Biochimica et Biophysica Acta 903 (1987) 411–416
Elsevier

BBA 73714

Characterization of an $Na^+/K^+/Cl^-$ co-transport in primary cultures of rat astrocytes

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(Received 12 May 1987)

Key words: Furosemide; Sodium/potassium/chloride co-transport; Ouabain; Hyperosmolarity; Primary culture; (Rat astrocyte)

The furosemide- and bumetanide-sensitive component of the $^{86}Rb^+$ uptake into primary cultures of rat astrocytes was fully dependent on the simultaneous presence of Na^+ and Cl^- in the incubation mixture and is therefore most likely an $Na^+/K^+/Cl^-$ co-transporter. As expected for such a co-transporter, its activity is insensitive to 0.1 mM amiloride and to 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, and of the tested anions, only Br^- could partly replace Cl^-. The $K_{0.5}$ values for K^+, Na^+ and Cl^- activation were 2.7, 35 and 40 mM, respectively. The activity of the co-transporter was stimulated 1.5-times in hyperosmolar (500 mosM) medium.

Introduction

Evidence is accumulating which indicates that the membrane functions of astrocytes are involved in the regulation of K^+ concentration in the extracellular space. These functions include (1) passive removal of potassium via spatial buffering [1–4], (2) active uptake of potassium via an ($Na^+ + K^+$)-ATPase [5–8] and (3) passive co-transport of potassium which chloride [9,10]. Controversy exists in the literature on the exact nature of the co-transporter in glial cells. An $Na^+/K^+/Cl^-$ co-transport has been demonstrated in the C6 BU-1 glioma cell line [11]; however, a K^+/Cl^- co-transport was found in LRM 55 rat glioma cells [12]. Other investigators, working with primary astrocyte cultures, concluded that the furosemide-sensitive K^+ accumulation is mediated by a K^+/Cl^- carrier in mouse [10], a K^+/Cl^- carrier with some suggestion of Na^+ involvement in rat [9] or a K^+/Cl^- carrier coupled to the Na^+-K^+ pump by a transmembrane sodium cycle in mouse [13]. In view of these data, it was considered necessary to investigate the nature of the co-transporter in more detail. For this study we used primary cultures of rat astrocytes since the permanent glioma cell lines might express properties not present in the parent cell line. The experimental data obtained in this study are in agreement with the presence of an $Na^+/K^+/Cl^-$ co-transporter in these cells.

Abbreviations: SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; DMEM, Dulbecco's modified minimal essential medium.

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Materials and Methods

Rubidium-86 was obtained from New England Nuclear. Bumetanide was a gift from Leo Pharmaceuticals (Ballerup, Denmark). Furosemide, SITS and amiloride were obtained from Sigma (St.

Louis, U.S.A.) and ouabain from Serva (Heidelberg, F.R.G.). All other chemicals were from Merck (Darmstadt F.R.G.). Cell culture petri dishes were from Nunc (Roskilde, Denmark).

Primary rat glial cultures were established as previously described [14]. 1-day-old Lewis rat pups were anesthesized with diethyl ether, washed with a surgical desinfectant, then decapitated. Brains were aseptically removed and brainstems, cerebella and meninges were carefully dissected from cerebral hemispheres and discarded. The pooled cerebral hemispheres were minxed with scissors in DMEM without serum and repeatedly aspirated through a Pasteur pipet to dissociate cells mechanically. Dissociated cells in DMEM (without serum) were centrifuged for 10 min at 1000 rpm and were suspended in 40 ml DMEM per hemisphere (the DMEM medium was without antibiotics and contained 0.6% dextrose and 15% non-heat-inactivated fetal bovine serum (Gibco)). The suspended cells were sieved through a 130 μm and then a 33 μm polyester screen without vacuum and plated onto 3 cm petri dishes (2 ml/well). Cultures were incubated at 37°C with 10% CO_2 at maximum humidity, fed 4 days after plating and then every other day thereafter. The cells were used between 2 and 3 weeks after plating.

Monolayers of primary astrocytes in 3 cm petri dishes were washed twice with DMEM (without serum) or with a medium as specified in the text. After a preincubation of 15 min at 37°C, the medium was replaced by identical medium containing 1 μCi/ml $^{86}Rb^+$ with or without inhibitor as specified in the text. The cells were allowed to take up the $^{86}Rb^+$ for 15 min, and were then quickly rinsed three times with ice-cold phosphate-buffered saline. After the last wash the cells were scraped off in 1 ml 5%trichloroacetic acid. After a low-speed centrifugation the radioactivity in the supernatant was measured by its Cerenkov radiation in a Beckman liquid scintillation counter. The sediment was dissolved in 1 M NaOH and the protein content was determined by the method of Lowry [15].

Results and Discussion

Primary cultures of rat astrocytes were obtained from neonatal Lewis rats and were virtually free of contaminating neurons and oligodendrocytes as demonstrated by immunological glial fibrillary acidic protein staining [16] of the fixed cultures (not shown).

The uptake of potassium into these astrocytes was studied with $^{86}Rb^+$ as substitute for K^+, since it has been shown that the two ions behave indistinguishably with respect to transport via the co-transport system [17]. The kinetics of the uptake of $^{86}Rb^+$ in the presence and absence of inhibitors are shown in Fig. 1A. The uptake into astrocytes consisted of three components: (1) a 1 mM ouabain-sensitive component which represents the $(Na^+ + K^+)$-ATPase; (2) a furosemide/bumetanide-sensitive component which represents the co-transporter; and (3) a component which was insensitive to ouabain and furosemide and which probably represents passive K^+ channels [10]. Fig. 1B shows the uptake by the three different pathways after 20 min of incubation. The furosemide-sensitive component of the $^{86}Rb^+$ uptake is severely reduced in the presence of 1 mM ouabain. If we assume that the driving force for the co-transporter is provided by the sodium gradient, which in turn is maintained by the $(Na^+ + K^+)$-ATPase [18--20], it seems likely that in the presence of ouabain the sodium gradient becomes less steep, resulting in a decreased driving force for the co-transporter. A similar observation was made with the $Na^+/K^+/Cl^-$ co-transporter in the seawater eel intestine [21]. Most $Na^+/K^+/Cl^-$ co-transporters, however, appear insensitive to the presence of ouabain [11,22,23]. The initial uptake rates of $^{86}Rb^+$ for our rat astrocytes varied from 35 to 55 nmol/min per mg protein, which are slightly higher as the 35 (Ref. 24) and 26 (Ref. 25) nmol K^+/min per mg protein found by others with astrocyte cultures of rat. The uptake rates, however, are about 50-fold lower than the uptake rats reported for mouse astrocytes [26]. These data suggest that species differences exist with respect to unidirectional K^+ fluxes.

In order to investigate the ionic requirements of the co-transporter we studied the furosemide sensitive component in two different experiments: (1) a medium in which all Na^+ ions were replaced by cholinium ions and (2) in a medium in which all Cl^- ions were replaced by gluconate. Fig. 2 clearly shows that in an Na^+-free as well as in a Cl^--free

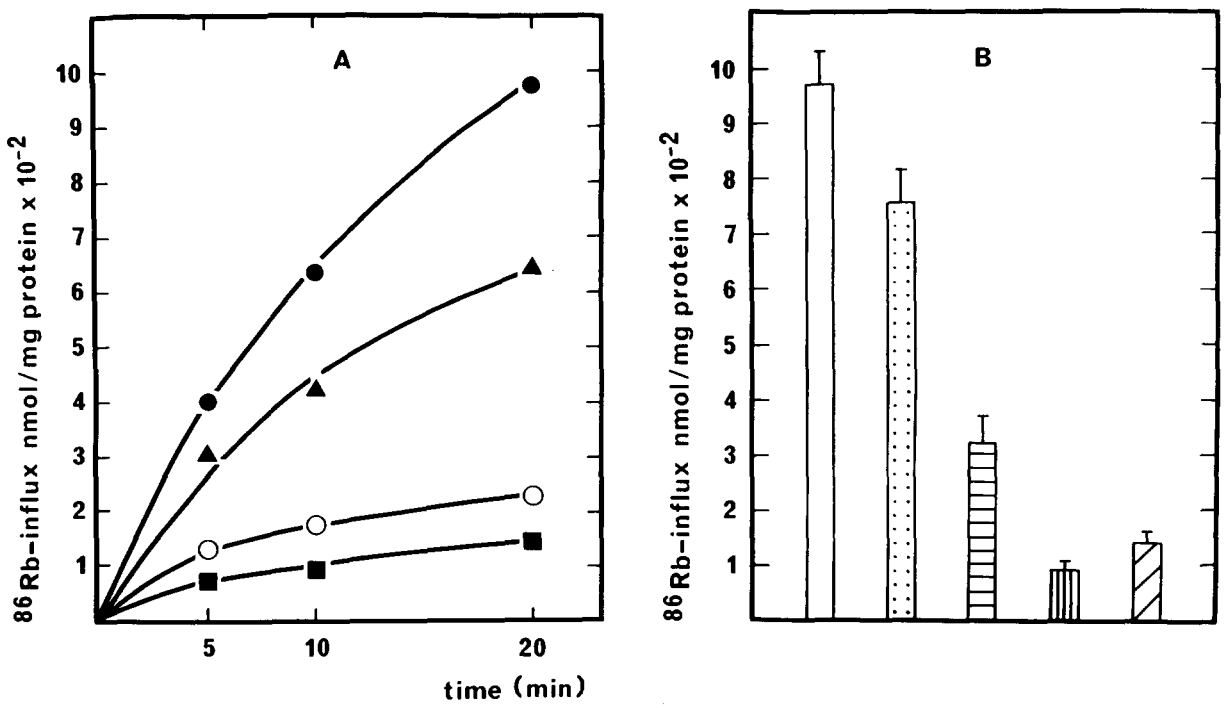

Fig. 1. (A) Kinetics of $^{86}Rb^+$ uptake into primary cultures of astrocytes in the presence and absence of inhibitors. ●, control; ▲, +2 mM furosemide; ○, +1 mM ouabain; ■, 1 mM ouabain+2 mM furosemide. Primary astrocytes in 3 cm petri dishes were preincubated in serum-free DMEM for 15 min. The incubation was started by replacing the medium with an identical medium containing 1 μCi $^{86}Rb^+$. (B) Comparison of the different transport activities over a period of 20 min (extrapolated from (A). □, total $^{86}Rb^+$ uptake; ▨, 1 mM ouabain-sensitive uptake; ▤, 2 mM furosemide-sensitive uptake in the absence of ouabain; ▥, 2 mM furosemide-sensitive uptake in the presence of ouabain; ▨, ouabain- and furosemide-insensitive $^{86}Rb^+$ uptake. Data are mean ± S.D. of duplicate determinations of a representative experiment.

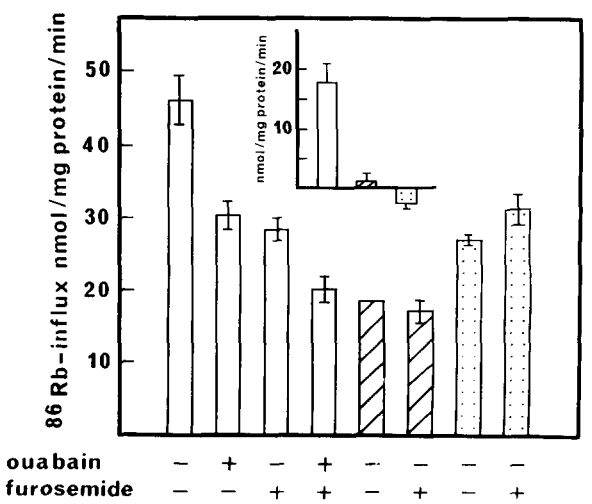

Fig. 2. Effect of sodium- and chloride-free medium on the $^{86}Rb^+$ influx in primary cultures of astrocytes. □, control medium; ▨, sodium-free medium; ▨, chloride-free medium. Control medium consisted of 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.4 mM magnesium sulfate, 25 mM glucose and 25 mM Hepes-Tris (pH 7.3). Sodium-free medium was identical to control medium except that NaCl was replaced by choline chloride. Chloride-free medium consisted of 150 mM sodium gluconate, 5 mM potassium gluconate, 2 mM calcium acetate, 0.4 mM magnesium sulfate, 25 mM glucose and 25 mM Hepes-Tris (pH 7.3). Preincubation with the different media was for 10 min followed by an incubation of 7 min in the same medium containing 1 μCi $^{86}Rb^+$, in the presence or absence of 1 mM furosemide. Data are means ± S.D. of triplicate determinations. Insets shows the furosemide-sensitive component in control medium (□), sodium-free medium (▨) and chloride-free medium (▨). Data are means ± S.D. of triplicate determinations.

medium the furosemide-sensitive component is virtually abolished, indicating that both Na^+ and Cl^- ions are involved in the co-transport activity. The co-transporter is therefore most likely a $Na^+/K^+/Cl^-$ co-transporter. Table I shows that only Br^- can partly replace Cl^- as transported

TABLE I

EFFECT OF DIFFERENT ION SUBSTITUTIONS AND INHIBITORS ON THE FUROSEMIDE SENSITIVE $^{86}Rb^+$ INFLUX IN PRIMARY ASTROCYTES

The medium consisted of 150 mM NaCl, 5 mM KCl, 2 mM calcium acetate, 0.4 mM magnesium sulfate, 25 mM glucose and 25 mM Hepes-Tris (pH 7.3). The Cl^- ions were replaced by gluconate, bromide or acetate, respectively, in the different incubations as indicated in the table. Incubations were in the presence and absence of 1 mM furosemide in the different media containing 1 μCi ^{86}Rb/ml. Data are means ± S.D. of triplicate determinations.

Medium	Furosemide-sensitive $^{86}Rb^+$ influx (nmol/mg protein per 15 min)
NaCl/KCl (control)	263 ± 11.5
Sodium gluconate/potassium gluconate	6 ± 2.9
NaBr/KBr	169 ± 22.4
Sodium acetate/potassium acetate	0 ± 4.65
Amiloride (0.1 mM)	226 ± 9.19
SITS (1 mM)	317 ± 44

anion. The co-transporter is inactive with gluconate or acetate as substitute for Cl^-. This ionic requirement seems to be a common feature of $Na^+/K^+/Cl^-$ co-transporters in different cell systems [27–29].

The furosemide-sensitive component of the $^{86}Rb^+$ uptake is not affected by the presence of 0.1 mM amiloride, an inhibitor of the Na^+/H^+ exchange and also not by the presence of SITS, which is an inhibitor of the Cl^-/HCO_3^- antiporter (Table I). The insensitivity to amiloride and SITS is also considered as a key feature of $Na^+/K^+/Cl^-$ co-transport [18,23,30].

The sensitivity of the co-transporter for the loop diuretics furosemide and bumetanide is shown in Fig. 3. Bumetanide (IC_{50} = 550 nM at 150 mM Cl^-) is a much stronger inhibitor of $Na^+/K^+/Cl^-$ co-transport than furosemide is (IC_{50} = 15 μM at 150 mM Cl^-), which is in agreement with published data of different cell species [11,22,29,31].

The dose-response curve for Na^+, K^+ and Cl^- activation of the co-transporter is shown in Fig. 4. To obtain the data points in these curves, experiments were performed in the absence of ouabain, since the compound severely reduces the furosemide-sensitive $^6Rb^+$ uptake (Fig. 1), which will make it extremely difficult to obtain meaningful

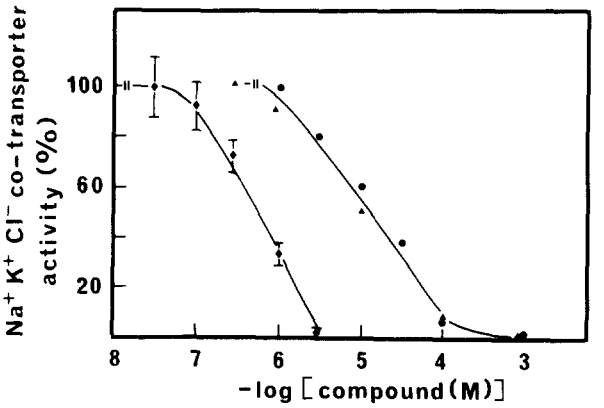

Fig. 3. Effect of bumetanide and furosemide on the activity of the $Na^+/K^+/Cl^-$ co-transporter in primary cultures of astrocytes. Preincubation for 15 min in medium without inhibitors was followed by an incubation of 15 min in medium containing the indicated amount of inhibitor (◆, bumetanide; ●, ▲, furosemide) and 1 μCi/ml $^{86}Rb^+$. 100% co-transport activity was taken as the difference between the $^{86}Rb^+$ influx in the absence and presence of 1 mM furosemide. The co-transport activity in the presence of different amount of furosemide and bumetanide was expressed as a percentage of this maximum activity. The bumetanide experiment was performed in duplicate; the furosemide experiment comprised single determinations of two different experiments.

data in the presence of this compound. Short incubation times (4 min) were used in order to obtain initial uptake rates. The data in Fig. 4 show that the activity of the co-transporter depends in a concentration-dependent manner on the presence of all three ions, Na^+, K^+ and Cl^-. Of interest is the fact that the co-transporter still increases in activity above 5 mM K^+, which suggests that this transport activity could play a role in removal of potassium from regions of increased K^+ concentration. The data in Fig. 4 were used to construct Hill plots for Na^+, K^+ and Cl^- (Fig. 5). From the slopes of the curves Hill coefficients of 1.06 for sodium, 0.99 for potassium and 1.90 for chloride were determined, indicating an electroneutral co-transport of one sodium, one potassium and two chloride ions. A $K_{0.5}$ of 35 mM for sodium, 2.7 mM for potassium and 40 mM for chloride were also deduced from the Hill plot. A low $K_{0.5}$ value for K^+ activation distinguishes $Na^+/K^+/Cl^-$ co-transport from other forms of co-transport [32]. The data are further in good agreement with those found for the $Na^+/K^+/Cl^-$ co-transporter in the C6 BU-1 glioma line [11] and

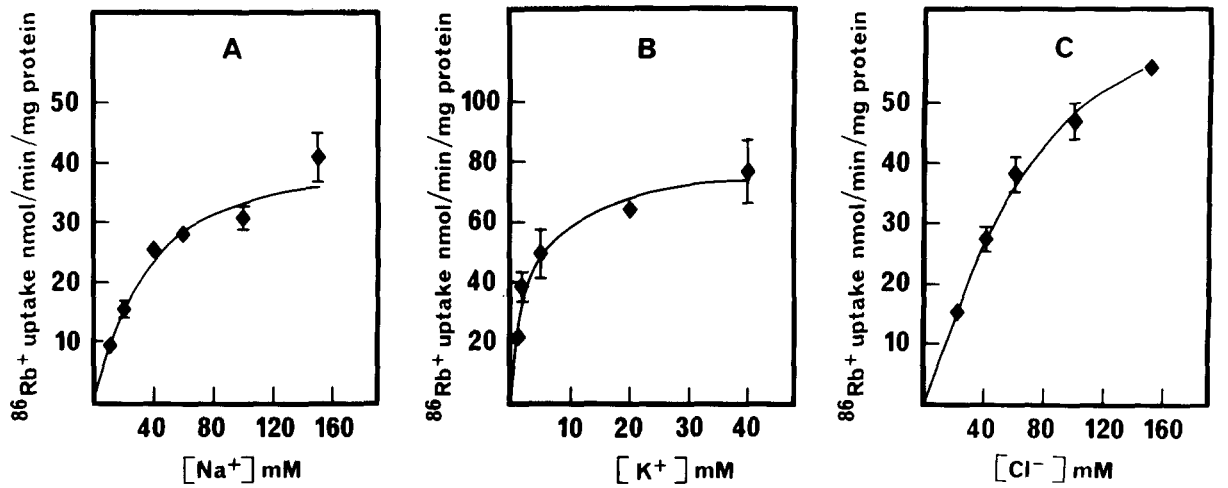

Fig. 4. Dependence of the activity of the $Na^+/K^+/Cl^-$ co-transporter on the extracellular Na^+ (A), K^+ (B) and Cl^- (C) concentrations. The medium consisted of 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.4 mM magnesium sulfate, 25 mM glucose and 25 mM Hepes-Tris (pH 7.3). Na^+ was replaced by choline in (A), K^+ was replaced by Na^+ at concentrations lower than 5 mM and added as potassium gluconate at concentrations above 5 mM in (B) and Cl^- was replaced by gluconate in (C). Preincubation was for 10 min in media without Na^+ (A), with 1 mM K^+ (B) and without Cl^- (C). The incubation was started by the addition of media containing different amounts of Na^+, K^+ or Cl^- and 1 μCi ^{86}Rb/ml. Incubation was for 4 min at 36 °C. Co-transport activity was measured as the 10 μM bumetanide-sensitive part of the total $^{86}Rb^+$ uptake. Data are means ± S.D. of duplicate determinations.

in rat glioma C6 cells (unpublished data). Co-transport activity in astrocytes remained virtually unchanged when 200–300 mM sorbitol was added to the incubation medium. The $Na^+/K^+/Cl^-$ co-transporter in astrocytes differs in this respect from the $Na^+/K^+/Cl^-$ co-transporter in avian

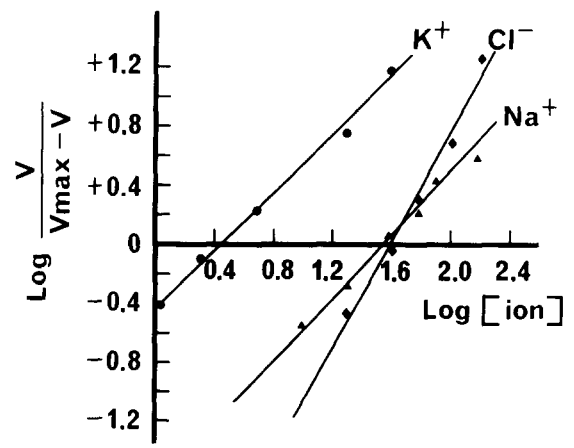

Fig. 5. Stoichiometry of the $Na^+/K^+/Cl^-$ co-transport in primary cultures of rat astrocytes. The data presented in Fig. 4 were used to construct Hill plots for Na^+, K^+ and Cl^-. The V_{max} values for Na^+ and K^+ activated co-transport, respectively, were determined using Lineweaver-Burk plots of the same data. The V_{max} for the Cl^- activated co-transport was determined using a plot of $1/v$ against $1/[Cl^-]^2$ [28,39]. The Hill coefficient for sodium is 1.06, for potassium 0.99 and for chloride 1.90. The intersection of the curves with the x-axis gives the $K_{0.5}$ values for activation of co-transport by the different ions.

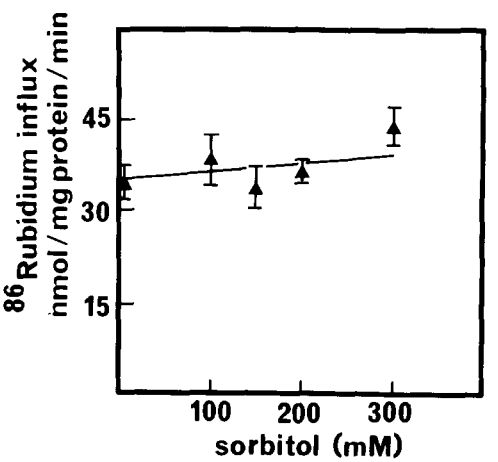

Fig. 6. Effect of hyperosmolar medium on the activity of the $Na^+/K^+/Cl^-$ co-transporter in primary cultures of rat astrocytes. Co-transporter activity was measured as the 10 μM bumetanide sensitive component of the total $^{86}Rb^+$ uptake. The normal osmolarity of the medium (300 mosM) was increased by the addition of sorbitol. Preincubation for 10 min in 300 mosM medium was followed by an incubation of 4 min in hyperosmolar medium. Data are means ± S.D. of quadruplicate determinations.

erythrocytes [33,34] and cardiac cells [22], where hypertonicity stimulates co-transport activity. In these latter cells osmotic shrinkage of the cells in hypertonic medium activates the co-transporter. Since the co-transported Na^+ is replaced by K^+ through the Na^+/K^+ pump, the overall result is a net accumulation of KCl with concomitant water uptake into the cell (regulatory volume increase). In this respect it is interesting that rat astrocytes do not show a regulatory volume increase upon incubation in hypertonic media [9,35] and also do not show any significant increase in co-transport activity under these conditions (Fig. 6). The presented data provide evidence for a $Na^+/K^+/Cl^-$ co-transport activity in primary cultures of rat astrocytes. Due to its low $K_{0.5}$ for co-transport activation, this co-transporter seems optimally suited to play a role in the maintenance of potassium homeostasis. Its exact role in the maintenance of potassium homeostasis and/or volume regulation, however, remains to be established. It has been shown in C6 BU-1 glioma cells [11], that an increase in potassium activates $Na^+/K^+/Cl^-$ co-transport with concomitant water uptake into the cells. It seems likely that this mechanism is involved in the glial swelling that has been documented both in brains slices and primary astrocyte cultures after exposure to increased K^+ concentrations [10,36–38].

Acknowledgements

We thank Mrs. Ruth Loeber, Ms. Birgit Schmitt and Ms. Ines Tschertner for skillful assistance, Ms. Helga Sennefelder for drawing the figures and Mrs. Christel Stoppe for typing. This project was supported by a grant from the Wilhelm-Sander-Stiftung, Neustadt/Donau, F.R.G. (Grant Nr. 83.007.2).

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