Extracellular osmolarity modulates G protein-coupled receptor-dependent ATP release from 1321N1 astrocytoma cells

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Blum AE, Walsh BC, Dubyak GR. Extracellular osmolarity modulates G protein-coupled receptor-dependent ATP release from 1321N1 astrocytoma cells. Am J Physiol Cell Physiol 298: C386–C396, 2010. First published November 11, 2009; doi:10.1152/ajpcell.00430.2009.—We previously reported that ATP release from 1321N1 human astrocytoma cells could be stimulated either by activation of G protein-coupled receptors (GPCR) or by hypotonic stress. Cheema et al. (Cheema TA, Ward CE, Fisher SK. J Pharmacol Exp Ther 315: 755–763, 2005) have demonstrated that thrombin activation of protease-activated receptor 1 (PAR1) in 1321N1 cells and primary astrocytes acts synergistically with hypotonic stress to gate the opening of volume-sensitive organic osmolyte and anion channels (VSOAC) and that hypotonic stress strongly inhibits PAR1 gating of VSOAC. We tested the hypothesis that a VSOAC-type permeability might comprise a GPCR-regulated pathway for ATP export by determining whether PAR1-sensitive ATP release from 1321N1 cells is similarly potentiated by hypotonicity but suppressed by hypertonic conditions. Strong hypotonic stress by itself elicited ATP release and positively modulated the response to thrombin. Thrombin-dependent ATP release was also potentiated by mild hypotonic stress that by itself did not stimulate ATP export. Notably, PAR1-sensitive ATP export was greatly inhibited in hypertonic medium. Neither the potency nor efficacy of thrombin as an activator of proximal PAR1 signaling was affected by hypotonicity or hypertonicity.

Mechanical stimuli including direct deformation of the surface membrane, physiological fluid shear stress, hypotonic stress-induced swelling, or agonists for G protein-coupled receptors (GPCR) that activate membrane-cytoskeletal rearrangements. Mechanical stress-triggered ATP release from multiple cell types has been mechanistically linked to the efflux of cytosolic ATP pools via three distinct types of nucleotide-permeable channels: 1) volume-regulated anion channels (VRAC) (25, 49); 2) maxi-anion channels (19, 39, 58, 59); and 3) hemichannels (64) composed of connexin (14) or pannexin subunits (27, 40, 54).

VRAC, also known as volume-sensitive outwardly rectifying anion channels (VSOR) or volume-sensitive organic osmolyte and anion channels (VSOAC), comprise a widely expressed, but molecularly undefined, channel activity that gradually develops within the first few minutes after the initial cell swelling in response to hypotonic stress (for reviews, see Refs. 46 and 49). It is an outwardly rectifying, anion-selective current with a single-channel conductance of 30–70 pS and an open-state pore of ~1.1 nm sufficiently large to accommodate ATP$^4^+$ or MgATP$^{2}$ (0.6–0.65 nm radius). Increased VRAC electrophysiological activity triggered by hypotonic stress is strongly correlated with the efflux of larger organic osmolytes, such as taurine and inositol. However, there is growing evidence that VRAC and volume-sensitive organic osmolyte and anion channels (VSOAC) may represent distinct permeability pathways that are coordinately activated by a common network of upstream signals and suppressed by an overlapping group of pharmacological inhibitors (26). Like VRAC/VSOAC, maxi-anion channels comprise a widely expressed, molecularly undefined, mechanosensitive permeability pathway for inorganic and organic anions (for review, see Ref. 60). They are characterized by a single-channel conductance of 200–400 pS and a pore diameter of ~1.3 nm. Finally, hemichannels composed of pannexin-1 (Panx1) or certain connexins (Cx43, Cx32, Cx37) have emerged as strong candidates for ATP release channels (3, 14, 15, 40, 41, 56, 69, 71). Although connexins are generally associated with the transcellular movement of molecules through gap junction channels, connexin (and pannexin) hemichannels at nonjunctional membrane sites can also be gated to the open state by diverse stimuli. In contrast to VRAC/VSOAC and maxi-anion channels, hemichannels are also permeable to inorganic and organic cations, including ethidium$^+$ and propidium$^+$ dyes. The role of Panx1 hemichannels as ATP release conduits has received particular attention given their widespread expression and susceptibility to activation by hypotonicity, direct mechanical stress, membrane depolarization, and increased cytosolic Ca$^{2+}$ (3, 40, 41, 54).

Although VRAC/VSOAC, maxi-anion channels, and hemichannels have been investigated in various cell types, many studies have utilized astrocytes as an experimental model. Astrocytes...
utilize nonexocytotic conductive mechanisms to release a range of so-called “gliotransmitters” including excitatory amino acids, such as glutamate, aspartate, and serine, which act as paracrine and autocrine modulators of nearby neurons, microglia, astroglia, and neurovascular cells. Gliotransmitters also include ATP released from different astrocyte models, and this ATP release has been ascribed to either maxi-anion channels or hemichannels, but not VRAC/VSOAC (14, 27, 29, 36, 38, 39). In contrast, there is considerable experimental support for VRAC/VSOAC as a major astrocytic pathway for the release of excitatory amino acids in response to strong hypotonic stress per se or to the activation of various GPCR under isotonic or mildly hypotonic conditions (37, 44, 45, 53, 66).

The possible convergence of osmotic regulatory responses and GPCR signaling at the level of VRAC/VSOAC function and ATP export in astrocytes has not been directly investigated. However, in previous studies we have reported that thrombin activation of protease-activated receptor 1 (PAR1) causes ATP release from 1321N1 astrocytoma cells under isotonic conditions and that PAR1 activation synergizes with hypotonic stress to elicit even greater ATP release (7, 28). This ability of PAR1 to induce robust ATP export required coordinate coupling to G_{q13 superfic} -> PLC -> Ca^{2+} and G_{12/13 superfic} -> Rho guanine nucleotide exchange factor -> Rho-GTPase signaling pathways known to modulate membrane/cytoskeletal interactions. 1321N1 cells are extensively used as a model system for characterizing intracellular signaling pathways and integrated cellular responses triggered by a wide range of GPCR agonists that regulate similar functions in primary astrocytes. Thrombin, acting via PAR1, stimulates similar VRAC/VSOAC-mediated increases in amino acid permeability in primary astrocytes (53) and 1321N1 cells (12). 1321N1 cells present an additional advantage for analysis of the signaling mechanisms that couple GPCR to VRAC/VSOAC activation (or other GPCR-regulated permeability pathways) because they lack endogenous expression of G protein-coupled P2Y receptors. This is germane because P2Y receptors also activate VRAC/VSOAC in primary astrocytes and other cell types (35, 44, 45, 57). Thus, ATP released in response to hypotonic stress or other GPCR agonists can act as an autocrine modulator or amplifier of VRAC/VSOAC and volume regulatory responses.

That ATP release from 1321N1 cells can be triggered by GPCR activation or hypotonic stress does not distinguish between hemichannels, maxi-anion channels, or VRAC/VSOAC as potential ATP conduits because each of these conductance pathways can be activated by reduced extracellular osmolarity (3, 4, 19, 39, 47, 54). Likewise, the use of small molecule inhibitors is complicated by the often overlapping actions of these reagents on the three channel families. Notably, the functional interaction between GPCR signaling and VRAC/VSOAC activity is distinguished by two critical features. 1) GPCR activation increases the efficacy of hypotonic stress stimulation to induce VRAC/VSOAC-mediated osmolyte and excitatory amino acid efflux in a graded manner depending on the magnitude of the hypotonic stress, such that significant efflux is induced even when cells are in isotonic medium. 2) GPCR activation induces no or only minor osmolyte and excitatory amino acid efflux in the absence of a permissive or “licensing” signal from a threshold amount of osmotic stress (11, 12, 24, 33, 37, 44).

Thus, hypotonic extracellular medium can be used as an alternative to pharmacological reagents to suppress GPCR-triggered VRAC/VSOAC responses. Fisher and colleagues observed that thrombin was able to stimulate taurine efflux from 1321N1 astrocytoma cells under isotonic conditions and that this PAR1-dependent osmolyte release was greatly increased under hypotonic conditions (12). Conversely, PAR1-dependent osmolyte release was abolished in hypotonic medium which causes cell shrinkage rather than swelling (12). In this study, we hypothesized that a similar positive and negative modulation of PAR1-dependent ATP release by hypotonic versus hypertonic conditions might be observed if VRAC/VSOAC comprises a quantitatively significant ATP efflux pathway in 1321N1 cells. Other experiments compared the effects of pharmacological inhibitors of VRAC/VSOAC, maxi-anion channels, and hemichannels. Our major new finding is that thrombin-stimulated ATP release is remarkably sensitive to extracellular osmolarity. Taken together, the observations support a model wherein GPCR stimulation and osmotic stress converge on an ATP release pathway that exhibits several features of VRAC/VSOAC-type channels.

METHODS

Reagents. Thrombin, ionomycin, mannitol, phorbol myristate acetate, β,γ-methylene-ATP (β,γ-MeATP), carbenoxolone (CBX), 1,2-dioleylphosphatidylcholine (dDf), probenecid (PB), and iohypophylized Firefly Luciferase ATP Assay Mix (FLAAM) containing luciferase, luciferin, MgSO4, dithiothreitol, EDTA, bovine serum albumin (BSA), and Tricine buffer were from Sigma-Aldrich. 1,2-Bis (2-aminoethoxyethane-N,N,N’,N’-tetraacetic acid tetrakis acetylamide) (BAPTA-AM) and fura-2 AM were from Invitrogen/Molecular Probes. Wild-type 1321N1 human astrocytoma cells were obtained from Drs. Ken Harden and Jose Boyer (University of North Carolina-Chapel Hill) and Sigma-Aldrich. Clostridial difficile toxin B (ToxB) was from the TechLab (diagnostic test kit).

Cell culture. 1321N1 human astrocytes were cultured in Dulbecco’s minimal essential medium (DMEM) containing 10% iron-supplemented bovine calf serum (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml). For ATP release experiments, 1321N1 cells were seeded on either 24-well plates or 35-mm culture dishes at densities of 4 × 10^4 cells/well or 3 × 10^5 cells/well, respectively. All experiments were conducted using confluent cell monolayers cultured for 6 to 7 days after plating.

Clostridial toxin loading. Confluent 1321N1 cell monolayers were treated with a 1:50 dilution of purified C. difficile ToxB for 3 h at 37°C until significant (>95%) cell rounding was observed. We previously reported that these conditions lead to >90% inhibition of Rho-GTPase activation in response to PAR1 activation (7).

Buffering of cytosolic calcium. The role of cytosolic Ca^{2+} concentration ([Ca^{2+}]) in thrombin-dependent and thrombin-independent ATP release was studied using 1321N1 cell monolayers incubated with the cell-permeable Ca^{2+} chelator BAPTA-AM for 60 min at 37°C.

Stimulation protocol for assay of ATP release. Confluent 1321N1 astrocytes were prepared for ATP release assays as described previously (7). Briefly, 1321N1 cell monolayers in 24-well plates were removed from growth media and washed twice with 0.5 ml basal saline solution (isotonic BSS) containing 130 mM NaCl, 5 mM KCl, 1.5 mM CaCl2, and 1 mM MgCl2, 25 mM NaH2PO4, and 1% BSA. 380 mM final calculated osmolarity. Cells were then allowed to equilibrate for 30–45 min in 250 μl isotonic BSS at 37°C and then rapidly (complete in 15 s) switched to test volume of 250 μl BSS with the indicated osmolarity. Table 1 shows the composition of the replacement BSS solutions used to generate
1321N1 cell monolayers in 24-well plates were allowed to equilibrate for 30–45 min in 250 µl isotonic 320 mosM basal salt solution (BSS) at 37°C and were then rapidly switched to test solutions with altered osmolarity by removal of 100 µl of the isotonic BSS and replacement with 100 µl of modified BSS with different NaCl concentration ([NaCl]) to regenerate a final test volume of 250 µl BSS with the indicated osmolality and [NaCl].

salines with the final indicated osmolalities. All replacement solutions contained different concentrations of NaCl but were otherwise identical to the isotonic BSS. For the experiments in Fig. 2B, an alternative 380 mosM BSS was generated by replacing 100 µl isotonic BSS with 100 µl of standard 130 mM NaCl BSS supplemented with 150 mM mannitol to yield 60 mM final mannitol.

We defined the 320 mosM BSS—rather than a 300 mosM BSS—as isotonic because Cheema et al. (12) have noted that 1321N1 astrocytes cultured in high glucose DMEM to a 300 mosM ATP release assay medium would per se comprise a mild hypertonic stress. The replacement solutions were additionally supplemented with the β,γ-MeATP ecto-ATPase inhibitor (300 µM final) and channel inhibitors at 2.5 times the desired final concentration in the 250 µl test incubation volume. The tested channel inhibitors were the following: 1) 1,9-dideoxyforskolin at 1–300 µM; 2) carbobenzoxyolone at 1–300 µM; 3) probenecid at 0.1–3 mM; or 4) GdCl₃ at 50 µM. In most experiments, the cells were simultaneously challenged by osmotic stress and PAR1 activation. The final thrombin concentration in most experiments was 10 nM (2 U/ml) except for concentration-response analyses. Test incubations were performed at 37°C, and samples of extracellular media (25 µl) were removed for immediate quantification of ATP at 2.5, 5, 10, and 15 min following the switch to replacement BSS plus or minus thrombin. For the experiments in Supplemental Fig. 1, 1321N1 cells were first transferred to BSS with altered osmolality, but lacking thrombin, and then incubated for 30 min before being challenged with various concentrations of thrombin for an additional 10 min (supplemental data for this article can be found online at the American Journal of Physiology-Cell Physiology website).

Luciferase-based quantification of ATP. ATP in each 25 µl sample of extracellular medium was quantified as previously described using a Turner Designs (TD 20/20) luminometer (7). Luminescence (as relative light units, RLU) was integrated over a 5-s photon counting period following which the sample was spiked with known amounts (0.1–10 pmol) of standard ATP for calibration and quantification. All quantifications of ATP release in the BSS formulations with altered NaCl concentrations were appropriately adjusted to account for the known inhibitory effects of chloride on the efficiency of the luciferase reaction. Because 2–3 mM PB and 50 µM Gd³⁺ attenuated luciferase activity, appropriate control calibrations were performed in the presence of these inhibitor concentrations to account for their effects on the luciferase reaction and quantification of ATP in extracellular media samples.

On-line assay of ATP release. Online measurements of ATP release from 1321N1 cell monolayers in 35-mm culture dishes were performed as previously described with minor modifications (7). After luciferase activity reached steady state, 1321N1 monolayers were stimulated either by addition of 10 nM thrombin (plus 300 µM β,γ-MeATP) or by strong hypotonic stress (rapid switch to 215 mosM by replacing 400 µl isotonic BSS with 400 µl of NaCl-free BSS supplemented with FLAAM and β,γ-MeATP).

Measurement of PAR1-activated Ca²⁺ mobilization. Thrombin-stimulated elevation in cytosolic [Ca²⁺] was assayed using fura-2 fluorescence measurements as previously described with minor modifications (28). Fura-loaded cell suspensions were resuspended in isotonic 320 mosM BSS; 3–5 min before experimentation, 0.75 ml of fura-loaded cells (~7.5 × 10⁵ cells) was mixed with 0.75 ml of 320 mosM BSS, 180 mosM BSS, or 380 mosM BSS to generate 1.5 ml isotonic 320 mosM BSS, hypotonic 250 mosM BSS, or 380 mosM BSS, respectively, with 5 × 10⁵ cells/ml. The cells were assayed for changes in fura-2 fluorescence (339-nm excitation and 500-nm emission) triggered by thrombin (0.3 pM-10 nM). Quantification of cytosolic [Ca²⁺] was performed following digitonin permeabilization of the cells using the equation [Ca²⁺] = Kᵣ × (F – Fₘ₉₆)/(Fₘ₉₆ – F), where F is fluorescence (in arbitrary units) and Kᵣ = 224 nM (22).

Data evaluation. RLU recordings were downloaded into Microsoft Excel using the Turner Designs spreadsheet interface software (version 2.0.1, Sunnyvale, CA). RLU values were converted to ATP concentrations using calibration with ATP standards for each experimental condition tested. All perturbations in each experiment were performed in duplicate, and all experiments were repeated three to eight times. (GraphPad Prism 3.0 software was used to compute the means ± SE and to generate graphs of the calculated ATP and [Ca²⁺] levels of the mean ± SE values from the three to eight separate experiments. The data were statistically analyzed using a two-tailed Student’s t-test for comparisons between means (±SE).

RESULTS

Extracellular osmolarity modulates thrombin-stimulated ATP release from 1321N1 astrocytes. We have reported that activation of PAR1 or exposure to hypotonic stress causes ATP release from 1321N1 astrocytes and that PAR1 activation in combination with hypotonic stress leads to greater ATP release than elicited by either stimulus alone (7, 28). However, these previous studies did not test how the opposite osmotic perturbation—hypertonicity—might modulate PAR1-regulated ATP export. Figure 1 compares the kinetics of basal versus thrombin-stimulated ATP release in isotonic (320 mosM, Fig. 1A)
versus hypertonic (380 mosM, Fig. 1B) media. Consistent with our previous findings, thrombin (10 nM) rapidly stimulated a time-dependent release of ATP under isotonic conditions that was near-maximal at 10 min following thrombin addition (Fig. 1A). Notably, this response to thrombin was completely suppressed at all time points when 1321N1 astrocytes were bathed in hypertonic medium (Fig. 1B).

We further defined the modulation of PAR1-stimulated ATP release by extracellular osmolarity by comparing the magnitudes of thrombin-independent versus thrombin-dependent ATP accumulation (assayed at 10 min poststimulation) over a broad range of osmotic conditions (Fig. 2A). In the absence of thrombin, extracellular ATP accumulation was similarly very low (<5 nM in 10 min) in hypertonic, isotonic, and moderately hypotonic (250–300 mosM) media, and increased ATP release was induced only by severe hypotonicity (<250 mosM). No significant thrombin-induced ATP release was observed when cells were bathed in strongly (380 mosM) or moderately hypertonic (350 mosM) salines. This contrasted with the approximately eightfold increase in thrombin-stimulated ATP release in isotonic saline and the progressive potentiation of this ATP export response by decreasing extracellular osmolarity. Because the hypertonic salines were generated by increasing extracellular NaCl concentrations, we considered the possibility that increased ionic strength, rather than increased osmolarity, was the cause of the markedly attenuated ATP release response to thrombin. However, an identical suppression was observed when mannitol was used to generate the hypertonic 380 mosM test saline (Fig. 2B).

The experiments in Figs. 1 and 2 were performed using 10 nM thrombin, which we previously determined was a maximally active concentration for stimulation of ATP release under isotonic conditions (28). We further characterized the concentration-response relationships describing thrombin (15 pM-15 nM) stimulation of ATP release in 1321N1 cells bathed in moderately hypotonic (250 mosM), isotonic (320 mosM), or hypertonic (380 mosM) salines (Fig. 3A). The major changes were a marked increase in thrombin efficacy by hypotonic stress over the entire range of thrombin concentrations. Hypertonic conditions effectively suppressed PAR1-dependent ATP release over the entire range of tested thrombin concentrations. Thrombin-induced ATP efflux was maximal at ~5 nM in isotonic saline versus 1.5 nM in hypotonic saline. The EC50 was ~1.5 nM under isotonic conditions versus ~500 pM for the hypotonic medium. Taken together, these data indicate that the potency and efficacy of thrombin as an ATP secretagogue varies inversely with the extracellular osmolarity (Figs. 1–3).

Most of our experiments involved simultaneous stimulation by altered osmolarity and thrombin for 10 min following a...
30-min preincubation in isotonic BSS. We additionally measured thrombin-dependent ATP release in 1321N1 cells that were preincubated for 30 min in the hypertonic 380 mosM, isotonic 320 mosM, or hypotonic 250 mosM salines before being stimulated by various concentrations of thrombin for an additional 10 min (Supplemental Fig. 1). Similar osmolarity-induced changes in thrombin efficacy and potency as an ATP release stimulus were observed under these experimental conditions. This suggests that the positive and negative modulatory effects of hypotonic versus hypertonic status on PAR1-dependent ATP release reflect stably maintained changes in the coupling of these receptors to the downstream ATP release machinery. However, an alternative possibility is that the altered osmotic conditions modulate the ability of thrombin to trigger the upstream signals (increased Ca\(^{2+}\), Rho-GTPase activation) required for activation of the downstream ATP release pathways (7, 28). We compared the concentration-response relationships for thrombin-dependent changes in intracellular Ca\(^{2+}\) mobilization in cells bathed in moderately hypotonic (250 mosM), isotonic (320 mosM), or hypertonic (380 mosM) salines (Fig. 3B). The EC\(_{50}\) values (~50 pM) and the peak magnitudes of the Ca\(^{2+}\) transients were identical in the three groups of cells (Fig. 3B). These observations established that PAR1 activation and its coupling to proximal second messenger pathways were not affected by changes in extracellular osmolarity.

Rho-GTPase activation and Ca\(^{2+}\) mobilization are required for stimulation of ATP release in response to PAR1 activation but not strong hypotonic stress. Our previous studies established that activation of thrombin-dependent ATP release from 1321N1 cells in isotonic saline requires coincident input from Rho-GTPase signals and increased cytosolic Ca\(^{2+}\). Therefore, we investigated the roles of these second messengers in the ATP release responses to strong hypotonic stress in the absence of thrombin, or to costimulation by thrombin and modest hypotonic stress, by pretreating cells with either BAPTA-AM for 60 min or C. difficile ToxB for 4 h before stimulation. BAPTA buffering blunts the increases in cytosolic Ca\(^{2+}\) triggered by PAR1 while ToxB glucosylates Rho family GTPases and prevents their activation by upstream PAR1-regulated GTP/GDP exchange factors. BAPTA produced similar 72%, 62%, and 60% decreases in thrombin-activated ATP release in isotonic, mild hypotonic, and strong hypotonic salines, respectively, but had no effect on the thrombin-independent ATP efflux induced by strong hypotonic stress (Fig. 4A). Likewise, ToxB caused 56%, 54%, and 64% reductions in PAR1-dependent ATP release under isotonic, mild hypotonic, and strong hypotonic conditions, respectively, while producing no inhibition of the response to strong hypotonicity (Fig. 4B).

Comparative effects of VRAC/VOAC, hemichannel, and maxi-anion channel inhibitors on ATP release responses to PAR1 activation or strong hypotonic stress. The strong inhibitory effect of hypotonicity on thrombin-induced ATP release is very similar to its suppressive action on GPCR-dependent release of organic osmolytes and excitatory amino acids described in previous studies of astrocyte VRAC/VOAC function. We also compared the effects of the VRAC/VOAC inhibitor ddF on the ATP release responses to PAR1-activation in isotonic and mild hypotonic salines (Fig. 5A) versus exposure to strong hypotonic stress (Fig. 5B). Notably, ddF attenuated thrombin-dependent and thrombin-independent ATP release with similar efficacy (maximal ~68% inhibition) and potency (IC\(_{50}\) ~50 \(\mu\)M) regardless of the osmotic conditions.

We next tested the effects of CBX on the ATP release responses under isotonic and mildly hypotonic conditions (Fig. 5C) versus strong hypotonic stress (Fig. 5D). Although CBX is a widely used inhibitor of Cx- and Panx-based hemichannels, it also blocks VRAC currents and VSOAC-mediated export of organic osmolytes in astrocytes (6, 73). CBX attenuated thrombin-dependent and thrombin-independent ATP release with similar efficacy (maximal ~66% inhibition) and potency (IC\(_{50}\) ~50 \(\mu\)M) regardless of the osmotic conditions.

Another set of experiments characterized the effects of PB, an efficacious inhibitor of Panx-1 hemichannel function (54). We observed no inhibitory effect of PB (0.1–3 mM) on ATP efflux from 1321N1 cells challenged by strong hypotonicity (Fig. 6B). In contrast, PB produced a dose-dependent attenuation (IC\(_{50}\) ~1.3 mM, ~55% maximal efficacy) of thrombin-stimulated ATP release under isotonic conditions (Fig. 6, A and C). PB (2.5 mM) did not inhibit thrombin-induced Ca\(^{2+}\) mobilization (Fig. 6D). Surprisingly, the inhibitory action of PB (2 mM) on PAR1-dependent ATP release was not observed when 1321N1 cells were bathed in progressively hypotonic
salines that positively modulated thrombin-triggered ATP efflux (Fig. 6C).

Finally, we compared the effects of Gd$^{3+}$/H11001, an inhibitor of maxi-anion channels, on the ATP release responses to PAR1-activation in isotonic saline (Fig. 7A) or exposure to strong hypotonic stress (Fig. 7B). Fifty micromolar Gd$^{3+}$, a concentration that inhibits swelling-induced ATP release in other astrocyte models (38, 39), had no effect on ATP release from 1321N1 cells in response to strong hypotonic stress or PAR1-activation.

**DISCUSSION**

This study demonstrates that GPCR-regulated ATP release in 1321N1 astrocytoma cells is an osmotically sensitive response wherein hypertonic stress suppresses and hypotonic stress potentiates PAR1-stimulated ATP export in the absence of effects on receptor activation and downstream Ca$^{2+}$/H11001 mobilization (Figs. 1–3). Importantly, the observed attenuation of thrombin-dependent ATP release by hypertonic conditions is very similar to the previously established hypertonic suppression of thrombin-stimulated taurine efflux in the 1321N1 cell model (12). Other studies have shown that hypertonicity suppresses, and hypotonicity potentiates, P2Y-receptor-induced release of aspartate from rat primary astrocytes (44, 45) and B2-bradykinin receptor-activated glutamate release from mouse primary astrocytes (37); both of these latter responses have been ascribed to VRAC/VSOAC activation. Notably, glutamate efflux from primary mouse astrocytes is stimulated by thrombin-activated PAR1 under isotonic conditions, and this response is further potentiated by mild hypotonicity (53). Thus, our data indicate that GPCR-dependent ATP release from astrocytes involves mechanisms that appear to be common to the GPCR-dependent release of other gliotransmitters and organic osmolytes. Our pharmacological studies also support, but do not prove, a role for VRAC or VSOAC, rather than Cx/Panx hemichannels or maxi-anion channels, as the conduit for GPCR-stimulated ATP release from 1321N1 human astrocytoma. This conclusion is necessarily tentative given 1) the modest and often overlapping selectivity of the existing pharmacological probes for VRAC/VSOAC, maxi-anion channels, and hemichannels; and 2) the molecularly undefined nature of VRAC/VSOAC.

VRAC/VSOAC exhibit several characteristics required of osmotically sensitive ATP release pathway because they are widely expressed, permeable to organic metabolites, and develop an outwardly rectifying current (single-channel conductance of 30–70 pS at +120 mV) within minutes in response to hypotonic stress (46, 65). However, it is important to stress that the electrophysiologically defined VRAC Cl$^{-}$/H11002 conductance, versus the VSOAC associated with the efflux of organic osmolytes (taurine and inositol) or excitatory amino acids, may represent distinct permeability pathways with overlapping regulation and pharmacology (26). VRAC-mediated Cl$^{-}$/H11002 currents are blocked in the presence of extracellular nucleotides (at millimolar concentrations), indicating that nucleotides can enter the permeability pore of the channels (18). Furthermore, several intracellular signaling pathways and second messengers, while not required for VRAC/VSOAC activation, modulate VRAC/VSOAC activity either by reducing the threshold for activation by hypotonic stress or by increasing the conductance of the gated channels (26, 49). Significantly, these signaling systems include Rho-GTPase and phospholipase C.
Stimulated by thrombin partially attenuates inositol phosphate pathways (67, 68). Notably, addition of apyrase to A549 cells decreased the maximal release in the presence of 0–3 mM PB. ATP release in the presence of PB was normalized to the maximal release in the absence of PB. C: 1321N1 monolayers were bathed in BSS with reduced osmotic pressure and then incubated for 10 min with 10 nM thrombin. Peak changes in cytosolic [Ca\(^{2+}\)] were determined. Values are the average ± range; n = 2.

Fig. 6. Concentration-inhibition relationships for the effects of probenecid (PB) on ATP release by thrombin versus strong hypotonic stress. A: 1321N1 monolayers bathed in isotonic 320 mosM (■) or moderately hypotonic 250 mosM (□) BSS were stimulated for 10 min with 10 nM thrombin in the presence of 0–3 mM PB. ATP release in the presence of PB was normalized to the maximal release in the absence of PB. B: 1321N1 monolayers were stimulated by transfer to strongly hypotonic 215 mosM BSS (○) for 10 min in the presence of 0–3 mM PB. ATP release in the presence of PB was normalized to the maximal release in the absence of PB. C: 1321N1 monolayers were bathed in BSS with the indicated osmolarity and then incubated for 10 min with no other additions (open bars), with 10 nM thrombin alone (filled bars), or with 10 nM thrombin plus 2 mM PB (hatched bars). Values in A–C are means ± SE (n = 4). *P < 0.05, significant differences from the normalized maximal release in the absence of PB. D: fura-2-loaded 1321N1 cells were suspended in BSS with or without 2.5 mM PB and then stimulated with 10 nM thrombin. Peak changes in cytosolic [Ca\(^{2+}\)] were determined. Values are the average ± range; n = 2.

The ATP release pathway elicited by PAR1 activation and hypotonic stress in 1321N1 cells exhibited a pharmacological profile consistent with an involvement of VRAC/VSOAC. VRAC-mediated Cl\(^{-}\} currents and VSOAC-mediated release of organic osmolytes and excitatory amino acids are sensitive to inhibition by a broad range of reagents including ddF, flufenamic acid, and CBX, but excluding Gd\(^{3+}\) (1, 6, 20, 47). The observed Gd\(^{3+}\) insensitivity of the ATP release responses in 1321N1 cells argues against a likely role for maxi-anion channels in this model (Fig. 7, A and B) (38, 39, 60). In contrast, the pharmacologic profile of both basal and thrombin-stimulated ATP release was similar with respect to the nonselective VRAC/VSOAC inhibitors ddF and CBX (Fig. 5, A–D). ddF, which is an inactive analog of forskolin with respect to adenyl cyclase activation, blocks volume-sensitive anion currents carried by the molecularly undefined VRAC channels or the VSOAC-mediated release of organic osmolytes and—as demonstrated for the first time in this report—ATP (47, 61). To our knowledge, ddF has not been directly investigated as a hemichannel blocker. However, 50 μM ddF did not mimic the production in response to PAR activation (62). Thus, 1321N1 cells provide a unique and useful model system for studying the coupling of hypotonic stress signals to ATP release channels in the absence of confounding signals from autocrine G protein-coupled P2Y receptors. While the hypotonic stress and PAR1 stimuli in 1321N1 cells are transduced by different signaling pathways, it is likely that the two stimuli overlap in their activation of a common ATP release conduit because the two stimuli together exert a synergistic rather than simply additive response. Moreover, both the Ca\(^{2+}\) and Rho-GTPase second messengers are known to modulate VRAC/VSOAC activities (26, 49).

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blocks Panx-, but not Cx-based hemichannels (63). Notably, recent studies used both small interfering RNA (siRNA) knockdown and PB-mediated blockade to demonstrate an important role for Panx1 hemichannels in the ATP release responses of human airway epithelial cells to strong hypotonic stress (54) or thrombin stimulation (via PAR3 rather than PAR1) (62). Effects of PB on VRAC/VSOAC or maxi-anion channels have not been reported. We found that PB did not block ATP release elicited by strong hypotonic stress in 1321N1 cells. This stands in contrast to findings in airway epithelia wherein PB markedly attenuates hypotonicity-induced ATP release and mimics the actions of Panx1 siRNA treatment (54). Thus, it appears unlikely that Panx1 hemichannels play a major role in hypotonic stress-stimulated ATP release in the 1321N1 cell model. However, PB did attenuate thrombin-induced ATP efflux from the 1321N1 cells (Fig. 6) but with an IC50 of 1.3 mM that was higher than the 150–350 μM IC50 values reported for the suppressive effects of PB on recombinant Panx1 hemichannel currents in Xenopus oocytes (63) or HEK293 cells (42). Importantly, there was no obvious effect of 2.5 mM PB on thrombin-induced Ca2+ mobilization (Fig. 6D).

We initially expected that Panx1 hemichannels would comprise the major GPCR-dependent ATP release pathway in the 1321N1 model given the role for this pathway in other cell types and because 1321N1 cells express Panx1 mRNA (51 and our data not shown). Pelegrin and Surprenant (51) also found that natively expressed Panx1 hemichannels mediated ATP-stimulated YoPro dye influx in 1321N1 cells engineered to express recombinant P2X7 receptors. Panx1 hemichannels provide the major conduit for the ATP release stimulated by P2X7 receptor activation in primary mouse astrocytes (27). However, our observations regarding the inhibitory actions of CBX and PB on thrombin-stimulated or hypotonic stress-stimulated ATP release from the 1321N1 astrocytoma cells were clearly different—with regard to potencies and efficacies—from the inhibitory effects of these reagents on molecularly defined Panx1 hemichannel activities in other cell types. We also found that the potency and efficacy of PB as an inhibitor of thrombin-stimulated ATP release was greatly reduced when the 1321N1 cells were bathed in mildly hypotonic medium (Fig. 3A). The reason for this loss of PB efficacy under hypotonic conditions is unclear but may reflect conformational changes in the ATP release channel (or channel complex) by swelling-associated changes in membrane organization or by reduced ionic strength. Regardless of mechanism, our atypical findings regarding the effects of CBX and PB on GPCR-dependent ATP release in 1321N1 cells are difficult to reconcile with canonical Panx1 hemichannel function. This is further supported by our previous observation that thrombin-triggered ATP efflux was not correlated with influx of ethidium Dye; this is inconsistent with the permeability of conventional Panx1-based hemichannels to small (<900 Da) organic cations (7).

The pharmacological and permeability characteristics of Panx1 hemichannels may vary with cellular background due to the interaction of Panx1 with other membrane proteins. Bunse et al. (9) recently reported that the efficacies and potencies of CBX and PB as inhibitors of recombinant Panx1 hemichannel currents were markedly attenuated when Panx1 was coexpressed with the potassium channel subunit Kvβ3. Thus, it remains possible that the osmotically sensitive, GPCR-gated...
ATP release channels in 1321N1 cells (and other cell types) are comprised of Panx1 hemichannels complexed with other modulatory proteins. The molecular compositions of VRAC, VSOAC, and maxi-anion channels have remained undefined despite significant efforts to identify candidate gene products. It is tempting to speculate that these functionally defined conductance pathways may be composed of Panx hemichannels in cell type-specific combinations with other membrane proteins or signaling proteins. This is an important experimental question for future studies.

The observed sensitivity of PAR1-dependent ATP release to the osmotic status of 1321N1 astrocytes is remarkably similar to that characterizing the effects of PAR1 on taurine efflux from 1321N1 cells (12) and glutamate efflux from primary mouse astrocytes (53). As we found for ATP release, increased Ca\(^{2+}\) mobilization was also a requisite signal for taurine release in response to PAR1 activation but not strong hypotonic stress alone. Ca\(^{2+}\) mobilization in response to thrombin results from a Gq−PLC signaling cascade shown to affect regulatory volume decrease (RVD) responses in astrocytes (5, 52). A common and defining feature of both the ATP release (this study) and taurine release (12) responses was that GPCR activation induced almost no efflux of these organic anions in the absence of a permissive or licensing signal from a threshold amount of osmotic stress. The mechanism whereby osmotic stress and consequent volume perturbation is transduced to the gating of VRAC/VSOAC or other osmotically sensitive channels is poorly understood (49) However, subtle cell swelling- or shrinkage-induced changes in the sub-plasma membrane cytoskeleton or organization of cytoskeletal-membrane lipid-channel protein complexes have been proposed (26, 30). This is certainly consistent with the roles of Ca\(^{2+}\) and Rho-GTPase as major second messengers in the modulation of VRAC/VSOAC function and PAR1-activated ATP release from 1321N1 cells.

In summary, we conclude that the thrombin-dependent ATP release pathway from 1321N1 cells is remarkably sensitive to osmotic conditions and, by implication, to cell volume. Our results add to a growing literature describing ATP release from different cell model systems in response to various types of mechanical stimuli, and support the involvement of multiple, mechanistically distinct ATP release pathways with overlapping pharmacology and regulation. Regardless of the molecular conduit for ATP release, the observed synergy between GPCR activation and hypotonic stress in regulating ATP export has likely physiological significance. One role of extracellular ATP is to accelerate cellular volume correction in response to changes in extracellular osmolality. For example, autocrine activation of P2 receptors in astrocytes, hepatocytes, or airway epithelial cells by endogenous ATP released in response to hypotonic stress accelerates the efflux of Cl\(^{-}\) and organic osmolytes that facilitate RVD responses. Scavenging of extracellular ATP by added nucleotidases or blockade of P2 receptors during exposure to hypotonic stress can interrupt these purinergic autocrine loops and attenuate or slow cell volume recovery from swelling (13, 16, 21, 34, 35, 43, 48, 70, 74). Protease-activated receptors and other sensors of local tissue damage/stress can modulate brain injury. Low concentrations of thrombin have been shown to attenuate brain cell death elicited by a number of different insults that result in cell swelling in vitro and in vivo (72). In addition to osmolality, the release of ATP as a paracrine modulator of neuronal and glial function might be further tuned by other biophysical conditions such as mild hypothermia, which is now an established brain protective therapy (17). However, under our defined in vitro conditions, the rate and extent of thrombin-stimulated ATP release from 1321N1 cells was identical at 37°C or 32°C, and only modestly slowed at 20°C (Supplemental Fig. 2). Our observation that PAR1-dependent ATP release was progressively potentiated by graded reductions in extracellular osmolality, but markedly suppressed by increased extracellular osmolality, may have important implications for the physiologic regulation of brain volume and response to injury.

REFERENCES


