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Na^+ entry via glutamate transporter activates the reverse Na^+/Ca^{2+} exchange and triggers Ca_i^{2+}-induced Ca^{2+} release in rat cerebellar Type-1 astrocytes

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Abstract

We have previously demonstrated that rat cerebellar Type-1 astrocytes express a very active genistein sensitive Na^+/Ca^{2+} exchanger, which accounts for most of the total plasma membrane Ca^{2+} fluxes and for the clearance of Ca_i^{2+} loads induced by physiological agonists. In this work, we have explored the mechanism by which the reverse Na^+/Ca^{2+} exchange is involved in agonist-induced Ca^{2+} signaling in rat cerebellar astrocytes. Microspectrofluorometric measurements of Ca_i^{2+} with Fluo-3 demonstrate that the Ca_i^{2+} signals associated long (> 20 s) periods of reverse operation of the Na^+/Ca^{2+} exchange are amplified by a mechanism compatible with calcium-calcium release, while those associated with short (< 20 s) pulses are not amplified. This was confirmed by pharmacological experiments using ryanodine receptors agonist (4-chloro-*m*-cresol) and the endoplasmic reticulum ATPase inhibitor (thapsigargin). Confocal microscopy demonstrates a high co-localization of immunofluorescent labeled Na^+/Ca^{2+} exchanger and RyRs. Low (< 50 μmol/L) or high (> 500 μmol/L) concentrations of L-glutamate (L-Glu) or L-aspartate causes a rise in Ca_i^{2+} which is completely blocked by the Na^+/Ca^{2+} exchange inhibitors KB-R7943 and SEA0400. The most important novel finding presented in this work is that L-Glu activates the reverse mode of the Na^+/Ca^{2+} exchange by inducing Na^+ entry through the electrogenic Na^+–Glu-co-transporter and not through the ionophoric L-Glu receptors, as confirmed by pharmacological experiments with specific blockers of the ionophoric L-Glu receptors and the electrogenic Glu transporter.

Keywords: Calcium-induced calcium release, glutamate, glutamate transporter, Na^+/Ca^{2+} exchange.

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The Na^+/Ca^{2+} exchanger, a counter-transport system working in concert with the plasma membrane Ca^{2+} pump, Ca^{2+} channels, and intracellular Ca^{2+} store system plays a critical role in the control of intracellular calcium. In its forward mode (Ca^{2+} efflux), the exchanger has an important physiological role for the rapid extrusion of large amounts of Ca^{2+} from the cell and the replenishment of intracellular Ca^{2+} stores (Blaustein and Lederer 1999). On the other hand, the physiological role of the reverse mode of the exchanger, (Ca^{2+} entry), is still controversial (Blaustein and Lederer 1999).

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Abbreviations used: 2-APB, 2-aminoethoxydiphenyl borate; CICR, calcium-induced calcium release; CMC, 4-chloro-*m*-cresol; ER, endoplasmic reticulum; GFAP, glial fibrillary acidic protein; RyRs, ryanodine receptors; SOCC, store operated Ca^{2+} channels; Tg, thapsigargin.

Numerous molecular biology, immunocytochemical, and functional studies have revealed the presence of the heart isoform of the Na^+/Ca^{2+} exchanger in astrocyte cells (Goldman *et al.* 1994; Takuma *et al.* 1994; Holgado and Beauge 1995; Verkhratsky *et al.* 1998; Benz *et al.* 2004). In a previous study (Rojas *et al.* 2004), we demonstrated that cerebellar Type-1 astrocytes functionally express a highly dynamic Na^+/Ca^{2+} exchanger, which under normal physiological conditions (Ca^{2+} extrusion mode), accounts for both the resting $[Ca^{2+}]_i$ and the Ca^{2+} clearance that occurs during agonist activation. In addition, we have presented evidence indicating that in these cells, the balance of membrane calcium fluxes at rest involves a component of Ca^{2+} entry through the Na^+/Ca^{2+} exchanger (Rojas *et al.* 2004). Nevertheless, the role of this exchange mode in the astrocyte physiological function remains unknown.

In principle, there are two non-exclusive possibilities for the involvement of the reverse exchange in Ca_i^{2+} signaling in cerebellar Type-1 astrocytes: (i) Ca_i^{2+} entering by the reverse exchanger directly triggers calcium dependent processes and/or (ii) Ca_i^{2+} entering through the exchanger serves as messenger for a Ca_i^{2+} signal amplification through a Ca_i^{2+}-induced-Ca_i^{2+}-release (CICR) mechanism. In favor of the first possibility are the reports that L-glutamate (L-Glu), through activation of kainate receptor channels, leads to the influx of Na^+ ions, which activate the reverse Na^+/Ca^{2+} exchange, thus leading to $[Ca^{2+}]_i$ increase (Goldman *et al.* 1994; Takuma *et al.* 1994). More recently, a similar mechanism has been proposed to explain the glutamate-induced homocysteic acid release from cortical astrocytes (Benz *et al.* 2004). On the other hand, the existence and functional significance of CICR coupled to ryanodine receptors (RyRs) is well documented in astrocytes (Verkhratsky and Kettenmann 1994; Golovina and Blaustein 2000; Matyash *et al.* 2002; Beck *et al.* 2004; Aley *et al.* 2006). Nevertheless, the existence and functional relevance if any, of RyRs in cerebellar Type-1 astrocytes, have not been demonstrated.

The experiments reported here examine the role of Ca^{2+} entry by the reverse Na^+/Ca^{2+} exchange as a mechanism for inducing amplification of Ca_i^{2+} signals that occur during conditions of agonist activation. Using microspectrofluorometric measurements, pharmacological tools, immunofluorescence labeling, and confocal fluorescence microscopy we present, for the first time, evidences that in rat cerebellar Type 1-astrocytes: (i) Ca^{2+}entry during operation of reverse Na^+/Ca^{2+} markedly increase $[Ca^{2+}]_i$ by a CICR mechanism, followed by the opening of store operated Ca^{2+} channels (SOCC), (ii) immunofluorescence labeling of both Na^+/Ca^{2+} exchanger and RyRs using confocal microscopy demonstrate that they are highly co-localized and (iii) unexpectedly, physiological agonist concentrations of L-Glu increase $[Ca^{2+}]_i$ through activation of the reverse exchange as a result of Na^+ entry through the electrogenic glutamate transporter.

Materials and methods

Materials

All solutions were prepared with deionized ultrapure (18 MΩ) water, (Milli-Q; Millipore, Beadford, MA, USA). L-glutamate, L-aspartate (L-Asp), caffeine, ryanodine, thapsigargin (Tg), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, digitonin, dimethylsulfoxide, glucose, and HEPES were from Sigma Co. (St Louis, MO, USA); 4-chloro-*m*-cresol (4-CmC) was from Fluka Chemical Corp (RonKonKoma, NY, USA); 2-aminoethoxydiphenyl borate (2-APB) and ionomycin were from Calbiochem (La Jolla, CA, USA); Fluo-3/AM, Rhod-2/AM, Na^+-sensitive fluorescent probe SBF sodium probe/AM, goat anti-mouse IgM antibody, Na^+/Ca^{2+} exchange Alexa-Fluor 546, BODIPY-FL-ryanodine, and BODIPY-FL-Tg were from Molecular Probes (Eugene, OR, USA); canine cardiac Na^+/Ca^{2+} exchanger monoclonal antibody was from Affinity Bioreagents (Golden, CO, USA); NaCl, LiCl, KCl, $MgCl_2$, $MnCl_2$, and $CaCl_2$ were from Merck (Darmstadt, Germany); L-trans-2,4-pyrrolidine dicarboxylate was from Tocris Cookson (Ballwin, MO, USA), and 6-cyano-7-nitroquinoxaline-2,3-dione and D,L-2-amino-5-phosphonopentanoic acid were from A.G. Scientific (San Diego, CA, USA). 2-[4-[(2,5-Difluorophenyl)methoxy]phenoxy]-5-ethoxy-aniline (SEA0400) was synthesized and supplied by Taisho Pharmaceutical Co. Ltd (Saitana, Tokyo, Japan). 2-[2-[4-(nitrobenzilloxi-phenyl ethyl] isothiourea metanesulfonate. (KB-R7943) was obtained from EMD Biosciences, Inc. (San Diego, CA, USA). Fluorescent dyes and many reagents were dissolved in dimethylsulfoxide (0.1% final concentration).

Cell dissociation and identification of Type-1 astrocytes

Cells were dissociated following the method of Mintz and Bean (1993)modified as reported (Rojas *et al.* 2003). In summary, 5- to 8-day-old Sprague-Dawley rats were beheaded and their brains was extracted and immersed in a solution containing a high K^+/high Na^+ ratio (in mmol/L: Na_2SO_4, 81.4; K_2SO_4, 30; $MgCl_2$, 5.8; glucose, 20.4; Hepes, 10; phenol red, 0.5%; [pH 7.4] at 4°C). The cerebellum vermis was dissected and minced into small (2 mm) pieces, then immediately transferred to the same solution with 1 mg/mL of protease (type XIII, Sigma) at 35–37°C within 6 min. The pieces were washed thoroughly with the high K^+/high Na^+ solution to remove the protease and then it is transferred to a Na^+–0Ca solution (Methods: solutions and extracellular perfusion), containing trypsine inhibitor (1 mg/mL soybean Type 1-S; Sigma) and bovine serum albumin (1 mg/mL; Sigma). The tissue was dissociated by gentle trituration with a fire-polished pipette. Subsequently, the cells were centrifuged at 660 *g* for 4 min and washed with tyrode containing no Ca^{2+}. This last procedure was repeated thrice. Dissociated cells were resuspended in a Dulbeco's modified Eagle's medium culture medium (Gibco, BRL, Rockville, MD, USA). Cells (200 μL) were plated onto 22 × 40 mm glass coverslips (0.15 mm thickness; Warner Instruments Corp. Hamden, CT, USA) containing poly-L-lysine (100 μg/mL, #1524; Sigma) and placed in 35 mm diameter culture plates. The plates were covered with the Dulbeco's modified Eagle's medium containing 70 ng/mL of growth factor (mNGF

grade II; Alomone Labs, Jerusalem, Israel) at 37°C in a 5% CO_2 incubator until used (3–5 days). We have used four methods to distinguish Type-1 astrocytes from Purkinje cells and other glial cells present in our preparation: (i) morphologically, Type-1 protoplasmic astrocytes were morphologically recognized under phase contrast optics (Raff *et al.* 1983). They were phase-dark, with a flattened polygonal, epithelioid look. They could be distinguished from stellate cells with radially distributed fine processes, most probably Type-2 astrocytes, and also from fusiform cells; (ii) Immunologically, immunocytochemistry of glial cells was carried out using a monoclonal antibody anti-glial fibrillary acidic protein (anti-GFAP; Bignami and Dahl 1977) and the monoclonal antibody anti-sodium/calcium exchanger (Frank *et al.* 1992; Affinity BioReagents Inc., Golden, CO, USA). Cells were washed thrice with a phosphate-buffered saline solution at pH 7.4 and permeabilized with methanol at −20°C for 5 min, fixed with 2% glutaraldehyde in phosphate-buffered saline for 15 min, and then treated with 2% bovine serum albumin for 15 min. Different preparations were exposed to the monoclonal antibodies, anti-GFAP (1 : 1000) and anti-sodium/calcium exchanger (1 : 100), for 60 and 240 min, respectively. Finally, the cells were incubated for 30 min at room temperature (22°C) with FITC conjugated anti-rabbit IgG or the anti-rabbit IgG conjugated to Alexa-Fluo 546 (1 : 50) for 1 h at room temperature (22°C) (1.5 μg/mL; Molecular Probes). The Fluorescence of GFAP-immunoreactive astrocytes was examined using an epifluorescence microscope. This procedure was repeated on culture days 1, 3, 5, 10, 14, and 21. More than 95% of all Type-1 astrocytes that were identified by morphological procedures gave positive immunological, pharmacological, and functional responses (see below). Cellular viability was measured the same days as the GFAP test using a commercial market kit: calcein-AM and ethidium homodimer-1 (kit viability/cytotoxicity; Molecular Probes). More than 80% of the cells in our primary co-culture were viable; (iii) pharmacologically, more than 95% of the cells morphologicaly identified as Type-1 astrocytes, responded to nanomolar concentrations of ET-3 with a fast transient rise in $[Ca^{2+}]_i$ followed by a plateau higher than the resting fluorescence (Supattapone *et al.* 1989; Rojas *et al.* 2004). Other glial cells with the morphological appearance of Type-2 astrocytes or oligodendrocytes did not respond to this agonist (Rojas *et al.* 2004); and (iv) functionally, in this work, we have added a new criterion for identifying a given glial cell as a Type-1 astrocyte; that is, the ability of these glial cells to respond with a rise in $[Ca^{2+}]_i$ upon activation of the reverse Na^+/Ca^{2+} exchange (increase in intracellular Ca_i^{2+} after removal of external Na^+ in the presence of external Ca^{2+}). In 45 cells with the morphological appearance of typical Type-1 astrocytes (from two different cultures, 4 days old) we found that 95% of them responded to short (< 20 s) pulses of 0Na,Ca solution with an increase in the Ca^{2+} dependent Fluo-3 signal, which was completely reversible upon re-addition of external Na^+. Furthermore, in all cells that gave positive responses to 0Na,Ca medium, addition of 30 nmol/L endothelin-3 caused the typical biphasic response in Ca_i^{2+}.Therefore, all cells used in the present study responded with an increase in $[Ca^{2+}]_i$ upon activation of the reverse Na^+/Ca^{2+} exchange.

Intracellular Ca^{2+} and Na^+ measurements

Measurement of $[Ca^{2+}]_i$ was carried out in Type-1 astrocytes, loaded for 45 min (20–22°C) with the fluorescent dye Fluo-3/AM dissolved in standard tyrode solution to a final concentration of 8 μmol/L. Measurement of the Na_i^+-dependent fluorescent signals was achieved with the Na^+ fluorescent probe SBFI sodium probe by pre-incubating the cells in standard tyrode solution with SBFI/AM (20 μmol/L) for 90 min (20–22°C). A three-point *in vitro* calibration of $[Na^+]_i$ was carried out at the end of the experiments. Because of the fact that Fluo-3 is a non-ratiometric dye, we chose the ionomycin-heavy metal (Mn^{2+})-digitonin method used by Kao *et al.* (1989) for calibrating the Fluo-3 signal in some cells (see Fig. 2b and Rojas *et al.* 2003). Coverslips with the loaded cells were placed in an open experimental chamber (RC-27; Warner Instruments Corp. Hamden, CT, USA), mounted on a T300 Nikon (Tokyo, Japan) inverted microscope connected to a fluorescence imaging apparatus (IonOptix, Co., Milton, MA, USA). The experimental procedure for measuring $[Ca^{2+}]_i$ in single cerebellar cells under temperature-controlled conditions has been previously described (Rojas *et al.* 2003). The fluorescence values reported in these experiments were normalized against the resting fluorescence ($\Delta F/F_0$). In most of the experiments reported here, raw fluorescence data show very little (less than 10%) bleaching of the signal over the course of 10 min recording. All calculations and graphics were carried out with Origin (Microcal Software Inc. MA, USA) and SigmaPlot (SPSS Inc. Chicago, IL, USA). Experimental values are given as mean ± SE.

Solutions and extracellular perfusion

The experimental chamber was superfused with a peristaltic pump at a rate of about 1 mL/min (36°C) with standard tyrode solution (control solution) containing (mmol/L): NaCl, 145; KCl, 4; $MgCl_2$, 2; $CaCl_2$, 2; glucose, 10; and HEPES, 10 (pH 7.4). Osmolarity was adjusted to 296 ± 1.5 mOsm. The control solution is identified as Na,Ca and test solutions as: 0Na,Ca and Na, 0Ca. Of these, the first (0Na,Ca) was prepared substituting Li^+ ions for Na^+, as the Na^+/Ca^{2+} exchanger under most physiological conditions does not transport Li^+, while the second (Na,0Ca) contained no added calcium plus 100 μmol/L EGTA. The control and test solutions were delivered close to the cells (200 μm) via a temperature-controlled pressure ejection from a 200 μm i.d. carbonated pipette, as previously described (Rojas *et al.* 2003). As Na^+/Ca^{2+} exchanger in astrocytes has high temperature sensitivity, most experiments were carried out at 35–37°C. The cells were continuously superfused with tyrode solution at 37°C at a rate of 1 mL/min.

Detection of subcellular Ca^{2+} signal by laser-scanning confocal microscope

Subcellular $[Ca^{2+}]$ was monitored in individual cells by using time-scan confocal microscopy. Type-1 astrocytes were incubated with Rhod-2/AM (10 μmol/L) for 50 min at 37°C in Tyrode solution, washed, and then incubated for an additional 10 min at 37°C with BODIPY-FL-ryanodine (500 nmol/L) or BODIPY-FL-Tg (500 nmol/L). The coverslip with the dual-loaded cells were placed in a superfusion open chamber on the laser-scanning confocal microscope stage (Nikon C1), mounted on an Eclipse TE-300 Nikon inverted microscope equipped with a Nikon 100/1.30 oil Ph4L oil-immersion objective coupled to a C1-LU2 unit with Neon (543 nm) and Argon cooled air (488 nm) lasers. These laser units were controlled by a D-eclipse C1 interface.

Results

Effect of short- and long-term pulses of free extracellular Na^+ solutions on the Ca_i^{2+} dependent Fluo-3 signal

Previous results from our laboratory indicate that manipulations of the Na^+ electrochemical gradient (decrease in extracellular or increase in intracellular Na^+) cause profound changes in the resting $[Ca^{2+}]_i$ in cerebellar Type-1 astrocytes (Rojas *et al.* 2004). In order to determine how the amount of Ca^{2+} entering through the reverse Na^+/Ca^{2+} exchanger affects the levels of $[Ca^{2+}]_i$, we exposed Type-1 astrocytes to 0Na,Ca solutions for short (< 20 s) or long (> 40 s) periods. Figure 1a shows a representative run in which three consecutive short (20 s) exposures to a Na^+-free solution caused changes in the $[Ca^{2+}]_i$ which rapidly returned to its resting level upon readmission of extracellular Na^+. This behavior was observed in 92% (n = 120) of the cells studied. Figure 1b shows a run in which a cell was exposed for 70 s to the 0Na,Ca solution, producing a much larger $[Ca^{2+}]_i$, increase. Readmission of external Na^+ in the presence of external Ca^{2+} (Na,Ca medium), caused the $[Ca^{2+}]_i$ to drop to a sloping plateau; $[Ca^{2+}]_i$ rapidly reached its base line value upon superfusing the cell with a Na^+-containing Ca^{2+}-free medium (Na,0Ca). These experiments suggest that in this preparation, substantial Ca^{2+} entry through the reverse exchange may activate the release of Ca^{2+} from intracellular Ca^{2+} stores, and therefore cause the opening of the SOCC. This possibility was further tested in the run of Fig. 1c. Also in this case, a robust Ca_i^{2+} increase was elicited by withdrawal of external Na^+ (30 s pulse). Readmission of Na^+ in the external medium causes a rapid drop to a new sloping level. Application of 80 μmol/L of 2-APB, an inhibitor of the SOCC (Van Rossom *et al.* 2000), brings the signal to its original base line. This behavior was observed in 89% (n = 39) of the cells studied. Figure 1c also shows that a second similar test pulse of 0Na,Ca medium causes a considerably smaller increase in $[Ca^{2+}]_i$ compared to the first one. In conclusion, it should be stressed that after short 0Na,Ca pulses, the Ca_i^{2+} responses which represents mostly Ca^{2+} entry via the reverse Na^+/Ca^{2+} exchange always return to the initial base line level upon readmission of external Na^+. On the other hand, after long (> 40 s) 0Na,Ca pulses, the Ca_i^{2+} signal is greatly augmented and readmission of external Na^+ causes the Ca_i^{2+} level to decay rapidly to a slowly sloping plateau, whose value varied between different cells (see Figs 1b and c and 2a and c).

Effect of depletion of intracellular Ca^{2+} stores on the Ca_i^{2+} signal induced by the reverse Na^+/Ca^{2+} exchange

As CICR may contribute to increase the $[Ca^{2+}]_i$ when the reverse Na^+/Ca^{2+} exchange mode is activated, depletion of intracellular Ca^{2+} stores, or inhibition of the CICR mechanism, should attenuate any $[Ca^{2+}]_i$ increase in response to 0Na,Ca. For this purpose, we have used: 4-CmC, a RyRs agonist (Herrmann-Frank *et al.* 1996); and Tg, a endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor (Kijima *et al.* 1991).

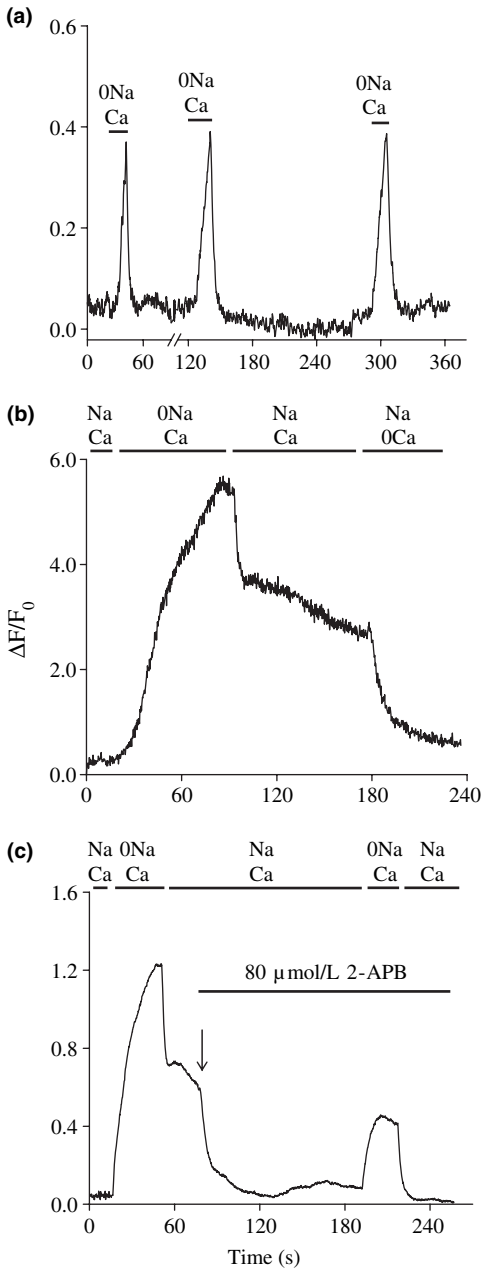

Fig. 1 The effect of short (< 20 s) and long (> 40 s) exposure to 0Na,Ca medium on $[Ca^{2+}]_i$.. (a) Short 0Na,Ca pulses cause a small and reversible increase in $[Ca^{2+}]_i$. (b) A long (90 s) 0Na,Ca pulse induces a larger Ca_i^{2+} signal, which, upon re-exposure to the normal medium (Na,Ca) is partially reversed reaching a sloping plateau. Base line level is rapidly reached after exposure to a Na,0Ca medium. (c) Shows a similar run in which the sloping plateau is abolished by the store operated Ca^{2+} channels blocker 2-aminoethoxydiphenyl borate (80 μmol/L). Note that a second pulse of 0Na,Ca of similar length cause a much smaller Ca_i^{2+} increase.

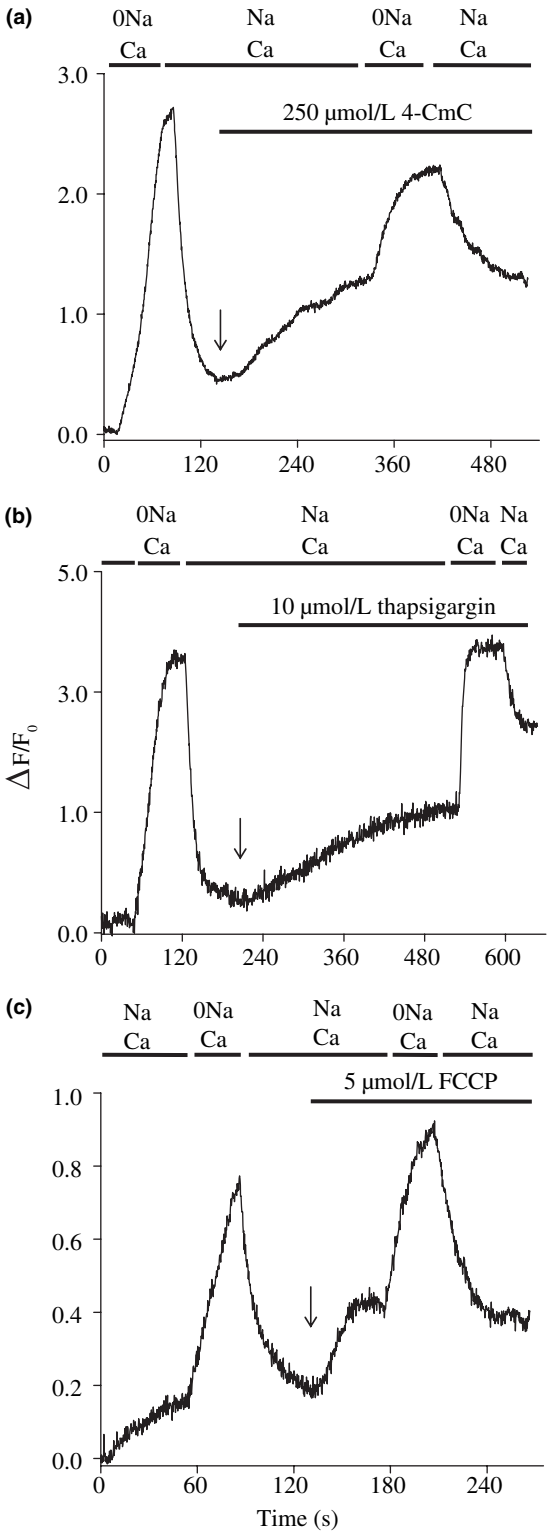

Fig. 2 The effect of 4-chloro-*m*-cresol (4-CmC), thapsigargin (Tg), and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) on $[Ca^{2+}]_i$ changes induced by the reverse operation of the Na^+/Ca^{2+} exchange. (a) After a control pulse in 0Na,Ca; 250 μmol/L 4-chloro-*m*-cresol cause a slow increase in Ca_i^{2+} to a new steady-state. A second pulse in 0Na,Ca after partial depletion of ryanodine sensitive stores causes a significantly reduced Ca_i^{2+} response. (b) After a test pulse in 0Na,Ca medium Tg causes a progressive increase in Ca_i^{2+} to a new steady-state level. Note that after depletion of Tg sensitive stores, the Ca_i^{2+} signal induced by a 0Na,Ca medium is considerably reduced. (c) The effect of depleting the mitochondrial Ca^{2+} stores with FCCP on the Ca_i^{2+} signal induced by the reverse Na^+/Ca^{2+} exchange. Note that application of 5 μmol/L FCCP in the presence of external Na^+ and Ca^{2+} induces an increase in the Ca^{2+}-dependent fluorescence to a new higher steady level. Nevertheless, application of the test 0Na,Ca pulse induces the usual increase in Ca_i^{2+}.

Figure 2a shows an experiment in which exposure of a cell to a 0Na,Ca pulse of 60 s duration causes a large increase in $[Ca^{2+}]_i$ which is partially reversed upon readmission of Na^+ (Na,Ca medium). After this response, the cell was exposed to a Na,Ca medium containing 250 μmol/L 4-CmC, an agonist of RyRs. This agent causes a slow increase in $[Ca^{2+}]_i$ to a new steady-state level. Subsequent application of an even longer 0Na,Ca pulse (80 s), causes a relatively much smaller $\Delta[Ca^{2+}]_i$, 34% of the response to the first pulse. In the experiment of Fig. 2b, after obtaining the usual Ca_i^{2+} response to a long (70 s) 0Na,Ca pulse, exposure of the cell to 10 μmol/L Tg, a potent inhibitor the ER Ca^{2+} transporting ATPase, caused a slow, progressive rise in Ca_i^{2+} to a new steady-state value. Under this condition, a 0Na,Ca pulse caused a smaller increase in Ca_i^{2+}-dependent fluorescence. In other experiments (not shown), application of 4-CmC or Tg in the absence of external Ca^{2+} resulted in an increase in the Ca_i^{2+} dependent Fluo-3 signal indicating that the increase in Ca_i^{2+} is of intracellular origin. These results strongly suggest that (i) RyRs are involved in Ca^{2+} release from intracellular stores and (ii) partial depletion of these stores significantly diminishes the increase in $[Ca^{2+}]_i$ induced by the operation of the reverse Na^+/Ca^{2+} exchange. Figure 2c is an experiment designed to test whether the mitochondrial Ca^{2+} compartment contributes to the increase in $Ca^{2+}{}_i$ during activation of the reverse Na^+/Ca^{2+} exchange. After obtaining the base line fluorescence in the presence of the standard Na,Ca medium, the astrocyte was rapidly superfused with 5 μmol/L carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone causing a significant increase in the Ca_i^{2+}-dependent Fluo-3 fluorescence. Subsequent application of a test pulse of 0Na,Ca causes the usual large increment in intracellular Ca^{2+} thus demonstrating that the mitochondrial Ca^{2+} pool, although present, is not directly involved in the Ca_i^{2+} signal induced by the operation of the reverse Na^+/Ca^{2+} exchange.

Intracellular Ca^{2+} stores depletion after repetitive Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchange

If Ca^{2+} entry through the reverse exchange is promoting CICR, then rapid activation of the forward exchange in the absence of external Ca^{2+} should in principle extrude part of the released Ca^{2+} and therefore contribute to deplete

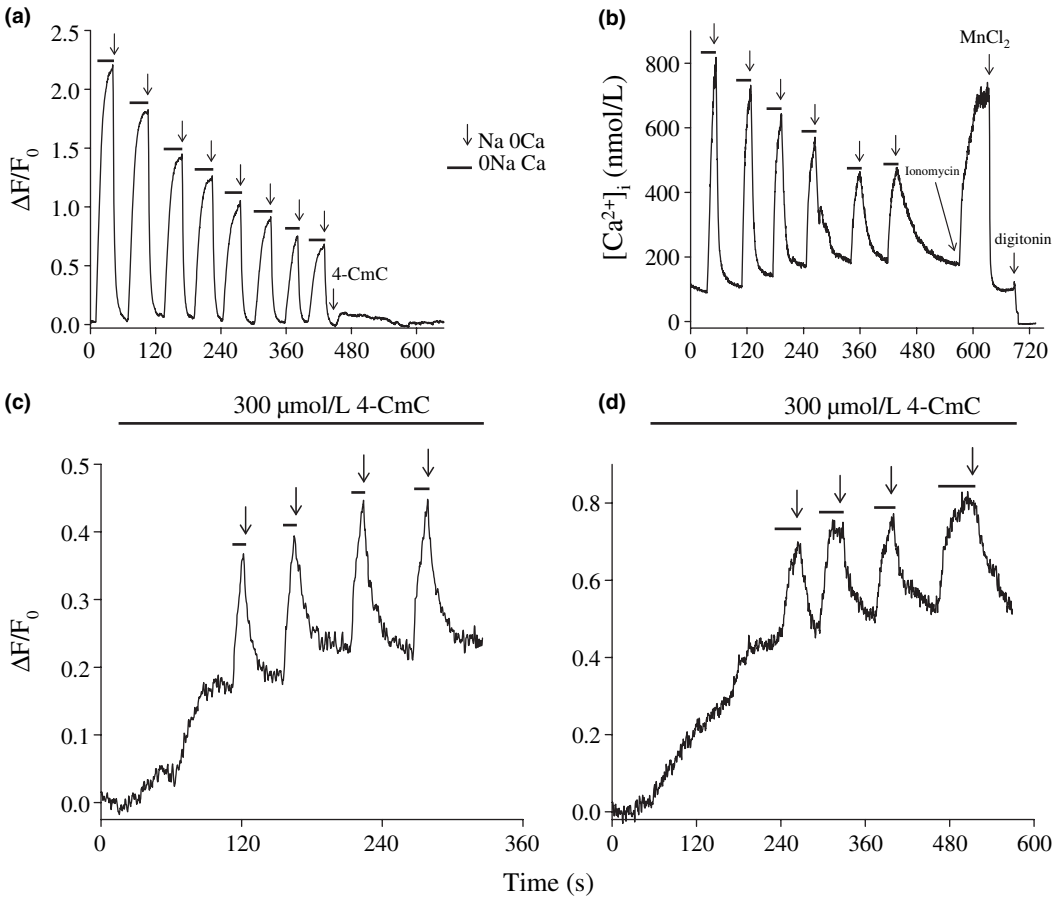

Fig. 3 The effect of consecutive cycles of activation of the reverse and forward Na^+/Ca^{2+} exchange modes on the Ca_i^{2+}-dependent Fluo-3 signal. (a) A protocol of eight consecutive reverse (0Na,Ca) and forward (Na,0Ca) pulses (30 s) causes a progressive decrease in the Ca_i^{2+} signal until it reaches a constant, small value. At the end of the run, the ryanodine agonist 4-chloro-*m*-cresol (4-CmC) fails to release Ca^{2+} from ryanodine sensitive Ca^{2+} stores. (b) Measurement of $[Ca^{2+}]_i$ during activation of the reverse Na^+/Ca^{2+} exchange. A protocol similar to that in (a) shows the same progressive decay of the peak of the Ca^{2+} signal induced by Ca^{2+} entry through the reverse exchange (four consecutive 30 s pulses in 0Na,Ca medium). After this, 2.5 μmol/L ionomycin, in the presence of 2 mmol/L Ca^{2+}, causes a large increase in fluorescence, equivalent to F_{max}. F_{min} was determined at the end of the experiment by adding 4 mmol/L $MnCl_2$ in the continuous presence of ionomycin. Finally, lysis of the cell with digitonin gives the background fluorescence, (F_{bkg}). $F_{max} = [(F_{Mn}{}^{2+} - F_{bkg})/0.2] + F_{bkg}$; $F_{min} = [(F_{max} - F_{bkg})/40] + F_{bkg}$. Cytosolic calcium at any given fluorescence (F) was estimated according to the equation: $[Ca^{2+}]_i = K_d\ [(F - F_{min})/(F_{max} - F)]$ where K_d is the Fluo-3 dissociation constant at vertebrate ionic strength (269 nmol/L (Kao *et al.* 1989; Rojas *et al.* 2003). (c) and (d) The effect of consecutive short (c) and long (d) 0Na,Ca test pulses on the relative Ca_i^{2+}-dependent fluo-3 fluorescence ($\Delta F/F_0$) after depleting the Ca^{2+} intracellular stores with 4-chloro-*m*-cresol (300 μmol/L). Note the absence of run down of the Ca^{2+} signal for short and long 0Na,Ca test pulses.

intracellular Ca^{2+} stores related to CICR. The experiment shown in Fig. 3a examine this possibility. The protocol of Fig. 3a was designed to first disable the forward Na^+/Ca^{2+} exchange with a 50 s pulse of 0Na,Ca (Ca^{2+} entry mode) and then rapidly enable the forward exchange (Ca^{2+} extrusion mode) for about 40 s by rapidly superfusing with the test Na, 0Ca medium (vertical arrows). This protocol was repeated during eight consecutive pulses. The results of Fig. 3a indicate that in the absence of external Ca^{2+} the forward mode of the exchange lowers the $[Ca^{2+}]_i$ faster to its original base line. More significantly, it shows that the peak of the Ca_i^{2+} dependent Fluo-3 signal induced by the reverse Na^+/Ca^{2+} exchange decreases progressively after each period of activation of the forward exchange. The fact that this decrease is due to depletion of ryanodine sensitive Ca_i^{2+} stores is confirmed at the end of the experiment by the failure of the ryanodine receptor agonist 4-CmC to release Ca_i^{2+}. It could be argued that the progressive decrease in Fluo-3 fluorescence during the above experimental protocol is the result of leakage/bleaching of Fluo-3 during the course of the consecutive pulses protocol, and not a direct consequence of depletion of intracellular Ca^{2+} stores. Figure 3b shows an experiment in which after only four consecutive pulses of 30 s duration the rise in Ca_i^{2+} induced by the test pulse in

0Na,Ca decreases by about 50%. At the end of the run, addition of 10 μmol/L ionomycin in the presence of external Ca^{2+} (2 mmol/L) causes a large increase in fluorescence thus demonstrating the unaltered capacity of Fluo-3 inside the cell to report $[Ca^{2+}]_i$ increases. The experiment also shows the quenching of the Fluo-3 signal by $MnCl_2$ and the loss of Fluo-3 in the presence of digitonin. The steady-state fluorescence levels in the presence of ionomycin, $MnCl_2$, and digitonin were used to calculate the $[Ca^{2+}]_i$ in Fig. 3b (see Kao *et al.* 1989 and Rojas *et al.* 2003, 2004). To further test the idea that intracellular Ca^{2+} stores are directly involved in the amplification of the Ca_i^{2+} signal induced by Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchanger, and that the decay of the Ca signals, shown in part A and B of Fig. 3, is due to depletion of intracellular Ca stores, the experiments shown in Figs 3c and d were carried out. Figure 3c shows a control run in which after depleting the ryanodine sensitive Ca^{2+} stores with 4-CmC, a series of four consecutive short (16 s) 0Na,Ca pulses causes equal increases in the relative Ca_i^{2+} Fluo-3 signal. Figure 3d shows a similar experiment in which the four consecutive pulses were of long (32 s) duration. The records clearly show that in the case of long 0Na,Ca pulses (Fig. 3d) the four consecutive responses are of equal magnitude, showing no decay as in runs A and B of the Fig. 3. Furthermore, they also show that the responses to long or short 0Na,Ca pulses are of similar magnitude thus indicating the absence of signal amplification.

Co-localization of the Na^+/Ca^{2+} exchanger and RyRs in cerebellar Type-1 astrocytes

Using confocal microscopy, we have further studied the relationship between Ca^{2+} entry during the reverse operation of the exchanger and Ca^{2+}release from intracellular stores. We took advantage of the fact that (i) under certain experimental conditions, the low affinity Ca^{2+} dye Rhod-2, a positively charged molecule, is largely retained within intracellular compartments in particular ER and mitochondria (Simpson *et al.* 1998) and (ii) the ER can be readily marked with BODIPY-FL-Tg (Hua *et al.* 2004). Figure 4a shows a typical confocal image showing that under control conditions (Na,Ca), Rhod-2 is internally compartmentalized in clusters, suggesting significant accumulations of Ca^{2+} within intracellular stores. Figure 4b shows the localization in the same cell of the ER Ca^{2+} stores marked with BODIPY-FL-Tg. Figure 4c represents the joint images of 4A and 4B clearly demonstrating the coincidence of the labeling of the ER calcium pool and the Tg receptors. Having shown that Rohd-2 is a good marker of the ER Ca^{2+} pool in this preparation, we tested whether activation of Ca^{2+} entry by the reverse Na^+/Ca^{2+} exchange could dissipate the compartmentalized Rhod-2 florescence signal as a result of CICR. Figure 4d shows clusters of Rhod-2 fluorescence in a Type-1 cerebellar astrocyte bathed in the control extracellular medium, Na,Ca (white arrow). As in Fig. 4a, compartmentalization of the dye is observed. Figure 4e shows that in the same cell activation of the reverse Na^+/Ca^{2+} exchange by a exposure to 0Na,Ca causes dissipation of the compartmentalized Ca^{2+}. Figure 4f shows the relative fluorescence of a compartmentalized Rhod spot (white arrow in Figs 4d and e) as a function of time during the 0Na,Ca pulse. The plots clearly illustrate that after activation of the reverse Na^+/Ca^{2+} exchange, Ca_i^{2+} is dissipated with a $t_{0.5}$ of about 50 s.

The pharmacological experiments of Fig. 2 indicated that in these glial cells RyRs are somehow involved in the amplification of the Ca^{2+} signal during reverse operation of the Na^+/Ca^{2+} exchange. Therefore, the next step was focused on the immunolocalization of this transporter in the plasma membrane of cerebellar Type-1 astrocytes, as well as on the spatial relationship between the plasmalemmal Na^+/Ca^{2+} exchanger and the underlying ER, in particular the RyRs. For this, cells were incubated first with a purified canine cardiac Na^+/Ca^{2+} exchanger mouse monoclonal antibody and second with a secondary labeled goat anti-mouse Igm antibody Na^+/Ca^{2+} exchange Alexa Fluor 546. The immunofluorescence of the Na^+/Ca^{2+} exchanger (red color) in a representative cell is presented in Fig. 5a. In all cells studied ($n = 6$) the labeling was punctual suggesting a cluster of Na^+/Ca^{2+} exchange molecules. Labeling of the exchanger was more intense at cell edges suggesting that the exchanger is distributed in an organized manner in the astrocyte plasmalemma. Figure 5b shows the localization of RyRs (green color) in the same cell using Bodipy-FL-Ryanodine, a specific ryanodine receptor marker (Hua *et al.* 2004). Figure 5c shows the co-localization (yellow color) of the Na^+/Ca^{2+} exchanger and the RyRs. From this analysis, shown in Fig. 5d, the observed overlap was found to be highly significant with a correlation of about 0.95 in this particular cell (see legend). In 20 different cells, the correlation between the Na^+/Ca^{2+} exchanger and the RyRs was 0.80 ± 0.01 thus indicating that the Na^+/Ca^{2+} exchanger is indeed co-localized with some of the ER, in particular with the RyRs.

Na^+ entry through the electrogenic glutamate transporter triggers for Ca^{2+} entry via the reverse Na^+/Ca^{2+} exchange

The influx of Na^+ through glutamate receptor channels can activate the reverse Na^+/Ca^{2+} exchange in cortical astrocytes thus leading to an intracellular Ca^{2+} increase (Goldman *et al.* 1994; Takuma *et al.* 1996; Benz *et al.* 2004). On the other hand, rat cerebellar Type-1 astrocytes (as opposed to Type-2 astrocytes) in culture up to 4 days, do not express glutamate receptor channels and the large glutamate inward current occurs through the electrogenic glutamate carrier mechanism (Wyllie *et al.* 1991). Moreover, even in old cerebellar cultures (at least 7 days) most of the inward depolarizing current is carried by the Na^+-glutamate co-transporter (Wyllie *et al.* 1991). In order to test the possibility that activation of the reverse Na^+/Ca^{2+} exchange may occur by Na^+ entry through the glutamate carrier, we have performed a series of

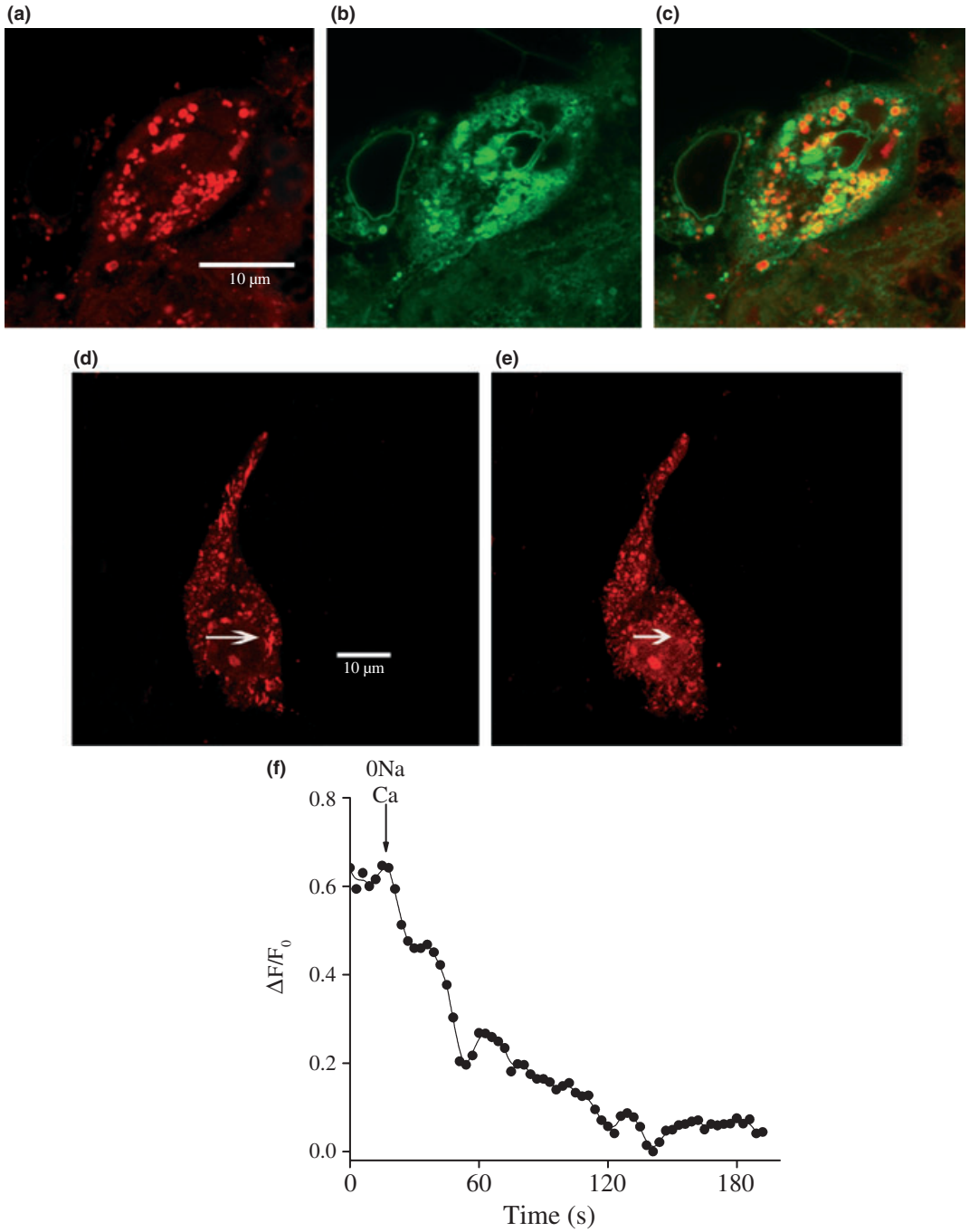

Fig. 4 Labeling of endoplasmic reticulum (ER) Ca^{2+} stores with Rhod-2 and immunofluorescence labeling of BODIPY-FL-thapsigargin (Tg) ER in Type-1 cerebellar astrocytes. (a) Cells labeled with Rhod-2. Note the compartmentalization of the dye. (b) BODIPY-FL-Tg receptors labeling. (c) Overlapping of the two images indicates the coincidence of the labeling of the intracellular ER Ca^{2+} pool and the Tg receptors. (d) Labeling of the ER Ca^{2+} pool by Rhod-2 in a control medium (Na,Ca). Observe the compartmentalization of the dye. (e) Effect of activation of the reverse Na^+/Ca^{2+} exchanger (pulse of 100 s in 0Na,Ca test medium) on the compartmentalization of the Rhod-2 signal. Note the release of the compartmentalized Rhod-2 to the cell interior. (f) Relative fluorescence ($\Delta F/F_0$) of a fluorescence spot (white arrow) as function of time in 0Na,Ca medium. Note the dissipation of about 50% of the compartmentalized Rhod-2 signal after 50 s in the 0Na,Ca test medium.

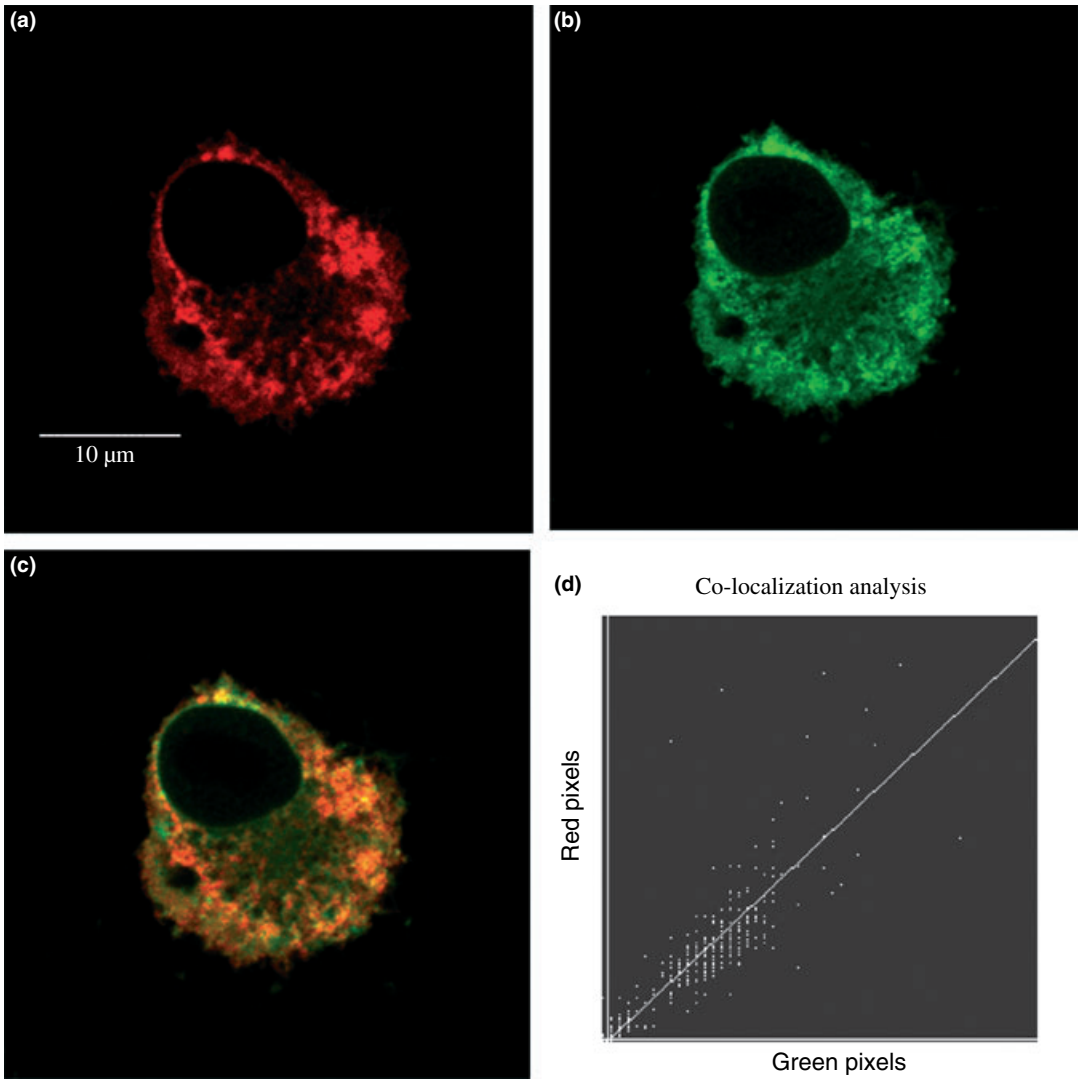

Fig. 5 ImmunoFluorescent labeling of Na^+/Ca^{2+} exchanger and ryanodine receptors in cultured rat cerebellar Type-1 astrocytes. The same cell in A and B was stained with affinity purified antibodies raised against the cardiac sarcolemmal Na^+/Ca^{2+} exchanger ALEXA-Fluor-546-Na/Ca (a) and BODIPY-FL-Ryanodine (b). The exchanger appears in red and ryanodine receptors in green. (c) Superposition of (a) and (b). The yellow color corresponds to regions in which the two labels overlaps. (d) Co-localization analysis indicates that there is a significant co-localization of the two labels. The co-localization mathematical analysis for this cell, gave a correlation of 0.95.

experiments using specific inhibitors of: the ionotropic glutamate receptor, the carrier mediated Na^+-glutamate uptake and the Na^+/Ca^{2+} exchanger.

Figure 6 shows two typical intracellular Fluo-3 responses (90% of cerebellar Type-1 astrocytes probed) to a low (< 50 µmol/L) L-Glu concentration. Figure 6a shows that after stimulation with 30 µmol/L L-Glu in the normal medium, (Na,Ca,), Ca^{2+} increases and then spontaneously decreases to a steady level higher than the level before stimulation. Figure 6b shows that in the absence of external Ca, 30 µmol/L L-Glu failed to increase $Ca^{2+}{}_i$. Addition of external Ca^{2+} in the continuous presence of L-Glu induces a substantial increase in the $[Ca^{2+}]_i$ similar to that shown in Fig. 6a. The result indicates that the Fluo-3 signal induced by L-Glu is mediated by Ca^{2+} entering from the extracellular medium. If the transient response to L-Glu was secondary to Na^+ and Ca^{2+} entry through the ionotropic glutamate receptors (Kainate-NMDA) then it should be abolished by antagonists of these receptors. Figure 7a shows that a cocktail of two specific blockers of these channels: D,L-2-amino-5-phosphonopentanoic acid and 6-cyano-7-nitroquinoxaline-2,3-dione do not affect the L-Glu-induced Ca_i^{2+}-dependent Fluo-3 signal. On the other hand, Fig. 7b shows that the specific blocker of the electrogenic Na^+-glutamate co-transporter, L-trans-2,4-pyrrolidine dicarboxylate, completely eliminates the L-Glu-induced increase in Ca_i^{2+}. To confirm that the increase in intracellular Ca^{2+} occurs through the activation of the electrogenic

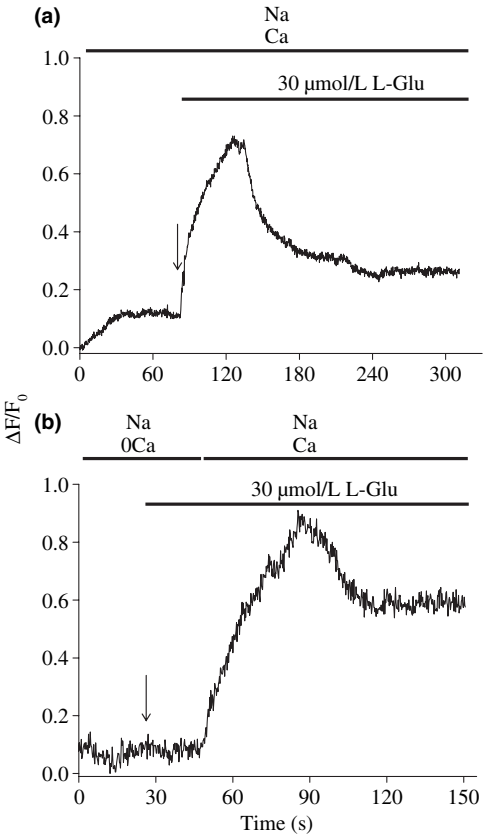

Fig. 6 The effect of low (< 30 μmol/L) L-Glu on the Ca_i^{2+}-dependent Fluo-3 signal in the presence and absence of extracellular Ca^{2+} (a) Consequence of fast superfusion of 30 μmol/L L-Glu in a control medium containing 2 mmol/L external Ca^{2+} (Na,Ca) on intracellular Ca^{2+}. Observe the rapid increase in the Fluo-3 signal followed by a spontaneous decay of the signal to a plateau level higher than the control base line. (b) Consequence of fast superfusion of 30 μmol/L L-Glu in a control medium containing 0 mmol/L external Ca^{2+} (Na,0Ca) on intracellular Ca^{2+}. This astrocyte was perfused from the beginning with a medium containing no external Ca^{2+}. Note that no effect of L-Glu is observed under this condition. Addition of 2 mmol/L external Ca^{2+} rapidly induces a biphasic response similar to that in (a).

Na^+-glutamate co-transporter, we carried out a series of experiments in which L-Asp was used instead of L-Glu. L-Asp is an effective substrate for the glutamate carrier in the CNS (Balcar and Johnston 1972), but it is known that it does not activate ionotropic receptor channels mediated currents (Wyllie *et al.* 1991). Figure 7c shows that in the presence of extracellular Na^+ and Ca^{2+}, L-Asp (100 μmol/L) evokes a Ca_i^{2+} response similar to that of L-Glu, except that it appears to be monophasic.

In the next series of experiments we further explored: first, whether low concentrations of glutamate could significantly increase the $[Na^+]_i$, and second, whether the increase in the Ca_i^{2+}-dependent Fluo-3 signal induced by low [L-Glu] is related to the operation of the reverse Na^+/Ca^{2+} exchange.

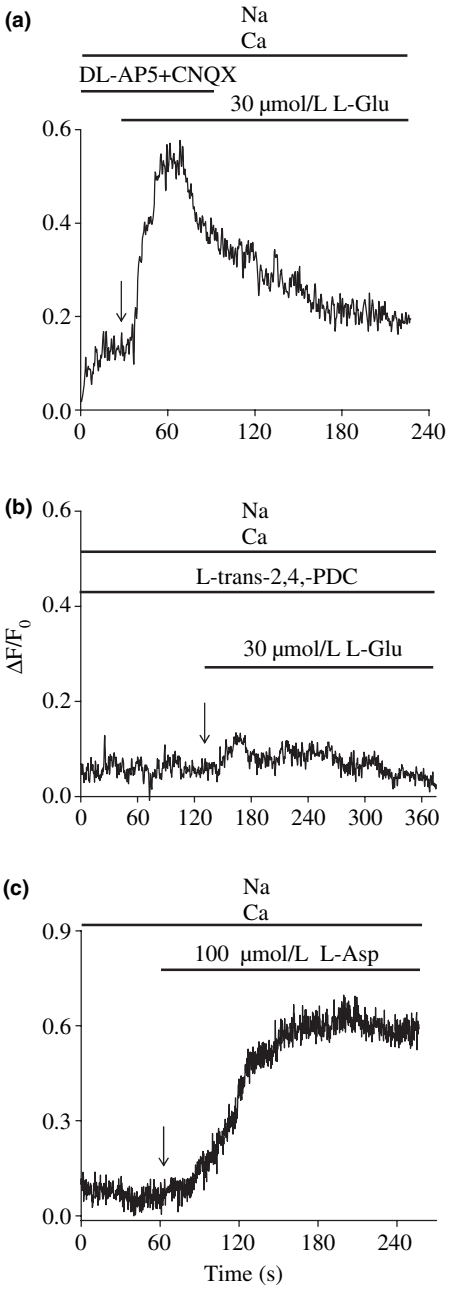

Fig. 7 The effect of inhibitors of ionotropic glutamate receptors and the glutamate transporter(s) on the Ca_i^{2+} signal induced by a low, 30 μmol/L, [L-Glu]. (a) 10 μmol/L D,L-2-amino-5-phosphonopentanoic acid (DL-AP5) plus 30 μmol/L 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) do not affect the increase in $[Ca^{2+}]_i$ induce by 30 μmol/L L-Glu. (b) 100 μmol/L L-trans-2,4-pyrrolidine dicarboxylate (L-trans-2,4-PDC), a blocker of the electrogenic glutamate transporter completely eliminates the L-Glu induced increase in Ca_i^{2+}. (c) The effect of L-Asp, an excitatory amino acid that is transported by the glutamate transporter but do not activate ionotropic glutamate receptors. This cell was superfused from the beginning with the control medium (Na,Ca) until it reaches a steady-state fluorescence signal. Note that application of 100 μmol/L L-Asp causes a significant increase in the Ca_i^{2+}-dependent Fluo-3 fluorescence signal.

Figure 8a shows an experiment in which the cell was loaded with the Na^+-sensitive fluorescent probe SBFI. After obtaining a resting steady base line of about 17 mmol/L in $[Na^+]_i$, the addition of 30 μmol/L L-Glu causes a rise in $[Na^+]_i$ to a new high steady-state level close to 25 mmol/L thus indicating that low concentrations of L-Glu are accompanied by a rise in the $[Na^+]_i$. In 20 different cells, the $\Delta[Na^+]_i$ induced by 30 μmol/L L-Glu was 12.6 ± 0.42. Figure 8(b and c) shows that two potent inhibitors of the Na^+/Ca^{2+} exchanger, SEA0400 (Matsuda *et al.* 2001) and KB-R7943 (Iwamoto *et al.* 1996), completely eliminate the Ca_i^{2+} response induced by L-Glu, thus strongly suggesting that Na^+ co-transported with L-Glu through the glutamate carrier is the trigger for the rise in $[Ca^{2+}]_i$ as consequence of activation of the reverse Na^+/Ca^{2+} exchange.

In further support of the idea that the entry of Na^+, which is co-transported with L-Glu, activates Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchange, which in turn triggers a CICR mechanism, we explored whether the L-Glu response occurred after blocking the RyRs with high ryanodine concentrations. Figure 9a shows that addition of 50 μmol/L ryanodine, a concentration known to block the ryanodine channel (Smith *et al.* 1988), induces a small but reproducible increase in intracellular Ca^{2+}, most probably as consequence of lower ryanodine concentrations reaching the cell at the beginning of the pulse. Subsequent superfusion with 50 μmol/L L-Glu in the presence of high ryanodine failed to induce the characteristic rise in intracellular Ca^{2+}. This type of experiment clearly indicates that functional RyRs are indeed required for the L-Glu-induced Ca^{2+} rise. Similar results were obtained when the intracellular stores were depleted with the ryanodine agonist 4-CmC or the ER ATPase inhibitor Tg (experiments not shown). Figure 9b shows an experiment designed to test whether the high concentrations of ryanodine used in the experiment of Fig. 9a may affect the activity of the reverse Na^+/Ca^{2+} exchanger. After obtaining a base line in $[Ca^{2+}]_i$, a short (13 s) pulse of 0Na,Ca was applied to the cell causing a rapid and reversible increase in $[Ca^{2+}]_i$.. Subsequently, addition of 50 μmol/L ryanodine caused a small increase in the $[Ca^{2+}]_i$ after which a second short 0Na,Ca test pulse produced the same increase in $[Ca^{2+}]_i$ thus indicating that no effect of ryanodine *per se* on the Na^+/Ca^{2+} activity occurs.

It is known that in the microenvironment of synapses, astrocytes can be exposed to glutamate concentrations as high as 1 mmol/L before it is recaptured (Bartol and Sejnowski 1993). We have therefore explored the effects of millimolar concentrations of L-Glu on the agonist-induced Ca_i^{2+} signal. Figure 10a shows that in the absence of extracellular Ca^{2+}, 1 mmol/L L-Glu has no effect on the basal level of intracellular Ca^{2+}. However, re-admission of 2 mmol/L external Ca^{2+} in the presence of millimolar [L-Glu] causes a rapid increase in the Fluo-3 fluorescence which

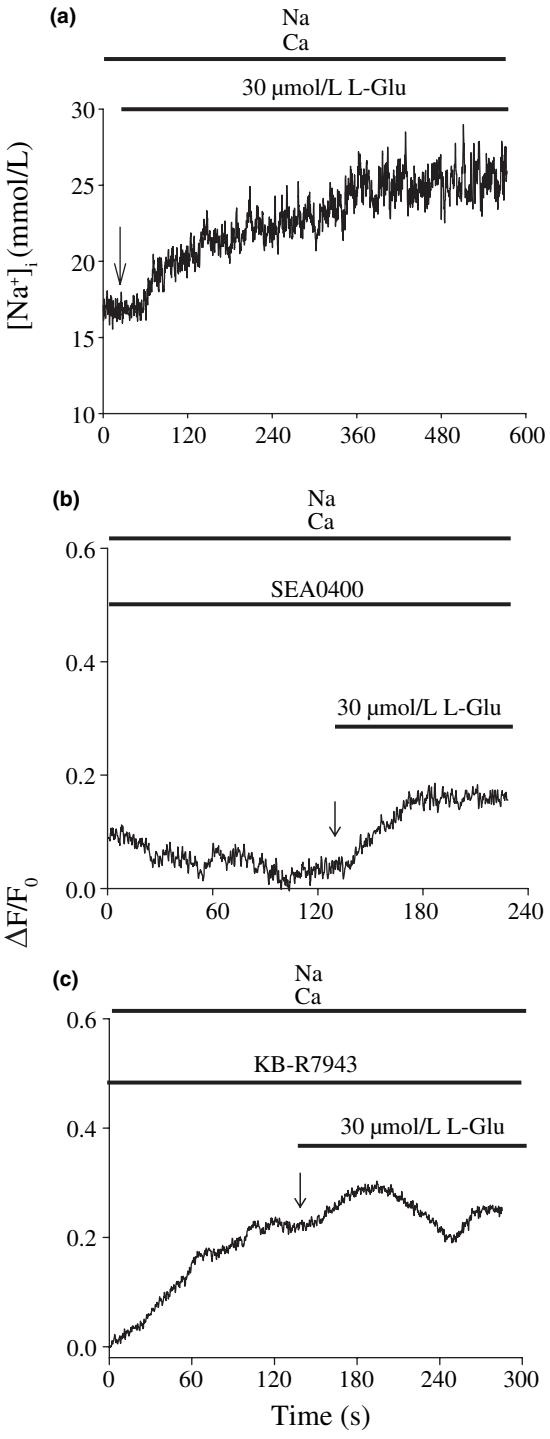

Fig. 8 The effect of low (30 μmol/L) [L-Glu] on intracellular Na^+ and the effect of Na^+/Ca^{2+} exchange inhibitors on the Fluo-3 Ca_i^{2+} signal induced by L-Glu. (a) After obtaining a base line in the $[Na^+]_i$, extracellular perfusion with 30 μmol/L L-Glu induces a significant rise to a new steady-state in the intracellular Na^+-dependent SBFI signal. (b) 3 μmol/L SEA0400 or (c) 10 μmol/L KB-R7943 added before the beginning of the experiment and maintained until the end. Observe that in the presence of the either inhibitor, 30 μmol/L L-Glu fail to induce any change in the level of intracellular Ca^{2+}.

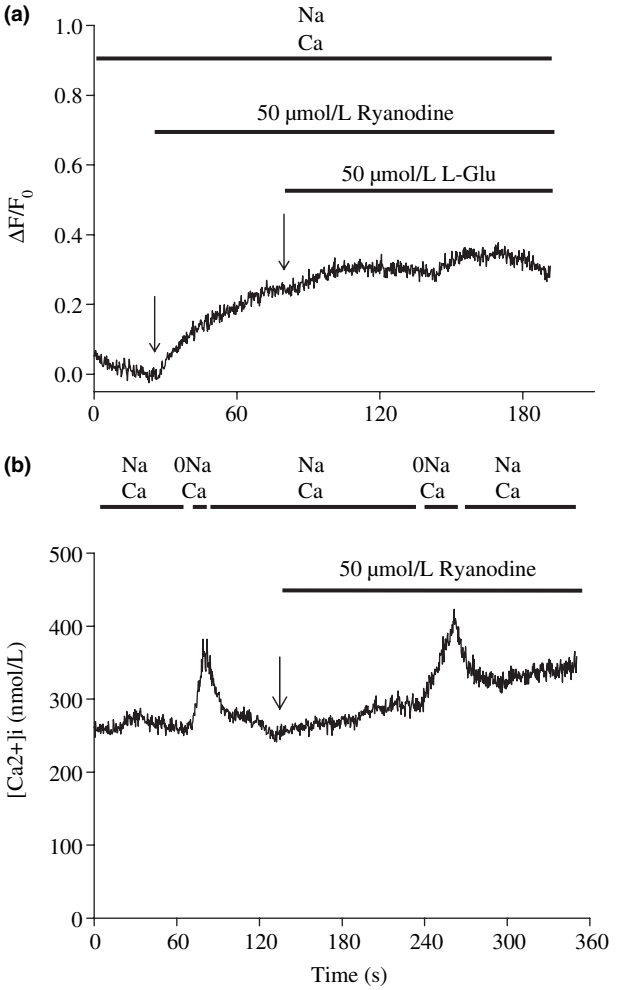

Fig. 9 (a) The effect of a high concentration of ryanodine on the Ca_i^{2+} signal induced by L-Glu. In this astrocyte after obtaining the fluorescence base line in the control medium (Na,Ca) addition of 50 μmol/L ryanodine causes a slow but significant rise in intracellular Ca^{2+}. Interestingly, at this concentration, ryanodine completely blocks the Ca_i^{2+} response to 50 μmol/L L-Glu. (b) The effect of high [ryanodine] on Ca_i^{2+} entry through the reverse Na^+/Ca^{2+} exchange. Note that for short pulses of 0Na,Ca (15 s) ryanodine has no effect on the $[Ca^{2+}]_i$ increases induced by the operation of the reverse exchange.

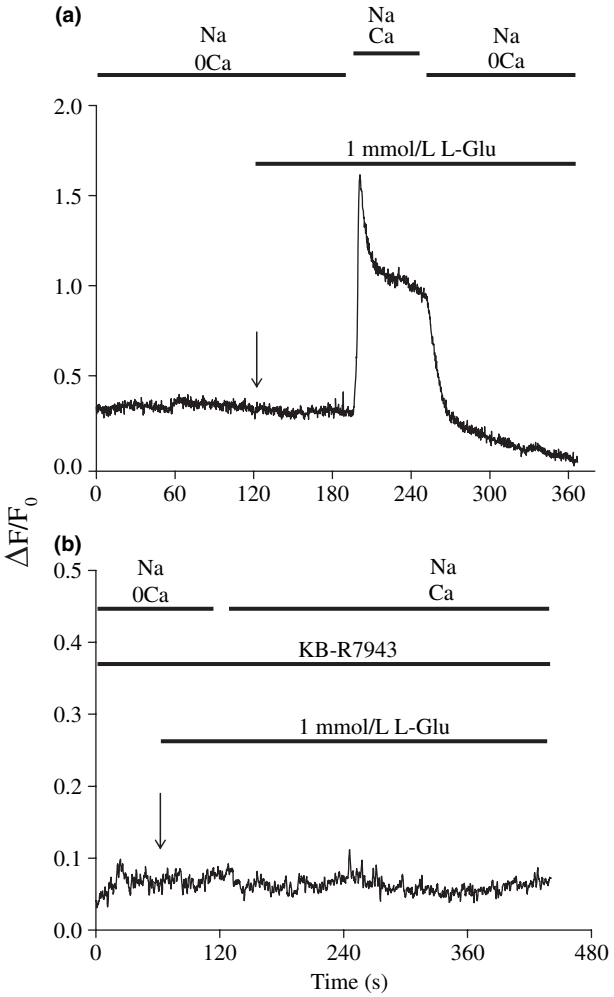

Fig. 10 The effect of millimolar concentrations of L-Glu on intracellular Ca^{2+} and their dependence on extracellular Ca^{2+} and the proper functioning of the Na^+/Ca^{2+} exchanger. (a) In this cell, the fluorescence base line was obtained in the absence of external Ca^{2+}. Addition of 1 mmol/L L-Glu has no effect whatsoever on the Ca_i^{2+}-dependent Fluo-3 signal. However, addition of 2 mmol/L external Ca^{2+} causes a rapid biphasic increase in Ca^{2+}. Note that the plateau component completely disappears upon external Ca^{2+} removal. (b) The Na^+/Ca^{2+} exchange inhibitor KB-R7943 (10 μmol/L) prevents the L-Glu-induced Ca_i^{2+} rise even at 1 mmol/L [L-Glu]. In this astrocyte, the fluorescence base line was obtained in the absence of external Ca^{2+} (Na, 0Ca medium). Additon of 1 mmol/L L-Glu in the presence of the exchange inhibitor has no effect independently of the presence of external Ca^{2+}.

decays to a lower plateau. This Ca_i^{2+} signal rapidly returns to the original base line when the external Ca^{2+} is removed. Figure 10b shows that as is the case with low glutamate concentrations, pre-incubation (60 s) with 10 μmol/L of the Na^+/Ca^{2+} exchange inhibitor KB-R7943, completely blocks the agonist response in the absence or presence of external Ca^{2+}.

Discussion

The present work demonstrates that in Type-1 cerebellar astrocytes in culture, the Ca^{2+} signal generated by Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchange is greatly amplified by a CICR mechanism, which involves RyRs and ryanodine sensitive Ca^{2+} stores. While the presence of RyRs has been demonstrated in this preparation, their physiological significance was not clear (Langley and Pearce 1994; Simpson *et al.* 1998; Golovina and Blaustein 2000; Matyash *et al.* 2002; Beck *et al.* 2004; Aley *et al.* 2006). Our experimental

results clearly demonstrate the presence of RyRs and provide evidence for a functional role of RyRs operated intracellular Ca^{2+} stores in cerebellar Type-1 astrocytes.

In addition, and most importantly this work, for the first time, demonstrates that the intracellular Ca^{2+} signal induced by physiological concentrations of the excitatory aminoacids L-Glu and L-Asp, is the result of Na^+ entry through the electrogenic glutamate transporter that activates the reverse Na^+/Ca^{2+} exchange and leads to Ca^{2+} entry, with a concomitant increase in $[Ca^{2+}]_i$. The finding of a functional co-expression of Na^+/Ca^{2+} exchangers with RyRs strongly supports the idea that the original Ca^{2+} signal due to Ca_i^{2+} entry through the exchanger is largely amplified by a CICR process involving RyRs.

Activation of reverse Na^+/Ca^{2+} exchange triggers CICR

Previous studies have shown that the Na^+/Ca^{2+} exchanger working in its reverse mode can induce Ca^{2+} entry in cultured astrocytes (Goldman *et al.* 1994; Takuma *et al.* 1994; Blaustein and Lederer 1999). Moreover Ca^{2+} influx via the exchanger may be responsible for $[Ca^{2+}]_i$ increase under certain pathological conditions (Kin-Lee *et al.* 1992; Matsuda *et al.* 1996). Cerebellar Type-1 astrocytes express a highly active Na^+/Ca^{2+}exchanger responsible for the balance of the plasma membrane Ca^{2+} fluxes under resting physiological conditions (Rojas *et al.* 2004). In different preparations, there is evidence of an intimate association between the Na^+/Ca^{2+} exchanger and internal Ca^{2+} stores (Juhaszova *et al.* 1996). Such association is well established in smooth muscle cells where the exchanger is in close proximity to the sarcoplasmic reticulum, so that Ca^{2+} release from the sarcoplasmic reticulum through RyRs is closely coupled to its extrusion by the exchanger (Nazer and van Breemen 1998). Furthermore, in neurons there is evidence for a spatial association of the exchanger with the intracellular Ca^{2+} stores (Juhaszova *et al.* 1996). Micci and Cristensen (1998) working in catfish retinal neurons have studied the interaction between the exchanger and ryanodine-sensitive Ca^{2+} stores showing that reverse operation of the Na^+/Ca^{2+} exchanger refills Ca^{2+} depleted ER. For the case of astrocytes however, the relationship between the Na^+/Ca^{2+} exchanger and the RyRs remained unknown.

One of the aims of the present work was to investigate whether the magnitude of the increase in $[Ca^{2+}]_i$ observed when the operation of the Na^+/Ca^{2+} exchanger was reversed, was solely dependent on Ca^{2+} entry or whether this entry could trigger further Ca^{2+} release from RyRs operated intracellular Ca^{2+} stores. The experiments (Figs 1, 2, and 3) involving time-dependent manipulations of the Na^+ electrochemical gradient clearly demonstrate that for short (< 20 s) Na^+ gradient reversal (0Na,Ca) pulses, the increase in intracellular Ca^{2+} reflects only Ca^{2+} entry through the exchanger. This is evidenced by the fact that the increase in Ca_i^{2+} is rapidly reverted when the normal Na^+ gradient is re-established with no activation of SOCC. For long (> 20 s) Na^+ gradient reversal pulses, the increase in $[Ca^{2+}]_i$ is much larger and leads to depletion of RyRs-operated intracellular Ca^{2+} stores, indicating the presence of a CICR mechanism. Furthermore, depletion of intracellular Ca^{2+} stores causes the activation of SOCC, as confirmed by the extracellular Ca^{2+} dependency (Fig. 1b) and sensitivity to 2-APB (Fig. 1c) of a late, residual component of the Ca^{2+} signal. The above conclusions are supported by the experiments in which the RyRs agonist 4-CmC and the ER Ca^{2+} pump inhibitor Tg were used (Figs 2a, b, and c). In this case, in the presence of these pharmacological agents, the increase in Ca_i^{2+} induced by a long pulse of Na^+ gradient reversion is significantly smaller after depletion of the intracellular Ca^{2+} stores. Figs 3c and d confirm that when the intracellular Ca^{2+} stores are depleted by 4-CmC, the CICR is abolished, thus causing the disappearance of the Ca^{2+} signal amplifying mechanism.

The presence of RyRs in Type-1 cerebellar astrocytes has been confirmed using conventional Ca^{2+} imaging confocal microscopy and immunocytochemistry techniques. The close proximity of the Na^+/Ca^{2+} exchanger to the ER membranes, where the RyRs are localized, allows the former to rapidly extrude Ca^{2+} ions released from the ER before their recapture by the ER Ca^{2+}-ATPase. This leads to depletion of the ER Ca^{2+} stores as demonstrated by the consecutive reverse-forward pulse experiments. In these experiments (Fig. 3a), the fact that no release of Ca^{2+} is observed at the end of the run in the presence of the ryanodine agonist 4-CmC demonstrates that the exchanger is capable of depleting the ER.

Role of the reverse Na^+/Ca^{2+} exchange on L-Glu-induce $[Ca^{2+}]_i$ rise in Type-1 cerebellar astrocytes

An important discovery in glial cell research is that $[Ca^{2+}]_i$ increase may trigger glutamate release from astrocytes which then mediates $[Ca^{2+}]_i$ increases in nearby neurones, thus indicating a cross-talk between neurons and astrocytes (Parpura *et al.* 1994; Pasti *et al.* 1995; Jeftinija *et al.* 1997; Calegari *et al.* 1999; Araque *et al.* 2000; Fellin and Carmignoto 2004). Recently Benz *et al.* (2004) have demonstrated the important role of the Na^+/Ca^{2+} exchanger in the glutamate response in cortical astrocytes from mice. Their experiments show that [L-Glu] (500 mol/L) induce a Ca_i^{2+}-dependent release of homocysteic acid from astrocytes through activation of glutamate receptors, leading to an influx of Na^+ and to an increase in Ca^{2+} entry through the reverse Na^+/Ca^{2+} exchange (Benz *et al.* 2004). Previous electrophysiological studies in rat cerebellar Type-1 astrocytes had shown that application of as low as 30 micromol/L L-Glu produced large inward currents which remained inward-going at potentials up to +80 mV, being the result of the presence of an electrogenic glutamate uptake carrier (Wyllie *et al.* 1991). In cells kept up to 4 days in culture, quisqualate, kainite, and NMDA failed to produce any

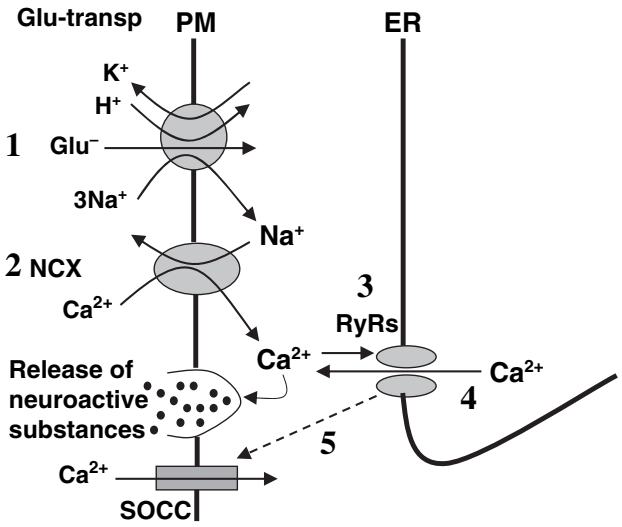

Fig. 11 Role of Na^+/Ca^{2+} exchanger in glutamate-induced rise in intracellular Ca^{2+} in rat cerebellar Type-1 astrocytes. The cascade of events that lead to glutamate-induced rise in intracellular Ca^{2+} involves: (1) Na^+ entry through the electrogenic glutamate co-transporter. (2) Activation of the reverse Na^+/Ca^{2+} exchange by the rise in intracellular Na^+ (Na^+ inward current through the glutamate transporter). (3) Rise in the $[Ca^{2+}]_i$ near the ryanodine receptors (RyRs). (4) Activation by Ca^{2+} of RyRs followed by Ca^{2+} release from ryanodine channels leading to an amplification of the original Ca^{2+} entry through the exchanger. (5) Opening of the store operated calcium channels (SOCC).

current indicating the absence at this early stage of glutamate ionotropic receptors in rat cerebellar Type-1 astrocytes (Wyllie *et al.* 1991). These authors showed that even in older cultures, in which ionotropic glutamate receptors are well expressed, most of the L-Glu-induced inward current can be ascribed to the Na^+-glutamate-co-transporter (Wyllie *et al.* 1991). Based on these findings, we considered the possibility that the electrogenic Na^+-glutamate co-transporter might be involved in the L-Glu dependent $[Ca^{2+}]_i$ increase in Type-1 cerebellar astrocytes through an increase in $[Na^+]_i$.

The major finding in the present work is that activation of the reverse Na^+/Ca^{2+} exchange by physiological [L-Glu] is not the consequence of Na^+ entry across ionotropic receptors as it occurs in other astrocytes preparations (Benz *et al.* 2004), but the result of Na^+ entry through the electrogenic glutamate transporter(s) (see the scheme of Fig. 11). An important role of the electrogenic glutamate transporter in the L-Glu-induced Ca_i^{2+} increase and its relationship with the reverse Na^+/Ca^{2+} exchange is supported by the demonstration that: (i) no effect of L-Glu is observed in the absence of external Ca^{2+}, (ii) inhibition of the ionotropic glutamate receptors does not impair the Ca_i^{2+} rise induced by L-Glu, (iii) inhibition of the Na^+/Ca^{2+} exchanger completely blocks the L-Glu effect, both at low and high [L-Glu], (iv) L-Glu effect is abolished by depletion of the ryanodine sensitive intracellular stores or by inhibition of the ryanodine release channels, and (v) specific inhibition of the electrogenic Na^+-glutamate co-transporter completely eliminates the L-Glu effect.

Considering that the transport current generated by the glutamate transporter is evoked by the inward movement of two positive charges per transported glutamate (1Glu : $1H^+$: $3Na^+$ entering vs. $1K^+$ moving outward; Greewer and Rauen 2005), and that the average inward current is about 800 pA/cm^2 for a 30 mol/L L-Glu (Wyllie *et al.* 1991) then, for a hypothetical Type-1 astrocyte resembling a rectangular triangle of 25 μm in the base and an approximate volume of 1.2×10^{-6} L, enough Na^+ will enter the astrocyte during L-Glu activation as to induce increases of the intracellular $[Na^+]$ in a time scale of seconds, sufficient to greatly activate the reverse mode of the Na^+/Ca^{2+}. It should be pointed out that the rise in $[Na^+]_i$ shown in Fig. 8a represents an average across the cell but it could be much higher close to the plasma membrane in the vicinity of the Na^+/Ca^{2+} exchanger molecules.

Finally, an interesting recent finding is the acute up-regulation of the Na^+-glutamate transporter mediated by metabotropic glutamate receptors in rat cortical astrocytes, in which activation of metabotropic glutamate receptors induces a protein kinase C-dependent up-regulation of GLT-1 activity (Vermeiren *et al.* 2005). Further experiments are necessary to link this cross-regulation with our proposed model.

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