Neurochem Res (2013) 38:472–485
DOI 10.1007/s11064-012-0938-3

ORIGINAL PAPER

Requirement of Glycogenolysis for Uptake of Increased Extracellular K^+ in Astrocytes: Potential Implications for K^+ Homeostasis and Glycogen Usage in Brain

Junnan Xu · Dan Song · Zhanxia Xue · Li Gu · Leif Hertz · Liang Peng

Received: 9 October 2012 / Revised: 9 November 2012 / Accepted: 20 November 2012 / Published online: 12 December 2012

Abstract The importance of astrocytic K^+ uptake for extracellular K^+ ($[K^+]_e$) clearance during neuronal stimulation or pathophysiological conditions is increasingly acknowledged. It occurs by preferential stimulation of the astrocytic Na^+,K^+-ATPase, which has higher K_m and V_{max} values than its neuronal counterpart, at more highly increased $[K^+]_e$ with additional support of the cotransporter NKCC1. Triggered by a recent DiNuzzo et al. paper, we used administration of the glycogenolysis inhibitor DAB to primary cultures of mouse astrocytes to determine whether K^+ uptake required K^+-stimulated glycogenolysis. KCl was increased by either 5 mM (stimulating only the Na^+,K^+-ATPase) or 10 mM (stimulating both transporters) in glucose-containing saline media prepared to become isoosmotic after the addition. DAB completely inhibited both uptakes, the Na^+,K^+-ATPase-mediated by preventing Na^+ uptake for stimulation of its intracellular Na^+-activated site, and the NKCC1-mediated uptake by inhibition of depolarization- and L-channel-mediated Ca^{2+} uptake. Drugs inhibiting the signaling pathways involved in either of these processes also abolished K^+ uptake. Assuming similar in vivo characteristics, partly supported by literature data, K^+-stimulated astrocytic K^+ uptake must discontinue after normalization of extracellular K^+. This will allow Kir1.4-mediated release and reuptake by the less powerful neuronal Na^+,K^+-ATPase.

Keywords Astrocyte · Cell signaling · Glycogen · Na^+ · K^+-ATPase · Ouabain · Potassium homeostasis

Introduction

It is gradually becoming accepted that *increased* extracellular K^+ ($[K^+]_e$) in the mammalian brain to a large extent is removed by rapid accumulation into astrocytes [1–6]. This applies both following K^+ release due to neuronal excitation and during pathological conditions. Uptake after the former is primarily based on a high-capacity, low affinity Na^+, K^+-ATPase in astrocytes, versus a higher-affinity, lower capacity enzyme in neurons [7–9], whereas uptake after the latter is also based on co-activation of an additional astrocytic transporter, NKCC1 [10, 11]. However, K^+ lost from neurons must get back to neurons, and there is experimental evidence that the increase in intracellular K^+ concentration $[K^+]_i$ in astrocytes in normal brain tissue is only transient [12]. In all probability it is followed by channel-mediated K^+ release and reuptake in neurons [13]. A relevant question is therefore how the powerful astrocytic uptake mechanisms are turned off, allowing astrocytic net release followed by net uptake in neurons.

The present work is an experimental study of the suggestion by DiNuzzo et al. [14] that K^+ uptake in astrocytes in response to an *elevated* $[K^+]_e$ may require glycogenolysis, which in the mammalian brain is stimulated by even very low increases in $[K^+]_e$ [15]. Resting $[K^+]_e$ was increased by addition of either 5 or 10 mM KCl to astrocyte cultures. Ours are traditionally grown at 5.4 mM

Junnan Xu and Dan Song contributed equally to this work.

J. Xu · D. Song · Z. Xue · L. Gu · L. Hertz · L. Peng (✉)
Department of Clinical Pharmacology, China Medical University, No. 92 Beier Road, Heping District, Shenyang, People's Republic of China
e-mail: lpeng@mail.cmu.edu.cn; hkkid08@yahoo.com

Present Address:
Z. Xue
Department of Pharmacology, University of Hebei North University, Zhangjiakou, People's Republic of China

$[K^+]_e$ (change of which would invalidate all previous results), and in order to maintain 'la fixité du milieu intérieur' [16], the same concentration (5.4 mM) was used in the control cultures. The importance of homeostatic mechanisms maintaining status quo is illustrated by the above-mentioned ability of even small increases in $[K^+]_e$ to activate glycogenolysis, whereas no glycogenolysis occurs without $[K^+]_e$ change [15].

Inhibition with ouabain has shown that under the conditions employed the Na^+,K^+-ATPase is the main K^+ transporter below a total $[K^+]_e$ of ~10 mM [17]. At higher $[K^+]_e$, NKCC1 plays the dominant role, as shown by inhibition with bumetanide, an inhibitor of the cotransporter NKCC1 [10, 11]. However, the selection of 10 mM $[K^+]_e$ as boundary below which K^+ uptake occurs exclusively by Na^+,K^+-ATPase activity, whereas NKCC1 activity is *also* involved at higher concentrations, may represent a slight overestimate for in vivo conditions since 'resting' $[K^+]_e$ normally amounts to 2.6–3.8 in the mammalian CNS [18]. NKCC1 is an inwardly directed Na^+, K^+, $2Cl^-$ cotransporter [19, 20] expressed both in cultured astrocytes [21–23] and mature astrocytes in vivo [24, 25]. It is also present in developing but *not* in mature central neurons [26, 27]. NKCC1 activity is driven by secondary active transport, i.e., the steep difference in ion, especially Na^+, concentrations across the cell membrane created by the Na^+,K^+-ATPase [19, 20], but at least in brain it also requires an increased $[K^+]_e$ [28]. During pathological conditions (brain trauma, stroke) its operation leads to potentially fatal cell swelling (cytotoxic edema).

Subbarao et al. [29] and Cai et al. [30] used pathologically high $[K^+]_e$ to stimulate glycogenolysis and NKCC1. This raises the question if the NKCC1 activation by all $[K^+]_e$ values above 10 mM is activated by a similar pathway. The pathway leading towards NKCC1 activation after K^+-mediated, depolarization-induced entry of Ca^{2+} [29, 31] has been identified in cultured astrocytes by the use of specific inhibitors, although only up to phosphorylation of extracellular regulated kinases 1 and 2 ($ERK_{1/2}$) [30, 32]. Ca^{2+} enters by opening of a nifedipine-inhibited L-channel for Ca^{2+} [31, 33, 34], probably $Ca_v1.3$, which is distinctly expressed in astrocytes in vivo [35, 36] or freshly isolated from normal brain [37, 38; B. Li and L. Peng, unpublished experiments].

Astrocytic L-channel expression in astrocytes is upregulated after brain injury, perhaps as a result of increased $[K^+]_e$ [39], reaching levels activating NKCC1. The pathway activated by excess $[K^+]_e$ is a transactivation pathway involving release of an epidermal growth factor (EGF) receptor ligand [40], which is detached from its membrane precursor by a metalloproteinase. The metalloproteinase used in NKCC1 activation is ADAM17, which in astrocytes otherwise only has been identified during transactivation induced by the vasopressinergic V1 receptor [32]. Preliminary experiments using cultured astrocytes depleted for ADAM17 after treatment with siRNA showed dependence on ADAM17 in response to $[K^+]_e$ as low as 15 mM $[K^+]_e$ suggesting activation of this pathway at all relevant $[K^+]_e$ values at and above 10–15 mM [32]. However, the pathway responding to addition of 5 mM K^+ to a final $[K^+]_e$ of 10 mM was not affected in the siRNA cells [32], indicating the use of a different pathway.

Nevertheless, the increase in $[K^+]_i$ after addition of 5 mM K^+ was also abolished by DAB. It has previously been shown that astrocytic Na^+,K^+-ATPase activity depends not only on the $[K^+]_e$ level but also on simultaneous availability of intracellular Na^+ ($[Na^+]_i$). Thus, the K^+-mediated stimulation of Na^+,K^+-ATPase activity in homogenates from cultured astrocytes depends on the ambient $[Na^+]_e$ [8], $[K^+]_i$ is higher in cells where $[Na^+]_i$ is increased [22], and $[K^+]_e$-dependent Na^+,K^+-ATPase-mediated K^+ uptake in cultured astrocytes is abolished by omission of Na^+ in the medium [5]. We therefore determined the potential ability of an increased $[Na^+]_i$ following either addition of monensin, a Na^+/H^+ exchanger [41], or an increase in $[Na^+]_e$ by 10 mM to counteract the effect of DAB. Either procedure rescued the increase in $[K^+]_i$ after addition of 5 mM K^+ in the presence of DAB. The Na^+ channel Na_X is present in astrocytes, both in culture [42] and in the brain in vivo [43, 44]. It shows little or no voltage dependence [45], but it is stimulated by increased $[Na^+]_e$ and is therefore also involved in Na^+ sensing in periventricular organs [46]. The effect of increasing $[Na^+]_e$ points towards opening of this channel. Furthermore, in our cultured astrocytes active uptake of ^{42}K is greatly reduced by the Na^+ channel inhibitors procaine, lidocaine and tetracaine (M. Shokeir, W. T. Code and L. Hertz, unpublished experiments). We therefore investigated the effect of the Na_X inhibitor, amiloride, on $[K^+]_e$ increase following addition of 5 mM K^+. This drug inhibits many Na^+ channels, including Na_X [47].

Cardiotonic steroids (ouabain, endogenous ouabain-like compounds) at nanomolar concentrations, far below those inhibiting the Na^+,K^+-ATPase, initiate downstream phosphorylation events. Since the Na^+,K^+-ATPase is the only known receptor for cardiotonic steroids, these observations led to the conclusion that the Na^+,K^+-ATPase functions not only as an ion pump but also as a signal transducer, mediating the signaling effect of the ouabains [48, 49]. The ouabain-initiated pathway involves many of the same factors as the pathway activating NKCC1, including $ERK_{1/2}$ phosphorylation and an increase in $[Ca^{2+}]_i$. However, IP_3 receptors, which are involved in the ouabain pathway [50], do not participate in the K^+-stimulated pathway leading to NKCC1 activation [30]. The effect of addition of 5 mM K^+ was therefore studied (1) on the increase in $[K^+]_e$ when

the IP_3 receptor was inhibited, and (2) on the increase in $[Ca^{2+}]_i$ with and without DAB.

Combined, these studies will test the hypothesis that uptake of elevated $[K^+]_e$ requires glycogenolysis, as suggested by DiNuzzo et al. [14]. They will allow determination of the effect of glycogenolysis inhibition on K^+ uptake in astrocytes by an increase in $[K^+]_e$ of either 5 mM, stimulating only Na^+,K^+-ATPase activity, or of 10 mM, stimulating also the operation of NKCC1. They will distinguish between the signal pathways operating in astrocytes in each of the two situations, and they will show whether each of these K^+-activated pathways require glycogenolysis, induced by the exposure to increased $[K^+]_e$, in order for intense K^+ accumulation to occur. If that is the case, K^+ uptake in astrocytes in the brain in vivo (provided they operate in a similar fashion) will only accumulate K^+ as long as $[K^+]_e$ is increased, facilitating subsequent and essential re-accumulation by neurons.

Materials and Methods

Reagents

Chemicals for preparation of medium and most other chemicals, including DAB (1,4-dideoxy-1,4-imino-D-arabinitol hydrochloride), Xestospongin C (1R-(1R,4aR,11R,12aS,13S,16aS,23R,24aS))-eicosahydro-5H,17H-1,23:11,13-diethano-2H,14H-[1,11]dioxacycloeicosino[2,3-b:12,13-b1]dipyridine), Amiloride (N-Amidino-3,5-diamino-6-chloropyrazinecarboxamide hydrochloride hydrate) and Monensin sodium salt were purchased from Sigma (St. Louis, MO, USA). Tyrphostin AG 1478 (N-[(2R)-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl]-L-tryptophan methylamide), GM 6001 (1,4-diamino-2,3-dicyano-1, 4-bis [2-aminophenylthio]butadiene), U0126 (1,4-diamino-2, 3-dicyano-1,4-bis[2-aminophenylthio]butadiene) and PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) and BAPTA-AM ((bis-(o-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (acetoxymethyl)-ester),were obtained from Calbiochem (La Jolla, CA, USA). P-1267 PBFI-AM (124549-23-1/1,3-Benzenedicarboxylic acid, 4,4′-[1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-7,16-diylbis(5-methoxy-6,2-benzofurandiyl)]bis-, tetrakis[(acetyloxy)methyl] ester) and Pluronic F-127 (polyethylene oxide (PEO)–polypropylene oxide (PPO)–polyethylene oxide block copolymer) for fluorescence determination of ($[K^+]_i$), fura-2 AM (bis(2-((acetylxoy) methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acid (acetyloxy)methyl ester) for intracellular calcium concentration ($[Ca^{2+}]_i$), Opti-MEMI and Oligofectamine were purchased from Invitrogen (Carlsbad, CA, USA). Santa Cruz Biotechnology (Santa Cruz, CA, USA) supplied the first antibodies, raised against ERK (K-23):sc-94 and against phosphorylated ERK (E-4):sc-7383, and the secondary antibody goat anti-rabbit IgG HRP conjugate. The secondary antibody Goat-anti-mouse IgG HRP conjugate was purchased from Promega (Madison, WI, USA).

Cell Culture

Primary cultures of astrocytes were prepared from the neopallia of the cerebral hemispheres as previously described [51, 52] with minor modifications [53] and grown in Dulbecco's Minimum Essential Medium (DMEM) with 7.5 mM glucose (to allow some decline between feedings) and the traditional 5.4 mM K^+. After the age of 2 weeks, 0.25 mM dibutyryl cyclic AMP (dBcAMP) was included in the medium. Such cultures are highly enriched in astrocytes (>95 purity of glial fibrillary protein- (GFAP-) and glutamine synthetase-expressing astrocytes [54]. The addition of dBcAMP at this specific stage of culturing is a crucial component of our culture preparation. It leads to a morphological and functional differentiation, as evidenced by the extension of cell processes, increases in several metabolic activities and expression of voltage sensitive L-channels for calcium (Ca^{2+}) [31, 55], features which are characteristic of astrocytes in situ. Use of astrocyte cultures has been authoritatively reviewed [56], and we can add that drug-induced changes and developmental alterations in gene expression originally described in our cultured cells have recently been confirmed in freshly isolated cells from mice treated with the same drugs [57–59].

Treatment with ADAM17 siRNA

Duplex of ADAM17 siRNA (sense 5′ AAGCTTGATTC TTTGCTCTCA 3′, and antisense 5′ AATGAGAGCAAA-GAATCAAGC 3′) [60] were synthesized by Sangon Co., Ltd. (Shanghai, China). To allow incorporation of siRNAs into astrocytes, 3-week-old astrocytes cultured in Primaria 24-well culture plates were incubated in Dulbecco's medium without serum for 24 h on the day before transfection. Transfection solution contained 2 mL Oligofectamine, 40 mL Opti-MEMI, and 2.5 mL siRNA (666 ng) and was added to the culture for 8 h. In siRNA(−) control cultures, transfection solution without siRNA was added. Thereafter, 87.5 mL DMEM with 37.5 mL serum was added to the cultures. The expression of mRNA of ADAM17 was measured by RT-PCR 3 days after transfection. It was greatly reduced and ADAM17 function was abolished [30].

Determination of $[K^+]_i$

For determination of $[K^+]_i$, an Olympus IX71 live cell imaging fluorescence microscope (Tokyo, Japan) was used to record fluorescence intensity of benzofuran isophtalate

(PBFI-AM) [61, 62], introduced in astrocyte cultures grown on coverslips coated with polylysine. The cells were loaded with 10 μM PBFI-AM with 0.2 % Pluronic F-127 [63] in saline solution (NaCl 137 mM; KCl 5.4 mM; KH_2PO_4 0.44 mM; $NaHCO_3$ 4 mM; $CaCl_2$ 1.3 mM; $MgSO_4$ 0.8 mM; $MgCl_2$ 5 mM and glucose 10 mM, pH 7.4) for 30 min at 37 °C. After 2 times wash with similar saline, the coverslip was incubated in 180 μL of the saline solution, and at a distance from the selected cells an additional 20 μL was carefully added of either similar saline (control) or a saline in which the KCl concentration had been increased by 50 mM or 100 mM, with a corresponding reduction of NaCl concentration. This addition iso-osmotically increases the final $[K^+]_e$ by 5, respectively 10 mM. Readings were performed at 340 nm and 380 excitation and 500 nm emission at 20 s intervals. Between thirteen and twenty cells were selected in each coverslip, and with a few exceptions (with only two coverslips) three-five coverslips were used in each experimental group. Changes in $[K^+]_i$ were indicated as changes in excitation ratios after (1) subtraction of the fluorescence ratio immediately before the treatment from that of each reading after the K^+ addition, and (2) correcting for potential differences in excitation ratio at the start between control and experimental cultures by moving all graphs to a start point of 1. The procedure is shown in a sample in Fig. 1 and its legend, with Fig. 1a showing readings of two cultures as actual excitation ratios, and Fig. 1b the same results starting from 1 (the procedure used in all remaining results). The graphs thus indicate the *increase* (or potential decrease) in $[K^+]_i$ during the experiments, not total $[K^+]$. Since the elevated $[K^+]_i$ rapidly declined (probably due to a strong outward driving force at a maintained elevated $[K^+]_e$), only the values of first two time points after the treatment were used for statistical analysis.

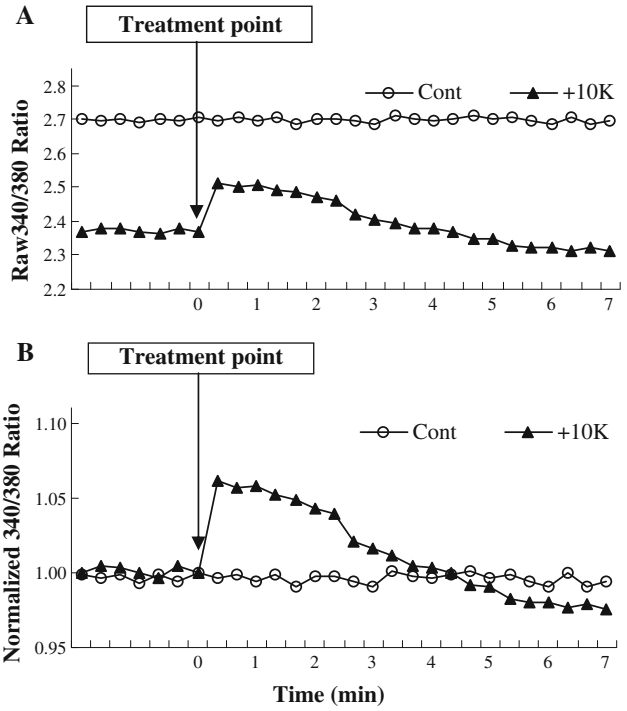


Fig. 1 Measurement of the effect of addition of 10 mM $[K^+]_e$ on $[K^+]_i$ in astrocytes using determination of fluorescence ratios following excitation at either 340 or 380 nm. **a** After loading with PBFI-AM for 30 min, cells were washed and incubated in saline solution for 2 min. Results from two independently run coverslips are shown. In one coverslip the cells were then stimulated by addition of 10 mM K^+, as described in "Methods" section, and in the second no K^+ addition was made. Each representative trace shows changes in 340/380 nm fluorescence ratios $[K^+]_i$ in ~20 cells, recorded simultaneously in each of 20 s during a 10 min experimental period. In order to convert the 340/380 ratios to measurements of induced changes in $[K^+]_i$, the average fluorescence ratio during the period before the addition of K^+ was assigned as value of 1, and was subtracted from each of the 340/380 values after K^+ addition. Thus, only alterations in $[K^+]_i$ after the addition of K^+ were determined and expressed as changes in 340/380 ratios. Moreover, a correction was made for potential differences in excitation ratio at the start between control and experimental cultures (as shown in **a**) by moving all graphs to a start point of 1. The results of these procedures are shown in **b**, which presents the results as changes in $[K^+]_i$ from the time K^+ was added (after 2 min) to some of the cultures (the procedure used in all remaining results). This time is in all remaining graphs indicated as zero time. The graphs accordingly indicate the increases (or potential decreases) in $[K^+]_i$ during the 7-min experiments, not total $[K^+]_i$

$ERK_{1/2}$ Phosphorylation

After a 20 min incubation in culturing medium under drug-free control conditions, in the presence of different concentrations of $[K^+]_e$, and/or specific inhibitors, the reaction was stopped by washing with ice-cold phosphate-buffered saline containing 7.5 mM glucose, and the cells were scraped off the dishes and harvested in 0.5 ml of ice-cold buffer (0.25 M sucrose, 10 mM HEPES, the phosphatase inhibitors alpha-mercaptoethanol [10 mM] and phenylmethyl sulfonyl fluoride [1 mM] and 1 mM sodium orthovanadate, pH 7.4) and homogenized to make a whole cell lysate. The protein content was determined in the homogenates by the Bradford method [64], using bovine serum albumin (BSA) as the standard. Samples containing 50 μg protein were applied on slab gels of 10 % polyacrylamide and electrophoresed. After transfer to nitrocellulose membranes, the samples were blocked by 5 % skimmed milk powder in TBS-T (30 mM Tris–HCl, 125 mM NaCl, 0.1 % Tween 20) for 2 h and transferred to nitrocellulose membranes. The nitrocellulose membranes were incubated with the first antibody, specific to either phosphorylated $ERK_{1/2}$ (p-$ERK_{1/2}$) or $ERK_{1/2}$ at 1 × 1,000 dilution for 1.5 h at room temperature. After washing, specific binding was detected by goat-anti-mouse (p-ERK) or goat-anti-rabbit (ERK) horseradish peroxidase-

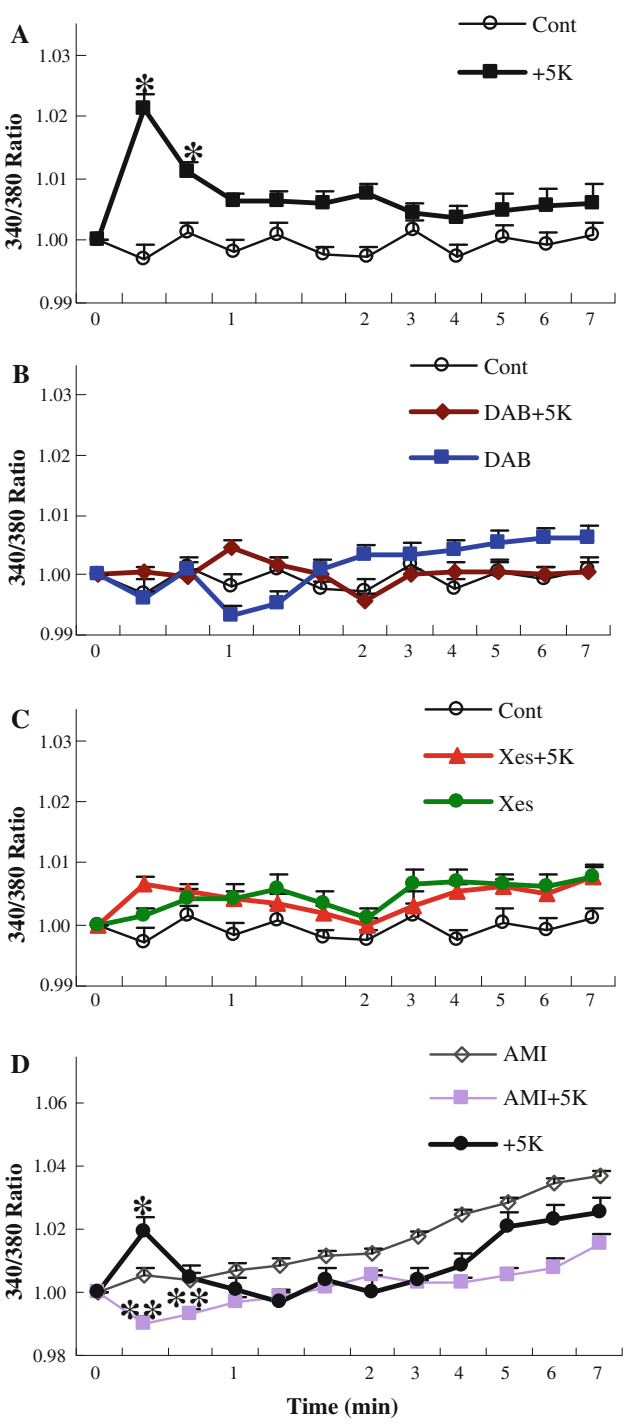


Fig. 2 K^+ uptake into astrocytes (measured as increase in $[K^+]_e$ as described in Fig. 1), after addition of 5 mM $[K^+]_e$ requires glycogenolysis. **a** After incubation of PBFI-AM-loaded cells in saline solution for 2 min and subsequent wash, the cells were from zero time incubated either in similar solution or in a solution to which an additional 5 mM KCl had been added as described in "Methods" section. **b** In some experiments, 10 mM DAB, an inhibitor of glycogenolysis, **c** 500 μM xestospongine, an inhibitor of IP_3 receptors, or **d** 200 μM amiloride, an inhibitor of Na^+ channels had been added at the time the measurement of fluorescence ratios began (2 min before K^+ was added to some cultures). With the exception of **d**, which for each group shows averages from 40 cells on two coverslips, results are averages from 42 to 77 cells on three-five individual coverslips. SEM values are indicated by *vertical bars*. *Statistically significant ($p < 0.05$) difference from other groups at the same time period. **Statistically significant ($p < 0.05$) difference from control group at the same time period (**d**)

conjugated secondary antibody at 1 × 1,000 dilution. Staining was visualized by aid of an enhanced chemoluminescence (ECL) reagent and ratios between p-ERK and ERK calculated.

Determination of $[Ca^{2+}]_i$

Similar equipment and kind of cultures were used as for determination of $[K^+]_i$, but fluorescence intensity was measured after incubation with fura-2 AM, and Pluronic F-127 was omitted. After the loading period, the coverslips were washed twice with similar saline and then incubated in the saline solution (control). Readings were made at 340 nm and 380 nm excitation and 510 nm emission at 20 s intervals, and the incubation continued for 8 min. In some samples, 5 mM K^+ and or DAB were added to the solution at time zero.

Statistics

The statistical values of the differences between individual groups were analyzed by one-way ANOVA followed by Fisher's LSD test. The level of significance was set at $p < 0.05$.

Results

Effects of Addition of 5 or 10 mM K^+ on Increase in $[K^+]_e$

Figure 2a shows the *increase* in $[K^+]_i$, when $[K^+]_e$ was raised from a control value of 5 mM by addition of another 5 mM KCl to the medium, stimulating the Na^+,K^+-ATPase, but not NKCC1. The stability of the system under resting conditions (no KCl added) is shown as control. $[K^+]_i$ is steady under control conditions, but shows a statistically significant, although transient increase after addition of 5 mM K^+. In spite of the presence of 10 mM glucose in all media the increase is virtually abolished after addition of DAB, which does not affect the control (Fig. 2b). Inhibition of the IP_3 receptor by xestospongin (Fig. 2c) had a similar effect. Addition of amiloride, blocking Na_X, acted differently. At a normal level of $[K^+]_e$ it caused an apparent increase in $[K^+]_e$, which progressed throughout the experiment. However, when $[K^+]_e$ was raised by 5 mM, there was statistically significant dip, followed by a gradual increase,

paralleling that without K^+ addition (Fig. 2d), and suggesting inhibition of the K^+ effect. The peculiar apparent increase in $[K^+]_e$ during continued incubation may have been be caused by K^+-independent effects of the inhibitor on fluorescence intensity of benzofuran isophtalate.

The importance of $[Na^+]_i$ was more directly shown by adding 0.5 μM of the Na^+/H^+ exchanger monensin. As it can be seen from Fig. 3a, addition of this concentration of monensin alone to control cultures had no effect, indicating that the evoked increase in $[Na^+]_i$ did not suffice to stimulate Na^+,K^+-ATPase activity. However, the response to addition of 5 mM K^+ in the presence of DAB was partly restored to its normal value, and with 1.0 μM monensin the recovery was complete (118.5 % ± 3.5 of response to 5 mM K^+ alone). Addition of 10 mM Na-pyruvate (Fig. 3b) or NaCl (Fig. 3c) had a similar rescue effect, probably by increased Na_X activity due to the higher $[Na^+]_e$, whereas it had no effect in the absence of DAB (not shown). Thus, Na^+,K^+-ATPase activity can be supported by glucose metabolism alone, provided sufficient $[Na^+]_i$ is secured.

Similar responses are shown in Fig. 4 after addition of 10 mM K^+. The control response was 2–3 times larger (Fig. 4a). This reflects the ability of NKCC1, which is now involved, to increase the effect of the Na^+,K^+-ATPase by taking advantage of the ion gradients across the membrane. However, as shown in Fig. 4b the inhibitory effect of DAB is the same as in Fig. 2b. Addition of monensin (Fig. 4c) or 10 mM Na-pyruvate partly rescued the response, but only up to the level of $[K^+]_i$ normally caused by addition of 5 mM K^+ (Fig. 4d). Thus, only the Na^+,K^+-ATPase response was rescued, whereas Na^+ supplementation does not restore any of the additional increase in K^+ uptake due to NKCC1 activity.

Effects on $ERK_{1/2}$ Phosphorylation by Addition of 5 or 10 mM K^+ and/or Inhibitors

Addition of ouabain (30 nM) increases $ERK_{1/2}$ phosphorylation, as shown in a representative Western plot in Fig. 5a. Scanning and averaging of three plots showed the effect to be statistically significant. It was inhibited by AG1478, the inhibitor of the EGF receptor, but not by GM6001, the metalloproteinase inhibitor. Addition of either 5 or 10 mM K^+ also caused an increase in $ERK_{1/2}$ phosphorylation. The increased $ERK_{1/2}$ phosphorylation by addition of 10 mM K^+ was abolished in astrocytes treated with siRNA against ADAM17 and must therefore occur via the pathway leading to NKCC1 activation, whereas that by 5 mM K^+ was not inhibited in the siRNA-treated cells (Fig. 5b), and accordingly probably activates the same pathway as ouabain. That addition of 5 mM K^+ stimulates this pathway and that the stimulation may be partly

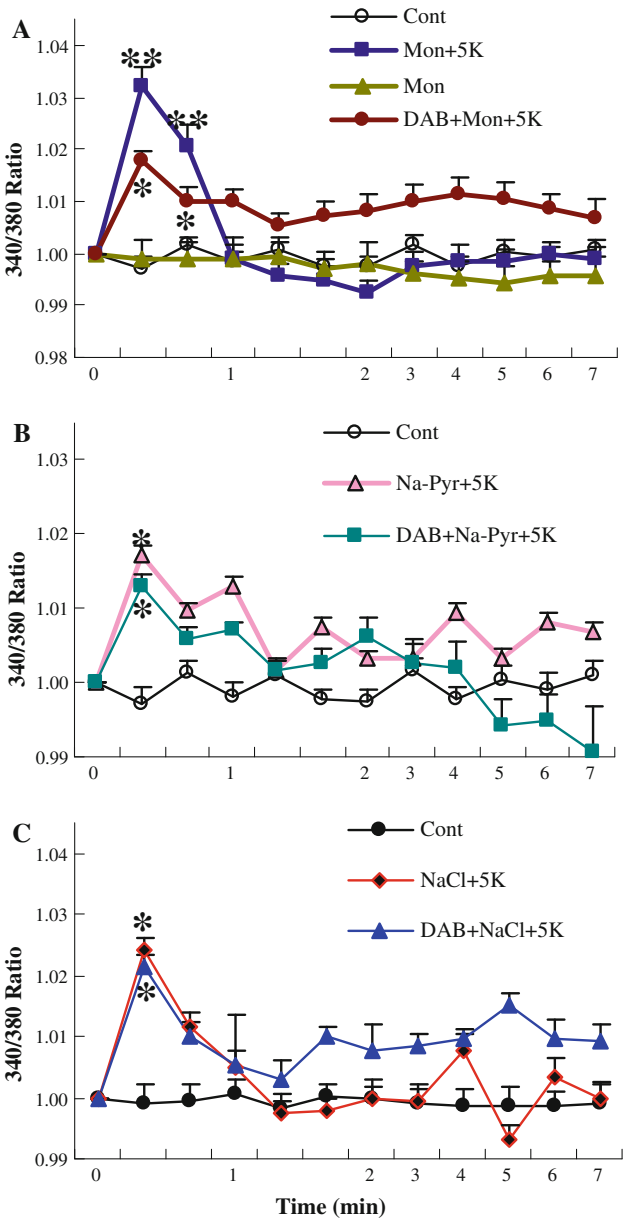
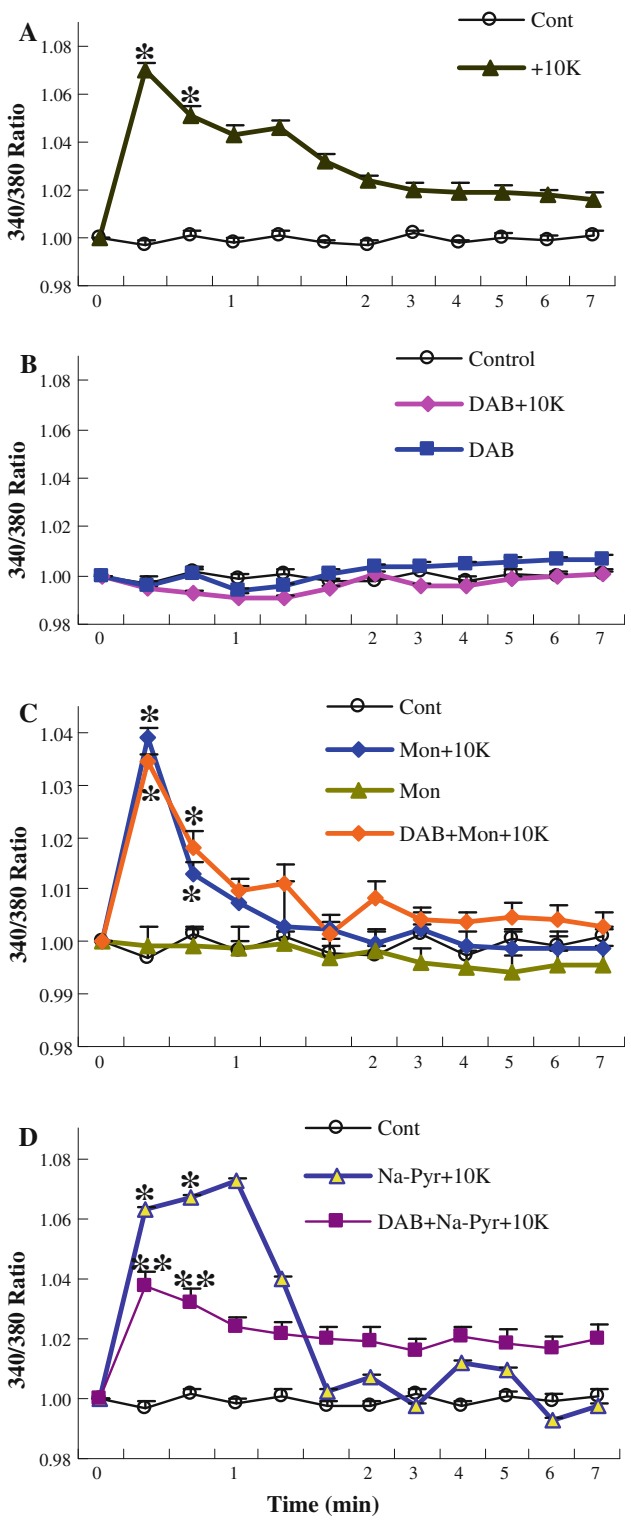


Fig. 3 Glycogenolysis is not required for K^+ uptake into astrocytes after addition of 5 mM $[K^+]_e$, when intracellular Na^+ has been increased. **a** After incubation of PBFI-AM-loaded cells in saline solution for 2 min and subsequent wash, the cells were from zero time incubated either in similar solution or in a solution to which an additional 5 mM KCl had been added (as described in "Methods" section), with or without 10 mM DAB, and/or 0.5 μM monensin, an Na^+/H^+ exchanger which had been added at the time the measurement of fluorescence ratios began. In other experiments 10 mM Na-pyruvate (**b**) or 10 mM NaCl (**c**) was added at this time, without osmotic compensation, i.e., increasing $[Na^+]_e$ by 10 mM, but with or without DAB. With the exception of **c**, which for each group shows averages from 29 to 41 cells on two coverslips, results are averages from 51 to 77 cells on three individual coverslips. SEM values are indicated by *vertical bars*. *Statistically significant ($p < 0.05$) difference from control group at the same time period. **Statistically significant ($p < 0.05$) difference from DAB + monensin + 5 K^+ group at the same time period



◀ **Fig. 4** Glycogenolysis is required for K^+ uptake into astrocytes after addition of 10 mM $[K^+]_e$. **a** After incubation of PBFI-AM-loaded cells in saline solution for 2 min, the cells were from zero time incubated either in similar solution or in a solution to which an additional 10 mM KCl had been added (as described in "Methods" section). **b** In some experiments, addition had been made of 10 mM DAB, an inhibitor of glycogenolysis, with or without addition of 10 mM K^+, **c** or of 0.5 mM monensin, with or without 10 mM K^+ and/or DAB, **d** or 10 mM Na-pyruvate, with or without 10 mM K^+ in the presence or absence of DAB. Results are averages from 42 to 77 cells on three-five individual coverslips. SEM values are indicated by *vertical bars*. *Statistically significant ($p < 0.05$) difference from control group at the same time period. **Statistically significant ($p < 0.05$) difference from DAB + Na-pyruvate + 10 K^+ group at the same time period

although not significantly inhibited by DAB is shown in Fig. 5c. However, significant inhibition of $ERK_{1/2}$ phosphorylation in this pathway was demonstrated after addition of the IP_3 receptor blocker xestospongin C, the Src blocker PP1, and the EGF receptor blocker AG1478, which on their own had no effect (Fig. 6a–c). Some of these transcription factors also participate in the pathway leading to NKCC1 activation, but IP_3 is selectively involved in the ouabain pathway.

Effects on $[Ca^{2+}]_i$ by Addition of 5 mM K^+ and/or DAB

In order to investigate if the ouabain-mediated pathway is inhibited by blockade of glycogenolysis we tested the effect on $[Ca^{2+}]_i$ by addition of 5 mM K^+, which activates the ouabain-induced pathway only. $[Ca^{2+}]_i$ was increased by this addition, and the increase was significantly reduced by DAB, which had no effect in the absence of added K^+ (Fig. 7).

Discussion

The present results show that glycogenolysis is required for K^+-mediated stimulation of both the Na^+,K^+-ATPase and the cotransporter NKCC1 in astrocytes. This is graphically illustrated in Fig. 8, which also shows that this inhibition occurs because the *signaling pathways* regulating the responses by the Na^+,K^+-ATPase to addition of 5 mM K^+ and of NKCC1 to addition of 10 mM K^+, both depend on glycogenolysis. Either of them may need glycogenolytically-derived energy for the multitude of phosphorylations, Ca^{2+} transport processes and other energy-requiring processes taking part during signaling. The ouabain signaling elicited by addition of 5 mM K^+ is necessary for cellular uptake of Na^+ to stimulate the Na^+,K^+-ATPase at its intracellular Na^+-stimulated site. Operation of the Ca^{2+} entry-mediated signaling initiated by L-channel opening after depolarization by addition of 10 mM K^+, which leads to NKCC1 activation, also requires K^+-stimulated glycogenolysis. That astrocytic pathways for utilization of glucose and of glycogen are segregated has been shown previously [65–67]. However, the requirement of glycogen to provide metabolic support for signaling processes

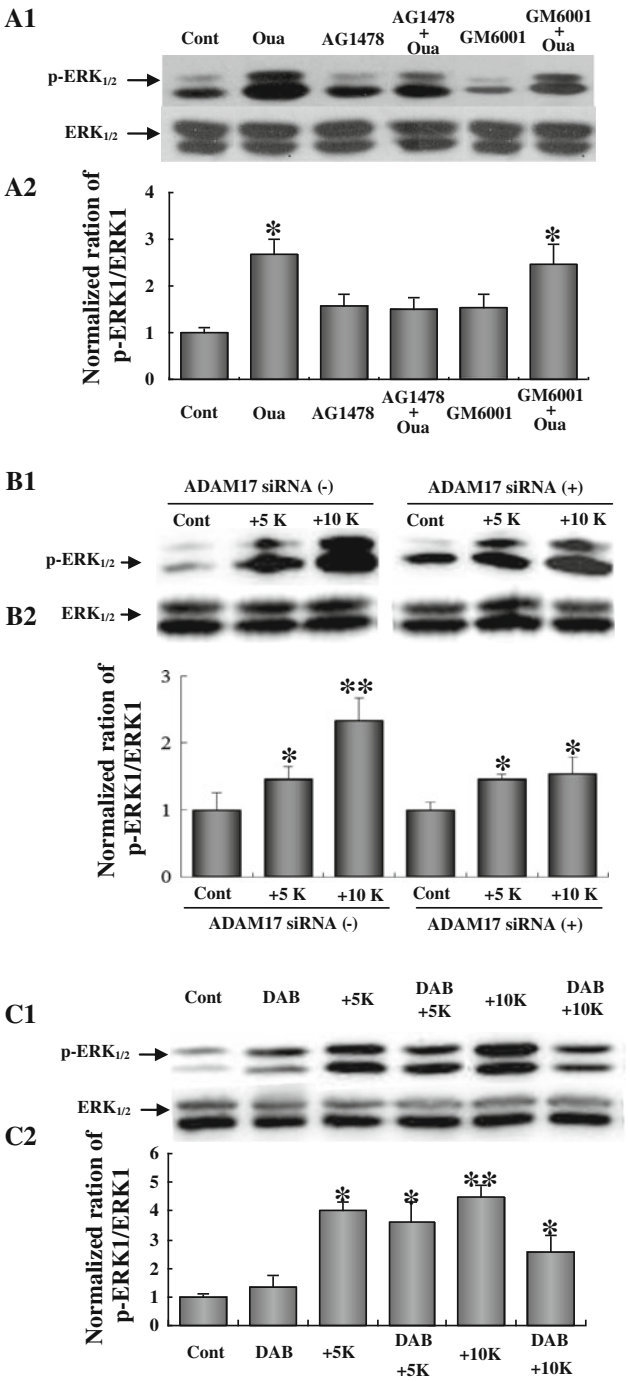


Fig. 5 $ERK_{1/2}$ phosphorylation, measured after incubation for 20 min in culturing medium (5.4 mM $[K^+]_e$) under drug-free control conditions, occurs in both the NKCC1 and the ouabain signal pathways, and can be counteracted by different inhibitors or gene knock-out. **a1** Representative Western blot showing that after exposure to 30 nM ouabain EGF receptor stimulation, inhibited by AG1478 is required for ERK phosphorylation in astrocytes, whereas metalloproteinase activity, inhibited by GM6001 is not involved. **b1** Representative Western blot showing that ADAM17 is required for ERK phosphorylation in astrocytes induced by addition of 10 mM, but not by addition of 5 mM K^+, from the beginning of the incubation. The cells had been treated for 3 days with either transfection solution *without* siRNA specific to ADAM17 (siRNA(−) (and therefore could serve as controls) (*left*) or with specific siRNA specific to ADAM17 (siRNA (+)). **c1** Representative Western blot showing that the increase in $ERK_{1/2}$ phosphorylation in normal cultures is more inhibited by DAB after addition of 10 mM than after addition of 5 mM K^+ (as described in "Methods" section). **a2**, **b2**, **c2** Mean ± SEM (n = 3) of scanned ratios between p-$ERK_{1/2}$ and $EPK_{1/2}$. *Statistically significant ($p < 0.05$) difference from all other groups. **Statistically significant ($p < 0.05$) difference from +5 K^+ group in siRNA(−) (**b**) or from DAB + 10 K^+ group (**c**)

involved in K^+ uptake (or indeed for any other signaling processes) had not been suspected, until pointed out by DiNuzzo et al. [14]. It is in further support of this point of view that inhibition of glycogen phosphorylation in pancreas tumor cells induces metabolomic and proteomic changes in posttranslational modification of signaling molecules [68]. Glycogenolysis is also needed for glutamate formation in astrocytes in the chick brain in vivo [66], and for maintenance of pyruvate carboxylase activity and glutamate content in rat brain and cultured astrocytes [69, 70]. These observations are consistent with stimulation of pyruvate carboxylation in cultured astrocytes by elevated $[K^+]_e$ [71]. In human brain approximately 2 % of the glucose utilization (0.3 mmol/g per min) proceeds via glycogen synthesis and degradation [72], but with astrocytes accounting for 20–25 % of total glucose metabolism [73] this corresponds to ~10 % of astrocytic glucose metabolism. This fraction is not large enough to account for direct incorporation of a glycogen-derived precursor into glutamate, since glutamate production (with associated production of energy) corresponds to at least one half of their glucose metabolism, and pyruvate carboxylation provides one half of the precursor molecule [73]. Thus metabolic support of signaling processes seems to account for ~10 % of astrocytic energy use.

Both K^+-accumulating astrocytic pathways operate via phosphorylation of $ERK_{1/2}$. DAB was demonstrated to significantly inhibit the phosphorylation of $ERK_{1/2}$ (in the total tissue) after addition of 10 mM K^+, but not after addition of 5 mM K^+, leading to the conclusion that it inhibited the NKCC1 pathway (upper part of Fig. 8). However, since NKCC1 is metabolically driven by the Na^+,K^+-ATPase, which is maximally activated after addition of K^+ concentrations of 5 mM K^+ or more, DAB must also have exerted at least some inhibition of $ERK_{1/2}$ phosphorylation in the ouabain-activated pathway after addition of 10 mM K^+. This may have contributed to the significance of the inhibition of $ERK_{1/2}$ phosphorylation by the addition of 10 mM K^+ in astrocytes from normal mice, versus the non-significance of the response to addition of 5 mM K^+, activating the ouabain pathway but not the NKCC1 pathway.

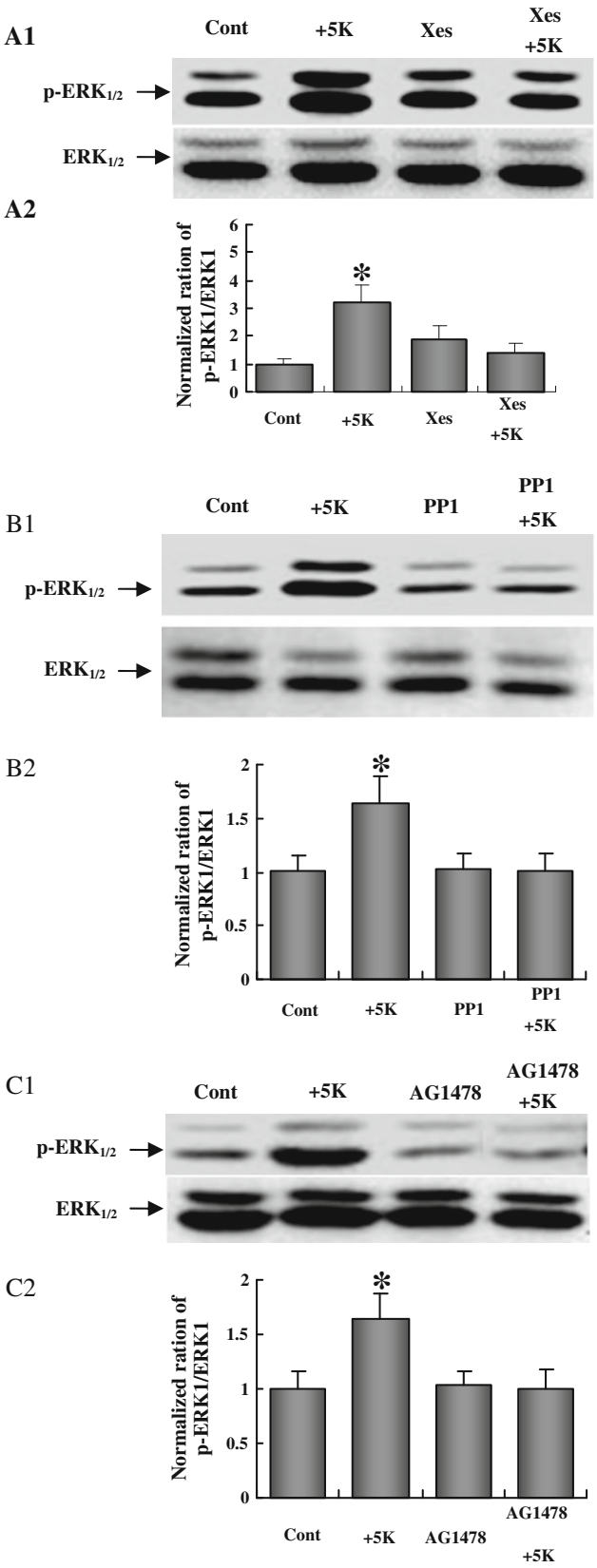


Fig. 6 Indications that $ERK_{1/2}$ phosphorylation by addition of 5 mM K^+ from the beginning of the incubation (as described in "Methods" section) to a medium with 5.4 mM $[K^+]_e$ and measured after 20 min of incubation in culturing medium occurs in the ouabain signal pathways, and can be counteracted by different inhibitors. **a1** Representative Western blot showing that IP_3 receptor activity, inhibited by xestospongin C, is required for $ERK_{1/2}$ phosphorylation after addition of 5 mM K^+. This receptor is especially important, because it is not also involved in the NKCC1 pathway. **b1** Representative Western blot showing that Src activity, inhibited by PP1 is required for $ERK_{1/2}$ phosphorylation after addition of 5 mM K^+. **c1** Representative Western blot showing that EGF receptor activity, inhibited by AG 1478, is required for $ERK_{1/2}$ phosphorylation after addition of 5 mM K^+. **a2**, **b2**, **c2** Mean ± SEM (n = 3) of scanned ratios between p-$ERK_{1/2}$ and $EPK_{1/2}$. *Statistically significant ($p < 0.05$) difference from all other groups

Metabolic support of the pathway activated by very low concentrations of ouabain was indicated as the reason for the glycogenolytic requirement after addition of 5 mM K^+. This was shown by the abolishment of the increase in $[K^+]_e$ by inhibition of IP_3, participating in this pathway, but not in that activated by addition of 10 mM K^+ (Fig. 8). The additional inhibition by Src or EGF receptor inhibition and the stimulation of $ERK_{1/2}$ phosphorylation by 30 nM ouabain support the involvement of this pathway. Na_X is present in astrocytes [42–44], and the ability of an increase in $[Na^+]_e$ by 10 mM to counteract DAB inhibition suggests the involvement of this Na^+-sensing Na^+ channel [46]. The inhibitory effect of the Na^+ channel inhibitor amiloride on K^+-induced increase in $[K^+]_i$ supports this point of view. A similar rescue effect could be observed by increasing $[Na^+]_i$ with monensin. The absolute increase in $[Ca^{2+}]_i$ is probably very small [74], but it may be sufficient to stimulate the glycogen phosphorylase kinase, the enzyme converting inactive to active phosphorylase [75].

The demonstration that K^+-induced glycogenolysis is required for both Na^+,K^+-ATPase stimulation (to supply sufficient $[Na^+]_i$) and for NKCC1 activation might be of special importance for the necessary return of K^+ to the neurons in the brain in vivo after its initial uptake in astrocytes. Bay and Butt [13] have shown that K^+ may be released from astrocytes through the K^+ channel Kir1.4 and become available for neuronal uptake, but they did not speculate how the powerful astrocytic K^+ uptake mechanism [7, 8] was neutralized. The present observations that stimulation of glycogenolysis is essential for both Na^+, K^+-ATPase- and NKCC1-mediated K^+ uptake, combined with K^+-mediated astrocytic glycogenolysis and increase in $[Ca^{2+}]_i$ [15, 30] may provide an answer to this question: it implies that once resting $[K^+]_e$ has been re-established the astrocytic uptake becomes inactivated. Previously astrocytically accumulated K^+ can therefore be released via Kir1.4 and be re-accumulated into neurons. The release does not necessarily occur from the same astrocytes that initially accumulated K^+, but K^+ may traverse through

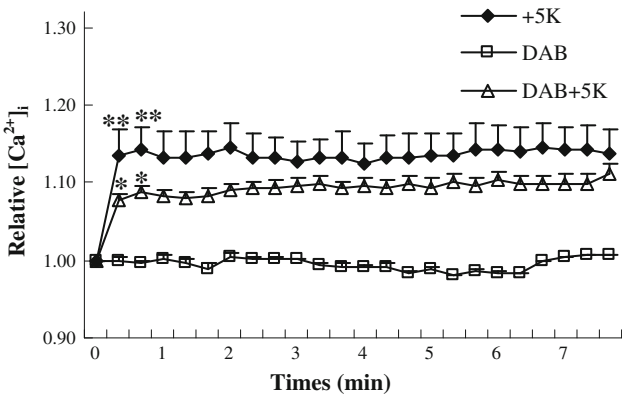


Fig. 7 Glycogenolysis is required for increase of $[Ca^{2+}]_i$ induced by addition of 5 mM K^+ (as described in "Methods" section) and measured by a similar approach as increase in $[K^+]_i$ (see Fig. 2). After incubation of fura-2-loaded cells in saline solution for 2 min and subsequent wash, the cells were incubated either in similar solution or in a solution to which an additional 5 mM KCl had been added at zero time (with a corresponding reduction of NaCl concentration). In some experiments, 10 mM DAB, an inhibitor of glycogenolysis, was added 2 min before the addition of K^+. Results are averages from 29 to 77 cells on 2–3 individual coverslips. SEM values are indicated by *vertical bars*. All results after addition of K^+ are significantly different ($p < 0.05$) from control conditions. *Statistically significant ($p < 0.05$) difference from drug-free group at the same time period

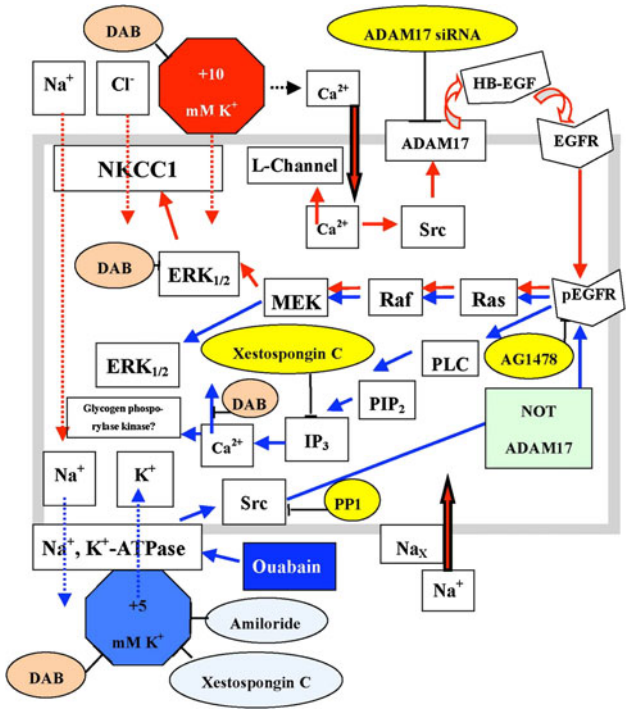


Fig. 8 Cartoon illustrating signal pathways activated in cultured astrocytes by (1) L-channel opening after addition of 10 mM KCl to reach a $[K^+]_e$ of 15 mM (*top*, *red solid lines*), which leads to K^+-mediated glycogenolysis, necessary for pathway function, and eventually to NKCC1 activation, and (2) opening of a prospective ouabain signal pathway, necessary for Na^+,K^+-ATPase stimulation by intracellular Na^+ in the non-excitable astrocytes after addition of 5 mM K^+. Activation of this pathway leads to an increase in $[Ca^{2+}]_i$ which promotes entry of Na^+ ions, able to act at the intracellular Na^+-sensitive site of the Na^+,K^+-ATPase, possibly by activation of glycogen phosphorylase activation and subsequent stimulation of glycogenolysis, necessary for pathway function. The NKCC1 pathway had previously been examined in detail by use of specific inhibitors up to phosphorylation of $ERK_{1/2}$. Therefore only the new results, showing its inhibition in ADAM17-depleted cultures and by DAB, an inhibitor of glycogenolysis, are indicated in the Fig. (in *yellow ovals* for all inhibitors, except DAB, always shown in *brown*), together with its inhibition of K^+ uptake following addition of 10 mM K^+ (*brown oval*). The ouabain signal pathway, shown at the *bottom* of the Figure (*blue solid lines*) is from literature data, but partly confirmed by our own previous studies and present inhibitor studies. Inhibitory effects on increase in $[K^+]_e$ after addition of K^+ by 5 mM are shown in light blue ovals, except for DAB (shown in *brown*), those on $ERK_{1/2}$ phosphorylation as yellow ovals, and that by DAB on K^+-induced increase in $[Ca^{2+}]_i$ by a *brown oval*

astrocytes via K^+-stimulated connexins and pannexins [76]. Such a mechanism would combine efficient removal of excess $[K^+]_e$ with subsequent return of K^+ to neurons and it would provide signaling opportunities, justifying the added energetic cost by dual K^+ uptake. It would in principle be quite similar to the manner in which extracellular glutamate is treated [73], again at a considerable metabolic cost. On the other hand, the dependence of K^+ uptake on K^+-induced glycogenolysis also suggests that astrocytes may not be involved in the K^+ uptake involved in housekeeping (accumulation of K^+ continuously released in the absence of specific stimulation), but that this uptake may occur mainly directly into neurons.

The ability of glycogenolysis to be stimulated by even small increases in $[K^+]_e$, [15], and by transmitters like noradrenaline [77–79] makes glycogen a molecule of choice for support of energy requiring processes stimulated by either elevated $[K^+]_e$ or noradrenaline, which may account for its absolute requirement during learning [66, 80–83]. Noradrenaline effects on glycogenolysis, including their potential ability to substitute for K^+-stimulated glycogenolysis were not discussed in the present paper. One important issue is whether noradrenergic signaling to astrocytes in the brain in vivo is activated at the time $[K^+]_e$ is normalized or reduced during the undershoot. To our knowledge this fundamental question has not been answered. In cultured astrocytes, the β_1/β_2-adrenergic agonist isoproterenol enhances the increase in $[K^+]_i$, although mainly when $[K^+]_e$ is not simultaneously increased (J. Xu and L. Peng unpublished experiments). This is in agreement with the ability of noradrenaline to cause maximum effect on the astrocytic Na^+,K^+-ATPase at the $[K^+]_e$ used under control conditions, including the previous culturing [8]. The observed immediate effect of noradrenaline is in contrast to findings by some other authors [70 and references therein], in the case of Obel

et al. perhaps because cerebellar astrocytes were used. The non-potent effect observed in the cerebellar cells (on $[Ca^{2+}]_i$, alanine and glutamate contents) is at variance with a much more potent glycogenolytic response [8] and completion of noradrenaline-stimulated glycogenolysis within 20 min [79] in the presently used cerebrocortical cultures. Gibbs and Hutchinson [83] also reported very fast turnover of not only glycogenolysis but also glycogen synthesis, stimulated by α_2-adrenergic activation, in the young chicken brain.

Requirement for glycogenolysis during signaling processes may not be selective for astrocytes, but analogous mechanisms may also be involved in other non-excitable cells carrying out cation transport mediated by the Na^+,K^+-ATPase. This applies to non-neuronal cells transporting K^+ in the cochlea/vestibular labyrinth and to cells achieving osmotic accommodation in the gills (and some other organs) of fish living in waters of changing salinity. Cells in both systems contain glycogen [84, 85] and express Na^+,K^+-ATPase and NKCC [86, 87]. Also, in tilapia transfer from fresh to salt water induced parallel increase in plasma osmolality and in ouabain immunoreactivity [88]. Ouabain concentrations comparable to or even lower than those used in the present study modulate junctions in the epithelial Madin-Darby canine kidney (MDCK) cells [89], and in cultured astrocytes they can prevent down-regulation of Na^+,K^+-ATPase by lipopolysaccharide (LPS) and restore actin filaments [90].

The present results need confirmation in the brain in vivo. This has already happened to some extent. Increased turnover of glycogen during increased neuronal activity was demonstrated 20 years ago [91]. More recent experiments have shown that Ca^{2+}- and IP_3-mediated signaling via astrocytes reduces stimulated increase in $[K^+]_e$ and enhances the undershoot following intense stimulation in hippocampal brain slices from mice [5]. In turn, this led to neuronal hyperpolarization, reduced frequency of excitatory synaptic activity, and improved signal-to-noise ratio of synaptic transmission. In the present context it is especially important that Wang et al. [5] demonstrated that brief high-frequency stimulation of hippocampal slices, causing an increase in $[K^+]_e$ of <1.0 mM, consistently induced a higher $[K^+]_e$ in slices from IP_3 knock-out mice than in slices from wild-type mice. This situation is essentially analogous to our observation (Fig. 2c) that IP_3 inhibition, specifically inhibiting the ouabain signaling, glycogenolysis-dependent pathway, abolishes the normal increase in $[K^+]_i$ in cultured astrocytes. The same group of authors [6] also established that transmitter-induced increases in $[Ca^{2+}]_i$ secondary to stimulation of P_2Y receptors in Bergmann glia by ATP or UTP increased astrocytic $[Ca^{2+}]_i$, and that the resulting decline in $[K^+]_e$ transiently increased spike activity of Purkinje cells both in cerebellar slices and in live anesthetized mice [6]. These findings further emphasize the question if transmitter-induced effects on $[Ca^{2+}]_i$ could have rescued K^+ uptake impaired by DAB. However, one important difference between our observations in cultured cortical astrocytes and that by Wang et al. [6] in cerebellum is that no preceding increase in $[K^+]_e$ had occurred in their study, whereas our studies indicated the necessity of an increase in $[K^+]_e$ to elicit the glycogenolysis-dependent effect. It cannot be excluded that transmitter-induced effects at normal $[K^+]_e$ may be functionally different from those evoked by elevated $[K^+]_e$. Alternatively, the increase in astrocytic $[Ca^{2+}]_i$ caused by the applied transmitters in the Wang study may have substituted for $[Ca^{2+}]_i$ increases after increases in $[K^+]_e$. Other potential substitutions for activation of the ouabain pathway may be Na^+ uptake during accumulation of glutamate. However, glutamate uptake rates in astrocytes are considerably lower than K^+ uptake rates [73], and even though the stimulation used by Wang et al. [5] is likely to have led to a release of glutamate, the $[K^+]_e$ responses were dependent on IP_3. A failure of Carmignoto et al. [92] to demonstrate voltage-dependent Ca^{2+} channels in brain slices, versus their clear-cut presence demonstrated by different means, can also be considered as an un-intended confirmation of the present findings, because the slices used by these authors must have been depleted of glycogen during the preincubation in a glucose-free saline (containing 2 mM pyruvate). A similar failure by Choi et al. [93] probably mainly reflects the use of a too low $[K^+]_e$ (total of 10 mM) for the opening of L-channels.

In conclusion, by following up on a suggestion by DiNuzzo et al. [14] the present study has clearly shown inhibition of uptake of excess extracellular K^+, when glycogenolysis is inhibited, and indicated that this inhibition results from the inability of the pathways involved to function without glycogenolytically provided energy. Literature evidence was presented that astrocytes in situ are likely to operate in a similar fashion. This would provide astrocytes with the ability initially to accumulate excess $[K^+]_e$, a process for which there is now considerable evidence, but later not to counteract processes aiming to return K^+ to neurons in order to not deplete these cells for $[K^+]_i$.

Acknowledgments The authors of DiNuzzo et al. [14] are cordially thanked for their contemplative suggestion that glycogenolysis may be involved in astrocytic K^+ uptake, without which this study would not have been undertaken. Our study was supported by Grant No. 31171036 to L. P. from the National Natural Science Foundation of China.

Conflict of interest Nothing to report.

References

1. Macaulay N, Zeuthen T (2012) Glial K^+ clearance and cell swelling: key roles for cotransporters and pumps. Neurochem Res 37:2299–2309
2. Ransom CB, Ransom BR, Sontheimer H (2000) Activity-dependent extracellular K^+ accumulation in rat optic nerve: the role of glial and axonal Na^+ pumps. J Physiol 522(Pt 3):427–442
3. Somjen GG, Kager H, Wadman WJ (2008) Computer simulations of neuron-glia interactions mediated by ion flux. J Comput Neurosci 25:349–365
4. Walz W (2000) Role of astrocytes in the clearance of excess extracellular potassium. Neurochem Int 36:291–300
5. Wang F, Smith NA, Xu Q et al (2012) Astrocytes modulate neural network activity by Ca^{2+}-dependent uptake of extracellular K^+. Sci Signal 5:ra26
6. Wang F, Xu Q, Wang W et al (2012) Bergmann glia modulate cerebellar Purkinje cell bistability via Ca^{2+}-dependent K^+ uptake. Proc Natl Acad Sci USA 109:7911–7916
7. Grisar T, Franck G, Schoffeniels E (1980) Glial control of neuronal excitability in mammals: II. Enzymatic evidence: two molecular forms of the Na^+, K^+-ATPase in brain. Neurochem Int 2C:311–320
8. Hajek I, Subbarao KV, Hertz L (1996) Acute and chronic effects of potassium and noradrenaline on Na^+, K^+-ATPase activity in cultured mouse neurons and astrocytes. Neurochem Int 28:335–342
9. Henn FA, Haljamae H, Hamberger A (1972) Glial cell function: active control of extracellular K^+ concentration. Brain Res 43: 437–443
10. Walz W (1992) Role of Na/K/Cl cotransport in astrocytes. Can J Physiol Pharmacol 70(Suppl):S260–S262
11. Walz W, Hertz L (1984) Intense furosemide-sensitive potassium accumulation in astrocytes in the presence of pathologically high extracellular potassium levels. J Cereb Blood Flow Metab 4: 301–304
12. Dufour S, Dufour P, Chever O et al (2011) In vivo simultaneous intra- and extracellular potassium recordings using a microoptrode. J Neurosci Methods 194:206–217
13. Bay V, Butt AM (2012) Relationship between glial potassium regulation and axon excitability: a role for glial Kir4.1 channels. Glia 60:651–660
14. Dinuzzo M, Mangia S, Maraviglia B et al (2012) The role of astrocytic glycogen in supporting the energetics of neuronal activity. Neurochem Res 37:2423–2438
15. Hof PR, Pascale E, Magistretti PJ (1988) K^+ at concentrations reached in the extracellular space during neuronal activity promotes a Ca^{2+}-dependent glycogen hydrolysis in mouse cerebral cortex. J Neurosci 8:1922–1928
16. Bernard C (1878) Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux, Tome 1. Edité par Albert Dastre. J.-B. Baillière et fils, Paris
17. Walz W, Hertz L (1982) Ouabain-sensitive and ouabain-resistant net uptake of potassium into astrocytes and neurons in primary cultures. J Neurochem 39:70–77
18. Sykova E (1992) K^+ homeostasis in the ECS. In: Sykova E (ed) Ionic and volume changes in the microenvironment of nerve and receptor cells in progress in sensory physiology. Springer, Berlin, pp 7–26
19. Epstein FH, Silva P (1985) Na-K-Cl cotransport in chloride-transporting epithelia. Ann N Y Acad Sci 456:187–197
20. Dawson DC (1987) Cellular mechanisms for K transport across epithelial cell layers. Semin Nephrol 7:185–192
21. Kimelberg HK, Frangakis MV (1985) Furosemide- and bumetanide-sensitive ion transport and volume control in primary astrocyte cultures from rat brain. Brain Res 361:125–134
22. Walz W, Hinks EC (1986) A transmembrane sodium cycle in astrocytes. Brain Res 368:226–232
23. Tas PW, Massa PT, Kress HG et al (1987) Characterization of an $Na^+/K^+/Cl^-$ cotransport in primary cultures of rat astrocytes. Biochim Biophys Acta 903:411–416
24. Kanaka C, Ohno K, Okabe A et al (2001) The differential expression patterns of messenger RNAs encoding K-Cl cotransporters (KCC1, 2) and Na-K-2Cl cotransporter (NKCC1) in the rat nervous system. Neuroscience 104:933–946
25. Mikawa S, Wang C, Shu F et al (2002) Developmental changes in KCC1, KCC2 and NKCC1 mRNAs in the rat cerebellum. Brain Res Dev Brain Res 136:93–100
26. Deisz RA, Lehmann TN, Horn P et al (2011) Components of neuronal chloride transport in rat and human neocortex. J Physiol 589:1317–1347
27. Zhang LL, Delpire E, Vardi N (2007) NKCC1 does not accumulate chloride in developing retinal neurons. J Neurophysiol 98:266–277
28. Pedersen SF, O'Donnell ME, Anderson SE et al (2006) Physiology and pathophysiology of Na^+/H^+ exchange and Na^+-K^+-$2Cl^-$ cotransport in the heart, brain, and blood. Am J Physiol Regul Integr Comp Physiol 291:R1–R25
29. Subbarao KV, Stolzenburg JU, Hertz L (1995) Pharmacological characteristics of potassium-induced, glycogenolysis in astrocytes. Neurosci Lett 196:45–48
30. Cai L, Du T, Song D et al (2011) Astrocyte ERK phosphorylation precedes K^+-induced swelling but follows hypotonicity-induced swelling. Neuropathology 31:250–264
31. Hertz L, Bender AS, Woodbury DM et al (1989) Potassium-stimulated calcium uptake in astrocytes and its potent inhibition by nimodipine. J Neurosci Res 22:209–215
32. Peng L, Du T, Xu J et al (2012) Adrenergic and V1-ergic agonists/antagonists affecting recovery from brain trauma in the lund project act on astrocytes. Curr Signal Transduct Ther 7:43–55
33. MacVicar BA (1984) Voltage-dependent calcium channels in glial cells. Science 226:1345–1347
34. Duffy S, MacVicar BA (1994) Potassium-dependent calcium influx in acutely isolated hippocampal astrocytes. Neuroscience 61:51–61
35. Brown AM, Westenbroek RE, Catterall WA et al (2001) Axonal L-type Ca^{2+} channels and anoxic injury in rat CNS white matter. J Neurophysiol 85:900–911
36. Cahoy JD, Emery B, Kaushal A et al (2008) A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. J Neurosci 28:264–278
37. Barres BA, Koroshet WJ, Chun LL et al (1990) Ion channel expression by whiteatter glia: the type-1 astrocytes. Neuron 5:527–544
38. Lovatt D, Sonnewald U, Waagepetersen HS et al (2007) The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. J Neurosci 27:12255–12266
39. Westenbroek RE, Bausch SB, Lin RC et al (1998) Upregulation of L-type $Ca2^+$ channels in reactive astrocytes after brain injury, hypomyelination, and ischemia. J Neurosci 18:2321–2334
40. Peng L (2004) Transactivation in astrocytes as a novel mechanism of neuroprotection. In: Hertz L (ed) Non-neuronal cells of the nervous system: function and dysfunction. Elsevier, Amsterdam, pp 503–518
41. Mollenhauer HH, Morre DJ, Minnifield N (1992) Swelling response of golgi apparatus cisternae in cells treated with monensin is reduced by cell injury. Cell Biol Int Rep 16:217–220
42. Gautron S, Dos Santos G, Pinto-Henrique D et al (1992) The glial voltage-gated sodium channel: cell- and tissue-specific mRNA expression. Proc Natl Acad Sci USA 89:7272–7276

43. Black JA, Dib-Hajj S, Cohen S et al (1998) Glial cells have heart: rH1 Na^+ channel mRNA and protein in spinal cord astrocytes. Glia 23:200–208
44. Watanabe E, Fujikawa A, Matsunaga H et al (2000) Nav2/NaG channel is involved in control of salt-intake behavior in the CNS. J Neurosci 20:7743–7751
45. Ogata N, Ohishi Y (2002) Molecular diversity of structure and function of the voltage-gated Na^+ channels. Jpn J Pharmacol 88:365–377
46. Shimizu H, Watanabe E, Hiyama TY et al (2007) Glial Na_x channels control lactate signaling to neurons for brain Na^+ sensing. Neuron 54:59–72
47. Mobasheri A, Barrett-Jolley R, Shakibaei M et al (2005) Enigmatic roles of the epithelial sodium channel (ENaC) in articular chondrocytes and osteoblasts: mechanotransduction, sodium transport or extracellular sodium sensing? In: Kamkin A, Kiseleva I (eds) Mechanosensitivity in cells and tissues. Academia, Moscow, pp 452–464
48. Reinhard L, Tidow H, Clausen MJ et al (2012) Na^+, K^+-ATPase as a docking station: protein–protein complexes of the Na^+, K^+-ATPase. Cell Mol Life Sci. doi:10.1007/s00018-012-1039-9
49. Xue Z, Li B, Gu L et al (2010) Increased Na, K-ATPase alpha2 isoform gene expression by ammonia in astrocytes and in brain in vivo. Neurochem Int 57:395–403
50. Zhang L, Zhang Z, Guo H et al (2008) Na^+/K^+-ATPase-mediated signal transduction and Na^+/K^+-ATPase regulation. Fundam Clin Pharmacol 22:615–621
51. Juurlink BHJ, Hertz L (1992) Astrocytes. In: Boulton AA, Baker GB, Walz W (eds) Neuromethods in cell cultures. Humana Clifton, New York, pp 269–321; Internet availability in Springer Protocols, Neuroscience
52. Hertz L, Peng L, Lai JC (1998) Functional studies in cultured astrocytes. Methods 16:293–310
53. Li B, Du T, Li H et al (2008) Signalling pathways for transactivation by dexmedetomidine of epidermal growth factor receptors in astrocytes and its paracrine effect on neurons. Br J Pharmacol 154:191–203
54. Hertz L, Juurlink BHJ, Szuchet S (1985) Cell cultures. In: Lajtha A (ed) Handbook of neurochemistry. Plenum, New York, pp 603–661
55. Meier E, Hertz L, Schousboe A (1991) Neurotransmitters as developmental signals. Neurochem Int 19:1–15
56. Lange SC, Bak LK, Waagepetersen HS et al (2012) Primary cultures of astrocytes: their value in understanding astrocytes in health and disease. Neurochem Res 37:2569–2588
57. Li B, Dong L, Wang B et al (2012) Cell type-specific gene expression and editing responses to chronic fluoxetine treatment in the in vivo mouse brain and their relevance for stress-induced anhedonia. Neurochem Res 37:2380–2495
58. Li B, Hertz L, Peng L (2012b) Aralar mRNA and protein levels in neurons and astrocytes freshly isolated from young and adult mouse brain and in maturing cultured astrocytes. Neurochem Int 61:1325–1332
59. Song D, Li B, Yan EZ et al (2012) Chronic treatment with antibipolar drugs causes intracellular alkalinization in astrocytes, altering their functions. Neurochem Res 37:2524–2540
60. Deshmukh HS, Case LM, Wesselkamper SC et al (2005) Metalloproteinases mediate mucin 5AC expression by epidermal growth factor receptor activation. Am J Respir Crit Care Med 171:305–314
61. Laskay G, Varhelyi T, Dale RE et al (1995) Role of interleukin-3 in the regulation of intracellular K^+ homeostasis in cultured murine hematopoietic cells. Biochem Biophys Res Commun 214:348–353
62. Andersson B, Janson V, Behnam-Motlagh P et al (2006) Induction of apoptosis by intracellular potassium ion depletion: using the fluorescent dye PBFI in a 96-well plate method in cultured lung cancer cells. Toxicol In Vitro 20:986–994
63. Yates SL, Fluhler EN, Lippiello PM (1992) Advances in the use of the fluorescent probe fura-2 for the estimation of intrasynaptosomal calcium. J Neurosci Res 32:255–260
64. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
65. Sickmann HM, Schousboe A, Fosgerau K et al (2005) Compartmentation of lactate originating from glycogen and glucose in cultured astrocytes. Neurochem Res 30:1295–1304
66. Gibbs M, Lloyd H, Santa T et al (2007) Glycogen is a preferred glutamate precursor during learning in 1-day-old chick: biochemical and behavioral evidence. J Neurosci Res 85:3326–3333
67. Obel LF, Muller MS, Walls AB et al (2012) Brain glycogen-new perspectives on its metabolic function and regulation at the subcellular level. Front Neuroenergetics 4:3
68. Ma D, Wang J, Zhao Y et al (2012) Inhibition of glycogen phosphorylation induces changes in cellular proteome and signaling pathways in MIA pancreatic cancer cells. Pancreas 41:397–408
69. Sickmann HM, Waagepetersen HS, Schousboe A et al (2012) Brain glycogen and its role in supporting glutamate and GABA homeostasis in a type 2 diabetes rat model. Neurochem Int 60:267–275
70. Obel LF, Andersen KM, Bar LK et al (2012) Effects of adrenergic agents on intracellular $Ca2^+$ homeostasis and metabolism of glucose in astrocytes with an emphasis on pyruvate carboxylation, oxidative decarboxylation and recycling: implications for glutamate neurotransmission and excitotoxicity. Neurotox Res 21:405–417
71. Kaufman E, Driscoll B (1992) Carbon dioxide fixation in neuronal and astroglial cells in culture. J Neurochem 58:258–262
72. Öz G, Tesfaye N, Kumar A et al (2012) Brain glycogen content and metabolism in subjects with type 1 diabetes and hypoglycemia unawareness. J Cereb Blood Flow Metab 32:256–263
73. Hertz L (2011) Astrocytic energy metabolism and glutamate formation—relevance for ^{13}C-NMR spectroscopy and importance of cytosolic/mitochondrial trafficking. Magn Reson Imaging 29: 1319–1329
74. Zhao Z, Hertz L, Code WE (1996) Effects of benzodiazepines on potassium-induced increase in free cytosolic calcium concentration in astrocytes: interactions with nifedipine and the peripheral-type benzodiazepine antagonist PK 11195. Can J Physiol Pharmacol 74:273–277
75. Ozawa E (2011) Regulation of phosphorylase kinase by low concentrations of Ca ions upon muscle contraction: the connection between metabolism and muscle contraction and the connection between muscle physiology and Ca-dependent signal transduction. Proc Jpn Acad Ser B Phys Biol Sci 87:486–508
76. Scemes E, Spray DC (2012) Extracellular K^+ and astrocyte signaling via connexin and pannexin channels. Neurochem Res 37:2310–2316
77. Magistretti PJ, Morrison JH, Shoemaker WJ et al (1983) Effect of 6-hydroxydopamine lesions on norepinephrine-induced [^3H]glycogen hydrolysis in mouse cortical slices. Brain Res 261:159–166
78. Cambray-Deakin M, Pearce B, Morrow C et al (1988) Effects of neurotransmitters on astrocyte glycogen stores in vitro. J Neurochem 51:1852–1857
79. Subbarao KV, Hertz L (1990) Noradrenaline induced stimulation of oxidative metabolism in astrocytes but not in neurons in primary cultures. Brain Res 527:346–349
80. Gibbs ME, Anderson DG, Hertz L (2006) Inhibition of glycogenolysis in astrocytes interrupts memory consolidation in young chickens. Glia 54:214–222
81. Hertz L, Gibbs ME (2009) What learning in day-old chickens can teach a neurochemist: focus on astrocyte metabolism. J Neurochem 109(Suppl 1):10–16

82. Suzuki A, Stern SA, Bozdaqi O et al (2011) Astrocyte-neuron lactate transport is required for long-term memory formation. Cell 144:810–823
83. Gibbs ME, Hutchinson DS (2012) Rapid turnover of glycogen in memory formation. Neurochem Res 37:2456–2463
84. Ito M, Spicer SS, Schulte BA (1994) Histochemical detection of glycogen and glycoconjugates in the inner ear with modified concanavalin A-horseradish peroxidase procedures. Histochem J 26:437–446
85. Tseng YC, Huang CJ, Chang JC et al (2007) Glycogen phosophorylase in glycogen-rich cells is involved in the energy supply for ion regulation in fish gill epithelia. Am J Physiol Requl Inteqr Comp Physiol 293:R482–R491
86. Tipsmark CK, Madsen SS, Borski RJ (2004) Effect of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). J Exp Zool A Comp Exp Biol 301:979–991
87. Chu HQ, Huang XW, Xiong H et al (2006) Role of ion channel Na-K-2Cl and alpha2 Na, K-ATPase in cochlear potassium cycling and auditory function. Zhonghua Er Bi Yan Hou Tou Jing Wai Ke Za Zhi 41:692–697
88. Kajimura S, Hirano T, Moriyama S et al (2004) Changes in plasma concentrations of immunoreactive ouabain in the tilapia in response to changing salinity: is ouabain a hormone in fish? Gen Comp Endocrinol 135:90–99
89. Larre I, Lazaro A, Contreras RG, Balda MS et al (2010) Ouabain modulates epithelial cell tight junction. Proc Natl Acad Sci USA 107:11387–11392
90. Forshammar J, Block L, Lundborg C et al (2011) Naloxone and ouabain in ultralow concentrations restore Na^+/K^+-ATPase and cytoskeleton in lipopolysaccharide-treated astrocytes. J Biol Chem 286:31586–31597
91. Swanson RA (1992) Physiologic coupling of glial glycogen metabolism to neuronal activity in brain. Can J Physiol Pharmacol 70(Suppl):S138–S144
92. Carmignoto G, Pasti L, Pozzan T (1998) On the role of voltage-dependent calcium channels in calcium signaling of astrocytes in situ. J Neurosci 18:4637–4645
93. Choi HB, Gordon GR, Zhou N et al (2012) Metabolic communication between astrocytes and neurons via bicarbonate-responsive soluble adenylyl cyclase. Neuron 75:1094–1104