/\{K^n$

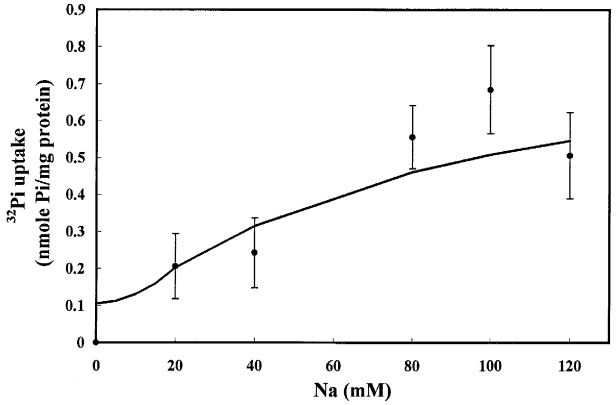

Fig. 1. Effect of sodium concentration on $^{32}P_i$ uptake to synaptic plasma membrane vesicles. Synaptic plasma membrane vesicles were loaded with 5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol, and 20 mM Tris-HCl (pH 7.4). The vesicles were added to a reaction medium containing 1.1 mM $MgCl_2$, 1.1 mM Tris-HCl, 78 mM mannitol, 0.1 mM KH_2PO_4 and 0.02 μCi $^{32}P_i$. NaCl was added at different concentrations. KCl was added in sufficient quantities to ensure that the total Na^+ plus K^+ concentration was 120 mM in each case. The reaction was stopped after 1 min. The data points are the mean $\pm$ S.E. of 10 experiments performed in quadruplicates on different membrane preparations. All data has been corrected for zero-time binding. The curve is a best fit to a Hill equation with $n = 2$; $K_{[Na]0.5} = 43$; $V(Na)_{max} =$ 0.5 nmol P_i/mg protein and $V_0 = 0.105$ nmol P_i/mg protein (see text).

$+ [Na]^n\}) + V_0$, where V is the total phosphate transport, $V(Na)_{max}$ is the maximal sodium-dependent phosphate transport, [Na] is the sodium concentration in the external medium, K is the Hill apparent dissociation constant of sodium binding to the transporter, n is the number of sites that have to be occupied by sodium in order for the transporter to transfer the phosphate to the other side of the membrane, and V_0 is the sodium-independent phosphate transport. The above equation corresponds to the simplest model of cotransporter based on the assumption that a mobile transporter is either a protein that is free of sodium and phosphate or a transporter that all the n sites of sodium and the site of phosphate are occupied. The curve shown in Fig. 1 corresponds to the following values of the parameters: $V(Na)_{max} = 0.5$ nmol P_i/mg membrane protein; $V_0 = 0.105$ nmol P_i/mg membrane protein; $K = 43$ mM; and $n = 2$. It is interesting to note that according to this equation the transporter is physiologically poised to drive phosphate into the cells, i.e., at external Na of 140 mM the probability of the transporter being occupied in both sites by sodium will be greater than 0.92 whereas with intracellular sodium concentration of 10 mM the probability of the transporter being occupied by sodium on both sites decreases to only about 0.05. Thus, at physiological conditions the transporter is set to transfer phosphate into cells at around 87% of its V_{max}.

3.2. Phosphate transport as a function of external phosphate concentration

Fig. 2 shows the sodium-dependent phosphate transport at various phosphate concentrations. Each experimental point in this figure was obtained by subtracting the phosphate transport in the presence of 110 mM sodium from the transport obtained with only 5 mM sodium in the external solution. The

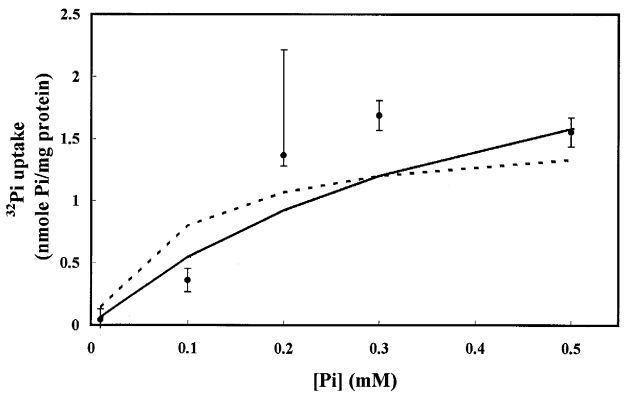

Fig. 2. Effect of phosphate concentration on $^{32}P_i$ uptake in synaptic plasma membrane vesicles. Synaptic plasma membrane vesicles were loaded with 5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol, and 20 mM Tris-HCl (pH 7.4). The vesicles were added to a reaction medium containing either 105 mM NaCl, 1.1 mM $MgCl_2$, 1.1 mM Tris-HCl, 78 mM mannitol (pH 7.4), or 5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 1.1 mM Tris-HCl, and 78 mM mannitol (pH 7.4). $^{32}P_i$ was added at 0.02 μCi/test-tube and phosphate concentration varied between 0.01 mM and 0.5 mM. The reaction was stopped after 1 min. The data points are the mean $\pm$ S.E. of three different experiments performed in quadruplicate on different membrane preparations. All data have been corrected for zero-time binding. The curves are the best fit to a hyperbolic equation based on the least sum of squares of absolute deviation (dotted line) and relative deviations (continuous line) from the experimental points. The K_m values were 0.1 M and 0.45 mM respectively, and the V_{max} values were 1.6 nmol P_i/mg protein and 3.0 nmol P_i/mg protein for the two fitting curves, respectively.

curves shown in the figure were obtained by a best approximation to a hyperbolic relationship between phosphate transport and phosphate concentration. This yielded the values of K_m of 0.1 mM and 0.45 mM and V_{max} of 1.6 nmol P_i/mg protein and 3.0 nmol P/mg protein by determining the least sum of squares of absolute and relative deviations respectively, from the experimental points. The results depicted in Fig. 1 and Fig. 2 are in good agreement. According to the theoretical curves of Fig. 2, the sodium-dependent phosphate transport at 0.1 mM phosphate would be 0.8 or 0.545 nmol P_i/mg membrane protein. According to the theoretical curve of Fig. 1, the sodium-dependent phosphate transport at 110 mM sodium should be 0.44 nmol P_i/mg membrane protein. Considering the spread of the results in each set of experiments and the fact that each set was performed on a different batch of membrane preparation, the figures of the calculated phosphate transport at 0.1 mM phosphate and 110 mM sodium from the curves fit reasonably well.

3.3. Effect of various inhibitors on the sodium-dependent phosphate transport

Table 1 summarizes the results of the effect of various compounds on the sodium-dependent phosphate transport in synaptic membrane vesicles. It is clear that arsenate, phosphonoformic acid (PFA) and 4,4′-diisothiocyanatestilbene-2,2′-disulfonic acid (DIDS) at high concentration inhibit sodium-dependent P_i transport. This is in agreement with previous studies on other tissues [23–29]. The sodium pump inhibitor, ouabain, did not inhibit the transport probably because that at these short intervals, i.e., less than 2 min of exposure, it did not affect significantly the sodium concentration within the vesicles. Surprisingly, amiloride significantly increased the transport. The mechanism of this enhancement may be related to the amiloride effects on the Na–H exchanger and warrants further investigation.

3.4. Sodium-dependent phosphate transport in other cells and preparations

Table 2 shows the values of sodium-dependent phosphate transport as determined in another set of synaptic membrane vesicles preparation in comparison to synaptosome preparation, to differentiated and undifferentiated PC12 cells and to pituitary primary cells. In all these neuronal preparations a significant sodium-dependent phosphate transport was observed, pointing to the presence of the Na–P_i co-transport system in these preparations. The sodium-dependent P_i transport in the vesicles preparation is 4-fold higher than in the synaptosomes probably due to the enrichment of transport proteins in the vesicles compared to the synaptosomes. It is noteworthy that the differenti-

Table 1
The effect of different inhibitors on phosphate uptake

Inhibitor	Sodium-dependent P_i uptake (nmol P_i/mg protein)	Sodium-dependent P_i uptake (% of control)
Control	0.475 ± 0.090 ($n = 77$)	100 ± 18.95
DIDS (0.5 mM)	0.182 ± 0.145 * ($n = 20$)	38.32 ± 30.53
PFA (10 mM)	0.017 ± 0.168 * ($n = 19$)	3.58 ± 35.37
PFA (1 mM)	0.379 ± 0.101 ** ($n = 15$)	79.79 ± 21.26
Na arsenate (50 mM)	-0.033 ± 0.190 * ($n = 24$)	-6.95 ± 40
Na arsenate (5 mM)	0.336 ± 0.189 ($n = 14$)	70.74 ± 39.79
Ouabain (1 mM)	0.728 ± 0.300 ($n = 23$)	153.26 ± 63.16
Amiloride (1 mM)	0.974 ± 0.205 *** ($n = 21$)	205.05 ± 43.16

Synaptic plasma membrane vesicles were loaded in 5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol, 20 mM Tris-HCl (pH 7.4), and incubated in media containing the desired inhibitor and either 110 mM NaCl, 1.1 mM $MgCl_2$, 1.1 mM Tris-HCl, 78 mM mannitol (pH 7.4) or 5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 1.1 mM Tris-HCl, and 78 mM mannitol (pH 7.4) (for sodium-independent phosphate uptake). Phosphate concentration was added at 0.11 mM with $^{32}P_i$ at 0.02 μCi/test-tube. The vesicles were incubated for 1 min at 37°C. Values are the mean ± S.E. of six experiments performed in quadruplicate on different membrane preparations.
* Significantly lower than control values ($P < 0.01$, paired comparative *t*-test). ** Significantly lower than control values ($P < 0.1$, paired comparative *t*-test). *** Significantly higher than control values ($P < 0.1$, paired comparative *t*-test).

Table 2
Sodium-dependent P_i uptake in different nervous cell preparations

Cell preparation	Sodium-dependent uptake ± S.E. (nmol P_i /mg protein)
Synaptosomes	0.280 ± 0.129
Synaptic plasma membrane vesicles	1.017 ± 0.215
Differentiated PC12 cells	0.199 ± 0.019
Non-differentiated PC12 cells	0.0065 ± 0.0027
Pituitary primary culture	0.037 ± 0.011

Synaptosomes were incubated with either 4 mM $CaCl_2$, 5.4 mM KCl, 130 mM NaCl, 30 mM Tris-HCl (pH 7.4), or 4 mM $CaCl_2$, 5.4 mM KCl, 130 mM choline-Cl, and 30 mM Tris-HCl; synaptic plasma membrane vesicles were incubated with either 110 mM NaCl, 1.1 mM $MgCl_2$, 1.1 mM Tris-HCl, 78 mM mannitol (pH 7.4), or 5 mM NaCl, 100 mM KCl, 1 mM $MgCl_2$, 1.1 mM Tris-HCl, and 78 mM mannitol (pH 7.4); PC12 cells differentiated and non-differentiated, and anterior pituitary cells were incubated in either 5 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol 18 mM, Tris-HCl, 100 mM NaCl, and 5 mM choline-Cl (pH 7.4), or 5 mM KCl, 1 mM $MgCl_2$, 78 mM mannitol 18 mM, Tris-HCl, 5 mM NaCl and 100 mM choline-Cl (pH 7.4). P_i was added at 0.1 mM and $^{32}P_i$ at 0.02 μCi per test-tube/well. The reaction was stopped at 1 min. Values are the mean ± S.E. of three experiments performed in quadruplicate on different membrane preparations.

ated PC12 cells have 30-fold higher sodium-dependent phosphate transport activity compared with the non-differentiated cells.

4. Discussion

The present study demonstrated unequivocally the presence of sodium-dependent phosphate transport in nervous tissue, in differentiated PC12 cells and to a lesser extent but still conspicuously enough in primary culture of pituitary cells. Thus, in agreement with previous studies [13–16], we demonstrated that in addition to the well known presence of the Na–P_i transporter in the luminal membranes of renal [1,14,28,30] and intestinal [24,31] epithelial cells, it is also present in neuronal and related cells. The Na–P_i transporter in the classical renal and intestinal epithelial cells plays a pivotal role in the active transport of phosphate from the lumen of these organs to the extracellular milieu of the organism. Obviously, in the non-epithelial cells the Na–P_i transporter is poised to enable active transport of phosphate into cells. This is probably the cells' means of ensuring a cytosolic phosphate concentration that would not decrease below a limit that will risk the cell function, e.g., the rate of production ATP in the cells. This is particularly important in excitable cells that posses a relatively high negative resting membrane potential that would tend to reduce the phosphate concentration in the cytosol of cells well below the concentration in the extracellular fluid. Our study complements other studies on liver cells, osteoblasts, red blood cells, and heart muscle cells [5–9] and imply that the Na–P_i transporter is ubiquitously expressed and active in many types of cells. It was shown that its activity can be modified in cells derived from various sources by manipulating the phosphate concentration in the culture medium; e.g., reducing the phosphate concentration to zero results in clear increase in the Na–P_i activity [32]. The way in which the Na–P_i activity can be modified in various types of cells remains to be elucidated. It is interesting that in liver cells insulin, among its other profound effects, also increases the sodium driven phosphate transport [5].

Another possible regulator of phosphate movement across cell membranes is the anion exchanger [32]. In cells devoid of an active inward transport of chloride this system cannot help in increasing the phosphate concentration in cells beyond the equilibrium value. However, in cells that posses a robust Na-driven Cl transport, the anion exchange system can be used to elevate also the phosphate concentration inside the cells beyond the equilibrium value. It will be of interest to compare the extent of the Na–P_i transporter activity in non-epithelial cells in relation to the presence of secondary active bumetanide-sensitive chloride transport.

The K_m for phosphate as determined in this study is in the range of the K_m concentrations obtained for other cell preparations studied in different tissues (between 0.1 and 1.0 mM) [5,6,8,13,16,25,33–35]. The V_{max}s for synaptic plasma membrane vesicles found in this study are 10–20-times lower than the V_{max} obtained for renal brush border membrane [34], 5–10-times higher than the V_{max} obtained for hepatocytes [5] and liver plasma membrane vesicles [6,25], and roughly similar to the value found in rat fetal cortical neurons [16]. The stoichiometry of 2Na:1P_i as obtained from the analysis of our experiments is in

agreement with the previous studies on other membranal preparations [1,5,6,25,33].

An important component of neuronal differentiation is the tightly controlled expression of a spectrum of plasma membrane proteins such as ion channels, transporters and receptors. The PC12 rat pheochromocytoma cell line is widely used to study neuronal differentiation by growth factors (for review see Ref. [36]). In response to NGF, PC12 cells differentiate into sympathetic-like neurons and become electrically excitable as a result of enhanced expression of a variety of sodium [37] and calcium channels [38]. The dramatic increase of the sodium-dependent phosphate transport in the differentiated PC12 cells in comparison to the non-differentiated cells, as shown in this study, may indicate the involvement of this transport system in processes underlying neuronal function.

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