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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ABSTRACT

The basal (swelling-induced) and receptor-stimulated effluxes 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. The addition of thrombin, 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 Cl\(^{−}\)-selective channel blocker (NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid) 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 deletion 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 after 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.

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 because of the restrictions of the skull. A common cause of brain swelling is hyponatremia, a condition that disproportionately affects 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) that 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 are 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 because of 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., 2000).
Materials and Methods

Materials. [1,2-3H]taurine (1.15 TBq/ml) and sodium iodide (125I)-labeled; 3885 MBq/ml) were obtained from GE Healthcare (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 Torrisi Cookson Inc. (Ellisville, MO). Thrombin receptor-activating peptides, TFLLRN, TFRGAP, and GYPGKF, were purchased from Bachem California (Torrance, CA). Fura 2/ace-toxymethyl ester was purchased from Invitrogen (Eugene, OR). Dulbecco’s modified Eagle’s medium (DMEM) and 50% 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) fetal calf serum with 1% penicillin/streptomycin. The osmolality of the medium was 330 to 340 mosM. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. The medium was aspirated, and the cells were detached from the flask with a TrypLE Express (Cambrex Bio Science, Walkersville, 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 to 6 days. Experiments were routinely conducted on cells that had reached 70 to 90% confluence.

Measurement of Efflux of Taurine or 125I-. Osmolyte efflux from SH-SY5Y neuroblastoma cells was monitored essentially as described previously (Heacock et al., 2004; Tomassen et al., 2004). In brief, cells were prelabeled overnight with 18.5 kBq/ml [3H]taurine or 92.5 kBq/ml 125I- at 37°C. After prelabeling, the cells were washed two or three 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, 30 mM HEPES, pH 7.4, and 1 mg/ml d-glucose; ~340 mosM). Cells were then allowed to incubate in 2 ml of hypotonic buffer A (295–195 mosM) for ~250–195 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 Osmometer (Shimadzu Scientific Instruments, Columbia, MD). At the times indicated, aliquots of the extracellular medium (200 µl for taurine and 1 ml for 125I-) were removed, and radioactivity was determined after the addition of 6 ml of Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer, and cells were lysed by the addition of 2 ml of ice-cold 6% (w/v) trichloroacetic acid for taurine or 1 ml of 0.1 M NaOH for 125I-. Efflux of taurine or 125I- 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 125I- efflux, radioactivity released at the zero time point was subtracted from the observed release of 125I-. Throughout this study, “basal” release of taurine or 125I- is defined as that which occurs at a specified osmolality in the absence of agonists.

Measurement of Phosphoinositide Turnover. To monitor phosphoinositide turnover, SH-SY5Y cells that had been prelabelled with 148 kBq/ml [3H]inositol for 96 h were incubated in hypertonic 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 described previously (Thompson and Fisher, 1990).

Measurement of Cytoplasmic Calcium Concentration. Cytoplasmic free calcium concentrations, [Ca2+]i, were determined in suspensions of SH-SY5Y neuroblastoma cells after preloading cells with the Ca2+ indicator, fura-2/ace-toxymethyl ester (Molecular Probes), as described previously (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 \( \pm \) 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 \( ^{125}\text{I}^- \) from SH-SY5Y Neuroblastoma Cells Is Enhanced by the Addition of Thrombin. When SH-SY5Y cells that had been prelabeled with \( [\text{H}]\text{taurine} \) were exposed to hypotonic buffer A (230 mOsm, \(-30\% \) reduction in osmolality), 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 (0.25 U/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 \(-7\% \) to \(-8\)-fold over basal (basal release is that monitored in the absence of thrombin). Likewise, exposure of the cells to hypotonic buffer A alone also resulted in an increase in \( ^{125}\text{I}^- \) efflux (Fig. 1A), and this was enhanced by the presence of thrombin (2- to 3-fold). Both the rate and magnitude of thrombin-stimulated \( ^{125}\text{I}^- \) efflux was greater than that of taurine release. Thus, the net increase in \( ^{125}\text{I}^- \) 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). Because the greatest difference in the magnitude of thrombin-stimulated \( ^{125}\text{I}^- \) 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 \( ^{125}\text{I}^- \) 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 PAR peptides were used. Addition of 100 \( \mu \text{M} \) 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 \( \mu \text{M} \) 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.
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 $^{125}\text{I}$ was 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 to 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 $^{125}\text{I}$ 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 $^{125}\text{I}$ efflux (183% of control) under mild hypotonic conditions (290 mOsM). The maximum enhancement of both taurine efflux (892% of control) and $^{125}\text{I}$ (319% of control) in the presence of thrombin was observed at an osmolarity of ~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 $^{125}\text{I}$.

Volume-Sensitive Efflux of Taurine and $^{125}\text{I}$ from SH-SY5Y Neuroblastoma Is Mediated via a VSOAC. Because a 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 $^{125}\text{I}$ 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 $^{125}\text{I}$ from SH-SY5Y cells (28–73 and 28–95% for basal and thrombin-stimulated effluxes, respectively) (Fig. 4, A and B). In general, the anion channel blockers, in particular DIDS, were less effective inhibitors of $^{125}\text{I}$ 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 $^{125}\text{I}$ efflux or on the basal release of either osmolyte (Fig. 4, A and B).

Because these anion channel inhibitors are relatively nonspecific, the ability of DCPIB, an agent that is considered highly selective for

Fig. 3. Basal- and thrombin-stimulated release of taurine and $^{125}\text{I}$ as a function of osmolarity. Cells prelabeled with $[^3\text{H}]$taurine (A) or $^{125}\text{I}$ (B) 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 four 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 comparisons 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 $[^3\text{H}]$taurine (A) or $^{125}\text{I}$ (B) were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsM) with 200 μM DIDS, 100 μM NPPB, 100 μM DDF, or 100 μM niflumic acid in the presence (filled bars) or absence (open bars) of 1.25 nM thrombin. Reactions were terminated after 5 min, and efflux of taurine and $^{125}\text{I}$ was monitored. Results are expressed as taurine or $^{125}\text{I}$ efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for three to five independent experiments. #, different from control basal, $p < 0.05$; ##, different from efflux in the presence of thrombin alone, $p < 0.05$ (by Student’s paired t test).
a 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. 5, A and B, respectively). Likewise, 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 [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 [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$^{2+}$]. The thrombin-mediated rise in [Ca$^{2+}$], occurred independently of phospholipase C activation because 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 elicited a robust increase in [Ca$^{2+}$], in these cells (Heacock et al., 2006), resulted in a significant increase in inositol phosphate release (250 ± 19% of control, n = 3).

**Fig. 5.** DCPIB inhibits the basal and thrombin-stimulated efflux of both taurine and $^{125}$I. Cells prelabeled with [3H]taurine (A) or $^{125}$I (B) 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 was monitored. Results are expressed as taurine or $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for four to five independent experiments. #, different from control basal efflux, p < 0.01 (taurine), p < 0.001($)^{125}$I); ##, 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 [3H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (230 mOsm) in the absence (pCa 7.0) 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) before 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 four independent experiments. ##, different from control basal efflux, p < 0.01 (by one-way ANOVA followed by Dunnett’s multiple comparisons test). B, cells prelabeled with $^{125}$I were treated as described for A. Results are expressed as $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for four independent experiments.

**Thrombin-Stimulated Efflux of Taurine but Not That of $^{125}$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), whereas 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 $^{125}$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 $^{125}$I, cells were preincubated with 10 μM chelerythrine before 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 $^{125}$I- was observed after 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, $^{125}$I- release elicited by the addition of thrombin was not attenuated under these conditions (Fig. 8B).

**Efflux of Taurine and $^{125}$I- after 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 $^{125}$I- observed after thrombin addition is differentially regulated by Ca$^{2+}$ and PKC prompted us to examine whether this relationship is also observed after 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 $^{125}$I- efflux was unaffected by removal of extracellular Ca$^{2+}$ and significantly less inhibited than taurine release after the additional depletion of intracellular Ca$^{2+}$ (31 ± 6% inhibition, $p < 0.005$) (Fig. 9B).

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% ($p < 0.001$ versus stimulated taurine release) (Fig. 11, A and B). Thus, a significant fraction (35–40%) of Oxo-M-stimulated 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 on 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 $^{125}$I- and taurine and that, for each osmolyte, stimulated release is mediated primarily by the PAR-1 subtype. However, recep-

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**Fig. 7.** Inhibition of the thrombin-stimulated efflux of taurine but not of $^{125}$I- by chelerythrine. Cells prelabeled with $[^3]$Htaurine (A) or $^{125}$I- (B) 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 was monitored. Results are expressed as taurine or $^{125}$I- efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for four independent experiments. #, different from control basal efflux, $p < 0.05$ ($^{125}$I-); ##, 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 $[^3]$Htaurine (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 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 (ext Ca + Chel + Thaps) 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 four to seven independent experiments. #, different from control basal efflux, $p < 0.05$ ($^{125}$I-); ##, different from thrombin-stimulated efflux under control conditions, $p < 0.05$ (taurine) (by Student’s paired t test).
tor-mediated $^{125}$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 $^{125}$I over that of taurine has previously been noted for HeLa and intestinal 407 cells after cell swelling (Stutzin et al., 1999; Tomassen et al., 2004). The threshold osmolality (set-point) at which the basal release of osmolytes occurs was the same for both $^{125}$I and taurine, i.e., ~200 mOsM (Fig. 4). This result is consistent with our previous studies with neurotumor cells in which a reduction in osmolality 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 osmolality for release of $^{125}$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-SYSY cells increased the set point for the efflux of both $^{125}$I and taurine from 200 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 nonspecific 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 inhibitors than DIDS, particularly for stimulated $^{125}$I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for four independent experiments. #, different from Oxo-M-stimulated efflux under control conditions, $p < 0.05$ ($^{125}$I); ##, different from thrombin-stimulated efflux under control conditions, $p < 0.05$ (taurine and $^{125}$I) (by Student’s paired $t$ test).

Inhibition of Oxo-M-stimulated efflux of taurine and $^{125}$I by chelerythrine. Cells prelabeled with $^{3}$Htaurine (A) or $^{125}$I (B) 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 was monitored. Results are expressed as taurine efflux under control conditions, $p < 0.05$ ($^{125}$I); ##, different from thrombin-stimulated efflux under control conditions, $p < 0.05$ (taurine and $^{125}$I) (by Student’s paired $t$ test).

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 $^{3}$Htaurine 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 the buffer and 50 μM EGTA was added) or presence of extracellular Ca$^{2+}$. Reactions were allowed to proceed for 5 min in the presence of 1 μM thapsigargin (Thaps) before 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 three 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 for A. Results are expressed as $^{125}$I efflux (percentage of total soluble radioactivity), and are the means ± S.E.M for five 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).
Ca^{2+}, depleted conditions, a result inconsistent with inhibition of the sons. First, DCPIB, a highly specific inhibitor of VSOAC, significance of this observation remains unclear for two reason.

5 min in the absence (open bars) or presence (closed bars) of 100 mOsM buffer A, that either contained Ca^{2+} or had Ca^{2+} omitted and 50 μM EGTA, 1 μM thapsigargin and 10 μM chelerythrine (− ext Ca + Chel + Thaps) 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 125I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for four independent experiments. #, different from Oxo-M-stimulated efflux under control conditions, p < 0.05 (taurine and 125I) (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 125I. Cells prelabeled with [3H]taurine (A) or 125I (B) were first preincubated for 15 min in the absence (control) or presence of 10 μM chelerythrine and 1 μM thapsigargin in isotonic buffer A. The medium was then aspirated and replaced with 230 mOsM buffer, that either contained Ca^{2+} (control) or had Ca^{2+} omitted and 50 μM EGTA, 1 μM thapsigargin and 10 μM chelerythrine (− ext Ca + Chel + Thaps) 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 125I efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for four independent experiments. #, different from Oxo-M-stimulated efflux under control conditions, p < 0.05 (taurine and 125I) (by Student's paired t test).

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, which 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 125I 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, after receptor activation, both 125I and taurine are released from SH-SY5Y cells via the same (or pharmacologically indistinguishable) VSOAC channels.

Although the release of 125I 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 had demonstrated that increases in [Ca^{2+}], or in PKC activity are not prereq-

uisites 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). Likewise, in the present study, we observed that, at least under mildly hypotonic conditions, the basal release of 125I also appears to be essentially independent of Ca^{2+} availability and PKC activity. However, PAR-1-mediated increases in taurine and 125I efflux differed in their dependence on Ca^{2+} availability and PKC activity. Thus, whereas taurine efflux was attenuated after the removal of extra- and intracellular Ca^{2+} or after inhibition of PKC activity with chelerythrine, thrombin-stimulated 125I efflux was unaffected by either treatment. Under conditions in which both Ca^{2+} depletion and inhibition of PKC activity occurred, stimulated taurine efflux was inhibited by >50%, whereas 125I release remained unchanged. Fura-2 fluorimetric studies indicated that the addition of thrombin to SH-SY5Y cells resulted in a significant increase in [Ca^{2+}], (from 100 to 250 nM), which was abolished when both extra- and intracellular sources of Ca^{2+} were depleted. Because the PAR-1-mediated increase in the release of 125I was not attenuated under these conditions, we conclude that the efflux of 125I (but not that of taurine) occurs independently of a rise in [Ca^{2+}], within these cells. This conclusion is consistent with the Ca^{2+} insensitivity of thrombin-stimulated Cl− currents previously observed in pulmonary artery endothelial cells (Manolopoulos et al., 1997). Further evidence that Ca^{2+} and PKC differentially modulate the release of these two osmolytes from SH-SY5Y cells was obtained after the addition of the muscarinic agonist, Oxo-M. Activation of mAChRs on SH-SY5Y cells elicits a large increase in [Ca^{2+}], (from 100 to 450 nM), which is sustained due to a continuous influx of extracellular Ca^{2+} (Lambert and Nahorski, 1990; Heacock et al., 2006). Although omission of extracellular Ca^{2+} and depletion of intracellular Ca^{2+} with thapsigargin resulted in a pronounced inhibition of mAChR-stimulated taurine release (60 and 81%, respectively), Oxo-M-stimulated 125I efflux was unaffected by removal of extracellular Ca^{2+} and much less inhibited (31%) by depletion of intracellular Ca^{2+} stores (Fig. 9). Likewise, inhibition of PKC resulted in a significantly greater loss of mAChR-stimulated taurine release (73%) than that of 125I efflux (47%). Two conclusions can be drawn from these results. The first is that, regardless of the receptor activated, the stimulated release of 125I is less dependent than taurine efflux on either Ca^{2+} availability or PKC activity. For the PAR-1 receptor, stimulated 125I 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 after 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 125I from SH-SY5Y cells is consistent with results previously obtained for hepatoma cells (Junker 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 \( \text{Ca}^{2+} \), however, although this rise in \( \text{Ca}^{2+} \) is required for the release of taurine, a stimulated efflux of \(^{125}\text{T}^-\) can occur in the absence of increased intracellular \( \text{Ca}^{2+} \). Conceivably, differences in \( \text{Ca}^{2+} \) and PKC requirements for taurine and \(^{125}\text{T}^-\) efflux in hepatoma and SH-SY5Y cells might reflect the following: 1) the receptor-specific activation of distinct signal transduction pathways (\( \text{Ca}^{2+}/\text{PKC-dependent or -independent} \)) that differentially contribute to the efflux of taurine and \(^{125}\text{T}^-\), both of which are released through a common membrane channel, 2) the presence of separate, but pharmacologically similar, efflux channels for \(^{125}\text{T}^-\) and taurine that differ in their degree of regulation by \( \text{Ca}^{2+} \) 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 \(^{125}\text{I}^-\) (data not shown). The possibility that separate efflux channels mediate the release of taurine and \(^{125}\text{I}^-\) 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, after 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


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