PROSTANOID RECEPTORS REGULATE THE VOLUME-SENSITIVE EFFLUX OF OSMOLYTES FROM MURINE FIBROBLASTS VIA A CYCLIC AMP-DEPENDENT MECHANISM

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Running Title: Prostanoid receptors regulate osmolyte efflux

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Non-standard abbreviations: DDF, 1,9-dideoxyforskolin; 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, protease-activated receptor; PGE1, prostaglandin E1; VSOAC, volume-sensitive organic osmolyte and anion channel; GPCR, G-protein coupled receptor; DCPIB, 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid; mAChR, muscarinic cholinergic receptor; IBMX, 3-isobutyl-1-methylxanthine; cAMP, adenosine 3’:5’-cyclic monophosphate; 8-CPT-cAMP, 8-(4-chlorophenylthio) adenosine 3’:5’-cyclic monophosphate; 8-bromo-cAMP, 8-bromo-cAMP, 8-bromoadenosine 3’:5’-cyclic monophosphate; PKA, cAMP-dependent protein kinase; butaprost, 9-oxo-11α,16S-dihydroxy-17-cyclobutyl-prost-13E-en-1-oic acid; 17-phenyl trinor PGE2, 9-oxo-11α,15S-dihydroxy-17-phenyl-18,19,20-trinor-5Z,13E-dien-1-oic acid; sulprostone, N-(methylsulfonyl)-9-oxo-11α,15R-dihydroxy-16-phenoxy-17,18,19,20-tetranor-prosta-5Z,13E-dien-1-amide; Gö 6983, 2-[1-(3-
dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide; \( I_{\text{Cl,swell}} \), an outwardly recifying Cl\(^-\) current activated by hypotonicity.

**Section Assignment:** Cell and Molecular Pharmacology
Abstract

The ability of prostanoid receptors to regulate the volume-dependent efflux of the organic osmolyte, taurine, from murine fibroblasts (L cells) via an adenosine 3'5'-cyclic monophosphate (cAMP)-dependent mechanism has been examined. Incubation of L cells under hypoosmotic conditions resulted in a time-dependent efflux of taurine, the threshold of release occurring at 250mOsM. Addition of prostaglandin E₁ (PGE₁) potently (EC₅₀=2.5 nM) enhanced the volume-dependent efflux of taurine at all time points examined and increased the threshold for osmolyte release to 290 mOsM. Maximal PGE₁ stimulation (250-300% of basal) of taurine release was observed at 250 mOsM. Of the PGE analogs tested, only the EP₂-selective agonist, butaprost, was able to enhance taurine efflux. Inclusion of 1,9-dideoxyfoskolin (DDF), 5-nitro-2-(3-phenylpropylamino benzoic acid (NPPB) or 4-[[(2-Butyl-6,7-dicloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]-butanoic acid (DCPIB) blocked the ability of PGE₁ to enhance taurine release, indicating the mediation of a volume-sensitive organic osmolyte and anion channel (VSOAC). The ability of PGE₁ to increase osmolyte release from L cells was mimicked by the addition of agents that inhibit cAMP breakdown, directly activate adenylyl cyclase or are cell-permeant analogs of cAMP. Taurine release elicited by either PGE₁ or 8-CPT-cAMP was attenuated by >70% in L cells that had been stably transfected with a mutant regulatory subunit of cAMP-dependent protein kinase (PKA). PGE₁-stimulation of taurine efflux was not attenuated by either depletion of intracellular calcium or inhibition of protein kinase C. The results indicate that activation of prostanoid receptors on murine fibroblasts enhances osmolyte release via a cAMP and PKA-dependent mechanism.
Introduction

The ability of cells to respond to alterations in their osmotic environment is of fundamental importance to their survival. 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 normalized following the exit of obligated water (McManus et al., 1995; Pasantes-Morales et al., 2000, 2002). Polyols, methylamines and amino acids are the principal organic osmolytes utilized by eukaryotic cells. Of these, the amino acid taurine appears to be ideally suited because of its metabolic inertness and abundance (Lambert, 2004). The extrusion of Cl⁻ and organic osmolytes such as taurine is thought to occur via a volume-sensitive organic osmolyte and anion channel (VSOAC) which is primarily permeable to Cl⁻ but is impermeable to cations (Nilius et al., 1997; Lang et al., 1998; Kimelberg, 2000; Nilius and Droogmans, 2003). The efflux of Cl⁻ generates an outwardly rectifying current known as $I_{\text{Cl,swell}}$. Regulatory volume decrease, organic osmolyte efflux and $I_{\text{Cl,swell}}$ can be blocked not only by non-selective Cl⁻ channel inhibitors such as DDF or NPPB but also by the highly selective inhibitor of VSOAC, DCPIB (Decher et al., 2001; Abdullaev et al., 2006). In addition to cell volume regulation, several other cell functions have been attributed to VSOACs. These include modulation of electrical activity, cell cycle progression, cell proliferation, apoptosis and metabolic regulation (for reviews, see Nilius et al., 1997; Lang et al., 1998; Stutzin and Hoffman, 2006).
When measured \textit{in vitro}, the efflux of organic osmolytes is relatively insensitive to hypoosmotic stress, often requiring substantial (non-physiological) reductions in osmolarity before a significant efflux of osmolytes occurs. This observation, along with previous reports that swelling-induced osmolyte release can be enhanced by \( \text{Ca}^{2+} \) ionophores, phorbol esters or agents known to elevate cAMP (Strange et al., 1993; Novak et al., 2000; Moran et al., 2001), raises the possibility that, \textit{in vivo}, the activity of VSOAC may be regulated by the activity of G-protein coupled receptors (GPCRs). In this context, we and others have recently identified a number of \( \text{Ca}^{2+} \)-mobilizing GPCRs that, when activated, enhance the volume-sensitive efflux of osmolytes from both neural and non-neural cells: P2Y purinergic receptors in rat primary astrocytes (Mongin and Kimelberg, 2002, 2005), \( \text{H}_1 \) histamine receptors in HeLa cells (Falktoft and Lambert, 2004), \( \text{m}_3 \) muscarinic cholinergic (mAChR), lysophosphatidic acid and sphingosine 1-phosphate receptors in human SH-SY5Y neuroblastoma (Loveday et al., 2003; Heacock et al., 2004, 2006) and the protease-activated receptor-1 (PAR-1) in myoblasts and human 1321N1 astrocytoma (Manopoulos et al., 1997; Cheema et al., 2005). Receptor activation has been demonstrated to facilitate the ability of the cells to release osmolytes under conditions of very limited reductions in osmolarity (5-10\%) via a mechanism that appears to involve intracellular \( \text{Ca}^{2+} \) and protein kinase C (PKC) activity.

A major signal transduction pathway utilized by a large number of GPCRs is the activation of adenylyl cyclase with the concomitant formation of cAMP. In this context, the addition of forskolin, a direct activator of adenylyl cyclase, has been reported to increase osmolyte release in some, but not all, tissues (Strange et al., 1993;
Manopoulos et al., 1997; Moran et al., 2001). Electrophysiological recordings indicate that cAMP can also increase $I_{\text{Cl,swell}}$ although inhibitory effects of the cyclic nucleotide have also been reported (Carpenter and Peers, 1997; Du and Sorota, 1997; Nagasaki et al., 2000; Shimizu et al., 2000). Although these results indicate a potential role for cAMP in osmoregulation, the ability of endogenously expressed adenylyl cyclase-linked receptors to regulate osmolyte efflux has not been systematically examined. In the present study, we have evaluated the ability of prostanoid receptors present in murine L fibroblasts to regulate osmolyte efflux under conditions of hypoosmotic stress. These cells are known to possess prostanoid receptors that robustly couple to adenylyl cyclase and PKA activation (Maganiello and Vaughn, 1972; Uhler and Abou-Chebl, 1992). The results indicate that activation of prostanoid receptors (principally of the EP$_2$ subtype) facilitates a volume-dependent increase in osmolyte release that is mediated via a VSOAC. The stimulatory effect of PGE$_1$ on taurine efflux can be mimicked by agents that elevate intracellular cAMP and are attenuated in an L cell line that exhibits reduced PKA activity. Moreover, in contrast to the responses elicited by agonist occupancy of Ca$^{2+}$-mobilizing receptors, osmolyte efflux triggered by prostanoid receptor activation is independent of both intracellular Ca$^{2+}$ and PKC.
Methods

Materials. [1,2,3H]Taurine (1.1 TBq/mmol) was obtained from Amersham Biosciences (Piscataway, NJ). [γ-32P]-ATP (111 TBq/mmol) was from Perkin Elmer Life Sciences, (Boston, MA). NPPB, sphingosine 1-phosphate (S1P), forskolin, thrombin, 8-CPT-cAMP, 8-bromo-cAMP, pepstatin A, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, dithiothreitol, cAMP, ATP and Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were purchased from Sigma-Aldrich (St. Louis, MO). Prostaglandin E1 was from Biomol (Plymouth Meeting, PA). DDF, Gö 6983 and IBMX were obtained from Calbiochem (San Diego, CA). DCPIB was purchased from Tocris Biosciences (Ellisville, MO). Guanidinethyl sulfonate was obtained from Toronto Chemicals (Toronto, ON). Fura-2/acetoxyethyl ester (Fura-2/AM) was from Molecular Probes (Eugene, OR). Butaprost (free acid), 17-phenyl trinor PGE2 and sulprostone were obtained from Cayman Chemical (Ann Arbor, MI). Dulbecco’s modified Eagle medium (DMEM), genetecin (G418), 50x penicillin/streptomycin and horse serum were obtained from Invitrogen (Carlsbad, CA). Universol was obtained from ICN biomedical (Urora, OH).

Cell culture conditions.

Murine Ltk− fibroblasts (L cells: passage numbers 5-19) and RAB-10 cells (an L cell derived cell line which exhibits reduced PKA activity: Uhler and Abou-Chebl, 1992, passage numbers 5-11) were grown in tissue culture flasks (75 cm2/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) horse serum and 1% penicillin/streptomycin. For the RAB-10 cells, 750 μg/ml of G418 (geneticin) was included. The osmolarity of the medium was 330-340mOsM. 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 trypsin-versene mixture (Biowhittaker, MD). Cells were then resuspended in DMEM/10% horse serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates at a density of 250-300,000 cells/well for 4-5 days. Cells that had reached 95-100% confluency were routinely used.

**Measurement of taurine efflux.**

Osmolyte efflux from L cells and RAB-10 cells was monitored essentially as previously described for SH-SY5Y neuroblastoma (Heacock et al., 2004, 2006). In brief, L cells were prelabeled overnight with 18.5 KBq/ml of [³H]taurine at 37°C. Under these conditions, approximately 90-95% of the added radiolabel was taken up into the cells. Uptake of radiolabel into L cells was time-dependent (t₁/₂ ~3h), temperature-sensitive (inhibited >98% by lowering the temperature to 4°C) and was inhibited >70% 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 x 2 ml of isotonic Buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 3.6 mM NaHCO₃, 1mM MgCl₂ and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 335 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 PGE₁. 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 (200 µl) of the extracellular medium were removed and radioactivity determined after the addition of 5
ml of 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. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released in the extracellular media 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 or cyclic AMP analogs.

**Measurement of PKA activity.**

PKA activity was determined essentially as described by Uhler and McKnight (1987). L cells or RAB-10 cells were harvested by detaching the cells from the flasks with a rubber policeman and centrifugation at 5,000 x g for 5 min. Cell pellets were then resuspended in homogenization buffer (10 mM NaPO₄ buffer (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 1 mM iodoacetic acid, 0.1 mM phenylmethysufonyl fluoride, 1 mM 1,10-phenanthroline, 1 mM pepstatin A and 250 mM sucrose), sonicated (6 x 1 s) and protein concentrations adjusted to 1 mg/ml. Assays (50 µl final volume) were conducted for 5 min at 30° C and contained (final concentrations) 100 µM ATP (250 dpm/ pmol), 5 mM Mg acetate, 15 µM Kemptide, 250 µM IBMX, 5 mM dithiothreitol, 2.5 mM NaF and 10 mM Tris-HCl (pH 7.4). When included in the assay, the concentration of cAMP was 5 µM. The phosphorylation of Kemptide was determined by spotting 25 µl of the incubation mixture on Whatman PE81 phosphocellulose filter papers (2 x 2 cm) and washing them with 4 x 200 ml of 10 mM orthophosphoric acid. After a final wash in 95%
ethanol, individual filters were allowed to dry at room temperature and radioactivity determined after the addition of 5 ml of Universol scintillation fluid.

**Measurement of Cytoplasmic Calcium Concentrations.**

Cytoplasmic free calcium concentrations, [Ca\(^{2+}\)]\(_i\), were determined in suspensions of L cells after preloading cells with the Ca\(^{2+}\) indicator, fura-2 AM, as previously described (Fisher et al., 1989). 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. Except where stated otherwise, 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 or unpaired) 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. EC\(_{50}\) values were obtained using Prism 4.0a (GraphPad Software, Inc. San Diego, CA).
Results

Osmosensitive efflux of taurine from L cells is enhanced by the addition of PGE₁.

When L cells that had been prelabeled with [³H]taurine were exposed to hypotonic buffer (250 mOsM), there was a time-dependent efflux of the radiolabeled amino acid, the initial rate of which (<5 min) exceeded that observed in more prolonged incubations (Fig. 1). Inclusion of PGE₁ (20 µM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of response to approximately 250-300 % of basal (basal release being that monitored in the absence of an agonist). As a result of these observations, both basal and agonist-stimulated taurine efflux were routinely monitored after 10 min of incubation in subsequent experiments. During the course of the present study, some inter-experimental variability in the magnitude of the basal release of taurine (5.2 ± 2.6% of total, mean ± SD, n=65) and in the extent of PGE₁-stimulated taurine efflux (274 ± 74% of control, mean ± SD, n=65) was observed. The addition of PGE₁ resulted in a concentration-dependent stimulation of taurine efflux with an EC₅₀ value of 2.5 ± 0.4 nM (n=3) and a Hill coefficient close to unity (1.1 ± 0.2, n=3, Fig. 2). To determine the subtype of prostanoid receptor coupled to osmolyte efflux, L cells were incubated in the presence of 1 µM concentrations of PGE₁, butaprost (EP₂-selective), 17-phenyl-trinor PGE₂ (EP₁- and EP₃-selective) or sulprostone (EP₃-selective). Of these analogs, only butaprost elicited a significant increase in taurine release (Fig. 3).

The ability of PGE₁ to enhance the volume-sensitive efflux of taurine from L cells is dependent upon osmolarity. The ability of PGE₁ to enhance the release of taurine
at different osmolarities was examined. Both basal and PGE\(_1\)-stimulated release of taurine were monitored under conditions of isotonicity (335 mOsM: defined by the osmolarity of the DMEM/horse serum medium in which the cells were grown), mild-severe hypotonicity (295-190 mOsM) or mild hypertonicity (370 mOsM). In the series of experiments conducted, the basal release of taurine (i.e. that monitored in the absence of an agonist) was not significantly enhanced until the osmolarity of the buffer had been reduced to 250 mOsM. In contrast, the addition of PGE\(_1\) resulted in a significant increase in taurine efflux under mildly hypotonic conditions (290 mOsM; Fig. 4). Moreover, as the osmolarity of the buffer was reduced, the ability of PGE\(_1\) to enhance taurine efflux over the basal component was further increased. The maximum enhancement of taurine efflux was observed at an osmolarity of 250 mOsM (350% of basal), but not under either isotonic or mildly hypertonic conditions (Fig. 4). As a result of these findings, an osmolarity of 250 mOsM was chosen for all subsequent experiments.

**PGE\(_1\)-mediated efflux of taurine from L cells is mediated via a VSOAC.** Previously, we demonstrated that osmolyte release triggered by the activation of mAChRs, PARs or lysophospholipid receptors is mediated via a VSOAC (Heacock et al., 2004,2006; Cheema et al., 2005). To determine whether taurine release elicited by the activation of prostaglandin receptors also occurred via the same channel(s), basal and PGE\(_1\)-stimulated taurine efflux were monitored in the presence of three putative blockers of VSOAC, namely NPPB, DDF and DCPIB. Each of these agents (at concentrations of 100 \(\mu\)M for DDF and NPPB or 30 \(\mu\)M for DCPIB) resulted in a significant inhibition of
both basal- and PGE$_1$-stimulated taurine release (45-62% and 74-90%, respectively, Fig. 5).

**Agents that elevate cAMP concentrations mimic the ability of PGE$_1$ to enhance taurine efflux.** Because PGE$_1$ is reported to substantially increase cAMP concentrations in L cells (Manganiello and Vaughn, 1972; Uhler and Abou-Chebl, 1992), we examined agents that are known to elevate cAMP concentrations for their ability to increase taurine efflux. Under hypoosmotic conditions (250 mOsM), the addition of 1 mM IBMX, a cAMP phosphodiesterase inhibitor, resulted in an increase in taurine release (174% of basal) that was approximately 65-70% of that elicited by PGE$_1$ (212% of control). In the combined presence of IBMX and PGE$_1$, taurine release was less than additive (235% of control; Fig. 6A). Taurine release could also be increased by the addition of 50 μM forskolin, a direct activator of adenylyl cyclase (195% of basal) or alternatively by the addition of 1 mM concentrations of cell permeant analogs of cAMP, namely 8-CPT-cAMP or 8-bromo-cAMP (179-208% of basal, Fig. 6B). When either forskolin or 8-CPT-cAMP were added to incubations that contained PGE$_1$, no further increase in osmolyte efflux was observed.

**PGE$_1$ stimulation of taurine efflux is mediated via PKA.** To investigate whether the stimulation of taurine efflux by PGE$_1$ and cAMP analogs was mediated via PKA, the ability of PGE$_1$ or 8-CPT-cAMP to enhance taurine release was monitored in L cells and RAB-10 cells. The latter are L cells that have been stably transfected with a mutant regulatory subunit of PKA that renders them less susceptible to activation by cAMP
(Uhler and Abou-Chebl, 1992). Preliminary data indicated that the time course of taurine release and sensitivity to osmotic stress were similar in the two cell lines. The ability of either PGE₁ or 8-CPT-cAMP to enhance taurine efflux under hypoosmotic conditions was reduced by <70% in the RAB-10 cells when compared to the untransfected L cells. Thus for L cells, the addition of PGE₁ or 8-CPT-cAMP increased taurine release to $372 \pm 42$ and $267 \pm 9\%$ of control, respectively, $n=5$, whereas the corresponding values for RAB-10 cells were $135 \pm 8$ and $127 \pm 10\%$ of control, $n=5$. In contrast, the ability of either thrombin or sphingosine 1-phosphate to elicit an increase in taurine efflux was not significantly reduced in RAB-10 cells (Fig. 7A). Measurement of PKA activity in extracts of L cells and RAB-10 cells revealed that enzyme activity was reduced by >45% when assayed either in the presence or absence of the cyclic nucleotide (Fig. 7B).

**PGE₁ stimulation of taurine efflux is independent of intracellular Ca²⁺ and PKC activity.** To date, a common characteristic of those agonists that have been demonstrated to promote the efflux of osmolytes from cells is their ability to elicit increases in [Ca²⁺]. Similarly, the addition of PGE₁ to fura-2 loaded L cells also resulted in a 2-3-fold rise in [Ca²⁺]. Thrombin addition also elicited an increase in [Ca²⁺]. (Fig. 8). The rise in [Ca²⁺], triggered by the addition of PGE₁ appears to be independent of cAMP formation since neither the addition of forskolin nor 8-CPT-cAMP had any significant effect on [Ca²⁺]. Furthermore, no significant increase in [Ca²⁺] was observed in the presence of butaprost (data not shown). The agonist-induced increases in [Ca²⁺] evoked by PGE₁ and thrombin were both markedly attenuated when extracellular Ca²⁺
was omitted (>75%) and abolished following depletion of the intracellular pool of Ca$^{2+}$ with thapsigargin (Fig. 8B).

The ability of PGE$_1$ to stimulate Ca$^{2+}$ mobilization in L cells was unexpected and prompted us to examine the role, if any, played by Ca$^{2+}$ in basal-, PGE$_1$- or thrombin-stimulated taurine efflux. Removal of extracellular Ca$^{2+}$ reduced the swelling-induced (basal) release of taurine and that elicited by the addition of either PGE$_1$ or thrombin, to the same extent, i.e. approximately 30-35%. However, when expressed relative to their controls, PGE$_1$-stimulated taurine efflux was only minimally reduced by the omission of Ca$^{2+}$ (203 ± 13 and 183 ± 8% of control for PGE$_1$ in the presence and absence of extracellular Ca$^{2+}$, respectively, n=10, Fig. 9A). Similarly, thrombin-stimulated taurine efflux was also unaffected by the removal of extracellular Ca$^{2+}$ (276 ± 28 and 289 ± 47% of control, in the presence and absence of Ca$^{2+}$, respectively, n=6, Fig. 9B). To examine the role of intracellular Ca$^{2+}$, cells were first preincubated for 5 min in the presence of 1 μM thapsigargin (in the absence of extracellular Ca$^{2+}$) to discharge the intracellular Ca$^{2+}$ pools and then challenged with either PGE$_1$ or thrombin. Under these conditions, PGE$_1$-stimulated taurine efflux was not significantly reduced (177 ± 6 and 183 ± 8% of control, in the presence and absence of thapsigargin, respectively). Basal release of taurine was also unaffected by depletion of intracellular Ca$^{2+}$. In contrast, thrombin-stimulated taurine efflux was diminished by 65% following depletion of intracellular Ca$^{2+}$ (167 ± 9% and 289 ± 47% of control, in the presence and absence of thapsigargin, respectively, p< 0.01, Fig. 9B).

To examine the involvement of PKC in PGE$_1$- and thrombin-stimulated taurine efflux, L cells were preincubated in isotonic buffer A for 15 min with 10 μM chelerythrine...
prior to agonist challenge under hypotonic conditions. Chelerythrine had no inhibitory effect on basal, PGE$_1$- or thrombin-stimulated taurine efflux and preincubation of L cells with the PKC inhibitor slightly enhanced all three parameters (Fig. 10). When calculated relative to their respective controls, the addition of PGE$_1$ increased taurine efflux to 306 ± 36 and 320 ± 44% of control in the absence or presence of chelerythrine, respectively, whereas the corresponding values for thrombin-stimulated taurine efflux were 428 ± 125 and 430 ± 136% of control, respectively, n=5, Fig.10. In addition, chelerythrine had no effect on mAChR-stimulated taurine release from L cells that had been stably transfected with the m$_3$ mAChR (data not shown). The ability of Gö 6983, a highly potent cell-permeant PKC inhibitor, to inhibit PGE$_1$-stimulated taurine efflux was also examined. When cells were pretreated for 15 min with 1 μM Gö 6983 and then challenged with PGE$_1$, agonist-stimulated release of taurine was not significantly reduced (278 ± 12 and 284 ± 27% of basal in the absence and presence of Gö 6983, respectively, n=6). Preincubation of L cells with 1 μM bisindolylmaleimide, another broad spectrum PKC inhibitor, also had no inhibitory effect on basal, PGE$_1$- or thrombin-stimulated taurine efflux (data not shown).
Discussion

Although the role of organic osmolyte release in volume regulation following hypoosmotic stress has been extensively studied, only recently has the possibility that this process is subject to neurohumoral control been systematically examined. To date, of the several GPCRs that have been demonstrated to facilitate the volume-dependent release of osmolytes from cells, all appear to share a common characteristic in that receptor activation triggers a mobilization of intracellular Ca\(^{2+}\), although the precise role of Ca\(^{2+}\) in osmolyte release remains to be determined. Furthermore, maximum receptor-stimulated osmolyte release also appears to require PKC activity (Mongin and Kimelberg, 2002, 2005; Falktoft and Lambert, 2004; Heacock et al., 2004, 2006; Cheema et al., 2005). In the present study, we demonstrate that a GPCR that couples to adenylyl cyclase activation is also able to facilitate osmolyte release via a mechanism that is distinct from that previously described. Evidence to support the conclusion that prostanoid receptors present on mouse fibroblasts regulate osmolyte efflux via a cAMP-dependent mechanism is based upon three series of experimental observations. First, the ability of PGE\(_1\) to enhance taurine efflux could be mimicked by the addition of agents that elevate intracellular cAMP concentrations via either the inhibition of cAMP breakdown or direct activation of adenylyl cyclase or, alternatively, by acting directly as cell-permeant analogs of cAMP (Fig. 6). Moreover, when these agents were included in incubations that contained PGE\(_1\), no further increase in osmolyte release was observed, a result consistent with a common mechanism of action. Second, of the PGE analogs tested, only butaprost, an analog that selectively activates the prostanoid EP\(_2\) receptor subtype that couples to activation of adenylyl cyclase, could facilitate osmolyte release.
Third, the ability of either PGE$_1$ or 8-CPT-cAMP (but not that of either thrombin or sphingosine 1-phosphate) to stimulate taurine efflux was significantly attenuated in RAB-10 cells, which exhibit a lower activity of PKA than L cells. Since PKA is the major downstream cellular target for cAMP action, this result is a further indication that the cyclic nucleotide is the mediator of osmosensitive increases in taurine efflux. The conclusion that prostanoid receptor-mediated changes in cAMP can regulate taurine release is consistent with a previous study in which isoproterenol, a β-adrenergic agonist, was observed to increase osmolyte efflux from glial cells (Moran et al., 2001). Taken collectively, the results indicate that receptor-mediated increases in cAMP are potentially linked to the process of osmoregulation in cells.

One complication in the interpretation of our results is that, in addition to its previously documented ability to increase the concentration of intracellular cAMP in L cells (Maganiello and Vaughn, 1972; Uhler and Abou-Chebl, 1992), PGE$_1$ was also observed to elicit an increase in [Ca$^{2+}$]$_i$ (Fig. 8). However, two lines of evidence suggest that the rise in [Ca$^{2+}$]$_i$ and increases in cAMP concentration are distinct events in L cells. First, the addition of forskolin, 8-CPT-cAMP or the EP$_2$-selective agonist, butaprost, (all of which elicit robust increases in taurine efflux) failed to mimic the ability of PGE$_1$ to increase [Ca$^{2+}$]$_i$. Second, the PGE$_1$–mediated increase in osmolyte release was essentially independent of both extra- and intracellular Ca$^{2+}$ (when calculated on a fold-stimulation basis) even though the agonist-mediated increase in Ca$^{2+}$ was either substantially inhibited or abolished under these conditions (see Fig. 8B). In contrast, although taurine release stimulated by thrombin addition was also independent of extracellular Ca$^{2+}$, depletion of intracellular Ca$^{2+}$ with thapsigargin strongly attenuated
the response, a result consistent with our previous findings in astrocytoma cells (Cheema et al., 2005). The most parsimonious interpretation of these results is that L cells possess two populations of prostanoid receptors, one that couples to the activation of adenylyl cyclase, PKA activation and osmolyte release whereas a second group of receptors is linked to an increase in Ca$^{2+}$ mobilization. It appears that the latter population of receptors does not make a significant contribution to osmolyte release in L cells. In this context, it should be noted that distinct differences in the susceptibility of GPCR-stimulated osmolyte release to depletion of intracellular Ca2+ have been observed. Thus whereas taurine release elicited by the addition of either lysophosphatidic acid or sphingosine 1-phosphate is reduced by 30-40%, the responses to ATP and muscarinic agonists are essentially abolished (Mongin and Kimelberg, 2005; Heacock et al., 2006).

PGE$_1$ stimulation of taurine release also appears to be independent of PKC, as determined from the inability of chelerythrine, Gō 6983 or bisindolylmaleimide to significantly inhibit either basal- or PGE$_1$-induced osmolyte release. The observation that inhibition of PKC also did not attenuate either thrombin- or mAChR-stimulated taurine release from L cells was unexpected and at variance with previous studies in which PKC activity was found to be necessary for the maximum release of osmolytes in response to either of these receptors (Cheema et al., 2005; Heacock et al., 2006). One interpretation of the present findings is that PKC activity may not invariably be a pre-requisite for agonist-stimulated osmolyte release, even for Ca$^{2+}$-mobilizing receptors.

Although taurine release elicited by prostanoid receptor stimulation appears to differ from that exhibited by previously studied receptors in terms of its apparent lack of
Ca\(^{2+}\) and PKC dependence, two features common to all receptors can be identified. The first is that, similar to osmolyte release induced by Ca\(^{2+}\)-mobilizing agonists, a VSOAC appears to mediate osmolyte efflux as indicated by the ability of non-selective anion channel inhibitors, such as DDF and NPPB, to block PGE\(_1\)-stimulated taurine efflux. This conclusion is strengthened by the observation that DCPIB, a highly selective inhibitor of VSOAC (Decher et al., 2001) also significantly inhibits PGE\(_1\)-stimulated taurine efflux (Fig.5). A second characteristic shared by both the prostanoid receptor and those receptors primarily linked to Ca\(^{2+}\)-mobilization is a reduction in the osmotic threshold for osmolyte release following receptor activation. Thus, in the absence of PGE\(_1\) addition, the ability of L cells to significantly respond to hypoosmotic stress is restricted to a relatively large reduction in osmolarity (>33%) whereas in the presence of the agonist, osmolyte release occurs when the osmolarity is reduced by <15% (Fig. 4). The present results are consistent with data previously obtained for osmolyte release following agonist activation of other GPCRs such as the P2Y purinergic, m3 mAChR, PAR-1, sphingosine 1-phosphate and lysophosphatidic acid receptors (Mongin and Kimelberg, 2002, 2005; Heacock et al., 2004, 2006; Cheema et al., 2005) and also that elicited following activation of the epidermal growth factor receptor (Franco et al., 2004). These results indicate that, regardless of the underlying mechanism(s) of activation, receptors coupled to osmolyte efflux share a common property in their ability to facilitate osmolyte release from cells in response to relatively small changes in osmolarity, such as those that are likely to be encountered in vivo.

In summary, the results in the present study provide evidence that prostanoid receptors coupled to an increase in cAMP concentration can facilitate osmolyte release
from L cell fibroblasts in a volume-dependent manner by a mechanism that is distinct from that previously described for Ca$^{2+}$-mobilizing receptors. These results raise the possibility that cells may utilize multiple cell signaling mechanisms to regulate their volume in the face of hypoosmotic challenge.
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References


Footnotes

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Legends for Figures

Fig. 1. Kinetics of basal- and PGE$_1$-stimulated taurine efflux from L cells. L cells that had been prelabeled overnight in the presence of [³H]taurine were washed twice with 2 ml of isotonic buffer A before incubation in 250 mOsM buffer A in the presence or absence of PGE$_1$ (20 µM). Reactions were terminated at the times indicated and taurine efflux measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and (where error bars are shown) are the means ± S.E.M. for three independent experiments, each performed in triplicate. Where errors bars are absent, the result from a single experiment is shown.

Fig. 2. Dose-response relationships for PGE$_1$-stimulated taurine efflux. Cells that had been prelabeled with [³H]taurine were washed with isotonic buffer A and then incubated in 250 mOsM buffer in the presence of PGE$_1$ at the concentrations indicated. Reactions were terminated after 10 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 triplicate replicates obtained from a single experiment, representative of three conducted. Where error bars are not shown, the S.E.M. fell within the symbol. In the experiment shown, the calculated EC$_{50}$ value for stimulated taurine efflux was 2.0 nM and the Hill coefficient was 0.8.
Fig. 3. Butaprost, an EP$_2$-selective agonist, mimicks the ability of PGE$_1$ to stimulate taurine release. Cells that had been prelabeled with [$^3$H]taurine were washed in isotonic buffer A and then incubated in 250 mOsM buffer in the presence or absence of 1 µM concentrations of PGE$_1$, butaprost, 17-phenyl trinor PGE$_2$ (17-PT-PGE$_2$) or sulprostone. Reactions were terminated after 10 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 5 independent experiments, each performed in triplicate. *, different from basal, $p<0.01$ (by paired student’s t-test).

Fig. 4. Basal- and PGE$_1$-stimulated release of taurine as a function of osmolarity. Cells prelabeled with [$^3$H]taurine were first washed in isotonic buffer A and then incubated for 10 min in buffer A at the osmolarities indicated in the absence (open bars) or presence of 20 µM PGE$_1$ (solid bars). Reactions were terminated after 10 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 4 independent experiments, each performed in triplicate. *, different from taurine release observed in cells incubated in isotonic buffer A (335 mOsM), $p<0.05$ (by one-way ANOVA followed by Dunnett’s multiple comparison test). **, different from basal release, $p<0.01$ (by paired student’s t-test).

Fig. 5. Inhibition of basal- and PGE$_1$-stimulated taurine release by anion channel blockers. Cells that had been prelabeled overnight with [$^3$H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (250 mOsM) with either (A)
100 µM NPPB or (B) 100 µM DDF or (C) 30 µM DCPIB in the absence (open bars) or presence (filled bars) of 20 µM PGE1. Reactions were terminated after 10 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of 6-8 independent experiments, each performed in triplicate. *, different from control basal, p<0.05. ***, different from PGE1-stimulated efflux under control conditions, p<0.03 (by paired Student’s t-test).

Fig. 6. Agents that increase cAMP concentrations in cells mimic the ability of PGE1 to enhance taurine release. Cells that had been prelabeled overnight with [3H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (250 mOsM) with either (A) IBMX (1 mM), PGE1 (20 µM) or both or (B) forskolin (FSK, 50 µM), 8-CPT-cAMP (1 mM) or 8-bromo cAMP (1 mM). Reactions were terminated after 10 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of 4-6 independent experiments, each performed in triplicate. *, ** different from control basal, p<0.05, p<0.01 (by paired Student’s t-test).

Fig. 7. The ability of PGE1 or 8-CPT-cAMP to stimulate taurine efflux is attenuated in RAB-10 cells that exhibit a reduced PKA activity. (A) L cells or RAB-10 cells that had been prelabeled overnight with [3H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (250 mOsM) with either PGE1 (20 µM), 8-CPT-cAMP (1 mM), thrombin (1.25 nM) or sphingosine 1-phosphate (S1P, 10 µM). Reactions were terminated after 10 min and efflux of taurine monitored. Results are expressed as
taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 5 independent experiments, each performed in triplicate. *, **different from control basal, p<0.03, p<0.006 (by paired Student’s t-test). (B) PKA activity was measured in extracts of L- or RAB-10 cells in the absence or presence of 10 µM cAMP. Results shown are means ± S.E.M. for 7 independent experiments, each performed in triplicate. *, different from L cells, p<0.001 (by paired Student’s t-test).

**Fig. 8. Addition of PGE1 or thrombin elicits an increase in cytoplasmic free calcium in L cells.** (A) Fura-2/AM loaded cells were first resuspended in 250 mOsM buffer A and then either PGE1 (20 µM) or thrombin (1.25 nM) added. Changes in [Ca2+]i were monitored after the addition of the agonists at 120 sec (indicated by the arrow). Traces shown are representative of 15-17 experiments. (B) For each agonist, the maximum increase in [Ca2+]i was monitored under control conditions (2.2 mM Ca2+; open bars), in the absence of extracellular Ca2+ (solid bars) or following pretreatment of the cells for 5 min with 1 µM thapsigargin in the absence of extracellular Ca2+ (hatched bars). Results shown are means ± S.E.M. for 15-17 experiments (+Ca2+) or 4 experiments (-Ca2+, -Ca2+/thapsigargin). *, **, different from control, p<0.05, p<0.001 (by paired Student’s t test).

**Fig. 9. The role of extracellular- and intracellular calcium in PGE1- or thrombin-stimulated taurine efflux.** Cells that had been prelabeled overnight with [3H]taurine were washed in isotonic buffer A and then incubated for 5 min in hypotonic buffer A (250 mOsM) in the presence (open bars) or absence (filled bars) of extracellular Ca2+.
(Ca\textsuperscript{2+} was omitted from the buffer and 50 µM EGTA added) prior to the addition of either (A) PGE\textsubscript{1} (20 µM) or (B) thrombin (1.25 nM), as indicated. In some experiments, cells were preincubated for 5 min in Ca\textsuperscript{2+}-free hypotonic buffer A (Ca\textsuperscript{2+} omitted and 50 µM EGTA added) in the presence (stippled bars) of 1 µM thapsigargin (TG) prior to the addition of agonist. Incubations were then allowed to proceed for an additional 10 min, reactions terminated and results expressed as taurine efflux (percentage of total soluble radioactivity). Values shown are the means ± S.E.M. for 10 independent experiments for PGE\textsubscript{1} or 6 independent experiments for thrombin, each performed in triplicate,\* different from Ca\textsuperscript{2+}-containing control incubations, p<0.05 (by one-way ANOVA followed by Dunnett’s multiple comparison test); **, different from Ca\textsuperscript{2+}-free incubations, p<0.01 (by one-way ANOVA followed by Dunnett’s multiple comparison test).

**Fig. 10. Inhibition of PKC does not attenuate either PGE\textsubscript{1}-or thrombin-stimulated taurine efflux.** Cells were pretreated in the absence or presence of 10 µM chelerythrine in isotonic buffer A for 15 min before incubation of cells in hypotonic buffer A (250 mOsm) in the absence (control: open bars) or presence of chelerythrine (filled bars) in the presence or absence of PGE\textsubscript{1} or thrombin, as indicated. Reactions were terminated after 10 min and taurine efflux monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for five independent experiments, each performed in triplicate. *, different from control release, p<0.01 (by paired Student’s \textit{t} test).
Fig. 1

Taurine Efflux (% of total)

- PGE$_1$
- Basal

Time (min)

0  5  10  15  20
Fig. 3

Taurine Efflux (% of total)

- Basal
- PGE₁
- Butaprost
- 17-PT-PGE₂
- Sulprostone

* Indicates statistical significance.
Fig. 4

![Graph showing taurine efflux (% of total) vs. osmolarity (mOsm).]

- Basal
- PGE₁

Values are presented as mean ± S.E.M. Significant differences are indicated by asterisks: * for p < 0.05, ** for p < 0.01.
Fig. 5

Panel A: Taurine Efflux (% of total)

- Basal
- PGE1

Panel B: Taurine Efflux (% of total)

- Basal
- PGE1

Panel C: Taurine Efflux (% of total)

- Basal
- PGE1
Fig. 7

A

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Fig. 9

A

Taurine Efflux (% of total)

+ Ca$^{2+}$
- Ca$^{2+}$
- Ca$^{2+}$/thapsigargin

Basal
PGE 1

B

Taurine Efflux (% of total)

+ Ca$^{2+}$
- Ca$^{2+}$
- Ca$^{2+}$/thapsigargin

Basal
Thrombin

* * *
Fig. 10

Taurine Efflux (% of total)

Control
Chelerythrine

Basal
PGE_1
Thrombin

* indicates significant difference from control.