Prostanoid Receptors Regulate the Volume-Sensitive Efflux of Osmolytes from Murine Fibroblasts via a Cyclic AMP-Dependent Mechanism

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ABSTRACT

The ability of prostanoid receptors to regulate the volume-dependent efflux of the organic osmolyte taurine from murine fibroblasts (L cells) has been examined. Incubation of L cells under hypotonic conditions resulted in a time-dependent efflux of taurine, the threshold of release occurring at 250 mOsM. 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 (9-oxo-11-cyclobutyl-1-prost-13E-en-1-ol) was able to enhance taurine efflux. 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 PGE1 to enhance taurine release, indicating the mediation of a volume-sensitive organic osmolyte and anion channel. The ability of PGE1 to increase osmolyte release from L cells was mimicked by the addition of agents that inhibit cAMP breakdown, directly activate adenyl cyclase, or are cell-permeant analogs of cAMP. Taurine release elicited by either PGE1 or 8-(4-chlorophenylthio)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.

The ability of cells to respond to alterations in their osmotic environment is of fundamental importance to their survival. In response to hypotonic 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 occurring 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., 2000, 2002). Polyols, methylamines, and amino acids are the principal organic osmolytes used by eukaryotic cells. 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 is thought to occur via a volume-sensitive organic osmolyte and anion channel (VSOAC), which is primarily permeable to CI− 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 ICl,swell. Regulatory volume decrease, organic osmolyte efflux, and ICl,swell can be blocked not only by nonselective 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

ABBREVIATIONS: VSOAC, volume-sensitive organic osmolyte and anion channel; ICl,swell, outwardly rectifying Cl− current activated by hypotonicity; DDF, 1,9-dideoxyforskolin; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; DCPIB, 4-[2-butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl]oxy]butanoic acid; GPCR, G protein-coupled receptor; EP, prostaglandin receptor; mACHR, muscarinic cholinergic receptor; PKC, protein kinase C; PKA, cAMP-dependent protein kinase; 8-CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; G69883, 2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl)maleimide; PG, prostaglandin; IBMX, 3-isobutyl-1-methylxanthine; AM, acetoxymethyl ester; DMEM, Dulbecco’s modified Eagle’s medium; ANOVA, analysis of variance.
progression, cell proliferation, apoptosis, and metabolic regulation (for reviews, see Nilius et al., 1997; Lang et al., 1998; Stutzen and Hoffman, 2006).

When measured in vitro, the efflux of organic osmolytes is relatively insensitive to hypoosmotic stress, often requiring substantial (nonphysiological) reductions in osmolality before a significant efflux of osmolytes occurs. This observation, along with previous reports that swelling-induced osmolyte release can be enhanced by 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, 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 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); H\(_1\) histamine receptors in HeLa cells (Falktoft and Lambert, 2004); m\(_2\) 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 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 osmolality (5–10%) via a mechanism that seems to involve intracellular Ca\(^{2+}\) and protein kinase C (PKC) activity.

A major signal transduction pathway used 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 prostanooid receptors present in murine L fibroblasts to regulate osmolyte efflux under conditions of hypoosmotic stress. These cells are known to possess prostanooid receptors that robustly couple to adenylyl cyclase and PKA activation (Maggiori and Vaughn, 1972; Uhler and Abou-Chebl, 1992). The results indicate that activation of prostanooid 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 that 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 prostanooid receptor activation is independent of both intracellular Ca\(^{2+}\) and PKC.

Materials and Methods

Materials. [1,2,3\(^{\text{H}}\)]Taurine (1.1 TBq/mmol) was obtained from GE Healthcare (Piscataway, NJ). [\(^{32}\)P]ATP (111 TBq/mmol) was from PerkinElmer Life and Analytical Sciences (Boston, MA). NPPB, sphingosine 1-phosphate, forskolin, thrombin, 8-CPT-cAMP, 9-bromo-cAMP, pepstatin A, phenylmethylsulfonyl fluoride, 1,10-phenanthroline, diithiothreitol, cAMP, ATP, and Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) were purchased from Sigma-Aldrich (St. Louis, MO). Prostaglandin E\(_2\) was from BIOMOL Research Laboratories (Plymouth Meeting, PA). DDF, G60983 and IBMX were obtained from Calbiochem (San Diego, CA). DCPIB was purchased from Tocris Cookson Inc. (Ellisville, MO). Guanidinethyl sulfonate was obtained from Toronto Chemicals (Toronto, ON, Canada). Fura-2/acetoxymethyl ester (Fura-2/AM) was from Invitrogen (Eugene, OR). Butaprost (9-oxo-11a,16\(\beta\)-dihydroxy-17-cyclobutyl-prost-13\(\beta\)-en-1-oxic acid, free acid), 17-phenyl trinor PGE\(_2\) (9-oxo-11a,15\(\beta\)-dihydroxy-17-phenyl-18,19,20-trinor-5Z,13\(\beta\)-dien-1-oxic acid), and sulprostone ([N-(methylsulfonyl)-9-oxo-11a,15\(\beta\)-dihydroxy-16-phenox-17, 18,19,20-tetranor-prosta-5Z,13\(\beta\)-dien-1-amide) were obtained from Cayman Chemical (Ann Arbor, MI). Dulbecco’s modified Eagle’s medium (DMEM), genetecin (G418), 50\% penicillin/streptomycin, and horse serum were obtained from Invitrogen. Universal was obtained from ICN (Aurora, OH).

Cell Culture Conditions. Murine Ltk\(^{–}\) fibroblasts (L cells; passage numbers 5–19) and RAB-10 cells (an L cell-derived cell line that expresses PKC and which has been inoculated with the gene for the taurine uptake transporter (Lambert, 2004). After prelabeling overnight with 18.5 KBq/ml [\(^{3}H\)]taurine at 37°C. Under these conditions, approximately 90 to 95% of the added radiolabel was taken up into the cells. Uptake of radiolabel into L cells was time-dependent (\(k_{1/2}\) of 3 h) and temperature-sensitive (reduced by 50% at 4°C) and was inhibited >70% by inclusion of 500 \(\mu\)M guanidinethyl sulfonate, an inhibitor of the taurine uptake transporter (Lambert, 2004). After prelabeling, the cells were washed twice with 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 d-glucose; ~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 PGE\(_1\). 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 \(\mu\)l) of the extracellular medium were removed, and radioactivity was determined after the addition of 5 ml of Universal scintillation fluid (MP Biomedicals, Solon, OH). 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. 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 then centrifuged at 5000g 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 isoacetic acid, 0.1 mM phenylmethyalsufonyl fluoride, 1 mM 1,10-phenanthroline, 1 mM pepstatin A, and 250 mM sucrose), sonicated (six times for 1 s each), and protein concentrations was 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 magnesium 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 × 2 cm; Whatman, Maidstone, UK) and washing them four times with 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 was determined after the addition of 5 ml of Univeros scintillation fluid.

**Measurement of Cytoplasmic Calcium Concentrations.** Cytoplasmic free calcium concentrations, [Ca²⁺]ᵢ, were determined in suspensions of L cells after preloading cells with the Ca²⁺ indicator Fura-2/AM, as described previously (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. EC₅₀ 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 20 μM PGE₁ significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of response to approximately 250 to 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 effluxes were routinely monitored after 10 min of incubation in subsequent experiments. During the course of the present study, some interexperimental variability in the magnitude of the basal release of taurine (5.2 ± 2.6% of total, mean ± S.D.; n = 65) and in the extent of PGE₁-stimulated taurine efflux (274 ± 74% of control, mean ± S.D.; 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 on Osmolarity.** The ability of PGE₁ to enhance the release of taurine at different osmolarities was examined. Both basal and PGE₁-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 hypertonicity (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 maximal 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, protease-activated receptors, 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 effluxes were monitored in the presence of three putative blockers of VSOAC, namely, NPPB, DDF, and DCPIB. Each of these agents (at concentrations of 100 μM for DDF and NPPB or 30 μM 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 (Maganiello 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 hypotonic 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 to 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 was 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 PGE$_1$ or 8-CPT-cAMP to enhance taurine efflux under hypotonic conditions was reduced by <70% in the RAB-10 cells compared with the untransfected L cells. Thus, for L cells, the addition of PGE$_1$ or 8-CPT-cAMP increased taurine release to 372 ± 42 and 267 ± 9% of control, respectively (n = 5), whereas the corresponding values for RAB-10 cells were 135 ± 8 and 127 ± 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

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**Fig. 3.** Butaprost, an E$_2$-selective agonist, mimics the ability of PGE$_1$ to stimulate taurine release. Cells that had been prelabeled with [3H]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$_1$ (17-PT-PGE$_1$), 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 five independent experiments, each performed in triplicate. *p < 0.01, different from basal (by paired Student’s t test).

**Fig. 4.** Basal and PGE$_1$-stimulated release of taurine as a function of osmolarity. Cells prelabeled with [3H]taurine were first 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$_1$ (17-PT-PGE$_1$), 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 four independent experiments, each performed in triplicate. *p < 0.01, different from basal (by paired Student’s t test).
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⁺]. Likewise, the addition of PGE₁ to Fura-2-loaded L cells also resulted in a 2- to 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, seems to be independent of cAMP formation, since neither the addition of forskolin nor the addition of 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 they were abolished following depletion of the intracellular pool of Ca²⁺ with thapsigargin (Fig. 8B).

The ability of PGE₁ to stimulate Ca²⁺ mobilization in L cells was unexpected and prompted us to examine the role, if any, played by Ca²⁺ in basal, PGE₁-, or thrombin-stimulated taurine efflux. Removal of extracellular Ca²⁺ reduced the swelling-induced (basal) release of taurine and that elicited by the addition of either PGE₁ or thrombin to the same
extent, i.e., approximately 30 to 35%. However, when expressed relative to their controls, PGE1-stimulated taurine efflux was only minimally reduced by the omission of Ca2+ (203 ± 13 and 183 ± 8% of control for PGE1 in the presence and absence of extracellular Ca2+, respectively; n = 10; Fig. 9A). Likewise, thrombin-stimulated taurine efflux was also unaffected by the removal of extracellular Ca2+ (276 ± 28 and 289 ± 47% of control, in the presence and absence of Ca2+, respectively; n = 6; Fig. 9B). To examine the role of intracellular Ca2+, cells were first preincubated for 5 min in the presence of 1 μM thapsigargin (in the absence of extracellular Ca2+) to discharge the intracellular Ca2+ pools, and then they were challenged with either PGE1 or thrombin. Under these conditions, PGE1-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 Ca2+. In contrast, thrombin-stimulated taurine efflux was diminished by 65% following depletion of intracellular Ca2+ (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 PGE1- and thrombin-stimulated taurine efflux, L cells were preincubated in isotonic buffer A for 15 min with 10 μM chelerythrine before agonist challenge under hypotonic conditions. Chelerythrine had no inhibitory effect on basal, PGE1- 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 PGE1 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 efflux (data not shown). The ability of Go<sup>−6983</sup>, a highly potent cell-permeant PKC inhibitor, to inhibit PGE<sub>1</sub>-stimulated taurine efflux was also examined. When cells were pretreated for 15 min with 1 μM Go<sup>−6983</sup> and then challenged with PGE<sub>1</sub>, agonist-stimulated release of taurine was not significantly reduced (278 ± 12 and 284 ± 27% of basal in the absence and presence of Go<sup>−6983</sup>, respectively; n = 6). Preincubation of L cells with 1 μM bisindolylmaleimide, another broad-spectrum PKC inhibitor, also had no inhibitory effect on basal, PGE<sub>1</sub>- 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 osmoles from cells, all seem to share a common characteristic in that receptor activation triggers a mobilization of intracellular Ca<sup>2+</sup>, although the precise role of Ca<sup>2+</sup> in osmolyte release remains to be determined. Furthermore, maximal receptor-stimulated osmolyte release also seems 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 adenyl cyclase activation is also able to facilitate osmolyte release via a mechanism that is distinct from that described previously. Evidence to support the conclusion that prostanoid receptors present on mouse fibroblasts regulate osmolyte efflux via a CAMP-dependent mechanism is based on three series of experimental observations. First, the ability of PGE<sub>1</sub> 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 adenyl 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<sub>1</sub>, 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
EP₂ receptor subtype that couples to activation of adenyl cyclase, could facilitate osmolyte release. Third, the ability of either PGE₁ 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 prostanooid 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), PGE₁ was also observed to elicit an increase in [Ca²⁺], (Fig. 8). However, two lines of evidence suggest that the rise in [Ca²⁺] and increases in cAMP concentration are distinct events in L cells. First, the addition of forskolin, 8-CPT-cAMP, or the EP₂-selective agonist butaprost (all of which elicit robust increases in taurine efflux) failed to mimic the ability of PGE₁ to increase [Ca²⁺]. Second, the PGE₁-mediated increase in osmolyte release was essentially independent of both extra- and intracellular Ca²⁺ (when calculated on a -fold stimulation basis), even though the agonist-mediated increase in Ca²⁺ was either substantially inhibited or abolished under these conditions (Fig. 8B). In contrast, although taurine release stimulated by thrombin addition was also independent of extracellular Ca²⁺, depletion of intracellular Ca²⁺ 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 prostanooid receptors, one population that couples to the activation of adenyl cyclase, PKA activation, and osmolyte release, and a second population of receptors that is linked to an increase in Ca²⁺ mobilization. It seems 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 to 40%, the responses to ATP and muscarinic agonists are essentially abolished (Mongin and Kimelberg, 2005; Heacock et al., 2006).

PGE₁ stimulation of taurine release also seems to be independent of PKC, as determined from the inability of chelerythrine, Go6983, or bisindolylmaleimide to significantly inhibit either basal or PGE₁-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 maximal 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 prerequisite for agonist-stimulated osmolyte release, even for Ca²⁺-mobilizing receptors.

Although taurine release elicited by prostanooid receptor stimulation seems to differ from that exhibited by previously studied receptors in terms of its apparent lack of Ca²⁺ and PKC dependence, two features common to all receptors can be identified. The first feature is that, similar to osmolyte release induced by Ca²⁺-mobilizing agonists, a VSOAC seems to mediate osmolyte efflux as indicated by the ability of nonselective anion channel inhibitors, such as DDF and NPPB, to block PGE₁-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 PGE₁-stimulated taurine efflux (Fig. 5). A second characteristic shared by both the prostanooid receptor and these receptors primarily linked to Ca²⁺ mobilization is a reduction in the osmotic threshold for osmolyte release following receptor activation. Thus, in the absence of PGE₁ 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, protease-activated receptor-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 osmolality, such as those that are likely to be encountered in vivo.

In summary, the results in the present study provide evidence that prostanooid 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 described previously for Ca²⁺-mobilizing receptors. These results raise the possibility that cells may use multiple cell signaling mechanisms to regulate their volume in the face of hypoosmotic challenge.

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