Receptor Regulation of the Volume-Sensitive Efflux of Taurine and Iodide from Human SH-SY5Y Neuroblastoma Cells: Differential Requirements for Ca$^{2+}$ and Protein Kinase C.

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Running Title: Differential regulation of $^{125}$I$^{-}$ and taurine release

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Non-standard abbreviations: $\text{Ca}^{2+}_i$, cytoplasmic calcium; DDF, 1,9-dideoxyforskolin; DIDS, 4,4'diisothiocyanatostilbene-2,2'-disulfonic acid; DMEM, Dulbecco's modified Eagle's medium; HEPES, N-[2 hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; PAR, proteinase-activated receptor; PKC, protein kinase C; VSOAC, volume-sensitive organic osmolyte and anion channel; RVD, regulatory volume decrease; mAChR, muscarinic cholinergic receptor; DCPIB, 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid; Oxo-M, oxotremorine-M; Y-27632, (R)-(+) -trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride.

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Abstract

The basal- (swelling-induced) and receptor-stimulated efflux of $^{125}$I and taurine have been monitored to determine whether these two osmolytes are released from human SH-SY5Y cells under hypotonic conditions via common or distinct mechanisms. Under basal conditions, both $^{125}$I$^-$ (used as a tracer for Cl$^-$) and taurine were released from the cells in a volume-dependent manner. Thrombin addition, mediated via the proteinase activated receptor-1 (PAR-1) subtype, significantly enhanced the release of both $^{125}$I$^-$ and taurine (3-6-fold) and also increased the threshold osmolarity for efflux of these osmolytes ('set-point') from 200 to 290 mOsM. Inclusion of a variety of broad spectrum anion channel blockers and of 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1$H$-inden-5-yl)oxy]butanoic acid (DCPIB) attenuated the release of both $^{125}$I$^-$ and taurine under basal- and receptor-stimulated conditions. Basal release of $^{125}$I$^-$ and taurine was independent of Ca$^{2+}$ or the activity of protein kinase C (PKC). However, although PAR-1-stimulated taurine efflux was attenuated by either a depletion of intracellular Ca$^{2+}$ or inhibition of PKC by chelerythrine, the enhanced release of $^{125}$I$^-$ was independent of both parameters. Stimulated efflux of $^{125}$I$^-$ following activation of muscarinic cholinergic receptors was also markedly less dependent on Ca$^{2+}$ availability and PKC activity than that observed for taurine release. These results indicate that, although the osmosensitive release of these two osmolytes from SH-SY5Y cells may occur via pharmacologically similar membrane channels, the receptor-mediated release of $^{125}$I$^-$ and taurine is differentially regulated by PKC activity and Ca$^{2+}$ availability.
Introduction

Cell volume is constantly subject to change as a consequence of solute accumulation, oxidative metabolism or fluctuations in the osmolarity of the extracellular fluid. To survive, cells need to regulate their volume within relatively narrow limits and this homeostatic mechanism is of particular importance to the brain due to the restrictions of the skull. A common cause of brain swelling is hyponatremia, a condition that disproportionately impacts the elderly, infants, marathon runners and military personnel (Upadhyay et al., 2006). Hyponatremia is associated with a variety of neurological symptoms, such as disorientation, mental confusion and seizures (Kimelberg, 2000; Pasantes-Morales et al., 2000, 2002).

In response to hypotonic stress, cells swell with a magnitude proportional to the reduction in osmolarity. This is followed by a homeostatic mechanism termed regulatory volume decrease (RVD) which involves the extrusion of intracellular ions such as K⁺, Cl⁻ and a number of organic osmolytes, which together facilitate the loss of water to normalize cell volume (Pasantes-Morales et al., 2000). Inorganic ions constitute two-thirds of the osmolytes released during RVD and the remainder is accounted for by “compatible” organic osmolytes such as polyols, methylamines and amino acids. Of these, taurine, an amino acid present in eukaryotic cells at concentrations of up to 40 mM, is considered to be an ideal osmolyte due to its metabolic inertness and abundance (Huxtable, 1992; Lambert, 2004).
It is proposed that extrusion of these osmolytes from the cell is mediated via a volume-sensitive organic osmolyte and anion channel (VSOAC), which is primarily permeable to Cl⁻ but impermeable to cations (for reviews, see Lang et al., 1998; Nilius and Droogmans, 2003). Evidence to support the involvement of VSOAC in response to hypotonic stress comes from studies in which RVD, volume-sensitive Cl⁻ current and organic osmolyte release can all be blocked by broad spectrum anion channel inhibitors, such as DDF or NPPB, and by a highly selective agent, DCPIB (Decher et al., 2001; Abdullaev et al., 2006). Similarities in the pharmacological inhibition profile of swelling-activated efflux of organic osmolytes and Cl⁻ in response to anion channel blockers has led to the suggestion that a common pathway exists for the extrusion of both Cl⁻ and organic osmolytes (Jackson and Strange, 1993; Banderali and Roy, 1992; Sanchez-Olea et al., 1996; Abdullaev et al., 2006). However this possibility is at variance with results obtained from some non-neural tissues in which Cl⁻ and taurine effluxes were found to exhibit differences in kinetics of release, osmotic sensitivity and/or degree of inhibition by anion channel blockers, results which suggest the existence of separate volume-sensitive channels for Cl⁻ and organic osmolytes (Lambert and Hoffman, 1994; Davis-Amaral et al. 1996; Shennan et al., 1996; Stutzin et al., 1999; Shennan and Thomson, 2000; Tomassen et al., 2004).

When measured in vitro, the efflux of organic osmolytes is relatively insensitive to hypotonic stress often requiring substantial (>25%) reductions in osmolarity. However, recent studies from this and other laboratories have
demonstrated that the volume-sensitive efflux of organic osmolytes from neural preparations can be enhanced following activation of cell-surface receptors. The latter include P2Y purinergic receptors in rat astrocytes (Mongin and Kimelberg, 2002, 2005), M3 muscarinic cholinergic (mAChR), lysophosphatidic and sphingosine 1-phosphate receptors in human SH-SY5Y neuroblastoma cells (Loveday et al., 2003; Heacock et al., 2004, 2006) and proteinase-activated receptor-1 (PAR-1) in human 1321N1 astrocytoma and rat astrocytes (Cheema et al., 2005). In each case, Ca2+ availability and PKC activity are required for the maximum release of organic osmolytes.

The goals of the present study were two-fold. First, to determine whether the release of 125I- (used as a tracer for Cl-) from hypotonically-stressed SH-SY5Y neuroblastoma cells was, like that of taurine, subject to receptor regulation and second, to evaluate whether these two osmolytes are released from the cells via similar or distinct mechanisms. The results indicate that the activation of either PAR-1 or mAChRs elicits a significant increase in the osmosensitive release of both 125I- and taurine and that the efflux of these osmolytes exhibits a similar, if not identical, inhibition profile in response to a variety of putative pharmacological inhibitors of VSOAC. However, the receptor-mediated efflux of 125I- can be readily differentiated from that of taurine on the basis of its more limited dependence on Ca2+ availability and, to a lesser extent, PKC activity. Thus, in SH-SY5Y cells, although both osmolytes may exit via a common (or pharmacologically similar) channel(s), distinct biochemical requirements exist for the receptor-stimulated release of 125I- and taurine.
Materials and Methods

Materials. [1,2-³H] Taurine (1.15 TBq/ml) and Na iodide (¹²⁵I labeled; 3885 MBq/ml) were obtained from Amersham Biosciences (Piscataway, NJ). Chelerythrine, thapsigargin, toxin B, Y-27632 and niflumic acid were obtained from Calbiochem (San Diego, CA). Thrombin, DIDS, NPPB, 1,9-Dideoxyforskolin and oxotremorine-M (Oxo-M) were purchased from Sigma-Aldrich (St. Louis, MO). DCPIB was obtained from Tocris Biosciences (Ellisville, MO). Thrombin receptor activating peptides; TFLLRN, TFRGAP, GYPGKF were purchased from BaChem (Torrance, CA). Fura 2/acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR). Dulbecco’s modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (Franklin Lakes, NJ). Universol was obtained from Valeant Pharmaceuticals (Costa Mesa, CA).

Cell culture conditions.

Human SH-SY5Y neuroblastoma cells (passages 75-90) were grown in tissue culture flasks (75 cm²/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330-340 mOsM. Cells were grown at 37°C in a humidified atmosphere...
containing 5% CO₂. The medium was aspirated and the cells detached from the flask with a trypLE express (Biowhittaker, MD) or sterile D1 solution (Heacock et al., 2004). Cells were then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates for 5-6 days. Experiments were routinely conducted on cells that had reached 70–90% confluency.

Measurement of efflux of taurine or ¹²⁵I⁻.

Osmolyte efflux from SH-SY5Y neuroblastoma cells was monitored essentially as previously described (Heacock et al., 2004; Tomassen et al., 2004). In brief, cells were prelabeled overnight with 18.5 KBq/ml of [³H]taurine or 92.5 KBq/ml of ¹²⁵I⁻ at 37°C. After prelabeling, the cells were washed 2 or 3 times with 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 3.6 mM NaHCO₃, 1 mM MgCl₂ and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 340 mOsM). Cells were then allowed to incubate in 2 ml of hypotonic buffer A (295-195 mOsM; rendered hypotonic by a reduction in NaCl concentration) in the absence or presence of thrombin or Oxo-M. In some experiments, buffer A was made hypertonic (370 mOsM) by the addition of NaCl. Osmolarities of buffer A were monitored by means of an Osmette precision osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots of the extracellular medium (200 µl for taurine and 1 ml for ¹²⁵I⁻) were removed and radioactivity determined after the addition of 6 ml Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 ml
of ice-cold 6% (wt/vol) trichloroacetic acid for taurine or 1 ml of 0.1 M NaOH for $^{125}$I. Efflux of taurine or $^{125}$I was calculated as a fractional release, i.e., the radioactivity released in the extracellular medium as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay. For $^{125}$I efflux, radioactivity released at the zero time point was subtracted from the observed release of $^{125}$I. Throughout this study, “basal” release of taurine or $^{125}$I is defined as that which occurs at a specified osmolarity in the absence of agonists.

**Measurement of Phosphoinositide Turnover.**

To monitor phosphoinositide turnover, SH-SY5Y cells that had been prelabeled with 148 KBq/ml of $[^3]$H]inositol for 96 hr were incubated in hypotonic buffer A (230 mOsM) that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher, 1990).

**Measurement of Cytoplasmic Calcium Concentration.**

Cytoplasmic free calcium concentrations, [Ca$^{2+}$], were determined in suspensions of SH-SY5Y neuroblastoma cells after preloading cells with the Ca$^{2+}$ indicator, fura-2 AM (Molecular Probes, OR), as previously described (Fisher et al., 1989; Cheema et al., 2005). The fluorometer used was a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD).
Data analysis.

Experiments were performed in triplicate and repeated at least three times. Values quoted are given as means ± S.E.M. for the number (n) of independent experiments indicated. A two-tailed Student’s t-test (paired) was used to evaluate differences between two experimental groups (level of significance, p<0.05). One-way or repeated measures Analysis of Variance (ANOVA) followed by Dunnett’s multiple comparisons test was used for statistical significance of differences between multiple groups (GraphPad Instat Software, Inc. San Diego, CA).
Results

Volume-sensitive efflux of taurine and $^{125}$I$^-$ from SH-SY5Y neuroblastoma cells is enhanced by the addition of thrombin. When SH-SY5Y cells that had been prelabeled with $[^3]$H]taurine were exposed to hypotonic buffer A (230 mOsM; ~30% reduction in osmolarity), there was a time-dependent release of the radiolabeled amino acid from the cells (Fig. 1A). Although the presence of a functionally coupled thrombin receptor on SH-SY5Y cells has not previously been reported, inclusion of thrombin (0.25U/ml, equivalent to 1.25 nM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of efflux by approximately 7-8 fold over basal (basal release is that monitored in the absence of thrombin). Similarly, exposure of the cells to hypotonic buffer A alone also resulted in an increase in $^{125}$I$^-$ efflux (Fig. 1A) and this was enhanced by the presence of thrombin (2-3 fold). Both the rate and magnitude of thrombin-stimulated $^{125}$I$^-$ efflux was greater than that of taurine release. Thus, the net increase in $^{125}$I$^-$ efflux over basal due to the addition of thrombin reached a maximum of 42% of the total radioactivity within 5 min, whereas the corresponding value for taurine was 25% (Fig. 1B). Since the greatest difference in magnitude of thrombin-stimulated $^{125}$I$^-$ and taurine release was observed in the first 5 min, the efflux of these osmolytes was subsequently routinely monitored after 5 min of incubation.

Thrombin enhances the volume-sensitive release of taurine and $^{125}$I$^-$ via the PAR-1 receptor. To determine whether the ability of thrombin to enhance
taurine and $^{125}\text{I}^-$ release was mediated via the same receptor, three specific Proteinase-Activated Receptor (PAR) peptides were used. Addition of 100 µM TFFLRN, a synthetic peptide specific for the PAR-1 subtype, significantly increased the release of both taurine (1226% of basal; Fig 2A) and $^{125}\text{I}^-$ (278% of basal; Fig. 2B). The enhancement of osmolyte release obtained with a PAR-1-specific peptide was similar in magnitude to that observed for thrombin. Although inclusion of a 500 µM concentration of the PAR-3 specific peptide (TFRGAP) also increased the release of taurine (269% of basal) and $^{125}\text{I}^-$ (142% of basal), the effect was markedly less than that obtained for the PAR-1 agonist. The addition of the PAR-4 peptide, GYPGKF, did not significantly increase taurine or $^{125}\text{I}^-$ release. Taken collectively, these data indicate that the ability of thrombin to enhance taurine and $^{125}\text{I}^-$ release in SH-SY5Y neuroblastoma cells is mediated primarily by the PAR-1 receptor subtype.

**Comparison of the volume-sensitive efflux of taurine and $^{125}\text{I}^-$ at various osmolarities.** Because the degree of receptor-mediated facilitation of osmolyte release appears to be dependent on the degree of hypoosmotic stress in SH-SY5Y cells (Heacock et al., 2004; 2006), the ability of thrombin to potentiate the release of taurine (Fig. 3A) and $^{125}\text{I}^-$ (Fig. 3B) at different osmolarities was examined. Both basal- and thrombin-stimulated release of taurine and $^{125}\text{I}^-$ were monitored under conditions of isotonicity (340 mOsM: defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild-severe hypotonicity (295-195 mOsM) or mild hypertonicity (370 mOsM). In the
series of experiments conducted, the basal release of taurine was not appreciably enhanced until the osmolarity of the buffer had been reduced to 195 mOsM (Fig. 3A). In contrast, the addition of thrombin resulted in a significant increase in taurine efflux (312% of control) even under mild hypotonic conditions (295 mOsM). Moreover, as the osmolarity of the buffer was reduced, the ability of thrombin to enhance taurine efflux over the basal component was further increased. A similar trend was observed for $^{125}$I efflux for which the basal release was not significantly enhanced until the osmolarity of the buffer had been reduced to 200 mOsM (Fig. 3B). The addition of thrombin resulted in a significant increase in $^{125}$I efflux (183% of control) under mild hypotonic conditions (290 mOsM). The maximum enhancement of both taurine efflux (892% of control) and $^{125}$I (319% of control) in the presence of thrombin was observed at an osmolarity of approximately 230 mOsM. In contrast, when cells were exposed to mildly hypertonic buffer A (370 mOsM), the addition of thrombin did not significantly enhance the release of either taurine or $^{125}$I.

**Volume-sensitive efflux of taurine and $^{125}$I efflux from SH-SY5Y neuroblastoma is mediated via a VSOAC.** Since VSOAC is considered to be primarily a chloride channel, the ability of a variety of broad spectrum chloride channel inhibitors to attenuate basal- and thrombin-stimulated taurine (Fig. 4A) and $^{125}$I release was examined (Fig. 4B). The addition of DIDS, NPPB or DDF resulted in a significant inhibition of the basal- and thrombin-stimulated release of both taurine and $^{125}$I from SH-SY5Y cells (28-73% and 28-95% for basal- and
thrombin-stimulated efflux, respectively; Fig. 4A,B). In general, the anion channel blockers, in particular DIDS, were less effective inhibitors of $^{125}$I release than that of taurine under both basal- and agonist-stimulated conditions. The inclusion of 100 µM niflumic acid, which, at this concentration is purported to inhibit Ca$^{2+}$-activated Cl$^{-}$ channels (Large and Wang, 1996) resulted in a 43% inhibition of thrombin-stimulated taurine release, but had no effect on either the thrombin-stimulated $^{125}$I efflux or on the basal release of either osmolyte (Fig. 4 A, B).

Because these anion channel inhibitors are relatively non-specific, the ability of DCPIB, an agent that is considered highly selective for VSOAC (Decher et al., 2001; Best et al., 2004; Abdullaev et al., 2006), was also examined for its ability to inhibit both taurine and $^{125}$I efflux. Inclusion of 20 µM DCPIB significantly inhibited the basal release of both taurine and $^{125}$I to a similar extent (49% and 58% inhibition; Fig. 5A,B, respectively). Similarly, DCPIB also inhibited the thrombin-stimulated taurine and $^{125}$I release (85-87% inhibition).

**Thrombin addition elicits an increase in the concentration of intracellular calcium in SH-SY5Y cells via a phospholipase C - independent mechanism.** As previously observed for 1321N1 astrocytoma cells (Cheema et al., 2005), the addition of thrombin to fura-2-loaded SH-SY5Y cells resulted in a significant increase in $[\text{Ca}^{2+}]$ (from a basal value of 100 nM to a peak value of 250 nM, n = 8). Removal of extracellular Ca$^{2+}$ diminished the thrombin-mediated increase in $[\text{Ca}^{2+}]$, from 150 to 75 nM, (n = 8) whereas depletion of intracellular Ca$^{2+}$ with
thapsigargin completely abolished the ability of thrombin to increase \([Ca^{2+}]_i\). The thrombin-mediated rise in \([Ca^{2+}]_i\) occurred independently of phospholipase C activation since no increase in release of inositol phosphates was observed in the presence of thrombin (104 ± 2% of control, \(n=3\)). In contrast, the addition of a 100 µM concentration of the muscarinic agonist, Oxo-M, which also elicits a robust increase in \([Ca^{2+}]_i\) in these cells (Heacock et al., 2006), resulted in a significant increase in inositol phosphate release (250 ± 19% of control, \(n = 3\)).

**Thrombin-stimulated efflux of taurine, but not that of \(^{125}\text{I}^-\), is dependent on Ca\(^{2+}\) availability and activation of PKC.** Activation of thrombin receptors on 1321N1 astrocytoma cells has been reported to elicit an increase in taurine release that is dependent on the intracellular concentration of calcium and activation of PKC (Cheema et al., 2005). In agreement with our previous observations, the magnitude of thrombin-stimulated taurine release from SH-SY5Y neuroblastoma cells is also dependent on Ca\(^{2+}\) availability. However in SH-SY5Y cells, removal of extracellular Ca\(^{2+}\) alone is sufficient to inhibit thrombin-stimulated taurine release (24% inhibition), while the basal release of taurine is unaffected. Depletion of intracellular Ca\(^{2+}\) stores with 1 µM thapsigargin did not further increase the extent of inhibition and no effect on basal release of taurine was observed (Fig. 6A). Neither the basal- nor thrombin-stimulated efflux of \(^{125}\text{I}^-\) efflux was attenuated by omission of extracellular Ca\(^{2+}\) or depletion of intracellular Ca\(^{2+}\) stores with 1 µM thapsigargin (Fig. 6B).
To evaluate a role, if any, for PKC in basal and thrombin-stimulated efflux of taurine and $^{125}\text{I}^-$, cells were preincubated with 10 µM chelerythrine prior to thrombin challenge under hypotonic conditions. Although chelerythrine had no effect on the basal release of taurine, it significantly attenuated thrombin-stimulated release (30% inhibition; Fig. 7A). In contrast, although a small reduction (9%) in the basal release of $^{125}\text{I}^-$ was observed following chelerythrine pretreatment, no effect on the magnitude of thrombin-stimulated efflux was observed (Fig. 7B). The combination of inhibition of PKC with 10 µM chelerythrine, along with depletion of intracellular Ca$^{2+}$ with 1 µM thapsigargin, resulted in a 54% inhibition of thrombin-stimulated taurine release but had no effect on basal efflux (Fig. 8A). In contrast, $^{125}\text{I}^-$ release elicited by the addition of thrombin was not attenuated under these conditions (Fig. 8B).

**Efflux of taurine and $^{125}\text{I}^-$ following the activation of mAChRs is also differentially sensitive to depletion of Ca$^{2+}$ and activation of PKC.** The observation that the efflux of taurine and $^{125}\text{I}^-$ observed following thrombin addition is differentially regulated by Ca$^{2+}$ and PKC prompted us to examine whether this relationship is also observed following the activation of mAChRs. As previously observed (Heacock et al., 2006), Oxo-M-stimulated taurine release was attenuated by omission of extracellular Ca$^{2+}$ (60% inhibition; Fig. 9A) and further in the presence of 1 µM thapsigargin to deplete intracellular Ca$^{2+}$ pools (81 ± 4% inhibition; Fig. 9A). However, Oxo-M-stimulated $^{125}\text{I}^-$ efflux was unaffected by removal of extracellular Ca$^{2+}$ and significantly less inhibited than taurine.
release following the additional depletion of intracellular Ca$^{2+}$ (31 ± 6% inhibition; Fig. 9B, p<0.005).

To examine the involvement of PKC, Oxo-M-stimulated taurine release was measured after preincubation of the cells with 10 µM chelerythrine. Basal taurine release was unaffected whereas that due to Oxo-M addition was significantly inhibited (73 ± 5% inhibition; Fig. 10A). Chelerythrine also significantly inhibited Oxo-M-stimulated $^{125}$I$^{-}$ release (47 ± 5% inhibition; Fig. 10B) but the degree of inhibition was significantly less than that observed for taurine release (p<0.05). The combination of inhibition of PKC with 10 µM chelerythrine, along with depletion of intracellular Ca$^{2+}$ with 1 µM thapsigargin, resulted in a 94 ± 3% inhibition of Oxo-M-stimulated taurine release whereas stimulated $^{125}$I$^{-}$ release was inhibited by 64 ± 1% (Fig. 11 A,B, p<0.001 vs stimulated taurine release). Thus, a significant fraction (35-40%) of Oxo-M-stimulated $^{125}$I$^{-}$ efflux, and all of that due to thrombin addition, is independent of both Ca$^{2+}$ availability and PKC activity.
Discussion

Previous studies of receptor-regulated osmolyte release from hypotonically-stressed neural cells have focused on the efflux of organic osmolytes, rather than that of Cl\(^-\). In the present study, we demonstrate that the addition of thrombin to SH-SY5Y neuroblastoma cells results in a significant enhancement of the volume-sensitive efflux of both \(^{125}\text{I}\) and taurine and that for each osmolyte, stimulated release is mediated primarily by the PAR-1 subtype. However, receptor-mediated \(^{125}\text{I}\) efflux occurs more rapidly and to a greater extent than that of taurine (Fig. 1B), an observation that may reflect differences in the respective molecular sizes of the two osmolytes. A similar preferential release of \(^{125}\text{I}\) over that of taurine has previously been noted for HeLa and Intestinal 407 cells following cell swelling (Stutzin et al., 1999; Tomassen et al., 2004). The threshold osmolarity (‘set-point’) at which the basal release of osmolytes occurs was the same for both \(^{125}\text{I}\) and taurine, i.e. approximately 200 mOsM (Fig. 4). This result is consistent with our previous studies with neurotumor cells in which a reduction in osmolarity of >25\% was required to elicit a significant increase in osmolyte release (Heacock et al., 2004, 2006; Cheema et al., 2005). In contrast, in Intestinal 407 cells, the threshold osmolarity for release of \(^{125}\text{I}\) (260 mOsM) is reported to be higher than that of taurine (225 mOsM; Tomassen et al., 2004). In the present study, thrombin addition to SH-SY5Y cells increased the ‘set point’ for the efflux of both \(^{125}\text{I}\) and taurine from 200 mOsM to 290 mOsM. Thus receptor activation facilitates the release of both
inorganic and organic osmolytes and this may constitute a mechanism whereby cells can respond to small changes in external osmolarity.

The possibility that the volume-sensitive release of Cl⁻ and organic osmolytes occurs via a common membrane channel (VSOAC) has received support primarily on the basis of the similarities of pharmacological inhibition profiles obtained in the presence of a variety of non-selective anion channel blockers (Banderali and Roy, 1992; Jackson and Strange, 1993; Sanchez-Olea et al., 1996; Abdullaev et al., 2006). However, in some tissues, the existence of separate Cl⁻ and taurine efflux pathways has also been proposed (Lambert and Hoffman, 1994; Stutzin et al., 1999; Shennan and Thomson, 2000; Tomassen et al., 2004). In addition, the issue of whether Cl⁻ and organic osmolytes are released from the cell under conditions of receptor activation via shared or distinct pathways has not yet been systematically addressed. In the present study we observed that the inclusion of three anion channel blockers, namely DIDS, NPPB and DDF, inhibited both basal- and receptor-stimulated release of ¹²⁵I⁻ and taurine. Of these, DDF and NPPB were more effective inhibitors than DIDS, particularly for stimulated ¹²⁵I⁻ release. The sole agent that was able to differentiate between taurine and ¹²⁵I⁻ release was niflumic acid, which, at the concentration employed (100 µM), is purported to inhibit Ca²⁺-activated Cl⁻ channels (Large and Wang, 1996). Although niflumic acid had no effect on the basal release of either osmolyte, it significantly inhibited thrombin-stimulated release of taurine, but not that of ¹²⁵I⁻ (Fig. 5). However, the significance of this observation remains unclear for two reasons. First, DCPIB, a highly specific
inhibitor of VSOAC that is without effect on Ca^{2+}-activated Cl^- channels and other
cation and anion channels (Decher et al., 2001; Best et al., 2004), was an equally
effective inhibitor of basal-and receptor-stimulated release of both taurine and
^{125}I^- from SH-SY5Y cells (Fig. 6). Second, niflumic acid attenuated thrombin-
stimulated taurine efflux even under Ca^{2+}-depleted conditions, a result
inconsistent with inhibition of the Ca^{2+}-activated Cl^- channel (data not shown).

Taken collectively, the most parsimonious interpretation of the current data is
that, following receptor activation, both ^{125}I^- and taurine are released from SH-
SY5Y cells via the same (or pharmacologically indistinguishable) VSOAC
channels.

Although the release of ^{125}I^- and taurine exhibited a similar
pharmacological inhibition profile, the receptor-mediated release of these two
osmolytes could be readily differentiated on the basis of their dependence on
Ca^{2+} availability and PKC activity. Previously, we and others have demonstrated
that increases in [Ca^{2+}]_i or in PKC activity are not prerequisites for the basal
(swelling-induced) release of organic osmolytes such as taurine and D-aspartate
from neurotumor cells, neurons or astrocytes (Moran et al., 1997; Mongin and
Kimelberg, 2002; Cardin et al., 2003; Loveday et al., 2003; Cheema et al.,
2005). Similarly, in the present study, we observed that, at least under mildly
hypotonic conditions, the basal release of ^{125}I^- also appears to be essentially
independent of Ca^{2+} availability and PKC activity. However, PAR-1-mediated
increases in taurine and ^{125}I^- efflux differed in their dependence upon Ca^{2+}
availability and PKC activity. Thus, whereas taurine efflux was attenuated
following the removal of extra- and intracellular Ca\textsuperscript{2+}, or following inhibition of PKC activity with chelerythrine, thrombin-stimulated \textsuperscript{125}I\textsuperscript{-} efflux was unaffected by either treatment. Under conditions in which both Ca\textsuperscript{2+} depletion and inhibition of PKC activity occurred, stimulated taurine efflux was inhibited by >50% whereas \textsuperscript{125}I\textsuperscript{-} release remained unchanged. Fura-2 fluorimetric studies indicated that the addition of thrombin to SH-SY5Y cells resulted in a significant increase in [Ca\textsuperscript{2+}], (100 nM to 250 nM), which was abolished when both extra- and intracellular sources of Ca\textsuperscript{2+} were depleted. Because the PAR-1-mediated increase in the release of \textsuperscript{125}I\textsuperscript{-} was not attenuated under these conditions, we conclude that the efflux of \textsuperscript{125}I\textsuperscript{-} (but not that of taurine) occurs independently of a rise in [Ca\textsuperscript{2+}] within these cells. This conclusion is consistent with the Ca\textsuperscript{2+} insensitivity of thrombin-stimulated Cl\textsuperscript{-} currents previously observed in pulmonary artery endothelial cells (Manolopoulos et al., 1997). Further evidence that Ca\textsuperscript{2+} and PKC differentially modulate the release of these two osmolytes from SH-SY5Y cells was obtained following the addition of the muscarinic agonist, Oxo-M. Activation of mAChRs on SH-SY5Y cells elicits a large increase in [Ca\textsuperscript{2+}], (from 100 to 450 nM), which is sustained due to a continuous influx of extracellular Ca\textsuperscript{2+} (Lambert and Nahorski, 1990; Heacock et al., 2006). Although omission of extracellular Ca\textsuperscript{2+} and depletion of intracellular Ca\textsuperscript{2+} with thapsigargin resulted in a pronounced inhibition of mAChR-stimulated taurine release (60 and 81% respectively), Oxo-M-stimulated \textsuperscript{125}I\textsuperscript{-} efflux was unaffected by removal of extracellular Ca\textsuperscript{2+} and much less inhibited (31%) by depletion of intracellular Ca\textsuperscript{2+} stores (Fig. 9). Similarly, inhibition of PKC resulted in a significantly greater loss
of mAChR-stimulated taurine release (73%) than that of $^{125}$I$^{-}$ efflux (47%). Two conclusions can be drawn from these results. The first is that, regardless of the receptor activated, the stimulated release of $^{125}$I$^{-}$ is less dependent than taurine efflux on either Ca$^{2+}$ availability or PKC activity. For the PAR-1 receptor, stimulated $^{125}$I$^{-}$ efflux is fully independent of Ca$^{2+}$ availability and PKC activity whereas for the mAChR, some degree of dependence upon these parameters is observed. The second conclusion is that although Ca$^{2+}$ and PKC are required for maximum receptor activation of taurine efflux from SH-SY5Y cells, the degree of dependence is receptor-specific. Thus Ca$^{2+}$ and PKC appear to play a quantitatively more significant role in mAChR stimulation of taurine release than that following the activation of either the PAR-1 or lysophospholipid receptors (Heacock et al., 2006).

Our observation that Ca$^{2+}$ availability (and PKC activity) differentially regulate the receptor-stimulated release of taurine and $^{125}$I$^{-}$ from SH-SY5Y cells is consistent with results previously obtained for hepatoma cells (Junankar et al., 2002). Osmotic swelling of these cells results in the release of intrinsic ATP, which subsequently activates P2Y receptors coupled to an increase in [Ca$^{2+}$]. However, although this rise in [Ca$^{2+}$] is required for the release of taurine, a stimulated efflux of $^{125}$I$^{-}$ can occur in the absence of an increased intracellular Ca$^{2+}$. Conceivably, differences in Ca$^{2+}$ and PKC requirements for taurine and $^{125}$I$^{-}$ efflux in hepatoma and SH-SY5Y cells might reflect the following: (1) the receptor-specific activation of distinct signal transduction pathways (Ca$^{2+}$/PKC-dependent or –independent) that differentially contribute to the efflux of taurine
and $^{125}$I$^-$, both of which are released through a common membrane channel, (2) the presence of separate, but pharmacologically similar, efflux channels for $^{125}$I$^-$ and taurine that differ in their degree of regulation by Ca$^{2+}$ and PKC or (3) a combination of both mechanisms (Fig. 12). In the context of multiple signaling pathways, one potential candidate, triggered by thrombin receptors, is rho-mediated remodeling of the cytoskeleton (Carton et al., 2002; Pederson et al., 2002). However, preincubation of SH-SY5Y cells with toxin B, or the rho kinase inhibitor Y-27632, had no effect on receptor-stimulated release of taurine or $^{125}$I$^-$ (data not shown). The possibility that separate efflux channels mediate the release of taurine and $^{125}$I$^-$ in SH-SY5Y cells has previously been suggested for non-neural cells (Lambert and Hoffman, 1994; Stutzin et al., 1999). Regardless of the pathways involved, our results indicate that, following receptor activation, the volume-dependent release of organic and inorganic osmolytes from SH-SY5Y cells does not occur by a common mechanism. This observation may ultimately be of relevance to our understanding of the different roles played by the two classes of osmolytes in cell volume regulation.
References


Heacock AM, Dodd MS and Fisher SK (2006) Regulation of volume-sensitive osmolyte efflux from human SH-SY5Y neuroblastoma cells following


Footnotes

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Legends for Figures

Fig. 1. Time course of basal- and thrombin-stimulated efflux of taurine and $^{125}$I from human SH-SY5Y neuroblastoma cells. (A) SH-SY5Y neuroblastoma cells that had been prelabeled in the presence of $[^3]$H]taurine or $^{125}$I were washed two or three times respectively with 2 ml of isotonic buffer A. The cells were then incubated in 230 mOsM buffer A in the presence or absence of 1.25 nM thrombin. Reactions were terminated at the times indicated and radioactivity measured (basal release, dotted lines; thrombin-stimulated release, solid lines). Unlike taurine, $^{125}$I was not tightly retained by SH-SY5Y cells and the isotonic release of this tracer under basal conditions was approximately 50% of that observed at 230 mOsM after a 5 min incubation and 75% at longer time intervals (10-15 min). Results are expressed as taurine or $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 independent experiments. (B) Net thrombin-mediated release (i.e. thrombin-stimulated minus basal) of taurine and $^{125}$I.

Fig. 2. Thrombin enhances taurine and $^{125}$I efflux from SH-SY5Y neuroblastoma cells via the PAR-1 subtype. Cells that had been prelabeled with (A) $[^3]$H]taurine or (B) $^{125}$I were first washed in isotonic buffer A and then incubated for 5 min in 230 mOsM buffer A in the presence or absence of either thrombin (1.25 nM) or synthetic peptides specific for PAR-1 (TFLLRN;100 µM), PAR-3 (TFRGAP; 500 µM) or PAR-4 (GYPGKF;500 µM) subtypes. Results are
expressed as taurine or $^{125}\text{I}^-$ efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 3 independent experiments. **, Different from basal control, p< 0.01 (by repeated measures ANOVA followed by Dunnett’s multiple comparisons test).

Fig. 3. Basal- and thrombin-stimulated release of taurine and $^{125}\text{I}^-$ as a function of osmolarity. Cells prelabeled with (A) [$^3\text{H}$]taurine or (B) $^{125}\text{I}^-$ were first washed in isotonic buffer A and then incubated for 5 min in buffers at the osmolarities indicated in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Results are expressed as taurine or $^{125}\text{I}^-$ efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 independent experiments. #, Different from $^{125}\text{I}^-$ release observed in cells incubated in isotonic buffer A (340 mOsM), p< 0.05 (by one-way ANOVA followed by Dunnett’s multiple comparison test). **, Different from basal release, p< 0.05 (by Student’s paired t-test).

Fig. 4. Inhibition of basal- and thrombin-stimulated efflux of taurine and $^{125}\text{I}^-$ by broad spectrum anion channel blockers. Cells that had been prelabeled with (A) [$^3\text{H}$]taurine or (B) $^{125}\text{I}^-$ were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsM) with 200 µM DIDS, 100 µM NPPB, 100 µM dideoxyforskolin (DDF) or 100 µM niflumic acid, in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux of taurine and $^{125}\text{I}^-$ monitored. Results are expressed as taurine or $^{125}\text{I}^-$ efflux (percentage of total soluble radioactivity) and are the means.
± S.E.M. for 3-5 independent experiments. #, Different from control basal, p<0.05 and ##, different from efflux in the presence of thrombin alone, p<0.05 (by Student’s paired t-test).

Fig. 5. DCPIB inhibits the basal and thrombin-stimulated efflux of both taurine and $^{125}$I$^{-}$ efflux. Cells prelabeled with (A) [$^{3}$H]taurine or (B) $^{125}$I$^{-}$ were first pretreated with 20 µM DCPIB in isotonic buffer A for 10 min before incubation in hypotonic buffer A (230 mOsM) containing 20 µM DCPIB in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux of taurine and $^{125}$I$^{-}$ monitored. Results are expressed as taurine or $^{125}$I$^{-}$ efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4-5 independent experiments. #, Different from control basal efflux, p<0.01 (taurine); p<0.001 ($^{125}$I$^{-}$) and ##, different from control thrombin-stimulated efflux, p<0.01 (taurine); p<0.001 ($^{125}$I$^{-}$) (by Student’s paired t-test).

Fig. 6. The role of extra- and intracellular Ca$^{2+}$ in thrombin-stimulated efflux of taurine and $^{125}$I$^{-}$. (A) Cells that had been prelabeled overnight with [$^{3}$H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsM) in the absence (-ext Ca: Ca$^{2+}$ was omitted from buffer and 50 µM EGTA added) or presence of extracellular Ca$^{2+}$. Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. In some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 1 µM thapsigargin (Thaps) prior to the 5 min incubation in hypotonic buffer A.
buffer. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 independent experiments. ###, Different from thrombin-stimulated efflux under control conditions, p<0.01 (by one-way ANOVA followed by Dunnett’s multiple comparisons test). (B) Cells prelabeled with $^{125}$I were treated as described in (A). Results are expressed as $^{125}$I efflux (percent of total soluble radioactivity), and are the means ± S.E.M. for 4 independent experiments.

**Fig. 7. Inhibition of the thrombin-stimulated efflux of taurine, but not of $^{125}$I, by chelerythrine.** Cells prelabeled with (A) $[^3H]$taurine or (B) $^{125}$I were pretreated with 10 µM chelerythrine in isotonic buffer A for 15 min before incubation in hypotonic buffer A (230 mOsM) in either the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 5 min and efflux monitored. Results are expressed as taurine or $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 4 independent experiments. #, Different from control basal efflux p< 0.05 ($^{125}$I) and ##, different from thrombin-stimulated efflux under control conditions, p< 0.01 (taurine) (by Student’s paired t-test).

**Fig. 8. Depletion of Ca$^{2+}$ and inhibition of PKC only attenuates thrombin-stimulated taurine efflux.** Cells prelabeled with (A) $[^3H]$taurine or (B) $^{125}$I were first preincubated for 15 min in the absence (control) or presence of 10 µM chelerythrine and 1 µM thapsigargin (Thaps) in isotonic buffer A. The medium
was then aspirated and replaced with 230 mOsM buffer A, that either contained Ca$^{2+}$ (control) or had Ca$^{2+}$ omitted and 50 µM EGTA, 1 µM thapsigargin and 10 µM chelerythrine added. Reactions were then allowed to proceed for 5 min in the absence (open bars) or presence (closed bars) of 1.25 nM thrombin. Results are expressed as taurine or $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4-7 independent experiments. #, Different from control basal efflux, p<0.05 ($^{125}$I) and ##, different from thrombin-stimulated efflux under control conditions, p< 0.05 (taurine) (by Student’s paired t-test).

**Fig. 9. The role of extra- and intracellular Ca$^{2+}$ in Oxo-M-stimulated efflux of taurine and $^{125}$I.** (A) Cells that had been prelabeled overnight with [$^{3}$H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsM) in either the absence (-ext Ca: Ca$^{2+}$ was omitted from buffer and 50 µM EGTA added) or presence of extracellular Ca$^{2+}$. Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (filled bars) of 100 µM Oxo-M. In some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 1 µM thapsigargin (Thaps) prior to the 5 min incubation in hypotonic buffer. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 independent experiments. ##, Different from Oxo-M-stimulated efflux under control conditions, p< 0.01 (by one-way ANOVA followed by Dunnett’s multiple comparisons test). (B) Cells prelabeled with $^{125}$I were treated as described in (A). Results are expressed as $^{125}$I efflux (percent of total soluble radioactivity), and are the means ± S.E.M. for
5 independent experiments. ###, Different from Oxo-M-stimulated efflux under control conditions, p< 0.01 (by one-way ANOVA followed by Dunnett’s multiple comparisons test).

**Fig. 10. Inhibition of Oxo-M-stimulated efflux of taurine and $^{125}$I by chelerythrine.** Cells prelabeled with (A) $[^{3}$H]taurine or (B) $^{125}$I$^{-}$ were pretreated with 10 µM chelerythrine in isotonic buffer A for 15 min before incubation in hypotonic buffer A (230 mOsM) in the absence (open bars) or presence (filled bars) of 100 µM Oxo-M. Reactions were terminated after 5 min and efflux monitored. Results are expressed as taurine or $^{125}$I$^{-}$ efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 4 independent experiments. #, Different from basal efflux under control conditions, p< 0.05 ($^{125}$I$^{-}$) and ###, different from thrombin-stimulated efflux under control conditions, p< 0.05 (taurine and $^{125}$I$^{-}$) (by Student’s paired t-test).

**Fig. 11. Depletion of Ca$^{2+}$ and inhibition of PKC abolishes Oxo-M-stimulated taurine efflux and attenuates that of $^{125}$I$^{-}$.** Cells prelabeled with $[^{3}$H]taurine (A) or $^{125}$I$^{-}$ (B) were first preincubated for 15 min in the absence (control) or presence of 10 µM chelerythrine and 1 µM thapsigargin (Thaps) in isotonic buffer A. The medium was then aspirated and replaced with 230 mOsM buffer A, that either contained Ca$^{2+}$ (control) or had Ca$^{2+}$ omitted and 50 µM EGTA, 1 µM thapsigargin and 10 µM chelerythrine added. Reactions were allowed to proceed for 5 min in the absence (open bars) or presence (closed bars) of 100 µM Oxo-M.
Results are expressed as taurine or $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 independent experiments. ##, Different from Oxo-M-stimulated efflux under control conditions, p< 0.05 (taurine and $^{125}$I) (by Student’s paired t-test).

Fig. 12. Potential mechanisms that may account for differences in Ca$^{2+}$ and PKC requirements for receptor-mediated release of osmolytes in SH-SY5Y cells. (A) PAR-1 and mAChRs, in a receptor-specific manner, can activate at least two different signal transduction pathways linked to osmolyte release. Signal transduction pathway ① is dependent upon Ca$^{2+}$ availability and PKC activity and is primarily linked to taurine efflux. Signal transduction pathway ② is independent of Ca$^{2+}$ and PKC activity and is primarily coupled to iodide efflux. The efflux of both taurine and iodide is mediated via a common membrane channel that is permeable to both organic osmolytes and iodide. The majority of taurine efflux elicited by both PAR-1 and mAChRs (and a fraction of iodide release following mAChR activation) is mediated by pathway ①. In contrast, all of the iodide efflux elicited by PAR-1 receptors, and a significant fraction of that resulting from mAChR activation, is mediated by pathway ②. (B) The receptor-specific release of taurine and iodide from the cell occurs via distinct membrane channels. Channel ① is primarily permeable to taurine and is regulated by Ca$^{2+}$ and PKC. In contrast, Channel ② primarily mediates the release of iodide and is independent of both Ca$^{2+}$ and PKC activity. Channel ① mediates the majority of
taurine release resulting from activation of both PAR-1 and mAChRs (and a fraction of iodide release resulting from mAChR activation) whereas Channel 2 mediates all of the iodide efflux elicited by the PAR-1 receptor and a fraction of that following mAChR activation. It remains possible that both mechanisms (A) and (B) operate concurrently.
Figure 2

A

Taurine efflux (% of total)

Basal

Thrombin

PAR-1

PAR-3

PAR-4

B

125I efflux (% of total)

Basal

Thrombin

PAR-1

PAR-3

PAR-4
Figure 7

A

Taurine efflux (% of total)

- Basal
- Thrombin

Control

Chelerythrine

B

$^{125}$I efflux (% of total)

- Basal
- Thrombin

Control

Chelerythrine
Fig. 8

Panel A: Taurine efflux (% of total) at different conditions: Basal and Thrombin. Control and -ext Ca + Chel + Thaps.

Panel B: 125I efflux (% of total) at different conditions: Basal and Thrombin. Control and -ext Ca + Chel + Thaps.
Figure 9

Panel A: Taurine efflux (% of total) in different conditions:
- Basal
- Oxo-M

- Control
- - ext Ca
- - ext Ca + Thaps

Panel B: 125I efflux (% of total) in different conditions:
- Basal
- Oxo-M

- Control
- - ext Ca
- - ext Ca + Thaps
Figure 11

A

![Graph A](image)

**Graph A**

**X-axis:**
- Control
- - ext Ca + Chel + Thaps

**Y-axis:**
- Taurine efflux (% of total)

**Basal**
- Control
- - ext Ca + Chel + Thaps

**Oxo-M**
- Control
- - ext Ca + Chel + Thaps

B

![Graph B](image)

**Graph B**

**X-axis:**
- Control
- - ext Ca + Chel + Thaps

**Y-axis:**
- $^{125}$I efflux (% of total)

**Basal**
- Control
- - ext Ca + Chel + Thaps

**Oxo-M**
- Control
- - ext Ca + Chel + Thaps

**Note:**

- **#** indicates statistical significance between groups.