Swelling-activated Taurine and Creatine Effluxes from Rat Cortical Astrocytes are Pharmacologically Distinct

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Abstract. Primary cultures of rat cortical astrocytes undergo a swelling-activated loss of taurine and creatine. In this study, the pharmacological characteristics of the taurine and creatine efflux pathways were compared, and significant differences were shown to exist between the two. Both taurine and creatine effluxes were rapidly activated upon exposure of astrocytes to hypo-osmotic media, and rapidly inactivated upon their return to iso-osmotic media. The relative rates of taurine and creatine efflux depended upon the magnitude of the hypo-osmotic shock. Anion-transport inhibitors strongly inhibited taurine efflux, with the order of potency being NPPB > DIDS > niflumic acid. DIDS and NPPB had less of an inhibitory effect on creatine efflux, whereas tamoxifen and niflumic acid actually stimulated creatine efflux. These data are consistent with separate pathways for taurine and creatine loss during astrocyte swelling.

Key words: Creatine — Taurine — Swelling-activated channel — Osmolyte — Cortical astrocytes

Introduction

Primary cultures of cortical astrocytes have been reported to swell to double their original volume within a few minutes of exposure to hypo-osmotic shock, and subsequently to return to their original volume over the next hour, displaying a volume regulatory response (Vitarella et al., 1994). This regulatory volume decrease is associated with a dramatic rise in the efflux rates of many small organic solutes, primarily amino acids (Kimelberg et al., 1990) and amino-acid derivatives such as taurine (Pasanet-Morales, Mopan & Schousbæe, et al., 1990). It is generally believed that the reduction in intracellular osmolarity brought about by this organic solute efflux makes a major contribution to the volume regulatory response, although this hypothesis remains controversial (Mountain Declercq & Van Drissche, 1996).

The mechanisms by which these solutes leave the cell during swelling have been the subject of extensive research, with taurine efflux, in particular, acting as a paradigm for solute efflux in general (Law, 1994). In most cell types studied to date, the swelling-activated efflux of taurine, and that of many other organic solutes, is sensitive to anion transport inhibitors (Kirk, 1997). This observation has led to the suggestion that, during volume regulation in these cell types, a broad-specificity solute channel is responsible for the greater part of organic solute efflux (Kirk, Ellory & Young, 1992; Roy & Malo, 1992). This proposed channel has been christened VSOAC, for volume sensitive organic anion channel (Strange et al., 1993) and it appears that this channel has been characterized under the name \( I_{Cl,swell} \), an outwardly rectifying chloride/anion conductance identified by Ackerman and co-workers (Ackerman, Wickman & Clapham, 1994).

Despite the focus on taurine in the study of cell volume regulation, it remains but one of a number of organic solutes lost from neural tissue following hypo-osmotic shock. Studies on rat brain in vivo (Verbalis & Gullans, 1993) and ex vivo (Bothwell et al., 2001) have shown swelling-activated decreases in the intracellular levels of, among other things, glutamate, glycerophosphorylcholine, creatine, and myo-
inositol. Earlier investigations suggested that, much as in other cell types, VSOAC mediated this efflux of solutes from neural tissue (Kimelberg et al., 1990; Levitan & Garber, 1998). However, recent work has called this conclusion into question. The pharmacology of both myo-inositol and aspartate effluxes differ noticeably from that of taurine efflux (Sánchez-Olea et al., 1995; Isaacks et al., 1999), and creatine efflux has not been examined at all, despite growing evidence that creatine can play a role in osmoregulation.

In this study, we present the first investigation into the swelling-activated efflux of [1H]-creatine from primary cultures of rat cortical astrocytes. The existence of a hitherto unremarked, rapidly swelling-activated, creatine pathway is reported. The physiological and pharmacological characteristics of this pathway are described and compared with VSOAC. The evidence is consistent with the existence of more than one channel for swelling-activated organic solute efflux in astrocytes.

Materials and Methods

Materials

Dulbecco’s modified Eagle medium (DMEM) was purchased from Life Technologies (Paisley, Scotland), and Path-O-cyte bovine serum albumin from ICN (Flow, UK). 2-[3H]-Taurine was purchased from New England Nuclear (Herts, UK) and [14C]-creatine from Tocris Cookson (Bristol, UK). All other enzymes and chemicals were purchased from either Sigma (Poole, UK) or Boehringer Mannheim (Lewes, UK) and were of analytical grade. Wistar rats and their pups were purchased from Harlan Olac (Bicester, UK). All animals were maintained in-house and handled in accordance with Home Office regulations.

Preparation of Rat Cortical Astrocytes

Enriched astrocytes were prepared by a modification of the method of Noble & Murray (1984). Cortices from one-day-old rat pups were removed and dissected free of meninges, chopped finely, suspended in EDETA solution (200 μg/ml solution of EDETA in Ca²⁺/Mg²⁺-free DMEM) containing 8,500 units/ml trypsin, and incubated at 37°C for 15 min. SBTI-DNase (a solution of 0.52 mg/ml of soybean trypsin inhibitor, 0.04 mg/ml of bovine pancreatic DNase, and 3 mg/ml of bovine serum albumin-fraction V) was added at a ratio of 2 ml for every 10 ml of cortical cell suspension and the mixture incubated for a further 5 min before being centrifuged at 200 × g for 5 min. The resulting pellet was suspended in DMEM-foetal calf serum (DMEM-FCS), which comprised DMEM, 10% v/v FCS, 2 mm glutamine and 25 μg/ml gentamicin. This suspension was further dissociated by repeated trituration through 21-gauge and 25-gauge needles. The dissociated cells were centrifuged at 200 × g for 10 min. The cells were suspended in DMEM-FCS, and then seeded into poly-γ-lysine (PLL)-coated flasks at a density of 10⁴ cells/175 cm² flask.

The cultures were grown in these flasks until confluence was reached, which usually took about 14 days. DMEM-FCS and cells on top of the monolayer were then removed by vigorous shaking in serum-free DMEM. The cell monolayer was then treated with 8 ml versine and 4 ml trypsin, giving a final concentration of 8,500 units trypsin/ml. This caused dissociation of cells from the PLL-coated flask. The resulting cell suspension was collected, resuspended in DMEM-FCS, and aliquoted onto 175-cm² dishes, or six-well plates (each well 38.5 mm²) which had been coated with 5 μg/ml PLL, prior to incubation at 37°C.

After 24 hr the cells were pulsed twice with a 10–20 μM solution of the anti-mitotic agent cytosine arabinoside, with a 24-hr gap between each pulse. This procedure routinely produced astrocyte cultures of >95% purity as assessed by staining with a polyclonal antiserum against glial fibrillary acid protein (DAKO, UK).

The day before an experiment, cells were subjected to a final medium change and incubated overnight in DMEM-B5. DMEM-B5 is a serum-free defined medium; a modification of the N2 medium described by Bottensteim and Sato (1979), containing DMEM supplemented with 0.0286% (v/v) Path-O-cyte BSA, 100 μg/ml human transferrin, 16.2 μg/ml putrescine, 5 μg/ml (0.234 units/ml) insulin, 0.4 μg/ml thryoxine, 0.337 μg/ml tri-iodothyronine, 0.62 μg/ml progesterone, 0.038 μg/ml selenium (as Na2SeO3), 2 mM glutamine and 25 μg/ml gentamycin.

Buffer Solutions

The basic buffer used in all experiments contained (in mm) 148 NaCl, 5 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄·7H₂O, 1.2 CaCl₂, 26 NaHCO₃, 10 glucose, and 2 sodium pyruvate. This had a final measured osmolality, using a freezing point osmometer (Roelbing, Germany), of 340–350 mOsm/kg H₂O. The buffer was gassed continually with 95% O₂/5% CO₂, giving a pH of 7.2–7.4. This buffer was made hypo-osmotic by a reduction in NaCl to give the desired measured osmolality.

Cell Culture Efflux Experiments

[1H]-Taurine and [14C]-creatine efflux from primary cell cultures were measured at 37°C. Cells in six-well plates were washed in buffer and incubated for 1 hr in iso-osmotic buffer containing 2 μCi/ml [1H]-taurine and/or 0.1 μCi/ml [14C]-creatine, and 2 mM of each of the unlabelled compounds. Prior to each experiment the loading solution was removed and cells were washed 5 times in iso-osmotic buffer to remove extracellular radiolabel. Each experiment was then started by the addition of a 1 ml aliquot of isotonic buffer to each well. At set time points after the start of the experiment the medium in each well was removed and replaced by another 1 ml aliquot of buffer. The solution removed from the well was transferred to a scintillation vial for determination of radioactivity. This operation was continued throughout the course of the experiment, with the removed solution being replaced with other solutions of interest as required.

At the end of each experiment 1 ml of 0.1 M NaOH was added to each well and left for 4 hr. This concentration of NaOH was chosen as it resulted in complete cell lysis but negligible quenching of radioisotope scintillation. The NaOH solution was removed, and each well washed 3 times with 1 ml of distilled water. The NaOH and water aliquots were pooled for radiolabel determination. The amount of radiolabel in each sample was determined by adding 2 ml of Ultima Gold (Canberra Packard, UK) to 1 ml of sample, and counting ¹H and/or ¹⁴C dpm on a Beckman LS 1701 scintillation counter.

Most experiments were performed using only one radio-labelled compound at a time. However, for the inhibitor dose-response experiments both [1H]-taurine and [14C]-creatine were present, and dual-label counting was employed. To ensure that ¹H-dpm were accurately distinguished from potentially overlapping ¹⁴C-dpm, external quench monitoring was carried out. Automatic quench compensation was then performed so that the ¹H and ¹⁴C windows varied as a function of the measured quench. Dual-labelled standards of varying ¹H/¹⁴C content were used to empirically test