Regulation of Volume-Sensitive Osmolyte Efflux from Human SH-SY5Y Neuroblastoma Cells following Activation of Lysophospholipid Receptors

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ABSTRACT

The ability of the lysophospholipids sphingosine 1-phosphate (S1P) and lysosphatidic acid (LPA) to promote the release of the organic osmolyte taurine in response to hypoosmotic stress has been examined. Incubation of SH-SY5Y neuroblastoma cells under hypoosmotic conditions (230 mOsM) resulted in a time-dependent release of taurine that was markedly enhanced (3–7-fold) by the addition of micromolar concentrations of either S1P or LPA. At optimal concentrations, the effects of S1P and LPA on taurine efflux were additive and mediated via distinct receptors. Inclusion of 1,9-dideoxyforskolin, 5-nitro-2-(3-phenylpropylamino) benzoic acid, or 4-[(2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid blocked the ability of both lysophospholipids to enhance taurine release, indicating the mediation of a volume-sensitive organic osmolyte and anion channel. Both S1P and LPA elicited robust increases in intracellular calcium concentration that were attenuated by the removal of extracellular calcium, abolished by the depletion of intracellular calcium with thapsigargin, and were independent of phosphoinositide turnover. Taurine efflux mediated by S1P and LPA was unaffected by the removal of extracellular calcium but was attenuated by depletion of intracellular calcium (34–38%) and by inhibition of protein kinase C (PKC) with chelerythrine (38–72%). When intracellular calcium was depleted and PKC was inhibited, S1P- or LPA-stimulated taurine efflux was inhibited by 80%. Pretreatment of the cells with pertussis toxin, toxin B, or cytochalasin D had no effect on lysophospholipid-stimulated taurine efflux. The results indicate that both S1P and LPA receptors facilitate osmolyte release via a phospholipase C-independent mechanism that requires the availability of intracellular calcium and PKC activity.

The maintenance of cell volume in response to hypoosmotic stress is of paramount importance to all tissues but is of particular significance to the central nervous system because of restrictions of the skull. Brain swelling is observed in clinical conditions that can lead to hyponatremia, such as polydipsia, inappropriate secretion of antidiuretic hormone, and renal or hepatic failure. Hyponatremia, a condition common in the elderly and young, is associated with a variety of neurological symptoms, such as disorientation, mental confusion, and seizures (Kimelberg, 2000; Pasantes-Morales et al., 2000, 2002), and if left untreated, it can result in death from cardiac and respiratory arrest (Pasantes-Morales et al., 2000).

In response to hypoosmotic stress, cells initially swell with a magnitude proportional to the reduction in osmolarity, but this is followed by a recovery process of regulatory volume decrease in which osmolytes (K⁺, Cl⁻, and “compatible” organic osmolytes) are extruded and cell volume is normalized following the exit of obligated water (McManus et al., 1995; Pasantes-Morales et al., 2002). Polyols, methylamines, and amino acids are the principal organic osmolytes utilized by the central nervous system. Of these, the amino acid taurine seems to be ideally suited because of its metabolic inertness and abundance (Lambert, 2004). The extrusion of Cl⁻ and organic osmolytes, such as taurine, occurs via a volume-
sensitive organic osmolyte and anion channel (VSOAC), which is primarily permeable to Cl\(^-\) but impermeable to cations (for review, see Nilius et al., 1997; Lang et al., 1998; Nilius and Droogmans, 2003). Both the VSOAC channel and regulatory volume decrease can be blocked by nonselective Cl\(^-\) channel inhibitors, such as DDF or NPPB, and by the highly selective agent, DCPIB (Decher et al., 2001).

Little is known regarding the cell-signaling mechanisms involved in swelling-induced opening of the VSOAC channel, with the exception that tyrosine kinase activity has been implicated in many cell types. However, it is evident that, when measured in vitro, osmolyte efflux is relatively insensitive to hypomosmotic stress, often requiring substantial (non-physiological) reductions in osmolarity before significant efflux of osmolytes occurs. This observation, along with previous reports that osmolyte release can be enhanced by Ca\(^{2+}\) ionophores, phorbol esters, or agents known to elevate cyclic AMP (Strange et al., 1993; Novak et al., 2000), raised the possibility that, in vivo, the activity of VSOAC may be under neurohumoral control. In this context, we and others have recently identified three pharmacologically distinct receptors that, when activated, enhance the volume-sensitive efflux of osmolytes from neural cells: P2Y purinergic in rat primary astrocytes (Mongin and Kimelberg, 2002, 2005), mA muscarinic cholinergic (mACHR) in human SH-SY5Y neuroblastoma (Loveday et al., 2003; Heacock et al., 2004), and protease-activated receptor-1 (PAR-1) in human 1321N1 astrocytoma (Cheema et al., 2005). In each case, receptor activation facilitates the ability of the cells to release osmolytes under conditions of very limited reductions in osmolarity (5–10%). Because all three receptors can potentially couple to phospholipase C (PLC) activation and Ca\(^{2+}\) mobilization, the possibility exists that there is a mechanistic link between this signaling pathway and osmolyte release.

In the present study, we have further evaluated the possibility of a link, if any, between receptor-mediated Ca\(^{2+}\) mobilization, phosphoinositide hydrolysis, and stimulated osmolyte release using SH-SY5Y neuroblastoma cells as a model system. Specifically, we have examined the ability of two lysophospholipids, namely sphingosine 1-phosphate (SIP) and lysophosphatidic acid (LPA), to enhance taurine efflux and have compared these responses to those previously characterized for the mAChR present in the same cell line (Loveday et al., 2003; Heacock et al., 2004). Both lysophospholipids are reported to elicit large increases in cytoplasmic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in SH-SY5Y cells (Young et al., 2000; Simpson et al., 2002; Villullas et al., 2003). The present results indicate that the activation of either SIP or LPA receptors can markedly enhance osmolyte efflux from these cells via a mechanism that is dependent on the maintenance of an intracellular store of Ca\(^{2+}\) and PKC activity, as previously demonstrated for the mAChR (Loveday et al., 2003). However, receptor-mediated increases in [Ca\(^{2+}\)]\(_i\) are not predictive of the magnitude of osmolyte efflux, and in contrast to mAChR-stimulated osmolyte efflux, a significant proportion (>60%) of SIP- and LPA-stimulated osmolyte release from the cells can still occur in the absence of Ca\(^{2+}\) mobilization. Furthermore, the present results indicate that stimulated osmolyte release can be elicited by Ca\(^{2+}\)-mobilizing receptors that operate via PLC-coupled (e.g., mAChR) or PLC-independent mechanisms (e.g., SIP and LPA). A preliminary account of part of this study has been reported previously (Heacock et al., 2006).

**Materials and Methods**

**Materials.** [1,2-\(^3\)H\]Taurine (1.15 TBq/ml) was obtained from Amersham Biosciences (Piscataway, NJ). NPPB, oxtremorine-M (Oxo-M), and SIP were purchased from Sigma-Aldrich (St. Louis, MO). Sphingosine, dihydrod sphingosine, dihydrosphingosine 1-phosphate, sphingosylphosphoryl choleline (SPhC), dimethyl sphingosine, ceramide, and cystealasin D were obtained from Biomol (Plymouth Meeting, PA). LPA, phosphatidic acid (PA), and lysophosphatidylcholine (LPC) were purchased from Avanti (Alabaster, AL). 1,2-Dideoxyforskolin, toxin B, and bisindolylmaleimide were obtained from Calbiochem (San Diego, CA). DCPIB was purchased from Tocris Biosciences (Ellisville, MO). Fura-2/acetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). Guanidinethyl sulfonate was obtained from Toronto Chemicals (Toronto, ON, Canada). 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). Universol was obtained from ICN biomedical (Uroha, OH). Dowex-1 resin 100- to 200-mesh; ×8 formate was obtained from Bio-Rad (Hercules, CA).

**Cell Culture Conditions.** SH-SY5Y cells (passages 75–90) were grown in tissue culture flasks (75 cm\(^2\)/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) fetal calf serum and 1% penicillin/streptomycin. The osmolarity of the medium was 330 to 340 mOsm. Cells were grown at 37°C in a humidified atmosphere containing 10% CO\(_2\). The medium was aspirated, and the cells were detached from the flask with a trypsin-verseine mixture (BioWhittaker, MD) or with Puck’s 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 2 to 3 days. Experiments were routinely conducted on cells that had reached 75 to 90% confluency.

**Measurement of Taurine Efflux.** Osmolyte efflux from SH-SY5Y cells was monitored essentially as described previously (Loveday et al., 2003; Heacock et al., 2004). In brief, cells were prelabeled overnight with 18.5 kBq/ml [\(^3\)H]taurine at 37°C. Under these conditions, approximately 40 to 50% of the added radiolabel was taken up into the cells. Uptake of radiolabel into SH-SY5Y cells was time-dependent (t\(_{1/2}\) ~ 6 h) and temperature-sensitive (inhibited >98% by lowering the temperature to 4°C) and was inhibited by >80% by inclusion of a 500 μM guanidinethyl sulfonate, an inhibitor of the taurine uptake transporter (Lambert, 2004). After prelabeling, the cells were washed with 2 × 2 ml of isotonic buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl\(_2\), 3.6 mM NaHCO\(_3\), 1 mM MgCl\(_2\), and 30 mM HEPES, pH 7.4, 1 mg/ml bovine serum; approximately 335 mOsm). Cells were then allowed to incubate in 2 ml of hypertonic buffer A (295–195 mOsm; rendered hypertonic by a reduction in NaCl concentration) in the absence or presence of SIP or LPA. 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 the times indicated, aliquots (200 μl) of the extracellular medium were removed, and radioactivity was determined after the addition of 5 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% (wt/vol) trichloroacetic acid. Taurine efflux 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 (Novak et al., 1999).

“Basal” release of taurine is defined as that which occurs at a specified osmolarity in the absence of agonists.
**Results**

**Osmosensitive Efflux of Taurine from SH-SY5Y Neuroblastoma Cells Is Enhanced by the Addition of Lysophospholipids.** When SH-SY5Y cells that had been prelabeled with [3H]taurine were exposed to hypotonic buffer (230 mOsM), there was a time-dependent release of the radiolabeled amino acid from the cells, the initial rate of which (t<sub>3 min</sub>) exceeded that observed in more prolonged incubations (Fig. 1). Inclusion of either S1P, LPA, or Oxo-M significantly enhanced the rate of release of taurine at all of the time points examined and increased the magnitude of response by approximately 3–5-fold over basal (basal release being that monitored in the absence of an agonist) with the rank order of efficacy being S1P > Oxo-M > LPA. As a result of these observations, both basal- and agonist-stimulated taurine effluxes were routinely monitored after a 5-min incubation in subsequent experiments. The addition of either S1P or LPA resulted in a concentration-dependent stimulation of taurine efflux with EC<sub>50</sub> values of 1.0 and 1.2 µM, respectively, with Hill coefficients of close to unity (Fig. 2, A and B). When S1P (5 µM) and LPA (10 µM) were added together, the stimulated release of taurine was approximately additive (83% of theoretical value when basal values are subtracted; Fig. 3A). Furthermore, a 30-min preincubation of the neuroblastoma with 10 µM LPA (in isotonic buffer A) diminished the subsequent responsiveness of the cells to the addition of LPA (in hypotonic buffer A) by 69%, whereas the responsiveness of the cells to S1P was essentially unaltered under these conditions, i.e., homologous desensitization. When the experimental paradigm was reversed, i.e., when cells were first pretreated with 5 µM S1P in isotonic buffer A and then challenged with either S1P or LPA in hypotonic buffer A, only the response to S1P was diminished (76% decrease; Fig. 3A).

The ability of a series of analogs of S1P and LPA to enhance volume-sensitive taurine efflux was also evaluated. Of the sphingosine analogs tested, SPC and dihydrosphingosine 1-phosphate (at concentrations of 5 µM) were as effective as S1P, whereas the addition of sphingosine, dihydrosphingosine, dimethylsphingosine, or ceramide had little or no effect on taurine efflux (Table 1). In addition to LPA, both PA and LPC (at concentrations of 10 µM) significantly enhanced taurine efflux (Table 1). Preincubation of the cells with S1P attenuated the ability of SPC to stimulate taurine efflux by >75% while having no effect on PA-mediated osmolyte release. Pretreatment of the cells with LPA reduced the subsequent response to LPC by 78% but had minimal effect on taurine release elicited by PA (Fig. 3B).

**The Ability of Both S1P and LPA to Enhance the Volume-Sensitive Efflux of Taurine from SH-SY5Y Cells Is Dependent upon Osmolarity.** Because the degree of facilitation of osmolyte release following mAChR activation is dependent on the degree of hypoosmotic stress (Loveday et al., 2003; Heacock et al., 2004), the ability of S1P and LPA to potentiate the release of taurine at different osmolarities was examined. Both basal- and lysophospholipid-stimulated release of taurine was monitored under conditions of isotonicity (335 mOsM; defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells were grown), mild-to-severe hypertonicity (295–195 mOsM), or mild hypertonicity (370 mOsM). In the series of experiments conducted, the basal release of taurine (i.e., that was monitored in the absence of an agonist) was not significantly enhanced until the osmolarity of the buffer had been reduced to 230 mOsM. In contrast, the addition of either S1P or LPA resulted in a significant increase in taurine efflux, even under isotonic conditions (S1P) or under mildly hypertonic conditions (LPA; 295 mOsM; Fig. 4). Moreover, as the osmolarity of the buffer was reduced, the ability of these lysophospholipids to enhance taurine efflux over the basal component was further increased. The maximal enhancement of taurine efflux was observed at an osmolarity of 230 mOsM (1021 and 526% basal for S1P and LPA, respectively). In contrast, when cells were exposed to mildly hypertonic buffer A (370 mOsM), neither lysophospholipid significantly enhanced taurine release. As a result of these findings, an osmolarity of 230 mOsM was chosen for all subsequent experiments.
by separate receptors. A, cells that had been prelabeled with [3H]taurine were washed with isotonic buffer A and then incubated in 230 mOsM buffer in the presence of either S1P (A) or LPA (B) at the concentrations indicated. No further increase in osmolyte release was observed with concentrations of S1P above 10 μM. Concentrations of LPA above 30 μM could not be tested, because they exceeded the critical micellar concentration. Reactions were terminated after 5 min, and taurine efflux was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for four to five independent experiments, each performed in triplicate. The calculated EC50 values for stimulated taurine efflux were 1.0 and 1.2 μM for S1P and LPA, respectively. The corresponding Hill coefficients were 0.8 and 0.7.

Table 1
Ability of S1P, its analogs, and other phospholipids to stimulate taurine efflux

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Taurine Efflux (% Total)</th>
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<tbody>
<tr>
<td>μM</td>
<td></td>
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<tr>
<td>Basal</td>
<td>5.6 ± 0.6 (11)</td>
</tr>
<tr>
<td>S1P</td>
<td>32.1 ± 1.5 (11)**</td>
</tr>
<tr>
<td>LPC</td>
<td>32.0 ± 4.4 (5)**</td>
</tr>
<tr>
<td>SPC</td>
<td>33.0 ± 2.5 (5)**</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>10.2 ± 0.6 (7)**</td>
</tr>
<tr>
<td>Dimethylsphingosine</td>
<td>6.3 ± 0.6 (5)</td>
</tr>
<tr>
<td>Dihydrosphingosine</td>
<td>6.6 ± 0.8 (4)</td>
</tr>
<tr>
<td>Ceramide</td>
<td>6.6 ± 0.9 (5)</td>
</tr>
<tr>
<td>LPA</td>
<td>22.0 ± 0.9 (4)**</td>
</tr>
<tr>
<td>LPC</td>
<td>18.6 ± 3.1 (4)**</td>
</tr>
<tr>
<td>PA</td>
<td>20.4 ± 3.1 (4)**</td>
</tr>
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</table>

Lysophospholipid-Mediated Efflux of Taurine from SH-SY5Y Neuroblastoma Is Mediated via a VSOAC. Previously, we demonstrated that mAChR-mediated osmolyte release was likely to be mediated via a VSOAC (Love-day et al., 2003; Heacock et al., 2004). To determine whether lysophospholipid-stimulated taurine release also occurred via the same channel, basal- and lysophospholipid-stimulated taurine efflux was monitored in the presence of two putative blockers of VSOAC, namely NPPB and DDF. Both of these agents (at a concentration of 100 μM) resulted in a significant inhibition of both basal- and lysophospholipid-stimulated taurine efflux (70–92%; Fig. 5, A and B). Because DDF and NPPB are nonspecific inhibitors of anion channels, the ability of DCPIB, an agent that has recently become commercially available and is highly selective for VSOAC (Decher et al., 2001), to inhibit taurine efflux was also examined. Inclusion of 10 μM DCPIB inhibited basal release by 30% and that induced by Oxo-M, S1P, and LPA by 70 to 100% (Fig. 5C). When the osmolarity of buffer A was reduced from 230 to 195 mOsM, DCPIB was a more effective inhibitor of basal taurine release (50–70%), a result that suggests that the efficacy of this agent to inhibit taurine release may depend on the degree of osmolyte flux through the channel.
Lysophospholipid-Stimulated Taurine Release from SH-SY5Y Cells Is Dependent on the Availability of Ca$^{2+}$ and PKC Activity. Agonist activation of S1P and LPA receptors on SH-SY5Y cells has been reported to elicit an increase in the concentration of cytoplasmic calcium [Ca$^{2+}$]i. (Young et al., 2000; Simpson et al., 2002; Villullas et al., 2003). In agreement with these previous observations, the addition of S1P, LPA, or Oxo-M to SH-SY5Y cells (incubated in hypotonic buffer A) resulted in a 1.5–4-fold rise in [Ca$^{2+}$]i, with a rank order of efficacy being Oxo-M > LPA > S1P (Fig. 6A). When the cells were first exposed to Oxo-M, there was no subsequent response to either LPA or S1P, indicating that all three agonists mobilize a common pool of Ca$^{2+}$ (data not shown). The agonist-induced increases in [Ca$^{2+}$]i were markedly attenuated when extracellular Ca$^{2+}$ was omitted (50–70%; Fig. 6B). In the absence of extracellular Ca$^{2+}$, the depletion of intracellular Ca$^{2+}$ stores with 1 μM thapsigargin essentially abolished the ability of all three agonists to increase [Ca$^{2+}$]i (≥95% inhibition; Fig. 6B).

Of the three Ca$^{2+}$-mobilizing agonists tested, only the addition of Oxo-M resulted in an increase in phosphoinositide turnover, as monitored by an increase in the accumulation of a total inositol phosphate fraction in the presence of Li$^+$ (Fig. 7). Further indication that neither LPA nor S1P elicited their effect on basal-, S1P-, or LPA-stimulated taurine efflux. In some experiments (C), cells were first preincubated for 10 min in hypotonic buffer A in the presence of 10 μM DCPIB. The medium was then aspirated and replaced with hypotonic buffer A (230 mOsM) containing 10 μM DCPIB in the presence or absence of S1P, LPA, or Oxo-M (100 μM). Reactions were terminated after 5 min, and efflux of taurine was monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of three to five independent experiments, each performed in triplicate. *p < 0.05, **p < 0.01 different from agonist-stimulated efflux under control conditions, p < 0.03 (by paired Student’s t test).

The observation that LPA and S1P could fully enhance osmolyte release under conditions in which the ability of these ligands to increase [Ca$^{2+}$]i was significantly reduced prompted us to systematically examine the role, if any, played by Ca$^{2+}$ in basal- and lysophospholipid-stimulated taurine efflux. Removal of extracellular Ca$^{2+}$ had little or no effect on basal-, S1P-, or LPA-stimulated taurine efflux. In contrast, the response to Oxo-M was reduced by 46% when Ca$^{2+}$ was omitted (Fig. 9A). To further examine the role of intracellular Ca$^{2+}$ in taurine release, the cells were preincubated with 1 μM thapsigargin (in the absence of extracellular Ca$^{2+}$) to discharge the intracellular Ca$^{2+}$ pools and then were challenged with the agonists under hypotonic conditions. Under these conditions, the ability of S1P and LPA to
stimulate taurine efflux was reduced by 34 and 38%, respectively, whereas the response to Oxo-M was attenuated by 86% (Fig. 9B).

To test the involvement of PKC in lysophospholipid-stimulated taurine efflux, cells were preincubated in isotonic buffer A for 30 min with 10 μM chelerythrine prior to agonist challenge under hypoosmotic conditions. Whereas chelerythrine had no effect on the basal efflux of taurine, it significantly attenuated S1P- and LPA-stimulated taurine release (38 and 72% inhibition, respectively; Fig. 10A). Stimulated taurine efflux in response to the addition of Oxo-M was also markedly inhibited (77%). Preincubation of SH-SY5Y cells with 1 μM bisindolylmaleimide, another broad-spectrum PKC inhibitor, also resulted in an inhibition of Oxo-M-, LPA-, and S1P-stimulated taurine release by 61 ± 2, 36 ± 2, and 27 ± 3%, respectively (n = 4, p < 0.004), whereas basal release of taurine was unaffected. The combination of inhibition of PKC with 10 μM chelerythrine, along with depletion of intracellular Ca2+ with 1 μM thapsigargin, resulted in an 80 to 100% inhibition of agonist-stimulated taurine release (Fig. 10B).

**Lysophospholipid-Stimulated Taurine Efflux Is Unaffected by Pertussis Toxin or Disruption of the Cytoskeleton.** In some tissues, the effects of lysophospholipids are mediated via activation of pertussis toxin-sensitive GTP-binding proteins and/or via the actin cytoskeleton. However, overnight pretreatment of SH-SY5Y cells with 100 ng/ml pertussis toxin did not significantly alter basal-, S1P-, or LPA-stimulated taurine efflux. Pretreatment of SH-SY5Y neuroblastoma with either cytochalasin D (1 μM) or toxin B (200 pg/ml), conditions previously established to disrupt the cytoskeleton in these cells (Linseman et al., 1998, 2000, 2001), also failed to reduce lysophospholipid-stimulated taurine efflux (data not shown).

**Discussion**

The lysophospholipids LPA and S1P have been linked to a diverse array of cellular functions, such as mitogenesis, apoptosis, and regulation, of the actin cytoskeleton in both neural and non-neural cells (Spiegel and Milstien, 2002, 2003; Moolenaar et al., 2004). In the central nervous system, these lysophospholipids have also been implicated in neuronal growth, survival, differentiation, and cell signaling (for review, see Toman and Spiegel, 2002). In the present study, we report an additional role for both LPA and S1P, that of osmoregulation, which to the best of our knowledge is the first such demonstration for these lysophospholipids.

The principal (if not sole) effects of both LPA and S1P are mediated via a family of edg (endothelial differentiation gene) cell-surface receptors, of which eight have been identified, three encoding receptors for LPA and five for S1P (Lynch and Im, 1999; Contos et al., 2000). SH-SY5Y cells express mRNA for edg 3, 4, 5, 7, and 8, which indicates the presence of LPA2 and LPA3 receptors (edg 4 and 7), and S1P,
S1P<sub>2</sub> and S1P<sub>3</sub> receptors (edg 8, 5, and 3, respectively) (Young et al., 2000; Villullas et al., 2003). The addition of micromolar concentrations of LPA or S1P to SH-SY5Y cells elicited robust increases in taurine efflux under hypoosmotic conditions, each ligand acting through its own set of specific receptors (Fig. 3A). The ability of S1P to increase osmolyte release was fully mimicked by either dihydrosphingosine 1-phosphate or SPC, whereas analogs devoid of a phosphate group, e.g., sphingosine, dimethylsphingosine, dihydrosphingosine, or ceramide, had little or no ability to stimulate taurine release. In addition to LPA, other glycerophospholipids, such as PA and LPC, were equally able to promote osmolyte efflux (Table 1). Because of the original identification of LPA and S1P receptors, several non-edg receptors that are phylogenetically closely related have been identified that respond to either sphingolipids and/or glycerophospholipids, such as SPC, LPC, or PA, in addition to S1P and LPA (Niedernberg et al., 2003; Kostenis, 2004). Whereas preincubation of the cells with S1P attenuated SPC-enhanced taurine release by 77%, osmolyte release elicited by PA addition was unaffected. A similar pretreatment of the cells with LPA reduced LPC-mediated taurine release by 78% but largely spared the response to PA. These results indicate that SH-SY5Y cells express multiple receptors that are activated by glycerophospholipids and sphingophospholipids that couple to the release of osmolytes.

Although the ability of LPA and S1P to enhance osmolyte release from SH-SY5Y cells was most pronounced at 230 mM, the addition of the lysophospholipids resulted in a significant increase in taurine release under isotonic (S1P) or mildly hypertonic conditions (295 mM; LPA). In contrast, neither ligand facilitated osmolyte release under hypertonic conditions. The present results, along with those previously obtained for the P2Y purinergic, m<sub>3</sub> mAChR, and PAR-1 receptors (Mongin and Kimelberg, 2002, 2005; Heacock et al., 2004; Cheema et al., 2005), indicate that receptors coupled to osmolyte efflux share a common property in their ability to facilitate osmolyte release in response to minimal changes in osmolarity. Two inferences can be drawn from these obser-
The ability of the cell to respond to hypoosmotic stress may be restricted to large (pathological) reductions in osmolarity. Second, because receptor-stimulated osmolyte release can occur under isosmotic (or mildly hypotonic) conditions, this indicates that the channel through which the organic osmolytes are released, i.e., the VSOAC, is partially open, even in the absence of a significant reduction in osmolarity. The involvement of a VSOAC in osmolyte release triggered by S1P or LPA is indicated by the ability of nonselective anion channel inhibitors, such as DDF and NPPB, to block lysophospholipid-stimulated taurine efflux. However, this conclusion is strengthened by the observation that DCPIB, a highly selective inhibitor of VSOAC (Decher et al., 2001), substantially inhibits osmolyte efflux elicited by the addition of S1P, LPA, or Oxo-M (Fig. 5C).

One feature common to the receptors that have been identified to enhance osmolyte release is that their activation leads to an increase in \([\text{Ca}^{2+}]\). In this regard, the ability of S1P and LPA receptors to elicit robust increases in both \([\text{Ca}^{2+}]\) and in osmolyte efflux is consistent with this linkage. However, no simple relationship exists between the magnitude of receptor-mediated increases in \([\text{Ca}^{2+}]\), and the extent of osmolyte release. For example, the rank order of efficacy for \(\text{Ca}^{2+}\) mobilization (Oxo-M > LPA > S1P) differs considerably from that of osmolyte release (S1P > Oxo-M > LPA). Furthermore, although the rise in \([\text{Ca}^{2+}]\), elicited by S1P or LPA resulted from both an influx of extracellular \(\text{Ca}^{2+}\) and mobilization of intracellular \(\text{Ca}^{2+}\), omission of extracellular \(\text{Ca}^{2+}\) did not significantly reduce the magnitude of taurine efflux in response to the addition of either lysophospholipid. In contrast, mAChR-stimulated taurine efflux was markedly dependent upon the availability of extracellular \(\text{Ca}^{2+}\), as observed previously (Loveday et al., 2003). A requirement for an intracellular pool of \(\text{Ca}^{2+}\) in S1P- and LPA-stimulated osmolyte release was indicated from the ability of thapsigargin pretreatment of cells (in the absence of extracellular \(\text{Ca}^{2+}\)) to partially inhibit the response (30–40%; Fig. 9B). However, under the same conditions, \(\text{Ca}^{2+}\) mobilization elicited by all three agonists was fully inhibited (Fig. 6B), and mAChR-mediated osmolyte release was reduced by >85% (Fig. 9B). Further evidence that S1P and LPA-stimulated osmolyte release is not directly proportional to a rise in \([\text{Ca}^{2+}]\), was obtained from experiments in which the cells were chronically exposed to Oxo-M. Under these conditions, the ability of LPA or S1P to enhance \([\text{Ca}^{2+}]\) was attenuated by ~70%, whereas stimulated osmolyte release was unaffected (Fig. 8, A and B). Taken collectively, these results suggest that, although \(\text{Ca}^{2+}\) availability is a prerequisite for maximal receptor-stimulated osmolyte release, both the degree of dependence and source of \(\text{Ca}^{2+}\) utilized may differ depending upon the cell type and/or receptor involved. Thus, for S1P and LPA receptors coupled to osmolyte release in SH-SY5Y cells (and PAR-1 receptors in astrocytes; Cheema et al., 2005), osmolyte efflux is independent of extracellular \(\text{Ca}^{2+}\), and there is only a limited dependence upon intracellular \(\text{Ca}^{2+}\) for taurine release. In contrast, osmolyte release elicited by P2Y purinergic receptors in primary astrocytes is severely inhibited (75–90%) by chelation of the intracellular \(\text{Ca}^{2+}\) pool of Ca2+ (Mongin and Kimelberg, 2005), and for mAChRs in SH-SY5Y cells, the removal of both intracellular and extracellular sources of \(\text{Ca}^{2+}\) essentially abolishes osmolyte efflux.

Previously, we and other investigators have demonstrated that PKC activity is also required for maximal receptor-mediated osmolyte release (Loveday et al., 2003; Cheema et al., 2005; Mongin and Kimelberg, 2005). LPA- and S1P-stimulated taurine release shares this property, as is evident from the ability of chelerythrine, a PKC inhibitor, to partially inhibit both responses. Under conditions in which PKC activity was inhibited and the intracellular \(\text{Ca}^{2+}\) pool was depleted, the ability of either lysophospholipid to enhance osmolyte release was reduced by >80%. The dependence on PKC activity and \(\text{Ca}^{2+}\) availability observed for osmolyte release elicited following the activation of LPA, S1P, and mAChR receptors in this cell line is in marked contrast to the
release of osmolytes observed in response to hypotonicity alone (i.e., basal or “swelling-induced”), which is independent of both parameters. In this context, our results are consistent with the conclusion of Mongin and Kimelberg (2005) who proposed that distinct mechanisms underlie basal- and receptor-mediated osmolyte efflux.

Although both Ca\(^{2+}\) availability and PKC activity play essential roles in LPA and S1P-stimulated osmolyte efflux, the present results indicate that neither lysophospholipid elicits increases in [Ca\(^{2+}\)]\(_i\) or PKC via activation of the phosphoinositide signaling pathway. Thus (in contrast to Oxo-M), the addition of LPA or S1P did not increase the formation of a total inositol phosphate fraction. Furthermore, the down-regulation of inositol 1,4,5-trisphosphate receptors following overnight pretreatment of the cells with Oxo-M failed to attenuate the magnitude of either LPA- or S1P-stimulated taurine release. Although both lysophospholipids have been reported to activate PLC in some neural cells (Toman and Spiegel, 2002 and references therein), an inability of LPA to activate the enzyme in SH-SY5Y cells has previously been noted by Young et al. (1999, 2000). Likewise, the addition of LPA or S1P failed to activate PLC in Jurkat cells (Takemura et al., 1996), bovine aortic endothelial cells (Meyer zu Her- ingdorf et al., 1996), and human embryonic kidney 293 cells (Meyer zu Heringdorf et al., 2001). The rise in [Ca\(^{2+}\)]\(_i\), elicited by LPA addition to SH-SY5Y cells has been attributed to the activation of sphingosine kinase (Young et al., 1999, 2000). Likewise, the addition of LPA or S1P did not increase the formation of a total inositol phosphate fraction. Furthermore, the down-regulation of inositol 1,4,5-trisphosphate receptors following overnight pretreatment of the cells with Oxo-M failed to attenuate the magnitude of either LPA- or S1P-stimulated taurine release. Although both lysophospholipid efflux or Ca\(^{2+}\) mobilization of G-protein-coupled sphingosine-1-phosphate receptors. Eur J Pharmacol 414:145–154.


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