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Swelling-induced activation of Na^+,K^+,2Cl^- cotransport in C6 glioma cells: kinetic properties and intracellular signalling mechanisms

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Abstract

Swelling of C6 glioma cells in hypotonic medium (180 mOsm) results in two- to three-fold activation of K^+ ($^{86}Rb^+$) influx suppressed by 10 μM bumetanide. Bumetanide-sensitive transport of $^{86}Rb^+$ is dependent on extracellular K^+, Na^+ and Cl^- both in iso-osmotic conditions and under hypo-osmotic shock, supporting the notion that it is mediated by Na^+,K^+,2Cl^- cotransport. Inhibitors of protein kinase C (10 μM polymyxin B and 1 μM staurosporine) had no significant effect on basal cotransport but reduced its hypotonic stimulation by 70–80%. Similar results were obtained with calmodulin antagonist R24571 (10 μM), indicating Ca^{2+}/calmodulin-dependence of the process. Influence of polymyxin B and R24571 was not additive. Swelling-activated Na^+,K^+,2Cl^- cotransport was also suppressed by protein kinase C activator PMA (1 μM). By contrast, preincubation of cells with inhibitors of protein phosphatases (100 μM vanadate, 5 mM fluoride and 0.5 μM okadaic acid) activated greatly the bumetanide-sensitive $^{86}Rb^+$ uptake in isotonic conditions, while a subsequent hypotonic swelling led to smaller or no increment. These results indicate the involvement of Ca^{2+}/calmodulin-dependent staurosporine/polymyxin B-sensitive protein kinase other than protein kinase C in swelling-induced activation of Na^+,K^+,2Cl^- cotransport in glial cells.

Keywords: Cell swelling; Regulatory volume decrease; Na^+,K^+,2Cl^- cotransport; Calmodulin; Protein kinase C; Protein phosphatase

1. Introduction

Na^+,K^+,2Cl^- cotransport is a major system of mammalian cells involved in maintenance of transmembrane K^+ and Cl^- gradients and volume regulatory processes especially in response to cell shrinkage [1–3]. This type of transport was extensively studied in brain glia [4–6] and its additional role in these cells was shown to be a clearance of extracellular space from K^+ released by excited neurons in physiological as well as in pathophysiological conditions [7,8].

Generally, Na^+,K^+,2Cl^- cotransport is stimulated under cell shrinkage and inhibited by cell swelling [9–14]. In vertebrate erythrocytes and epithelial cells, an increase in cell volume is accompanied by activation of alternative K^+,Cl^- cotransport system operating mainly in an efflux mode [9,10,15,16]. However, recently we have found a hypotonic activation of the

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inward-directed $Na^+,K^+,2Cl^-$ cotransport identified as bumetanide-inhibited $^{86}Rb^+$ influx in two types of cultured glial cells, viz. C6 glioma [17] and primary astrocytes from rat brain [18]. A similar phenomenon was established in Ehrlich ascites tumor cells [19–21].

It is well recognized that several types of intracellular signalling pathways are involved in volume regulation of ion transport systems including electroneutral cotransporters of cations and chloride [9,10,22,23]. While the nature of a volume sensor remains obscure, there are some reasons to suspect the existence of multiple volume detectors and volume regulatory effector pathways that respond specifically to the mechanisms and magnitude of volume change [24]. Swelling-induced activation of K^+,Cl^- cotransport in erythrocytes is due to dephosphorylation of transport protein by serine/threonine protein phosphatases (PPs) [25,26], while shrinkage-induced activation of $Na^+,K^+,2Cl^-$ cotransport is determined by protein phosphorylation through protein kinase C (PKC) or some other Ca^{2+}/calmodulin-dependent protein kinase (Ca^{2+}/CM PK) [12,13]. Recent findings of the laboratory of E.K. Hoffmann confirm the involvement of cytoskeleton rearrangements and PKC in both shrinkage- and swelling-induced activation of $Na^+,K^+,2Cl^-$ cotransport in Ehrlich ascites tumor cells [19–21]. Our results support the role of membrane cytoskeleton in volume-dependent regulation of K^+,Cl^- and $Na^+,K^+,2Cl^-$ cotransport in rat erythrocytes [27].

The present study was aimed at investigation of kinetic properties and intracellular signalling mechanisms of swelling-activated $Na^+,K^+,2Cl^-$ cotransport in cultured C6 glioma cells.

2. Materials and methods

2.1. Reagents

Fetal calf serum and Dulbecco's modified minimum essential medium (DMEM) were obtained from Gibco, NY. Ouabain, bumetanide, polymyxin B, staurosporine, phorbol 12-miristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4α-PDD) and gentamycine were from Sigma Chemicals, St. Louis, MO. N-(2)-hydroxyethylpiperasine-N'-2-ethanesulfonic acid (Hepes), Tris(hydroxymethyl)aminomethane (Tris), glucose, N,N,N',N'-ethylendiamine tetraacetic acid disodium salt (EDTA), sodium dodecylsulfate (SDS) were purchased from Serva, Heidelberg, Germany. Na_3VO_4, NaF and other salts were from BDH Chemicals, Poole, UK. $^{86}RbCl$ (spec. act. 3.5 mCi/mg) was obtained from Isotope, St.-Petersburg, Russia.

2.2. Cell cultures

Glioma C6 cells were obtained from cell culture collection of Institute of Cytology (St.-Petersburg, Russia). All studies were performed on nonconfluent monolayers between passages 20 to 25. Cells were grown in 24-well multiwell dishes (Costar) on polylysine support at 37°C in a humidified 5% CO_2–95% air atmosphere. The final density of cultures was 250 000–350 000 cells/well. The cell culture medium was prepared on the basis of DMEM with addition of 10% fetal calf serum and 100 μg/ml gentamycin.

2.3. Measurements of K^+ ($^{86}Rb^+$) influx

Cells in 24-well plates were washed from the culture medium with medium A containing (in mM): 140 NaCl, 5 KCl, 0.8 NaH_2PO_4, 1.2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 20 Hepes-Tris (pH 7.4, 37°C) and preincubated for 30 min at 37°C in 0.4 ml of the same medium. After preincubation, the medium A was aspirated and 0.25-ml aliquots of prewarmed isotonic or hypotonic media containing 5 μCi/ml $^{86}RbCl$ were added. The medium A was used as isotonic (310 mOsm). Hypotonic medium B (180 mOsm) contained 75 mM NaCl, the concentrations of other components being the same as in medium A. K^+ transport in isotonic and hypotonic conditions was determined as accumulation of its radioactive analogue $^{86}Rb^+$ in the cells. Reaction was terminated by adding 1.5 ml cooled washing medium W consisting of (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 5 Hepes-Tris (pH 7.4, 4°C) followed by 4 washes with 1.5 ml of the same medium. Cells were lysed during 1 h in 1 ml of solution containing 1% SDS, 2 mM EDTA at 37°C. Aliquots of lysate (0.5 ml) were placed into scintillation vials with 5 ml of Bray scintillator [28]. 0.3-ml samples were used for protein assay according to Lowry et al. [29]. Radioactivity was measured on a scintillation counter Rackbeta 1217 (LKB, Sweden).

The rate of K^+ transport was calculated as $V = A/(a\ c\ t)$, where V is transport rate (nmol/mg protein/min), A is radioactivity of a sample (cpm), a is specific radioactivity of $^{86}Rb^+$ related to the content of its analogue K^+ (cpm/nmol), c is protein content in a sample (mg), t is the time of incubation of cells in the medium containing isotope (10 min). The rate of $Na^+,K^+,2Cl^-$ cotransport was calculated as a difference between rates of isotope accumulation in media containing 1 mM ouabain and a mixture of 1 mM ouabain with 10 μM bumetanide.

2.4. Treatment of cells with modulators of intracellular signalling

To evaluate the involvement of intracellular signalling systems in regulation of $Na^+,K^+,2Cl^-$ cotransport, C6 glioma cells were preincubated in medium A for 30 min with calmodulin antagonist R24571 (10 μM), protein kinase inhibitors, polymyxin B (10 μM) [30] and staurosporine (1 μM) [31], PKC activator, PMA (1 μM) or its non-active analogue 4α-PDD (1 μM). PP inhibitors fluoride (5 mM) [32], vanadate (100 μM) [33], and okadaic acid (0.5 μM) [34]. The preincubation time was chosen on the basis of preliminary experiments and corresponded to maximal effects of the drugs used. The same concentrations of inhibitors were added to media in which measurements of ion transport were performed.

3. Results

3.1. Time course of basal and swelling-induced $^{86}Rb^+$ influx in C6 glioma cells

Fig. 1 presents kinetics of $^{86}Rb^+$ accumulation in cultured glioma C6 cells. In basal iso-osmotic conditions (medium A) 50–55% of K^+ influx was suppressed by 1 mM ouabain and up to 35% by 10 μM bumetanide (Fig. 1A). The effects of two inhibitors were additive, indicating the involvement of two independent transport pathways, the sodium pump and electroneutral cotransport of cations and chloride. Cell swelling in medium B (180 mOsm) activated both ouabain- and bumetanide-sensitive components of K^+ ($^{86}Rb^+$) transport by 1.4–1.6 and 2.4–2.6

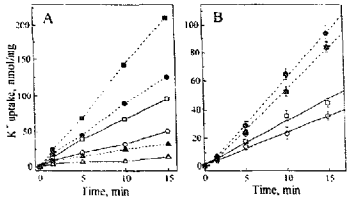

Fig. 1. Time course of K^+ ($^{86}Rb^+$) influx into C6 glioma cells under isotonic conditions (open symbols) or after hypoosmotic shock (solid symbols). A: Total uptake of $^{86}Rb^+$ (squares), uptake in the presence of 1 mM ouabain (circles), and 1 mM ouabain plus 10 μM bumetanide (triangles). B: Calculated values of ouabain-sensitive (squares) and ouabain-resistant bumetanide-sensitive (circles) K^+ influx. Fitting was performed by linear regression. Isotonic medium (310 mOsm) contained (in mM): 140 NaCl, 5 KCl, 0.8 NaH_2PO_4, 1.2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 20 Hepes-Tris (pH 7.4, 37°C). Hypotonic medium (180 mOsm) contained 75 mM NaCl, the concentration of other components being the same as in isotonic one. Data are means of three experiments ± S.E.M.

times, respectively. As shown in Fig. 1B, the sodium pump and $Na^+,K^+,2Cl^-$ cotransport reached a fully activated state no later than 1.5–2 min after the placement of cells in hypotonic medium.

3.2. Dependence of bumetanide-sensitive $^{86}Rb^+$ influx on extracellular K^+, Na^+ and Cl^-

Sensitivity to low concentrations of bumetanide is good but not conclusive evidence of $Na^+,K^+,2Cl^-$ cotransport. In particular, Ehrlich ascites tumor cells, epithelial cells and erythrocytes from many mammalian species along with $Na^+,K^+,2Cl^-$ cotransport possess the K^+,Cl^- cotransport [9,22,35] which may also be partly inhibited by bumetanide [36]. Therefore, in order to reveal the nature of bumetanide-sensitive $^{86}Rb^+$ transport, we studied its interaction with $Na^+,K^+,2Cl^-$ cotransport substrates Na^+ (Fig. 2A), K^+ (Fig. 2B) and Cl^- (Fig. 2C) under isotonic and hypotonic conditions. A full replacement of external K^+ with choline completely suppressed bumetanide-sensitive $^{86}Rb^+$ uptake in basal conditions as well as after hypo-osmotic swelling (Fig. 2A).

Fig. 2B presents dependence of $^{86}Rb^+$ transport

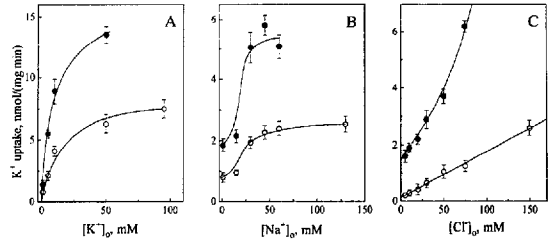

Fig. 2. Dependence of bumetanide-sensitive K^+ ($^{86}Rb^+$) influx on external K^+ (A), Na^+ (B) and Cl^- (C) concentrations in isotonic conditions (open symbols) or under hyposmotic shock (solid symbols). In A: media contained (in mM) 45 NaCl, 1–95 KCl, 0.8 NaH_2PO_4, 1.2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 20 Hepes-Tris (pH 7.4, 37°C). In B: media contained (in mM) 0–140 NaCl, 4.2 KCl, 0.8 KH_2PO_4, 1.2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, 20 Hepes-Tris (pH 7.4, 37°C). In C: media contained (in mM) 0–140 NaCl, 5 KCl, 0.8 NaH_2PO_4, 1.2 $CaCl_2$, 1 $MgSO_4$, 5 glucose, 20 Hepes-Tris (pH 7.4, 37°C). Osmolality was adjusted to 310 or 180 mOsm by adding choline chloride (A and B) or Na glucuronate (C). Data are means of 3–4 experiments ± S.E.M.

activity on external Na^+. In iso-osmotic conditions we observed that choline substituting for external Na^+ reduced the rate of bumetanide-sensitive $^{86}Rb^+$ uptake by 70%. The same phenomenon was shown for $^{86}Rb^+$ accumulation under hypo-osmotic shock.

In iso-osmotic conditions the rate of bumetanide-sensitive $^{86}Rb^+$ uptake was linearly related to the external Cl^- concentration up to 150 mM (Fig. 2C). A full replacement of external Cl^- with glucuronate suppressed $^{86}Rb^+$ influx by 93% in iso-osmotic me-

Table 1
Influence of intracellular signalling modulators on ouabain-resistant bumetanide-sensitive (Na^+,K^+,2Cl^- cotransport) and ouabain- and bumetanide-resistant (K^+ leak) K^- influx (nmol/mg/min) in C6 glioma cells under isotonic conditions and after hypoosmotic shock

Drugs	Na^+,K^+,2Cl^- cotransport		K^+ leak		Number of exper
	310 mOsm	180 mOsm	310 mOsm	180 mOsm	
No additions	2.51 ± 0.05	5.91 ± 0.05	1.35 ± 0.02	1.62 ± 0.04	12
10 μM R24571	1.89 ± 0.11 **	2.21 ± 0.17 **	1.40 ± 0.06ns	1.74 ± 0.08ns	5
10 μM Polymyxin B	2.41 ± 0.07ns	3.49 ± 0.15 **	1.21 ± 0.08	1.45 ± 0.12ns	5
1 μM Staurosporine	2.20 ± 0.12ns	2.91 ± 0.21 **	1.25 ± 0.08ns	1.55 ± 0.10ns	3
1 μM PMA	2.07 ± 0.08 *	3.74 ± 0.32 **	2.09 ± 0.15 **	2.34 ± 0.31 *	4
1 μM 4α-PDD	2.81 ± 0.21ns	6.32 ± 0.25ns	1.45 ± 0.16ns	1.79 ± 0.14ns	3
R24571 + polymyxin B	1.65 ± 0.29 **	2.36 ± 0.27 **	1.21 ± 0.08ns	1.27 ± 0.34ns	3
5 mM Fluoride	6.01 ± 0.15 **	7.50 ± 0.43 **	1.59 ± 0.18ns	1.51 ± 0.13ns	6
100 μM Vanadate	6.31 ± 0.32 **	8.21 ± 0.26 **	1.14 ± 0.09ns	1.47 ± 0.10ns	5
0.5 μM Okadaic acid	5.68 ± 0.21 **	5.65 ± 0.22ns	1.13 ± 0.17ns	1.25 ± 0.24ns	3

$^{86}Rb^+$ was used as tracer for K^+ fluxes. Cells were preincubated for 30 min with R24571, polymyxin B, staurosporine, PMA, 4α-PDD, fluoride, vanadate or okadaic acid in isotonic medium A. The measurements of fluxes were initiated by adding of isotonic (A, 310 mOsm) or hypotonic (B, 180 mOsm) media containing $^{86}Rb^+$ and the same concentrations of inhibitors. 10 min later incubation was terminated by 4-fold washing with ice-cold medium W. For composition of media see Section 2. * $P < 0.05$; ** $P < 0.01$, significantly different vs isoosmotic/hypoosmotic control. ns, not significant.

dia and by 80% after hypotonic swelling as compared by maximal values observed. In addition, unlike iso-osmotic conditions, Cl^--dependence under hypo-osmotic shock was non-linear.

3.3. Effect of modulators of intracellular signalling on Na^+, K^+, $2Cl^-$ cotransport in iso-osmotic conditions and after hypotonic shock

We studied the effects of different modulators of intracellular signalling on ouabain-resistant bumetanide-sensitive $^{86}Rb^+$ influx (Na^+,K^+,$2Cl^-$ cotransport) and ouabain- and bumetanide-resistant $^{86}Rb^+$ influx (K^+ leak) (see Table 1). Calmodulin antagonist R24571 inhibited bumetanide-sensitive K^+ uptake by 25 and 73% in isotonic and hypotonic medium, respectively. The swelling-induced but not basal cotransport of cations and chloride was also significantly decreased by PKC inhibitors polymyxin B and staurosporine. The effects of R24571 and polymyxin B were not additive. To clarify further if PKC is involved in volume regulation of Na^+,K^+,$2Cl^-$ cotransport, we tested PKC activator PMA and its non-active analogue 4α-PDD. PMA suppressed Na^+,K^+,$2Cl^-$ cotransport by 20 and 35–40% in basal conditions and after hypo-osmotic swelling, respectively. By contrast, 4α-PDD had no significant influence on the transport rate both in iso-osmotic and hypo-osmotic conditions, indicating rather specific action of PMA.

Preincubation of C6 cells with putative PP inhibitors, fluoride and vanadate as well as with specific inhibitor of PP 1 and 2a, okadaic acid led to about 2.5-fold activation of Na^+,K^+,$2Cl^-$ cotransport (Table 1). This action was comparable in magnitude with that from swelling. After treatment with fluoride and vanadate, hypo-osmotic swelling resulted in a lower rise of cotransport activity (not more that 1.4-fold). No effect of hypo-osmotic shock was noted after preincubation of C6 cells with okadaic acid.

Unlike the Na^+,K^+,$2Cl^-$ cotransport, K^+ leak (ouabain- and bumetanide-resistant $^{86}Rb^+$ influx) was unaffected by all the inhibitors tested except PMA. This drug activated ouabain- and bumetanide-resistant $^{86}Rb^+$ accumulation both in basal conditions and under hypo-osmotic shock (Table 1).

4. Discussion

Swelling of cultured glial cells in hypo-osmotic medium results in activation of bumetanide-sensitive K^+ uptake as we described earlier in C6 glioma cells [17] and primary astrocytes from rat brain [18]. Both basal and swelling-induced bumetanide-sensitive $^{86}Rb^+$ transport are dependent on external Na^+, K^+ and Cl^- and this is strong evidence for the Na^+,K^+,$2Cl^-$ cotransport in C6 cells and its activation upon the increase of cell volume. The Na/K/Cl cotransport system with stoichiometry of 1/1/2 was described earlier in these cells [6]. As in our case the authors [6] showed a partial dependence of $^{86}Rb^+$ accumulation on extracellular sodium that probably reflects a function of cotransporter in a mode of Na_o^+-independent K^+,Cl^- symport or K_o^+/K_{in}^+ exchange. A similar phenomenon was established in chick cardiac cells [37] and macrophage-like cells [38]. Unlike Chassande et al. [6], we observed that Cl^- replacement by glucuronate did not decrease bumetanide-sensitive $^{86}Rb^+$ influx to a zero level, the remaining flux being 7%. Such a tendency was even more expressed in hypotonic medium. This may be further evidence of a K_o^+/K_{in}^+ exchange. Nevertheless, our data show that at least 2/3 of bumetanide-sensitive $^{86}Rb^+$ influx in isotonic and hypotonic conditions depends on external Na^+ and Cl^- and therefore may be attributed to the Na^+,K^+,$2Cl^-$ cotransport.

Depending on animal species and tissues, activity of Na^+,K^+,$2Cl^-$ cotransport is modulated by various hormonal factors and respective intracellular messengers such as cAMP [39,40], cGMP [41,42] and Ca^{2+}/calmodulin [43–45]. It has also been reported that Na^+,K^+,$2Cl^-$ cotransport in vascular smooth muscle cells and BALB/c 3T3 fibroblasts is regulated both by growth factors and PKC [46–48]. In our experiments PKC inhibitors polymyxin B and staurosporine had no significant effect on basal Na^+,K^+,$2Cl^-$ cotransport but reduced its swelling-induced stimulation by 70–80%. An even larger inhibition was observed with calmodulin antagonist R24571, the effects of R24571 and polymyxin B not being additive. That indicates that Ca^{2+}/calmodulin and PK pathways are probably linked in series rather than in parallel. Earlier the PKC was shown to be involved in RVD- and volume-dependent potassium

channel regulation in primary astrocytes [49] and proximal tubule cells from frog [50]. Larsen et al. [21] discovered that hypotonic swelling of Ehrlich ascites tumor cells was accompanied by activation of the PKC while inhibitors of this enzyme, H7 and chelerythrine, blocked the swelling-induced Na^+,K^+,$2Cl^-$ cotransport. More recent work [12] confirmed a key role of PKC in shrinkage-induced activation of this transport system in pneumocytes. However, in our experiments PKC activator PMA suppressed swelling-induced cotransport in a similar manner to PKC inhibitors. A similar phenomenon was observed by Clerici et al. [12] for shrinkage-stimulated cotransport in alveolar epithelial cells. A maximal effect was shown to be at about 10^{-6} M. Such a concentration (5 to 10-fold higher than usually used) might result in down-regulation of PKC followed by inhibition of PKC-mediated protein phosphorylation. In general PKC desensitization needs 12–24 h of incubation with PMA. We failed to notice any significant differences in PMA effects at 10, 30 and 60 min of preincubation (data not shown). Therefore a mechanism based on PKC down-regulation seems to be unlikely. It is possible that the inhibitory effect of PMA is connected with the existence of a separate regulatory pathway involving PKC as described in vascular smooth muscle and fibroblasts [46–48], whereas swelling-induced activation of Na^+,K^+,$2Cl^-$ cotransport in C6 glioma cells is mediated by Ca^{2+}/calmodulin-dependent protein kinase other than PKC. Apart from PKC, staurosporine and polymyxin B was also shown to suppress other types of PKs including tyrosine-dependent [51]. Evidence was presented that Ca^{2+}/CM-dependent PK II is involved in swelling-induced activation of K^+ and amino acid efflux in cultured astrocytes [52]. Using orthovanadate as an inhibitor of PPs, tyrosine kinase inhibitors herbimycin A and genistein and antibodies to phosphotyrosine, Tilly et al. [53] showed that the tyrosine-dependent phosphorylation is involved in RVD process and hypotonicity-induced K^+ and Cl^- efflux in the cultured human intestine 407 cell line. Recent work of O'Donnell et al. [13] demonstrates directly the phosphorylation of transport protein in both hormone- and shrinkage-induced activation of Na^+,K^+,$2Cl^-$ cotransport in vascular endothelial cells. The volume-dependent regulation of cotransporters in these cells was affected by inhibitors of myosin light chain kinase. Identification of protein kinase involved in the regulation of Na^+,K^+,$2Cl^-$ cotransport during glial RVD needs further studies.

In many cell types PP inhibitors were shown to be potent stimulators of Na^+,K^+,$2Cl^-$ cotransport in resting conditions [13,54,55]. In our case preincubation of C6 cells with putative inhibitors of PPs, vanadate and fluoride caused a large activation of Na^+,K^+,$2Cl^-$ cotransport in iso-osmotic medium. After treatment with vanadate and fluoride hypotonic swelling results in a lower stimulation of bumetanide-sensitive K^+ influx. It is known that fluoride in complex with endogenous Al^{3+} is also a potent nonspecific activator of G-proteins and able to change activity of several ion transport systems by this pathway [56]. However, earlier we have shown that adding Al^{3+} to preincubation medium was without effect on fluoride-induced stimulation of Na^+,K^+,$2Cl^-$ cotransport, thus indicating that fluoride acts through inhibition of PPs rather than activation of G-proteins [57]. The influence of fluoride and vanadate on Na^+,K^+,$2Cl^-$ cotransport was previously described in a giant squid axon [58] in which both inhibitors did not affect the basal activity of transport but increased the period of its inactivation caused by ATP depletion under intracellular dialysis. Further, the inhibitor of PPs 1 and 2a, okadaic acid stimulated Na^+,K^+,$2Cl^-$ cotransport in isotonic medium like fluoride and vanadate and no additional stimulation of cotransport in hypo-osmotic conditions was observed. The reasons for some additional effect of hypotonic swelling after vanadate or fluoride pretreatment in activation of $^{86}Rb^+$ influx are unknown.

Thus, inhibitors of PPs affect mainly the basal activity of Na^+,K^+,$2Cl^-$ cotransport in C6 glioma cells, whereas inhibitors of PKs and calmodulin antagonist suppress its swelling-induced activation. These data may be viewed as evidence for involvement of protein phosphorylation in the regulation of Na^+,K^+,$2Cl^-$ cotransport under hypo-osmotic shock. Like a shrinkage-induced activation of this transport system registered in many cell types, a swelling-induced activation requires a shift of phosphorylation/dephosphorylation equilibrium to a phosphorylated protein state, while swelling-induced stimulation of K^+,Cl^- cotransport in mammalian erythrocytes is due to protein dephosphorylation.

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