J. Membrane Biol. 185, 157–164 (2002)
DOI: 10.1007/s00232-001-0121-2

The Journal of
Membrane
Biology

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

J.H. Bothwell, P. Styles, K.K. Bhakoo
MRC Biochemical and Clinical Magnetic Resonance Unit, Department of Biochemistry, University of Oxford, South Parks Rd., Oxford, OXI 3QU, UK

Received: 22 May 2001/Revised: 29 September 2001

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 (Pasantes-Morales, Mopan & Schousbae, 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-*

Correspondence to: K.K. Bhakoo; email: k.bhakoo@bioch.ox.ac.uk

Abbreviations: DMSO, dimethylsulphoxide; DIDS, 4,4′-Di-isothiocyanatostilbene-2,2′-disulphonic acid; NPPB, 5-Nitro-2-(3-phenylpropylamino)benzoic acid; VSOAC, Volume Sensitive Organic Anion Channel

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 [^{14}C]-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-[^{3}H]-Taurine was purchased from New England Nuclear (Herts, UK) and [^{14}C]-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 EDTA solution (200 μg/ml solution of EDTA in Ca^{2+}/Mg^{2+}-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 gentamycin. 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-L-lysine (PLL)-coated flasks at a density of 10^7 cells/175-cm^2 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-cm^2 dishes, or six-well plates (each well 38.5 mm^2) 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-BS. DMEM-BS is a serum-free defined medium; a modification of the N2 medium described by Bottenstein 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 thyroxine, 0.337 μg/ml tri-iodothyronine, 0.62 μg/ml progesterone, 0.0383 μg/ml selenium (as Na_2SeO_3), 2 mM glutamine and 25 μg/ml gentamycin.

Buffer Solutions

The basic buffer used in all experiments contained (in mM) 148 NaCI, 5 KCI, 1.2 KH_2PO_4 1.2 $MgSO_4 \cdot 7H_2O$, 1.2 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, and 2 sodium pyruvate. This had a final measured osmolality, using a freezing point osmometer (Roebling, Germany), of 340–350 mOsm(kg $H_2O)^{-1}$.The buffer was gassed continually with 95% O_2/5% CO_2, 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

[^{3}H]-Taurine and [^{14}C]-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 [^{3}H]-taurine and /or 0.1 μCi/ml [^{14}C]-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 radiolabel. 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 radiolabel 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 ^{3}H and/or ^{14}C dpm on a Beckman LS 1701 scintillation counter.

Most experiments were performed using only one radiolabelled compound at a time. However, for the inhibitor dose-response experiments both [^{3}H] taurine and [^{14}C]-creatine were present, and dual-label counting was employed. To ensure that ^{3}H-dpm were accurately distinguished from potentially overlapping ^{14}C-dpm, external quench monitoring was carried out. Automatic quench compensation was then performed so that the ^{3}H and ^{14}C windows varied as a function of the measured quench. Dual-labelled standards of varying ^{3}H/^{14}C content were used to empirically test

that the ^3H and ^{14}C contributions could be accurately resolved at the specific activity and counting times used.

Anion Transport Inhibitors

4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), niflumic acid, 5-Nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and tamoxifen were added to iso- and hypo-osmotic buffers in a stock solution in DMSO, such that the concentration of DMSO in the buffer was constant at 0.5% (v/v). The inhibitors were added after the first wash to remove excess radiolabel, and were maintained at a constant concentration throughout the experiment.

Influx Experiments

[^{14}C]-Creatine influx into primary astrocyte cultures was measured at 37°C. Cells in six-well plates were washed twice in iso-osmotic buffer and incubated for various lengths of time in iso- or hypo-osmotic KHB supplemented with 0.1 μCi/ml [^{14}C]-creatine. At the end of the experiment cells were washed three times in iso-osmotic buffer to remove extracellular radiolabel. The amount of [^{14}C]-creatine remaining in the cells was determined by adding 1 ml of 0.1 M NaOH to each well. After 4 hr the NaOH was removed and each well washed two times with 1 ml of distilled water. The NaOH suspension and the two washes were transferred directly to scintillation vials for determination of radioactivity. Parallel six-well plates that had been grown from the same cell suspension, but which had not been exposed to radioactivity, were used for estimation of protein content using the method of Lowry et al. (1951).

Statistical Analysis

Statistical analysis was performed using the StatView 512+ software (Brainpower Inc., CA) in all cases where the number of determinations (n) was 3 or more. All results are expressed as means ± SD. Unidirectional efflux rate constants were calculated using the following formula:

$$\text{Rate Constant} = -[\ln(X_1/X_2)]/(t_1 - t_2) \quad (1)$$

where X_1 and X_2 are the percentage of counts remaining in the cells at times t_1 and t_2, respectively. Where comparisons were made, mean rate constants were quantified over a period of 6–12 min. Rate constants are assumed to follow normal distributions, and are compared using one- or two-way ANOVA, as appropriate. The null hypothesis, which is tested, is that two or more compared means are from the same population. A significant difference in levels is claimed when the null hypothesis is rejected at a level of 5% or less.

Results

Increases in [^3H]-Taurine and [^{14}C]-Creatine Efflux Rates Following Hypo-Osmotic Shock

There was an increase in the rate of [^3H]-taurine and [^{14}C]-creatine release following hypo-osmotic shock (Fig. 1). This increase was reversible, returning to basal levels within 4 min of return to iso-osmotic buffer. The rates of [^3H]-taurine and [^{14}C]-creatine efflux were also measured during iso-osmotic incubation, immediately after hypo-osmotic (200 mOsm (kg H_2O)$^{-1}$) shock, and 2 hr after the onset of hypo-osmotic shock, and the efflux rate constants are shown in Fig. 2. Astrocytes in iso-osmotic buffer displayed low resting efflux of [^3H]-taurine and [^{14}C]-creatine, with rate constants of 0.12 ± 0.02 hr^{-1} (n = 16) and 0.12 ± 0.03 hr^{-1} (n = 16) respectively. Upon exposure to hypo-osmotic buffer, efflux of [^3H]-taurine was increased significantly within four minutes of shock, with an increased rate constant of 1.48 ± 0.14 hr^{-1} (n = 16; $p < 0.001$). After 2 hr of exposure to hypo-osmotic buffer, the rate constant for [^3H]-taurine efflux had significantly decreased to 0.39 ± 0.07 hr^{-1}, but was still significantly higher than in iso-osmotic buffer (n = 4; $p < 0.001$). The rate constant for [^{14}C]-creatine efflux rose less dramatically following exposure to hypo-osmotic buffer, to 0.31 ± 0.03 hr^{-1} (n = 16; $p < 0.001$). However, this efflux rate constant did not decrease during 2 hr of exposure to hypo-osmotic buffer, remaining at 0.28 ± 0.04 hr^{-1} (n = 4) after 2 hr.

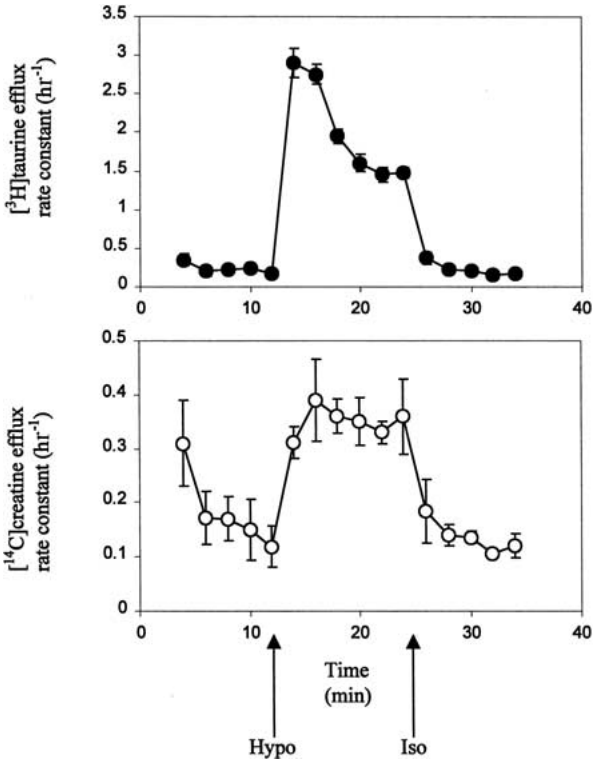

Fig. 1. Swelling-activated [^3H]-taurine (●) and [^{14}C]-creatine (○) efflux from primary cultures of rat cortical astrocytes. Astrocytes, which had been preloaded with either [^3H]-taurine or [^{14}C]-creatine, were incubated with iso-osmotic buffer (340 mOsm/kg H_2O). At t = 12 min the incubating buffer was changed to hypo-osmotic buffer (200 mOsm/kg H_2O), and then back to iso-osmotic buffer after 24 min; this is marked by an arrow in the diagrams. All values are expressed as means ± SD (n = 4).

Effect of Magnitude of Osmotic Shock on [^3H]-Taurine and [^{14}C]-Creatine Efflux

The initial rate constants for both [^3H]-taurine and [^{14}C]-creatine efflux depended upon the magnitude of

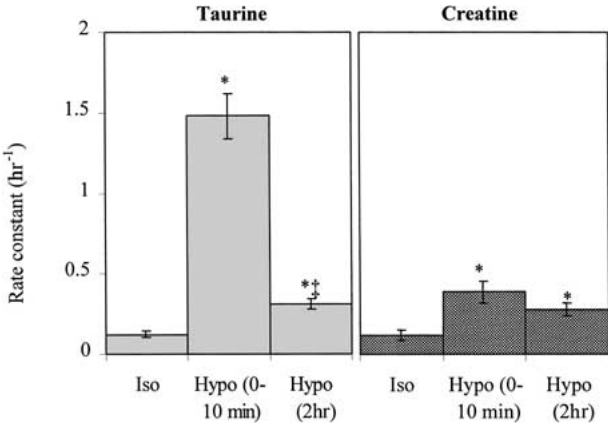

Fig. 2. Time dependence of rate constants for [^{3}H]-taurine and [^{14}C]-creatine efflux rates in primary cultured cortical astrocytes. Astrocytes were preloaded with either [^{3}H]-taurine or [^{14}C]-creatine and incubated for 10 min in iso-osmotic buffer (340 mOsm/kg H_2O), followed by 120 min in hypo-osmotic buffer (200 mOsm/kg H_2O). Rate constants (in hr^{-1}) are expressed as mean ± SD. Rate constants were compared using one-way ANOVA and were considered statistically different from iso-osmotic values if * $p < 0.001$, and from 0–10 min hypo-osmotic values if ‡ $p < 0.001$.

the hypo-osmotic shock. A significant increase in the rate of [^{3}H]-taurine efflux was not seen unless the osmolality of the hypo-osmotic buffer was 290 mOsm (kg $H_2O)^{-1}$ or lower (i.e. 80% of iso-osmolal). By contrast, a significant increase in the rate of [^{14}C]-creatine efflux was not seen unless the buffer osmolality was 260 mOsm (kg $H_2O)^{-1}$ or lower (i.e. 70% of iso-osmolal). At lower osmolalities the rates of efflux rose sharply, and had not reached a plateau at hypo-osmotic insults of 175 mOsm (kg $H_2O)^{-1}$ (50% of iso-osmolal), as shown in Fig. 3*a*.

In addition to determining the absolute values of the efflux rate constants, the magnitude of the hypo-osmotic shock also affected the relative values of the taurine and creatine efflux rate constants (Fig. 3*b*). The efflux rate constant for [^{3}H]-taurine in iso-osmotic conditions was similar in value to that for [^{14}C]-creatine. As the osmolality of the hypo-osmotic buffer was decreased the [^{3}H]-taurine efflux rate constant rose more rapidly than the [^{14}C]-creatine efflux rate constant, being about four times greater than the [^{14}C]-creatine efflux rate constant when the buffer osmolality was reduced to 50% of iso-osmolal.

Anion Transport Inhibitors

Dose-response curves for the changes in the rate constants of the swelling-activated [^{3}H]-taurine and [^{14}C]-creatine conductances are shown in Fig. 4. None of the "traditional" (Kirkt 1997) anion transport inhibitors (DIDS, NPPB, and niflumic acid) affected iso-osmotic [^{3}H]-taurine or [^{14}C]-creatine efflux at any of the concentrations used. At high concentrations, all three of these inhibitors caused a reduction in the rate constant for [^{3}H]-taurine efflux. NPPB and DIDS also inhibited [^{14}C]-creatine efflux, but niflumic acid stimulated [^{14}C]-creatine efflux as shown in Fig. 5. The dose response curves of [^{3}H]-taurine and [^{14}C]-creatine efflux differed significantly for DIDS and niflumic acid, but did not for NPPB. The results of preliminary experiments using tamoxifen as a channel blocker are also shown in Fig. 5. Although low micromolar concentrations of tamoxifen stimulated hypo-osmotic [^{14}C]-creatine efflux, tamoxifen concentrations of 10 μM or more resulted in a greatly increased rate of iso-osmotic [^{14}C]-creatine efflux, and so a complete dose-response comparison was not possible.

[^{14}C]-Creatine Influx

To investigate the nature of the hypo-osmotically activated creatine release mechanism, [^{14}C]-creatine influx into cortical astrocytes incubated in iso- and hypo-osmotic buffer was measured. Astrocytes exposed to iso-osmotic buffer showed negligible uptake of [^{14}C]-creatine. In contrast, exposure to hypo-osmotic buffer caused a rapid increase in the unidirectional rate of [^{14}C]-creatine uptake, which increased almost linearly as the external creatine concentration was raised, and which was not saturated at external creatine concentrations of up to 50 mM (Fig. 6).

Discussion

The Properties of the Taurine Efflux Pathway Correspond to Those of VSOAC

Most cell types are thought to respond to swelling by loss of a variety of organic solutes, the most prevalent of these being taurine. It has previously been proposed that the swelling-activated efflux of taurine from astrocytes is mediated by a broad-specificity channel (Pasantes-Morales et al., 1994; Cardin Peña-Segura & Pasantes-Morales, 1999). This channel is also thought to be ubiquitous to cells in higher organisms, and has been named VSOAC (Strange et al., 1993). The properties of [^{3}H]-taurine efflux in this study confirm those previously reported, namely the existence of a rapidly activating volume-sensitive efflux pathway (Pasantes-Morales & Schousboe, 1988), which is sensitive to DIDS at concentrations between 100–400 μM (Sánchez-Olea, et al., 1993; Pasantes-Morales et al., 1994), to niflumic acid at concentrations between 50–300 μM (Sánchez-Olea et al., 1993), and which inactivates following changes in cell volume (Cardin et al., 1999). We have, therefore, assumed [^{3}H]-taurine efflux to be a marker of VSOAC activity.

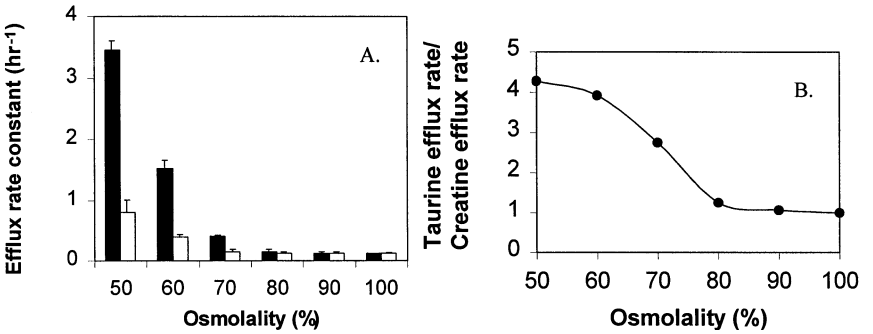

Fig. 3. Rates of [^{3}H]-taurine and [^{14}C]-creatine efflux from primary cultures of rat cortical astrocytes at different osmolarities. Astrocytes, which had been preloaded with either [^{3}H]-taurine or [^{14}C]-creatine were incubated with buffer at a range of osmolalities (340–175 mOsm/kg H_2O), corresponding to 100–50% of the iso-osmolal value. (*A*) The absolute values of [^{3}H]-taurine (■ bars) or [^{14}C]-creatine (□ bars) are shown as a function of osmolality. (*B*) The ratio of [^{3}H]-taurine to [^{14}C]-creatine efflux rates is shown as a function of osmolality. All values are expressed as means ± SD (n = 4).

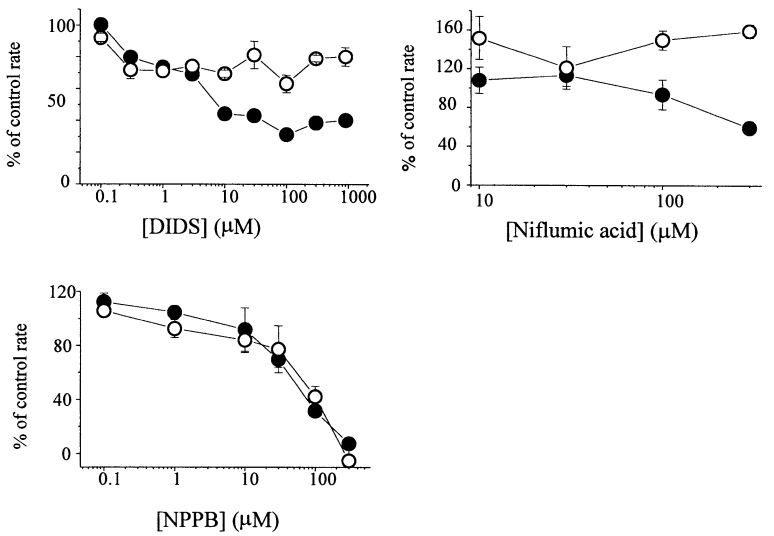

Fig. 4. Dose-response curves for (●) [^{3}H]-taurine and (○) [^{14}C]-creatine efflux from primary cultures of rat cortical astrocytes. Astrocytes were preloaded with either [^{3}H]-taurine or [^{14}C]-creatine. Inhibitors, made up as stock solutions in DMSO, were added to iso-osmotic buffer for 12 min prior to a hypo-osmotic shock of 200 mOsm/kg H_2O, and were maintained at the same concentration throughout hypo-osmotic incubation. All values are expressed as means ± SD (n = 6).

A Volume-Sensitive Creatine-Efflux Pathway Exists in Primary Astrocyte Cultures

In the present study we have observed a rapidly activating, volume-sensitive efflux of [^{14}C]-creatine from primary cultures of rat cortical astrocytes. At most magnitudes of hypo-osmotic shock, the rate constant for [^{14}C]-creatine efflux is much lower than that for [^{3}H]-taurine. However, it is important to emphasize that the values given here for the rate constants of [^{14}C]-creatine efflux are, if anything, underestimates. This is because, in brain tissue, the main biochemical fate of creatine is its conversion to phosphocreatine by creatine kinase. We may, therefore, expect a sizeable proportion of the [^{14}C]-creatine loaded into the astrocytes to be converted into phosphocreatine, which may be inaccessible to the creatine efflux pathway. Hence the total intracellular [^{14}C] radioactivity, which is used as a measure of the total intracellular free creatine in the calculation of efflux rate constants, may overestimate the free creatine levels, and thus underestimate the rate of [^{14}C]-creatine efflux.

Pharmacological Comparison of the Creatine Efflux Pathway with VSOAC

In contrast to the wealth of information available on the properties of VSOAC, the pathway by which creatine leaves the cell during volume regulation has only rarely been investigated. We report, for the first time, a rapidly activating volume-sensitive efflux of [^{14}C]-creatine from primary cultures of rat cortical astrocytes. Moreover, the pharmacology and kinetics of this [^{14}C]-creatine efflux do not correspond with those of [^{14}C]-taurine efflux. There are two possible reasons for this.

Firstly, although dose-response curves are often used to distinguish between pathways (Kirk et al., 1992; Lewis, Bursell & Kirk, 1996), neither the methods of action of the inhibitors nor the selectivity mechanism of the pathways are fully understood. It is often assumed that charged inhibitors such as NPPB, DIDS, and niflumic acid, exert their effects via the steric block of transporter pores. If this is the case, then differences in the sizes or charges of the solutes

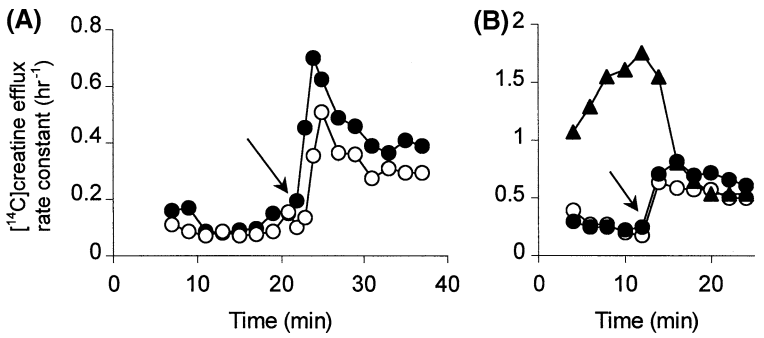

Fig. 5. Stimulation of [^{3}C]-creatine efflux from primary cultures of rat cortical astrocytes by niflumic acid and Tamoxifen. Representative plots of: (*A*) stimulation of [^{14}C]-creatinine efflux by 300 μM niflumic acid (○, untreated and ● treated); (*B*) Effects of Tamoxifen on [^{14}C]-creatinine efflux (○, untreated; ● + 3 μM Tamoxifen). Astrocytes were preloaded with [^{14}C]-creatine and inhibitors, made up as stock solutions in DMSO, were added to iso-osmotic buffer prior to a hypo-osmotic shock of 200 mOsm/kg. H_2O (indicated by an arrow in the figures). The inhibitor concentrations were maintained throughout the hypo-osmotic incubation.

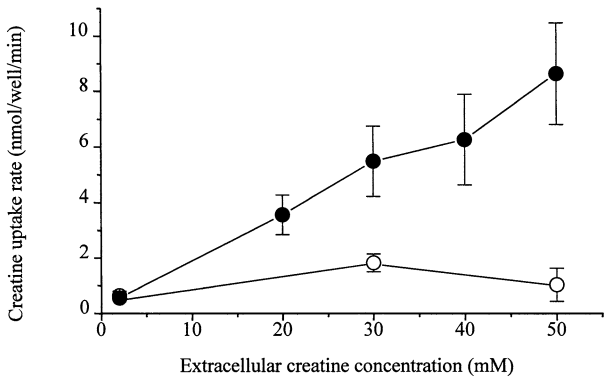

Fig. 6. Dependence of swelling-activated [^{14}C]-creatine influx on extracellular creatine concentration in primary cultures of rat cortical astrocytes. Astrocytes were incubated with (○) iso-osmotic buffer (340 mOsm/kg H_2O) or (●) hypo-osmotic buffer (200 mOsm/kg H_2O), supplemented with varying concentrations of creatine and trace [^{14}C]-creatine. All values are expressed as means ± SD (n = 6).

and inhibitors, rather than any difference in the pathways used, may give rise to the observed differences in dose-response curves. However, in this there is little to choose between creatine and taurine, as both have similar hydrodynamic radii (creatine's being about 20% larger) and both are found predominantly in the zwitterionic form at physiological pH (7.4).

Second, different pathways may exist for the efflux of taurine and creatine. This may take the form of two separate, volume-activated channels. The uncertainties about inhibitor action notwithstanding, we think that this is the more likely reason for the observed pharmacological differences. The idea that swelling-activated organic solute release from neural tissue occurs through more than one pathway is not a new one. In fact, evidence for the existence of more than one such pathway has been accumulating over the past decade (Pasantes-Morales et al., 1994; Vitarella et al., 1994; Mongin et al., 1999) and a small-magnitude, slowly deactivating, swelling-activated efflux has already been reported for *myo*-inositol (Gonzalez, Sanchez-Olea & Pasantes-Morales 1995), another organically active solute.

We note two points in passing. First, that [^{14}C]-creatine efflux is, paradoxically, stimulated by certain inhibitors. This observation cannot be conclusively explained, but if different volume-regulatory pathways exist in astrocytes, with differential sensitivities to inhibitors, then inhibition of one pathway may lead to a compensatory stimulation of another, as the cell attempts to effect the same overall reduction in intracellular osmolarity. Second, previous work carried out in our laboratory has shown that endogenous taurine and creatine loss from adult rat cortical brain slices show equal sensitivity to anion transport inhibitors (Bothwell et al., 2001), consistent with the existence of a common permeability pathway. This is at variance with the findings of the present study, in which [^{14}C]-creatine and [^{3}H]-taurine effluxes show divergent pharmacology and kinetic properties within a single cellular population. However, this observation is not unexpected. Brain slices consist of a heterogeneous cellular preparation, with a correspondingly heterogeneous population of membrane transporters, and preserve many of the neuronal/glial interactions, which occur in vivo (Griffin et al., 1998). In contrast, the astrocyte cultures used in the present study are homogenous (>95%). Cell-cell cooperation in the maintenance of ion homeostasis is well documented (Magistretti et al., 1999), and it is not unreasonable to suppose that such cooperation may occur in the maintenance of osmolyte homeostasis. Indeed, morphological studies have also shown that the volume-regulatory response of cells in brain slices is not necessarily the same as that of cells in culture (Andrew, Labinowich & Oseholo, 1997).

The Nature of the Creatine-Efflux Pathway

Channels are not always implicated in the efflux of solutes from cells. Glutamate efflux from neurons has been reported to occur by reversal of a Na^+-independent glutamate transporter that normally mediates glutamate uptake (Attwell, Barbour & Szatkowski, 1993; Rossi, Oshima & Attwell, 2000). A Na^+-independent creatine-uptake transporter has

been cloned by Sora and coworkers (1994), and it is possible that reversal of this transporter is responsible for the swelling-activated [^{14}C]-creatine efflux reported in this study. However, most Na^+-dependent carriers saturate at extracellular substrate concentrations of a few micromolar, and the creatine-uptake transporter is no exception, having a k_m for creatine of 45 μM (Möller & Hamprecht, 1989). The failure of the hypo-osmotically activated creatine-uptake pathway to saturate at millimolar external creatine concentrations is characteristic of the behavior of a channel, rather than a reversed cotransporter. Hence, we propose that the swelling-activated pathway that allows non-saturable creatine influx may be similar to that which mediates creatine efflux and it is therefore likely that swelling-activated creatine efflux occurs through a channel.

An Osmoregulatory Role for Creatine in Vivo?

It is tempting to ascribe a role for creatine in the volume-regulatory response of astrocytes. The levels of creatine in neural tissue are certainly high enough to support such a hypothesis. Urenjak and coworkers (1993) found that the level of creatine in cultured rat astrocytes was comparable to that of taurine (65 nmol/mg protein *vs*. 71 nmol/mg protein respectively). Indeed, in whole rat brain, creatine levels may be twice as high as taurine levels (Verbalis & Gullans, 1993). Moreover, we recognize that the osmotic-shock protocol, which has been used in the present study, is unlikely to provide a precise model of osmotic insults in vivo. The behavior of [^{3}H]-taurine and [^{14}C]-creatine effluxes under severe hypo-osmotic shocks serve to distinguish one from the other as the rate constant for creatine efflux is several-fold lower than for taurine and as a more extreme shock is required to fully activate creatine efflux. However, at smaller, and more physiological, magnitudes of osmotic shock (i.e. reductions down to around 80% of original osmolality) the efflux rates for taurine efflux and creatine are similar. In addition, we have shown that the [^{14}C]-creatine efflux does not rapidly inactivate in the same way as [^{3}H]-taurine efflux, so during a prolonged hypo-osmotic insult creatine may well contribute as much as taurine to the reduction in intracellular osmolarity. Even if creatine itself is not of major importance in the control of astrocyte volume, the pathway we describe may be. VSOAC is a broad-specificity channel, and it is conceivable that some of the other organic solute pathways are likewise.

It is perhaps more relevant that the total metabolite availability determines the potential of any given metabolite to eventually correct osmolar imbalance. Certainly, creatine has a quite separate and vital role to play in maintaining cellular energetics, and so taurine, with a higher efflux rate constant, might be expected to be the preferred osmolyte in an acute event. However, creatine could have a role as a low-level but persistent osmolyte over the longer term. In this model, we might expect taurine and creatine efflux to occur through different pathways to allow for differential regulation of solute loss depending upon the exact magnitude of the osmotic stress. Such a system has recently been proposed to operate in fish erythrocytes, separating inorganic and organic osmolyte effluxes (Guizouarn & Motais, 1999).

In conclusion, we have demonstrated the acute, swelling-activated, efflux of [^{14}C]-creatine from primary astrocytes. It is likely that this efflux occurs through a channel. This efflux was a small, rapidly activating pathway, which did not inactivate after 2 hr of exposure to hypo-osmotic buffer. The pharmacology of [^{14}C]-creatine efflux was significantly different from that of [^{3}H]-taurine efflux, implying that [^{14}C]-creatine efflux did not occur through VSOAC. This suggests that, in rat astrocytes, more than one pathway contributes to organic osmolyte decrease.

Many of the earlier studies of volume regulation have used extremely large osmotic shocks, and this may be leading to an exaggeration of the importance of taurine and, by extension, VSOAC in cell volume regulation in vivo. It may now be more profitable to consider the operation of several volume-regulatory pathways, the contributions of which vary depending upon the nature and duration of the osmotic insult.

The authors thank Julian Griffin and Kiaran Kirk for their invaluable advice. This work was funded by the Medical Research Council of Great Britain. JHB was the recipient of an MRC Studentship, with additional funding from Merton College, Oxford.

References

Ackerman, M.J., Wickman, K.D., Clapham, D.E. 1994. Hypotonicity activates a native chloride current in *Xenopus* oocytes. *J. Gen. Physiol.* **103:**153–179

Andrew, R.D., Lobinowich, E., Osehobo, E.P. 1997. Evidence against volume regulation by cortical brain slices during acute osmotic stress. *Exp. Neurol.* **143:**300–312

Attwell, D., Barbour, B., Szatkowski, M. 1993. Nonvesicular release of Neurotransmitter. *Neuron* **11:**401–407

Bothwell, J.H., Rae, C., Dixon, R.M., Styles, P., Bhakoo, K.K. 2001. Hypo-osmotic swelling-activated release of organic osmolytes in brain slices: implications for brain oedema in vivo. J. Neurochem. **77:**1632–1640.

Bottenstein, J.H. and Sato, G.H. 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* **76:**514–517

Cardin, V., Peña-Segura, C., Pasantes-Morales, H. 1999. Activation and inactivation of taurine efflux in hyposmotic and isosmotic swelling in cortical astrocytes: Role of ionic strength and cell volume decrease. *J. Neurosci. Res* **56:**659–667

González, E., Sánchez-Olea, R. and Pasantes-Morales, H. 1995. Inhibition by Cl- channel blockers of the volume-activated,

diffusional mechanism of inositol transport in primary astrocytes in culture. *Neurochemical Res.* **20:**895–900

Griffin, J.L, Rae, C., Dixon, R.M., Radda, G.L.-afld Matthews, P.M. 1998. Excitatory amino acid synthesis in hypoxic brain slices; does alanine act as a substrate for glutamate production in hypoxia? *J. Neurochem.* **71:**2477–2486

Guizouarn, H., Motais, R. 1999. Swelling activation of transport pathways in erythrocytes: effects of Cl^-, ionic strength, and volume changes. *Am. J. Physiol.* **276:**C210–C220

Isaacks, R.E., Bender, A.S., Kirn, C.Y., Shi, Y.F. and Norenberg, M.D. 1999. Effect of osmolarity and anion channel inhibitors on myo-inositol efflux in cultured astrocytes. *J. Neurosci. Res.* **57:**866–871

Kimelberg, H.K., Goderie, S.K., Higman, S., Pang, S., Waniewski, R.A. 1990. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J. Neurosci.* **10:**1583–1591

Kirk, K., Ellory, J.C., Young, J.D. 1992. Transport of organic substrates

via a volume-activated channel. *J. Biol. Chem.* **267:**23475–23478

Kirk, J., Kirk, K. 1994. Inhibition of volume-activated I- and taurine efflux from HeLa- cells by p-glycoprotein blockers correlates with calmodulin inhibition. *J. Biol. Chem.* **269:**29389–29394

Kirk, K. 1997. Swelling-activated organic osmolyte channels. *J. Membrane Biol.* **158:**1–16

Law, R.O. 1994. Regulation of mammalian brain-cell volume. *J. Exp. Zool.*, **268:**90–96

Levitan, I., Garber, S.S. 1998. Anion competition for a volume-regulated current. *Biophys. J.* **75:**226–235

Lewis, R.A., Bursell, J.D.H., Kirk, K. 1996. Anion-selectivity of the swelling-activated osmolyte channel in eel erythrocytes. *J. Membrane Biol.* **149:**103–111

Lowry, O.H., Rosebrough, N.J., Lewis Farr, A., Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265–275

Magistretti, P.J., Pellerin, L., Rothman, D.L., Shulman, R.G. 1999. Energy on demand. *Science* **283:**496–497

Möller, A., Hamprecht, B. 1989. Creatine transport in cultured cells of rat and mouse brain. *J. Neurochem.* **52:**544–550

Mongin, A.A., Reddi, J.M., Charniga, C., Kimelberg, H.K. 1999. [^{3}H]- taurine and D-[^{3}H]-aspartate release from astrocyte cultures are differently regulated by tyrosine kinases. *Am. J. Physiol.* **276:**C1226–C1230

Mountian, I., Declercq, P.E., Van Driessche, W. 1996. Volume regulation in rat brain cells: lack of a substantial contribution of free amino acids. *Am. J. Physiol.*, **270:**C1319–1325

Noble, M., Murray, K. 1984. Purified astrocytes promote the in vitro division of a bipotential glial progenitor cell. *EMBO J.* **3:**2243–2247

Pasantes-Morales, H., Schousboe, A. 1988. Volume regulation in astrocytes: A role for taurine as an osmoeffector. *J. Neurosci. Res.* **20:**505–509

Pasantes-Morales, H., Moran, J., Schousboe, A. 1990. Volume-sensitive release of taurine from cultured astrocytes: properties and mechanism. *Glia* **3:**427–432

Pasantes-Morales, H., Murray, R. A., Sánchez-Olea, R., Morán, J. 1994. Regulatory volume decrease in cultured astrocytes II. Permeability pathway to amino acids and polyols. *Am. J. Physiol.* **266:**C172–C178

Rossi, D.J., Oshima, T., Attwell, D. 2000. Glutamate release in severe brain ischaemia is mainly by reversed uptake. *Nature* **403:**316–321

Roy, G., Malo, C. 1992. Activation of amino acid diffusion by a volume increase in cultured kidney (MDCK) cells. *J. Membrane Biol*, **130:**83–90

Sánchez-Olea, R., Pena, C., Morán, J., Pasantes-Morales, H. 1993. Inhibition of volume regulation and efflux of osmoregulatory amino acids by blockers of Cl-transport in cultured astrocytes. *Neurosci. Lett.* **156:**141–144

Sánchez-Olea, R., Morales-Mulia, M., Morán, J., Pasantes-Morales, H. 1995. Inhibition by polyunsaturated fatty acids of cell-volume regulation and osmolyte fluxes in astrocytes. *Am. J. Physiol.* **38:** C96–C102

Sora, I., Richman, J., Santoro, G., Wei, H., Wang, Y., Vanderah, T., Horvath, R., Nguyen, M., Waite, S., Roeske, W.R., Yamamura, H.I. 1994. The cloning and expression of a human creatine transporter. *Biochem. Biophys. Res. Commun.* **204:**419–427

Strange, K., Morrison, R., Shrode, L., Putnam, R. 1993. Mechanism and regulation of swelling-activated inositol efflux in brain cells. *Am. J. Physiol.* **265:** C244–C256

Urenjak, J., Williams, S.R., Gadian, D.G., Noble, M. 1993. Proton nuclear magnetic resonance spectroscopy unambiguously identies different neural cell types. *J. Neurosci.* **13:**981–989

Verbalis, J.G., Gullans, S.R. 1993. Rapid correction of hyponatremia produces differentiasl effects on brain osmolyte and electrolyte reaccumulation in rats. *Brain Res.* **606:**19–27

Vitarella, D., DiRisio, D. J., Kimelberg, H. K., Aschner, M. 1994. Potassium and taurine release are highly correlated with regulatory volume decrease in neonatal primary rat astrocyte cultures. *J. Neurochem.* **63:**1143–1149