Glutamate-induced calcium increase in myotubes depends on up-regulation of a sodium-dependent transporter

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Abstract **We report a study on the regulation by 2-chloro adenosine (2CA) of a glutamate (Glu) transporter in myogenic C2C12 cells. Long-term 2CA exposition significantly increased the V_{max} of the Glu transporter. Moreover, 2CA-treated cells responded to Glu challenge by a rapid and transient increase in their intracellular calcium level. The above reported effects were totally abolished by treating C2C12 cells with the Na^+-dependent Glu transporter inhibitors DL-threo-b-hydroxyaspartic acid and L-*trans*-pyrrolidine-2,4-dicarboxylic acid. We propose that the possible link between the Glu uptake increase and the Glu induction of calcium rise could be the depolarizing currents carried by Na^+ coupled with transporter activity.** **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Myogenic cell; Adenosine; Glutamate transporter; Calcium

1. Introduction

The metabolically stable adenosine analogue 2-chloro adenosine (2CA) is able to induce, in the myogenic cell line C2C12, severe changes in cell morphology and eventually cell death [1] through a strategy not mediated by purinergic receptors [2]. We have recently demonstrated that 2CA sensitizes C2C12 cells to the excitatory amino acid glutamate (Glu) that increases the intracellular level of calcium [3]. The data reported in our previous paper ruled out an involvement of the main Glu receptor subtypes, in the 2CA-evoked response to Glu [3].

To elucidate the nature of the element responsive to Glu we carried out a study in C2C12 myogenic cells taking into account the possibility that this element, induced by 2CA, could be a Glu transporter. In fact, the Glu uptake is paralleled by an influx of Na^+ ions into the cells that might activate a reverse Na^+–Ca^{2+} exchange, that may in turn trigger Ca^{2+} release from internal stores, as described in other muscle cells [4].

Glu transport into and out of cells is an essential part of intra- and extracellular Glu homeostasis. This amino acid plays a fundamental role in a variety of functions including protein synthesis, production of energy metabolism, ammonia fixation, glutathione synthesis and nerve transmission [5]. Altered Glu metabolism and/or decreased Glu uptake by skeletal muscle have been observed in several pathologies, such as malignant diseases [6], emphysema [7]. In mammalian cells, the uptake of Glu is mediated by specialized energy-dependent and/or passive transporters, most of which are coupled to the cotransport of Na^+ or to countertransport of K^+ [5]. The best characterized sodium-dependent Glu transporters, belonging to the system XAG activity, are the excitatory amino acid transporters (EAATs). The four cloned transporters (EAAC1, GLT-1, GLAST, EAAT4) have been found mainly in the brain where they represent the principal factor that allows the termination of Glu-mediated signal transmission. Recently, sodium-dependent Glu uptake systems have been identified in several cells and tissues such as fibroblasts [8], epithelial cells [9], macrophages [10], heart [11], placenta [12] and in skeletal muscle as well [13,14]. Despite the limited data available on Na^+-dependent Glu transporters in skeletal muscle, the presence of these systems was demonstrated in different models both in vivo [13,14] and in vitro [15]. These transporters, although not well-characterized, share features with Na^+/K^+-coupled Glu transporter belonging to EAAC1 type [15]. The principal difference between EACC1 and skeletal muscle Glu transporters resides in the different K_m shown by the two carriers (EACC1, K_m = 5–20 μmol; skeletal transporters, K_m = 500–700 μmol). The existence of low affinity Na^+-coupled Glu uptake has been observed in a cloned member of the Glu transporter family ASCT2 that shows a K_m = 1.6 mM. Interestingly, the molecularly well-characterized ECC1 present in the epithelial cells line CaCo-2 shows a K_m around 120 μM [9], a value in between the low and high affinity values displayed by brain and muscle, respectively.

In this paper, we report the effect of a prolonged exposure of differentiated myocytes to 2CA on the extracellular Glu-induced intracellular Ca^{2+} response and propose a role for purines also in muscle cells.

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Abbreviations: 2CA, 2-chloro-adenosine; Glu, glutamate; PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; THA, DL-threo-b-hydroxy-aspartic acid; EAATs, excitatory amino acid transporters

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2. Materials and methods

2.1. Cell cultures and myotube differentiation

C2C12 myoblasts (American Type Culture Collection, ATCC no. CRL-1772) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Experiments were performed on cells with no more than 20 passages in culture (passage 0 corresponding to that provided by the cell supplier). Myoblasts were seeded at the concentration of 5×10^5/ml in 30 mm Petri dishes and cultivated for 48 h to reach the confluent state. Thereafter, cells were incubated in DMEM supplemented with 2% horse serum (Gibco) (differentiating medium) for a further 48 h to induce cell differentiation. At this time point, well-organized myotubes were observed and differentiation was assessed by analysis of myosin heavy chain (MHC) expression, as described below. All experiments were carried out with cells grown for 48 h in differentiating medium and bearing the features above defined.

2.2. Drugs and cell treatments

After 48 h in differentiating medium, myotubes were exposed to 100 μM 2CA for a further 48 h (96 h total time in differentiating medium). Time of exposure and 2CA concentration were chosen on the basis of previous studies on this adenosine analogue [1,2]. Following addition of Glu (Research Biochemicals International) to both control and 2CA-treated cells the intracellular calcium level was evaluated as described in Section 2.3.

To inhibit protein synthesis, myocells treated with 2CA for 30 h were challenged with 5 μg/ml cycloheximide (CHX) (Sigma) for 18 h in the presence of 2CA (altogether 48 h of 2CA treatment). A specific adenosine kinase inhibitor, 5-iodotubercidin (Sigma), was added (10 nM) to cells 30 min before addition of 2CA and maintained in the medium along the experiment. The Glu transporter inhibitors used were: L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC; Sigma), DL-threo-b-hydroxyaspartic acid (THA; Sigma). Both were applied to the cells by dropping in the bath solution immediately before challenge with Glu.

The depletion of internal calcium stores was obtained by challenging 2CA-treated cells with 100 μM thapsigargin (Sigma) 10 min before addition of 1 mM Glu. In experiments in Na^+-free medium, choline chloride (Sigma) (130 mM) substituted NaCl.

2.3. Intracellular calcium measurement

Optical fluorimetric recordings with Fura 2AM (Molecular Probe) were utilized to evaluate the intracellular calcium concentration ($[Ca^{2+}]_i$). The bath solution contained (mM): NaCl 125, KCl 1, $CaCl_2$ 5, $MgCl_2$ 1, HEPES 20, pH 7.35. Cells were bathed for 60 min at room temperature with 5 μg of Fura 2AM solved in 5 μl of a solution 75% DMSO+25% pluronic acid (Molecular Probe) in 1 ml of bath solution, at a final concentration of 5 μM. This solution was then removed, replaced with bath solution, and the dishes were quickly placed on the microscope plate. Glu (1 mM) was applied by directly dropping in the bath.

To measure fluorescence changes, an Hamamatsu Argus 50 computerized analysis system was used, recording each 6 s the ratio between the values of light intensity at 340 and 380 nm stimulation.

2.4. Immunocytochemistry

C2C12 cells, grown as described above, were fixed with ethanol (70%) or methanol (70%) at room temperature before incubation with anti-MHC (Sigma). Immunocytochemistry analysis was performed using Dako EnVision System Peroxidase (AEC) (Dako). Finally, coverslips were mounted and examined with a phase contrast microscope.

2.5. Glu uptake determination

Cells for Glu uptake determination were seeded (5×10^4 cells/plate) in 24-well plates and grown as described above. All experiments were carried out at 37°C. After incubation for 48 h in DM, myotubes were treated for 48 h with 100 μM 2CA. Before the experiments, cells were washed with phosphate saline buffer and incubated in an uptake medium (137 mM NaCl, 0.7 mM K_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.4) for 10 min. The medium was aspirated and replaced with 250 μl of the same buffer containing 0.025–3.2 mM L-Glu (Sigma) trace-labeled with 25 μCi of [U-^{14}C]Glu (≥250 mCi/mmol, Amersham). After 15 min, the medium was removed, cells were rapidly washed twice, then lysed with 300 μl of 20 mM NaOH in SDS 0.1%. The samples (250 μl) were transferred to vials containing liquid scintillation fluid (Optifluor – LKB) and the retained radioactivity was quantified by a liquid scintillation counter (LKB 1500). Glu uptake was expressed as picomoles of Glu per milligram of protein per minute. The protein content of 50 μl of cell lysate was determined according to Lowry [16].

Initial assays confirmed that the uptake was for 95% Na^+-dependent (by utilizing Na^+-free medium choline-substituted buffer) and linear for at least 20 min with respect to time and protein content.

3. Results

3.1. 2CA induces morphological changes and a calcium response to Glu in C2C12 myotubes

2CA induced a dramatic alteration of the differentiation phenotype in cultured C2C12 muscle cells. We used C2C12 cells maintained in differentiating medium for 48 h until the formation of myotubes and further grown for 48 h in differentiating medium either in the presence or in the absence of

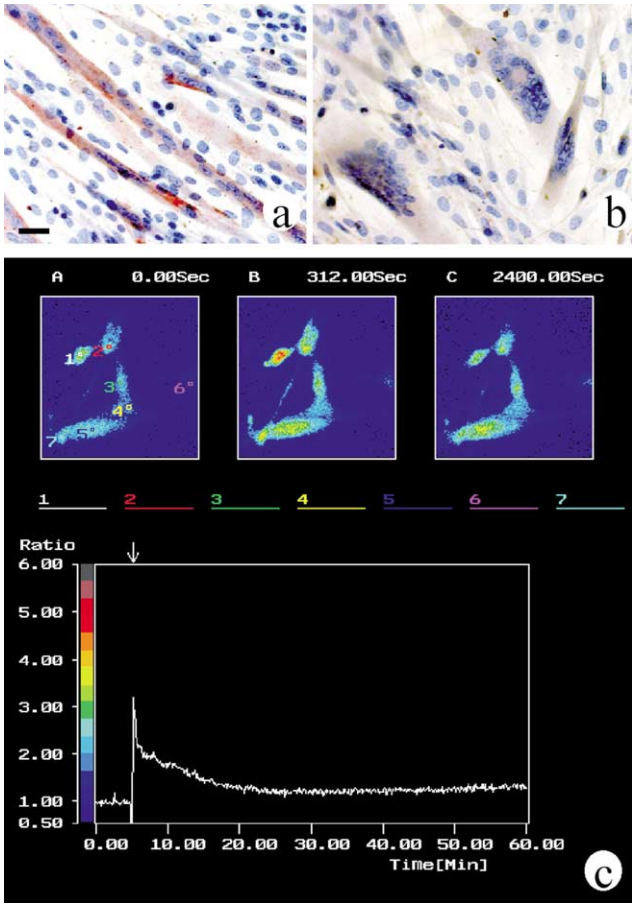

Fig. 1. 2CA induces alterations of myotube phenotype and a Glu-evoked calcium response in C2C12 myotubes. a: After 96 h in DMEM with 2% horse serum, C2C12 showed the typical features of differentiated myotubes. b: 2CA exposure provokes a dramatic alteration of the myotube morphology with the formation of enlarged structures. c: Addition of Glu (1 mM) (white arrow) induces a fast transient calcium response in 2CA-treated cells (graph). The scale on the left indicates the values of ratio (340/380) and the equivalent pseudo-color. The small pictures (A, B and C) represent the selected cells at three different times (ca. 0, 5 and 40 min) and the amount of calcium loading (pseudo-color). Scale bar: 10 μM.

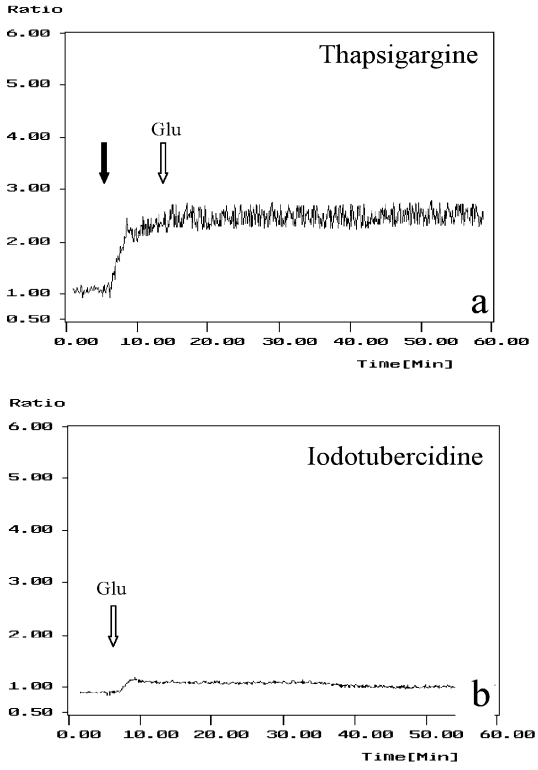

Fig. 2. Glu mobilizes calcium from intracellular stores and its activity is mediated by adenosine kinase activity in 2CA-treated C2C12 cells. a: Depletion of internal calcium stores by 100 μM thapsigargin (black arrow) induces cytosolic calcium rise. The subsequent application of Glu (white arrow) fails in provoking an additional $[Ca^{2+}]_i$ increase. b: Pretreatment of cells with 10 nM 5-iodotubercidin, a potent inhibitor of adenosine uptake into brain, before application of 2CA for 48 h, significantly prevents Glu-induced calcium increase. White arrow: Glu application.

2CA. As shown in Fig. 1, while C2C12 control cells maintained in the DM for 96 h formed multinucleated myotubes displaying aligned nuclei (Fig. 1a), 100 μM 2CA treatment profoundly altered the myotube morphology. In fact, the cells lost the phenotype of differentiated myotubes and formed enlarged structures containing randomly distributed nuclei (Fig. 1b).

As previously reported [3], exposure to 2CA for 48 h rendered differentiated myocells sensitive to the excitatory amino acid Glu (Fig. 1c). The application of 1 mM Glu, in fact, evoked a powerful, fast and transient intracellular calcium increase in 95% of cells. The number of Glu-responsive cells dramatically decreased to 24% at a Glu concentration of 750 μM. Lower Glu concentrations (starting from 500 μM to below) were totally ineffective in stimulating a calcium response by 2CA-treated cells (data not shown).

3.2. Glu-evoked calcium increase depends on the outflow of calcium from the intracellular calcium stores

To define whether intracellular calcium increase was due to an influx from the external medium or to a depletion of intracellular calcium stores, we first substituted the calcium in the medium with barium or alternatively added EGTA directly to the medium. In both cases, however, 2CA-treated cells rapidly detached from the substrate because of the loss of extracellular calcium, therefore impairing the possibility to properly perform the experiments (data not shown). Therefore, we utilized thapsigargin, the most widely used inhibitor of the ubiquitous sarco-endoplasmic reticulum Ca^{2+}-ATPases in mammalian cells [17] and we monitored the exocytosis of calcium from intracellular stores in myotubes exposed to 2CA for 48 h (Fig. 2). Further application of Glu failed in increasing $[Ca^{2+}]_i$, suggesting that only calcium from intracellular stores was involved.

As previously reported [2], 2CA treatment induces apoptotic cell death in myotubes, and cytotoxicity depends on an intracellular phosphorylation/activation of 2CA. In this study, we observed that a phosphorylation/activation of 2CA is also involved in the increase of $[Ca^{2+}]_i$ since when the adenosine kinase inhibitor 5-Itub (10 nM) was applied before and during the 48 h lasting treatment with 2CA, the Glu-induced calcium increase was significantly prevented (Fig. 2b).

3.3. A Na^+-dependent transporter is necessary for Glu-dependent calcium increase in differentiated C2C12 cells

The Glu-evoked calcium increase in C2C12 cells required the presence of Na^+ in the extracellular medium (Fig. 3). Cells pretreated with 2CA for 48 h were challenged with 1 mM Glu and $[Ca^{2+}]_i$ increase was monitored by changes of Fura fluorescence values in a medium containing 140 mM of Na^+ or an equimolar content of choline (Fig. 3a,b). The cells responded to Glu with a massive increase of $[Ca^{2+}]_i$, only when Na^+ was present in the medium (Fig. 3a). Otherwise, a negligible effect was recorded when Na^+ was substituted by choline (Fig. 3b).

Considering that Glu-induced calcium increase in C2C12 cells is not inhibited by any of the Glu receptor antagonists used [3] and that Na^+ is an essential component of the response to Glu, we have hypothesized that the responsive element to Glu could be a Na^+/Glu synport system. In order to verify this hypothesis, we inhibited the Na^+/Glu cotransporter by using the specific inhibitors PDC and THA. Both compounds totally inhibited the Glu-induced $[Ca^{2+}]_i$ rise at 1 mM concentration (Fig. 3c,d). PDC exerted a dose-dependent inhibitory activity in a concentration frame ranging from 1000 to 250 μM, showing an IC_{50} of about 500 μM. In contrast, THA retained its inhibitory activity at all the concentrations tested (from 1000 to 250 μM) (data not shown).

3.4. Sensitivity of differentiated C2C12 cells to Glu is dependent on the synthesis of new proteins

Na^+/Glu transporter activity is finely regulated in the cells by both translational and/or post-translational activity. To clarify the level of the induction by 2CA of the element responsive to Glu we blocked protein synthesis by CHX. C2C12 cells pre-incubated with 100 μM 2CA in the presence of 5 μg/ml CHX for 18 h did not respond to challenge of Glu by increasing their calcium concentrations (data not shown). These data are indicative for a 2CA-induced neosynthesis of the element responsive to Glu.

3.5. Effect of 2CA on Glu transport in C2C12 cells

The data obtained by monitoring the $[Ca^{2+}]_i$ increase were confirmed by experiments carried out with radioactively labeled Glu (Fig. 4). To determine whether the K_m or V_{max} of the Glu transporter were affected by 2CA, C2C12 cells were treated with 2CA and the kinetics of Glu uptake from cells were measured. The V_{max} of Glu uptake was significantly increased after 48 h of treatment with 100 μM 2CA, from

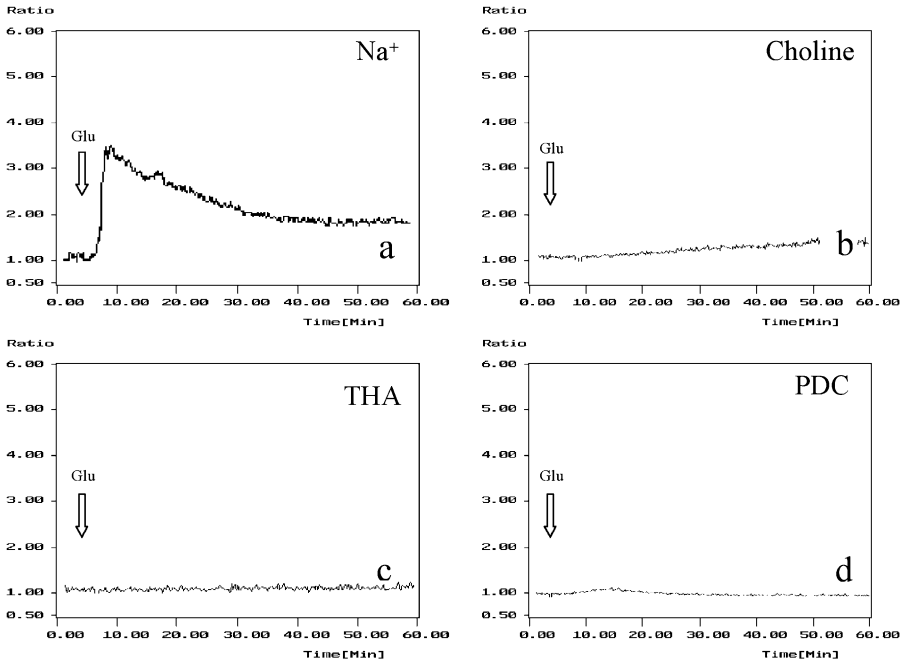

Fig. 3. Effect of Glu transporter inhibition on Glu-induced calcium increase in 2CA-treated C2C12 cells. Addition of Glu (1 mM) (white arrows) induces a fast transient calcium response in 2CA-treated cells, when Na^+ ions are present in the bath solution. b: When Na^+ is substituted with 130 mM choline, Glu fails in inducing $[Ca^{2+}]_i$ increase. c, d: Pretreatment of cells with the Na^+/Glu cotransporter inhibitors THA (c) or PDC (d) prevents the Glu-induced $[Ca^{2+}]_i$ rise.

1.72 ± 0.32 nmol/min/mg cell protein in the control to 3.45 ± 0.28 nmol/min/mg cell protein in 2CA-treated cells. However, the K_m for Glu uptake (640 ± 170 μM) in sensitized cells was not significantly different from control values (590 ± 200 μM). A percentage of about 5% of the total Glu uptake was not dependent on Na^+, as demonstrated by experiments carried out in choline buffer. The Na^+-independent Glu uptake was not influenced by cell incubation with 2CA (data not shown).

Our data of kinetic values of Ca^{2+} transporter in myogenic cells are in good agreement with those previously reported [15].

4. Discussion

The uptake of Glu from the extracellular media mainly depends on ion-coupled transporters. Energy for this process is provided by the electrochemical potentials of Na^+ and the process is electrogenic [18]. It is therefore difficult to determine, solely on the basis of functional experiments, which transporters are responsible for Glu uptake in C2C12 cells. However, the requirement of sodium in the transport process may indicate that the Glu uptake occurs through the widespread XAG system. The main disagreement between Glu transport system in myotubes with respect to the XAG system concerns the lower affinity of the Glu transporter in C2C12 myotubes. Notably, Low and coworker hypothesized the existence of this transport system also in rat myotubes in culture [15].

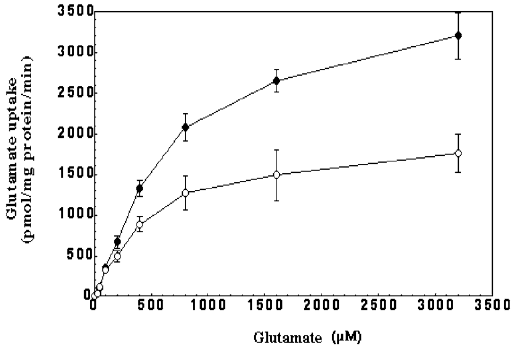

Fig. 4. Exposure to 2CA increases Glu uptake in C2C12 cells. Glu uptake was measured in myotubes growth in the absence (○) or presence (●) of 100 μM 2CA. Representative values (mean ± S.E.M.) from a single preparation with individual points measured in triplicate.

Moreover, a depolarizing current related to the transport activity induced by the flux of Na^+ into the cell ('transport-associated current') has been observed in several cells, such as glia [19], isolated salamander Muller cells [20] and Purkinje cells [21]. We could argue that the $[Ca^{2+}]$ rise is a result of the current flux induced by Na^+ entry. Although the movement of ions (mainly protons and K^+) has been described in different cells as a response to transporters' activity [22], no evidence has so far been reported that calcium ions are involved in Glu transport. We propose that in differentiated C2C12 cells, the Glu uptake-associated current could be sufficient to open a 'limited' number of voltage-activated calcium channels. The calcium entry triggers the opening of calcium channel of the endoplasmic reticulum and, finally, a massive increase of the intracellular level of the ions. An alternative interpretation of our data could be that the increase of Na^+ entry induces a reverse Na^+–Ca^{2+} exchange and finally triggers Ca^{2+} release from internal stores. Experiment carried out in rabbit ventricular myocytes demonstrated that reversion of the Na^+–Ca^{2+} exchange can function as a trigger promoting Ca^{2+} release from sarcoplasmic reticulum [23].

Different evidences support our statement of a link between Glu transport activity increase and Glu-induced Ca^{2+} increase. First, a relevant percentage of free cytosolic Ca^{2+} that increases after Glu addition to the cells derives from the endoplasmic reticulum storage as demonstrated by thapsigargin experiments. A possible link between the current flowing through the opening of C2C12 pores and the $[Ca^{2+}]_i$ increase has been proposed by Grassi and coworkers [24]. Second, the Glu-induced $[Ca^{2+}]$ increase seems to follow an 'all or none' mechanism. In fact, a concentration of Glu less than 1 mM did not induce any change in the calcium entry. A possible explanation is that a 'threshold' current is necessary to induce the opening of endoplasmic Ca^{2+} channels, which respond synchronously to the voltage change. Notably, both inhibitors used in this study (*t*-PDC and THA) induce in mouse astrocytes an increase in Na^+ inward current that is comparable in shape to the response to Glu-induced Na^+ inward current but is about two-fold lower in amplitude [25]. However, both inhibitors were able to block the Glu action on C2C12 cells, a result that can be explained only by assuming the existence of a threshold current. Third, the data on Glu uptake and Glu-induced calcium increase show compatible time-courses and dose-responses. It is worth noting that the results obtained by monitoring a restricted number of cells (fluorescence method) were totally overlapped by those gained examining the whole cell population (Glu uptake). This confirms the link between Glu uptake and Glu-induced calcium increase.

The inhibitory effect of CHX on the 2CA stimulation and the apparent increase of V_{max} of Glu transporters are both consistent with the neo synthesis of 'new' Glu transporters. The increase in V_{max} of Glu uptake (see Section 3) might be caused by the insertion of transporters into the plasma membrane [26] or by newly synthesized transporter molecules [27]. We did not observe any changes neither in the Na^+-dependent Glu uptake and the Glu-induced Ca^{2+} increase after 24 h of C2C12 challenge with 2CA. This increase in Glu uptake suggested synthesis of new proteins. The modulation of Glu uptake by differential expression of EAATs is a well-studied topic in many cells and tissues [5]. Probably, the most important factor for the induction of glial GLAST is the activation of a Glu receptor [28]. However, the expression of other Glu transporters, such as GLT from astrocytes, is not up-regulated by Glu but rather by chronic treatment with growth factors [29]. In peripheral tissues, Rimaniol et al. recently demonstrated that inflammatory molecules, such as tumor necrosis factor-α, can stimulate the expression of members of the EATTS family [30]. On the basis of our data we cannot rule out the idea that the up-regulation of Glu uptake after 2CA administration could depend on other newly synthesized proteins other than Glu transporters. In fact, the mechanism of membrane insertion of a cytosolic pool of transporters is in itself a complex and sophisticated mechanism, which involves a transduction system not yet well-defined. Thus, these regulatory proteins might be the target of 2CA. Interestingly, Low and coworkers demonstrated in cultured rat myotubes the existence of adaptive up-regulation of Glu transporter in response to glutamine deprivation. In Low's paper it is not clear whether the up-regulation of Glu transporter is dependent on a stress situation or on a more finely regulated mechanism.

Taking into account the physiological relevance of Glu uptake in muscle cells, a more detailed study on the molecular nature of the 2CA-inducible Glu-responsive element is mandatory. The up-regulation of Glu transport might represent the target mechanism for a pharmacological approach to certain muscular disorders.

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