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ORIGINAL PAPER

Basic Mechanism Leading to Stimulation of Glycogenolysis by Isoproterenol, EGF, Elevated Extracellular K^+ Concentrations, or GABA

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Abstract Glycogenolysis, in brain parenchyma an astrocyte-specific process, has changed from being envisaged as an emergency procedure to playing central roles during brain response to whisker stimulation, memory formation, astrocytic K^+ uptake and stimulated release of ATP. It is activated by several transmitters and by even very small increases in extracellular K^+ concentration, and to be critically dependent upon an increase in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), whereas cAMP plays only a facilitatory role together with increased $[Ca^{2+}]_i$. Detailed knowledge about the signaling pathways eliciting glycogenolysis is therefore of interest and was investigated in the present study in well differentiated cultures of mouse astrocytes. The β-adrenergic agonist isoproterenol stimulated glycogenolysis by a β_1-adrenergic effect, which initiated a pathway in which cAMP/protein kinase A activated a G_i/G_s shift, leading to Ca^{2+}-activated glycogenolysis. Inhibition of this pathway downstream of cAMP but upstream of the G_i/G_s shift abolished the glycogenolysis. However, inhibitors operating downstream of the Ca^{2+}-sensitive step, but preventing transactivation-mediated epidermal growth factor (EGF) receptor stimulation, a later step in the activated pathway, also caused inhibition of glycogenolysis. For this reason the effect of EGF was investigated and it was found to be glycogenolytic. Large increases in extracellular K^+ activated glycogenolysis by a nifedipine-inhibited L-channel opening allowing influx of Ca^{2+}, known to be glycogenolysis-dependent. Small increases (addition of 5 mM KCl) caused a smaller effect by a similarly glycogenolysis-reliant opening of an IP_3 receptor-dependent ouabain signaling pathway. The same pathway could be activated by GABA (also in brain slices) due to its depolarizing effect in astrocytes.

Keywords Astrocyte · β-Adrenergic receptor · GABA · EGF · Glycogenolysis · Potassium

Introduction

Glycogenolysis is important for many signaling processes (e.g., [1]). It is ideal for such a role partly because it is rapidly degraded without initial phosphorylation (e.g., [2]), but perhaps even more because its degradation is initiated in brain by many transmitters and by increases in extracellular K^+ concentration even slightly above the resting level [3–5]. Glycogenolysis is induced brain, muscle and liver by activation of the enzyme glycogen phosphorylase with profound differences in terms of activation by allosteric and phosphorylation mechanisms, which also are very important in brain [5]. Nevertheless, the glycogen phosphorylase shows a basically identical requirement for an increase in free cytosolic Ca^{2+} $[Ca^{2+}]_i$ concentration. Briefly, the inactive phosphorylase a is converted to the active phosphorylase b by phosphorylation catalyzed by phosphorylase kinase. The absolute Ca^{2+} dependence of this process was first shown by Ozawa [6] in muscle. Cyclic AMP (cAMP), often believed sufficient to induce glycogenolysis (e.g., [7]) can increase

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glycogenolytic rate but only when $[Ca^{2+}]_i$ is simultaneously increased [6, 8]. Results consistent with this conclusion have been obtained in mouse brain slices by Ververken et al. [9], who investigated the relative roles of cAMP and of Ca^{2+} for the activation of glycogen phosphorylase induced by noradrenaline (100 μM) or by exposure to 25 mM K^+ in mouse brain cortical slices. Short-term treatment with EGTA or $LaCl_3$ abolished the noradrenaline-mediated activation of the phosphorylase, showing a critical role of extracellular Ca^{2+}, and the K^+-induced depolarization produced a rapid activation of phosphorylase without increasing cAMP levels.

In the present study we have further investigated the signaling pathways leading to glycogenolysis by isoproterenol, a $\beta_{1,2}$-adrenergic agonist, and by elevated K^+ concentrations. From previous studies by Du et al. [10] it is known that isoproterenol concentrations ≥1 μM stimulate β_1-adrenergic receptors and via a G_s-mediated pathway activate cAMP and protein kinase A (PKA). However, PKA induces a G_s/G_i switch [10] followed by an increase in $[Ca^{2+}]_i$, a release of an agonist of the epidermal growth factor (EGF) receptor, stimulation of this receptor, and eventually phosphorylation of extracellular regulated kinases 1 and 2 ($ERK_{1/2}$). In the present study we confirmed the involvement of a β_1-adrenergic effect, whereas inhibition of β_2-adrenergic signaling was of no consequence, and we tested the influence on stimulated glycogenolysis by the PKA inhibitor H-89, the G_i inhibitor PTX, GM6001 an inhibitor of the release of EGF receptor agonist, and AG 1478, an EGF receptor antagonist, as well as of administration of the EGFR agonist EGF.

From Xu et al. [1] it is known that addition of 5 mM K^+ stimulates a pathway initiated by nM concentrations of ouabain (simulating an effect of endogenous ouabains), whereas very high extracellular K^+ concentration (≥15 mM) stimulate a pathway initiated by depolarization-mediated opening of L-channels for Ca^{2+}. Although many second messengers are identical in the two pathways, the inositoltrisphosphate (IP_3) receptor is an intermediate only in the ouabain-activated pathway, and L-channels are only involved in the pathway opened by K^+ concentrations ≥15 mM. We therefore tested the IP_3 receptor inhibitor xestospongin and the L-channel inhibitor nifedipine on glycogenolysis evoked by addition of 5 and of 45 mM K^+ as well as a ouabain inhibitor, canrenone, on the effect of addition of 5 K^+.

Materials and Methods

Reagents

Chemicals for preparation of medium and most other chemicals, including isoproterenol, the β-adrenergic receptor antagonists, betaxolol (4-(2-cyclopropylmethoxyethyl)-1-phenoxy-3-isopropylaminopropan-2-ol) and ICI118551 (erythro-(±)-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol), pertussis toxin (PTX), the PKA inhibitor H-89 (N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulphonamide), nifedipine, xestospongin, canrenone, γ-aminobutyric acid (GABA), BAPTA-AM, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide phosphate (NADP), hexokinase, glucose-6-phosphate dehydrogenase, and amyloglucosidase were purchased from Sigma (St. Louis, MO, USA). EGFR tyrosine kinase inhibitor, Tyrphostin AG 1478 (N-[(2R)-2-(hydroxamidocarbonymethyl)-4-methylpentanoyl]-L-tryptophan methylamide), metalloproteinase inhibitor, GM 6001 (1,4-diamino-2,3-dicyano-1, 4-bis[2-aminophenylthio]butadiene), and the inhibitor of mitogen-activated kinase (MEK) U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) were obtained from Calbiochem (La Jolla, CA, USA). Epidermal growth factor was purchased from Invitrogen (Carlsbad, CA, USA).

Cell Culture

Primary cultures of mouse astrocytes were prepared from the neopallia of the cerebral hemispheres as previously described [11] with minor modifications [1] and planted in 24-well plates in Dulbecco's Minimum Essential Medium (DMEM) with 7.5 mM glucose (to allow some decline between feedings) and the 5.4 mM K^+ traditionally used in our cultures. 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 [12]. The addition of dBcAMP at this specific stage of culturing is a crucial component of the culture preparation. It leads to a morphological and functional differentiation, as evidenced by the extension of cell processes and increases in several metabolic activities, and it induces expression of voltage sensitive L-channels for calcium (Ca^{2+}), features which are characteristic of astrocytes in situ [13–15] and necessary for K^+-induced stimulation of glycogenolysis [16]. Use of astrocyte cultures has recently been authoritatively reviewed [17], and in our own cultures drug-induced changes in gene expression and editing have recently been confirmed in freshly isolated cells from mice treated with the same drugs (reviewed in [18]) and the development of the glutamate/aspartate exchanger component aralar shows similar developmental patterns in the two preparations, with an increase in the cultured cells after dBcAMP administration [19].

Brain Slices

All experiments were carried out in accordance with the USA National Institute of Health Guide for the Care and Use of Laboratory Animals, and all experimental protocols

were approved by the Institutional Animal Care and Use Committee of China Medical University. CD-1 mice, weight 25–35 g, were housed in cages on a 12 h light/dark cycle in a temperature-controlled (23–25 °C) colony room with free access to food and water. Two brain slices of 300 μm thickness were cut from the lateral surface after removing the frontal 3 mm of each brain hemisphere of CD-1 mice with a Leica LV1000S slice cutter. The brain slices were incubated in artificial cerebral spinal fluid, (ACSF, containing in mM: 126 NaCl, 2 KCl, 1.25 NaH_2PO_4, 1 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 glucose) for 5 h following slicing to allow recovery of glycogen. Thereafter one slice was incubated in ACSF under control conditions and the other in the presence of 100 μM GABA. Tissues were homogenized with 0.3 ml 65 % ethanol/35 % phosphate-buffered saline (PBS containing in mM: 137 NaCl, 2.5 KCl, 10 Na_2HPO_4, 2 KH_2PO_4, 0.5 MgCl, and 1 $CaCl_2$), and after centrifugation at 9,000 g for 10 min, the precipitate was acidified in 0.4 ml 30 mM HCl.

Determination of Glycogenolysis

For determination of glycogen in either cultured cells or brain slice homogenates, a TECAN infinite M200 Microplate Analyzer (Lausanne, Switzerland) was used to record fluorescence intensity of NADPH, generated from $NADP^+$ by the action of glucose-6-phosphate dehydrogenase. This was done during a 20 min incubation period in DMEM in untreated control cultures (or slices) and in GABA-treated slices or cultures treated with the glycogenolytic agents isoproterenol, GABA [20], EGF or elevated K^+ concentrations in the presence or absence of specific inhibitors. The relatively long time period was chosen in order also to be able to visualize a slowly occurring glycogenolysis, e.g., after addition of only 5 mM K^+. After the incubation the astrocytes were washed three times with ice-cold phosphate-buffered saline (PBS) and sonicated in 30 mM HCl (similar solution as the homogenized slices). The suspension was used to measure non-hydrolyzed glycosyl units of glycogen. Briefly, three 50 μl aliquots were sampled. In the first aliquot, 150 μl of acetate buffer (0.1 M, pH 4.65) was added. In the second, 150 μl of a solution containing 1 % amyloglucosidase (10 mg/ml) in the acetate buffer was added in order to degrade remaining glycogen to glucose, and the mixture was incubated at room temperature for 30 min. Subsequently the two aliquots were treated identically. Two ml of Tris–HCl buffer (0.1 M, pH 8.1) containing 3.3 mM $MgCl_2$, 0.2 mM ATP, 25 μg/ml NADP, 4 μg/ml hexokinase, and 2 μg/ml glucose-6-phosphate dehydrogenase was added to each, and the mixture was incubated at room temperature for 30 min. The fluorescence of the NADPH formed in amounts equivalent to glucose metabolized by hexokinase was then read (excitation 340 nm; emission 450 nm). The first aliquot measures the sum of glucose and glucose-6-phosphate in the tissue, whereas the second aliquot in addition to those also measures the glycosyl units from glycogen remaining in the tissue. Determination of the difference between these two aliquots provides a measurement of the amount of the latter. The third aliquot was used to measure the protein content by the Lowry method to normalize the glycogen contents (nmol) per mg protein. A standard curve was made to show fluorescence intensity at different glucose concentrations and glycogen content was calculated, using a conversion factor based on a molecular weight of 180 g/mol for free glucose, but 162 g/mol per glycosyl unit of glycogen.

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 $p < 0.05$. Some of the graphs show results from several different experiments, but the statistical analysis always compared experimental values with the control(s) from the same experiment(s).

Results

Under control conditions the glycogen content averaged from all experiment was 90 ± 8.1 nmol/mg protein, with only minor differences between different experiments. One μM isoproterenol stimulates both the higher affinity β_2-adrenergic and the lower affinity β_1-adrenergic receptors [10]. However, Fig. 1a shows that the β_1-adrenergic antagonist betaxolol inhibited glycogenolysis in response to isoproterenol, whereas the β_2-adrenergic antagonist ICI118551 had no effect. Among the β_1-adrenergic inhibitors tested neither PTX nor H-89 had any glycogenolytic effect on their own, but either of these inhibitors completely inhibited the pronounced glycogenolytic effect of 1 μM isoproterenol (Fig. 1b). GM6001, an inhibitor of release of EGF receptor ligand(s), and AG1478, an inhibitor of the receptor itself, also inhibited isoproterenol-induced glycogenolysis substantially (Fig. 1c), although their inhibition of the isoproterenol-activated pathway is downstream of the effect on $[Ca^{2+}]_i$ [10]. However, it should be noted in Fig. 1b and C that glycogen content was 25–30 % higher in cultures treated with by PTX or H89 than in those treated with GM6001 and AG1478. Moreover, it cannot be excluded that inhibition of downstream signaling inhibits signaling further upstream, perhaps especially in the case of GM6001, which inhibits release of an EGF receptor agonist before the transactivation.

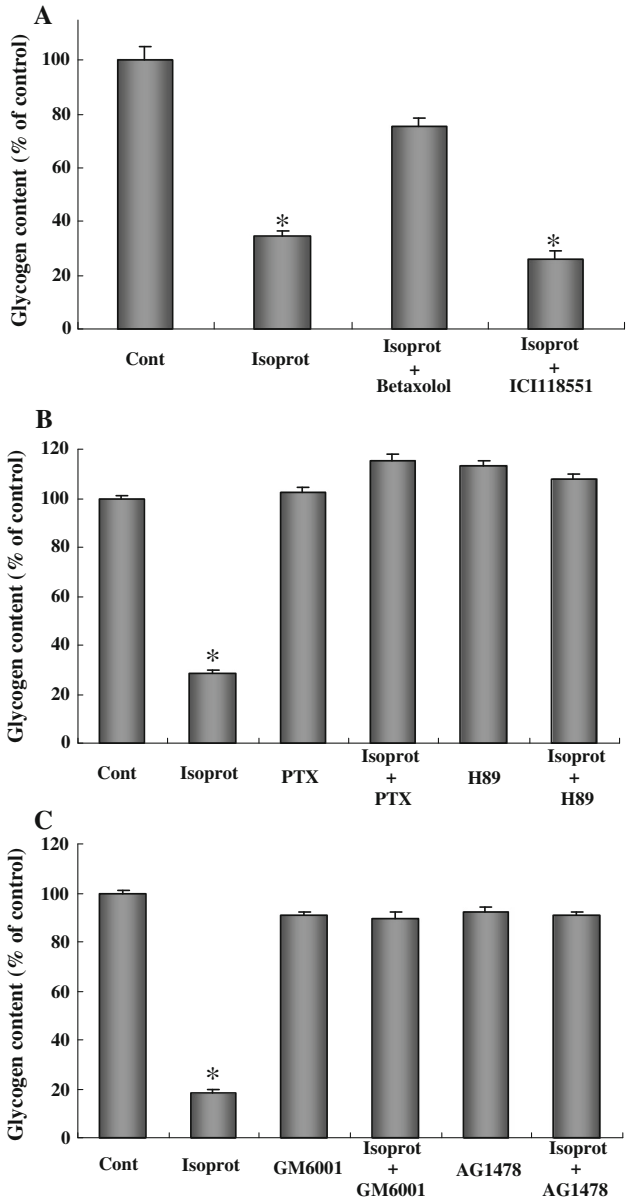

Fig. 1 Effect of isoproterenol on glycogenolysis indicated as reduction of glycogen content in astrocytes. **a** Cultured astrocytes were incubated in DMEM (containing 7.5 mM glucose) for 20 min without any addition (Cont) or in the presence of 1 μM isoproterenol (Isoprot), activating both β_1 and β_2 receptors, with or without 15 min pre-treatment with the β_1 receptor antagonist betaxolol or the β_2 receptor antagonist ICI118551. **b** Cultured astrocytes were incubated in DMEM for 20 min under control conditions (Cont) or in the presence of 1 μM isoproterenol (Isoprot), with or without 15 min pre-treatment with the $G_{i/o}$ inhibitor PTX or the PKA inhibitor H89. **c** Cultured astrocytes were incubated in DMEM for 20 min under control conditions (Cont) or in the presence of 1 μM isoproterenol (Isoprot), with or without 15 min pre-treatment with the inhibitor of release of EGF receptor ligand(s) GM6001 or the EGF receptor inhibitor AG1478. After the experiment glycogenolysis was determined by measuring glucose content fluorometrically before and after breakdown of remaining glycogen in the cells. Average glycogen contents are indicated as percentages of those under control conditions. All values are expressed as mean ± SEM, indicated by vertical bars and are from 3 to 5 individual cultures. *Statistically significant ($p < 0.05$) difference from control

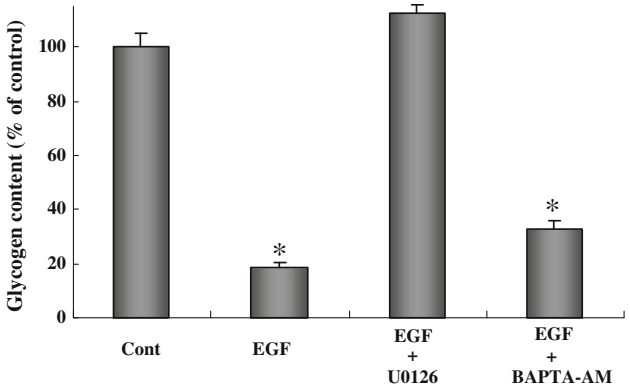

Fig. 2 Effect of EGF on glycogenolysis, indicated as reduction of glycogen content, in astrocytes. Cultured astrocytes (treated as described in Fig. 1) were incubated in DMEM for 20 min under control conditions (Cont) or in the presence of 10 ng/ml EGF, with or without 15 min pre-treatment with the MEK antagonist (and therefore inhibitor of $ERK_{1/2}$ phosphorylation) U0126, or the Ca^{2+} chelator BAPTA-AM. After the experiment glycogenolysis was determined by measuring glucose content fluorometrically before and after breakdown of remaining glycogen in the cells. Average glycogen contents are indicated as percentages of those under control conditions. All values are expressed as mean ± SEM indicated by vertical bars and are from 3 to 5 individual cultures. *Statistically significant ($p < 0.05$) difference from control

Nevertheless, the effect of 10 ng/ml EGF was tested and found to exert a large stimulation of glycogenolysis (Fig. 2), which was unaffected by the intracellular Ca^{2+} chelator BAPTA-AM but inhibited by U0126 an inhibitor of phosphorylation of $ERK_{1/2}$. These kinases are well known downstream targets of EGF, but their role(s) in eliciting glycogenolysis do not seem to be have been established.

Addition of 45 mM K^+ stimulated glycogenolysis as much as isoproterenol. The stimulation was greatly, but not completely inhibited by nifedipine, but only slightly and non-significantly by xestospongin (Fig. 3a). Addition of only 5 mM K^+ caused somewhat less glycogen degradation than addition of 45 mM. It was untouched by nifedipine but inhibited, although incompletely by xestospongin and completely by the spironolactone derivative canrenone (Fig. 3b), acting as a ouabain inhibitor [21].

γ-Aminobutyric acid (GABA) administration to astrocytes causes a depolarization because of the high intracellular Cl^- concentration in these cells [22, 23]. $GABA_A$ receptor-mediated increase of Cl^- efflux, results in depolarization and $[Ca^{2+}]_i$ increases [24, 25] and in glycogenolysis [20]. Like the effect of addition of only 5 mM K^+, GABA-induced glycogenolysis was untouched by nifedipine but inhibited by xestospongin (Fig. 4a). Since a glycogenolytic effect of GABA does not seem to have documented before, it was also tested, and shown, in brain slices (Fig. 4b). No obvious differences were seen from the results in the cultured cells.

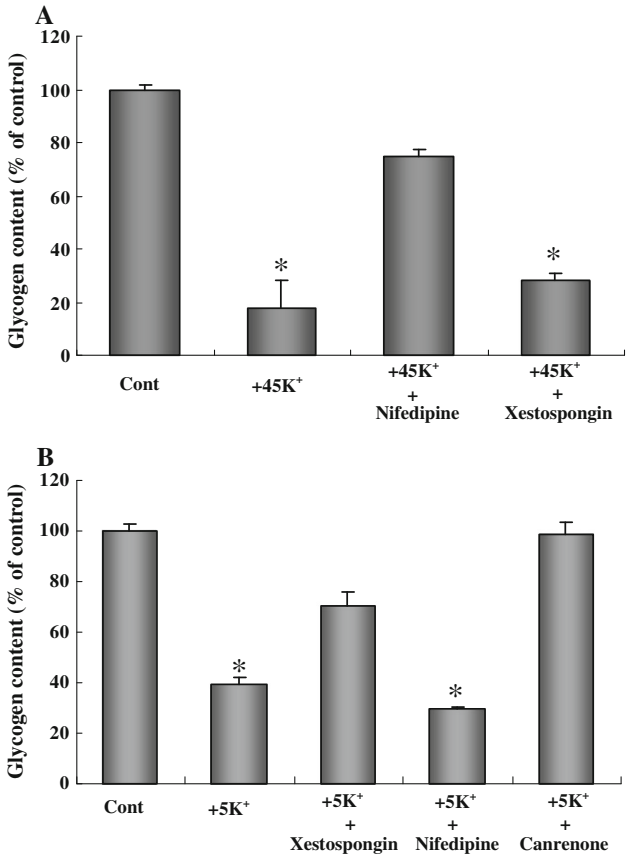

Fig. 3 Glycogenolytic effect of addition of 45 mM K^+ or addition of 5 mM K^+, indicated as reduction of glycogen content, in astrocytes. **a** Cultured astrocytes (treated as described in Fig. 1) were incubated in DMEM for 20 min under control conditions (Cont) or with addition of 45 mM K^+ to a final extracellular concentration of 50 mM (+45 K^+), with or without 15 min pre-treatment with the L-channel inhibitor nifedipine or the IP_3 receptor inhibitor xestospongin. **b** Cultured astrocytes (treated as described in Fig. 1) were incubated in DMEM for 20 min under control conditions (Cont) or with addition of 5 mM K^+ to a final extracellular concentration of 10 mM (+5 K^+), with or without 15 min pre-treatment with xestospongin, nifedipine or the ouabain inhibitor canrenone. After the experiment glycogenolysis was determined by measuring glucose content fluorometrically before and after breakdown of remaining glycogen in the cells. Average glycogen contents are indicated as percentages of those under control conditions. All values are expressed as mean ± SEM indicated by vertical bars and are from 3 to 5 individual cultures. *Statistically significant ($p < 0.05$) difference from control

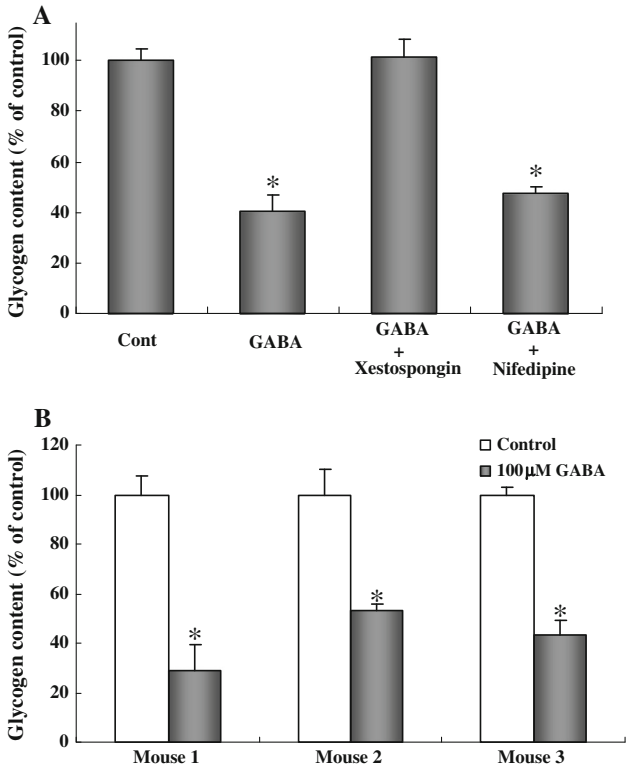

Fig. 4 Glycogenolytic effect of γ-aminobutyric acid (GABA), indicated as reduction of glycogen content, in astrocytes or in brain slices. **a** Cultured astrocytes (treated as described in Fig. 1) were incubated in DMEM for 20 min under control conditions (Cont) or in the presence of 100 μM GABA, with or without 15 min pre-treatment with the IP_3 receptor inhibitor xestospongin or the L-channel inhibitor nifedipine. **b** Brain slices were prepared from CD-1 mice and incubated in artificial cerebrospinal fluid (ACSF) for 5 h to recover their content of glycogen. Thereafter a brain slice from one hemisphere was maintained in ACSF under control conditions for another 20 min (□, Cont), whereas a slice from the other hemisphere was exposed for 20 min to 100 μM GABA in ACSF (■, GABA). After the experiment glycogenolysis was determined by measuring glucose content fluorometrically before and after breakdown of remaining glycogen in the cells. Average glycogen contents are indicated as percentages of those under control conditions. All values are expressed as means from three mice. *Statistically significant ($p < 0.05$) difference from those controls

Discussion

The concentration of glycogen in mammalian brain is generally reported to be ~3 μmol/g wet wt, but values up to 8 μmol/g can be measured in normal rat brain following gentle animal handling and non-destructive analysis [26]. In mammalian brain, glycogen is located almost exclusively in astrocytes [27], which constitute 20–25 % of normal brain cortical volume in both rat and man (reviewed in [28]). Accordingly the content of glycogen in astrocytes in the brain in vivo may be as high as ≥30 μmol/g wet wt. Consistent with this, incubation of cultured mouse astrocytes with 3 mM glucose leads, on the assumption of 200 μg protein per g wet wt [29] to a glycogen content of ~20 μmol/g wet in our cultured astrocytes [30]. In the present cultures, incubated with 7.5 mM glucose, the glycogen content was found to be 90 nmol per mg protein, which in a similar manner can be calculated to correspond to 18 μmol/g wet wt.

Although glycogenolysis can be fast both in intact brain tissue [4] and in astrocyte cultures [30, 31] a period of 20 min was used in order include effects of conditions, e.g., addition of a low extracellular K^+ concentration with less pronounced

effect [4]. The different efficacy of 10 and 45 mM K^+ was also evident in the present study, but on the other hand the effect of the low concentration was sufficiently pronounced that inhibitor effects could be easily determined. It seems reasonable that a lower concentration of extracellular K^+ causes slower glycogenolysis than a higher concentration, since the glycogenolysis-requiring the K^+ uptake pathways [1] must be activated to a lesser extent. The higher but not the lower K^+ concentrations also causes a glycogenolysis-dependent release of ATP from astrocytes [32]. The higher concentration was inhibited by nifedipine-mediated blockade of L-channels and Ca^{2+} entry [15] and the lower by the IP_3 receptor antagonist xestospongine, pointing towards participation of a pathway initiated by endogenous ouabains [1] and this hypothesis was verified by canrenone inhibition. Xestospongine also abolished the glycogenolytic effect of GABA, but nifedipine had no effect. GABA-induced glycogenolysis, only briefly mentioned in ref. [20], was documented and confirmed in brain slices, and seems to be a new observation. It may be of special interest because astrocytes are now known to be both GABA-ceptive and GABA-ergic cells [33]. Benzodiazepines are known to increase K^+-induced glycogenolysis [31], and in ref [20] it had been suggested that benzodiazepines and $GABA_A$ agonists might exert anxiolytic effects secondary to facilitation of K^+-mediated glycogenolysis or to induction of glycogenolysis, respectively.

The importance of PKA activity and G_i function for the effect of isoproterenol was expected and is in agreement with other findings that an increase in $[Ca^{2+}]_i$ is essential for the glycogenolytic response. That inhibitors of EGF receptor function also appeared to have an effect on isoproterenol-induced glycogenolysis was unexpected, but confirmed by the large glycogenolytic effect of EGF itself. Nevertheless, besides having an immediate powerful and rapid [30] effect on glycogenolysis in astrocytes isoproterenol also exerts an effect due to EGF release, that probably is slightly delayed and increases with time. The former effect is crucial for glutamate formation in day-old chicken, required during learning [34, 35], for glutamate formation in cultured astrocytes [36] and for astrocytic accumulation of K^+ [1]. These processes are essential during normal brain function and require fast and appropriately triggered and regulated glycogenolysis. This is in agreement with the pioneering observation by Swanson et al. [37] that whisker stimulation leads to glycogenolysis. A few years later it was suggested that stress may induce glycogenolysis in astrocytes via transmitter- or hormone-mediated processes [38]. The role of the delayed glycogenolysis in response to EGF is presently unknown. When the response to isoproterenol is determined experimentally, its role may be misjudged because of glycogen depletion by the fast preceding response to isoproterenol itself, and it would be of interest to perform time courses using lower concentrations of isoproterenol. Delayed glycogenolytic effects are also seen in response to other transmitters, e.g., ATP, where it may at least partly be a response to arachidonic acid or its metabolite(s) [39], including prostaglandins, which have glycogenolytic effect in liver cells [40]. That the effect of EGF was unaffected by BAPTA-AM does not indicate Ca^{2+}-independence, since EGF in another cell type is known to increase Ca^{2+} influx through TRPC1, a component of store-operated Ca^{2+} channels (Socs) [41]. Very recent studies have also shown that SOC operation is astrocytes stimulates glycogenolysis [42]. The conclusion in ref. [7] that cAMP alone can activate glycogenolysis without any increase in $[Ca^{2+}]_i$ may be due to a low glycogen content in the slices used (all data for glycogenolysis are given as percentages without absolute values) after only 1 h of incubation. In contrast, we used 5 h to secure glycogen recovery. A low content of glycogen might prevent pathways activated by small increases in extracellular K^+ concentration [1] to increase $[Ca^{2+}]_i$ measurably. Moreover, the actual increase in cAMP measured in the study was very small.

Concluding remarks

The days are gone when the primary role of astrocytic glycogenolysis was regarded to be that of an emergency energy reservoir, although such a role can come into play when energy metabolism is stressed or other substrates are lacking [43]. The present study has shown that glycogenolysis can be elicited by a number of different compounds. This does not necessarily mean that all of them exert a direct stimulation of glycogen phosphorylase, because stimulation of processes requiring glycogenolysis can also stimulate glycogen breakdown.

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Conflict of interest None.

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