Subnanomolar Concentrations of Thrombin Enhance the Volume-Sensitive Efflux of Taurine from Human 1321N1 Astrocytoma Cells

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ABSTRACT
The ability of subnanomolar concentrations of thrombin to protect both neurons and glia from ischemia and other metabolic insults has recently been reported. In this study, we demonstrate an additional neuroprotective property of thrombin; its ability to promote the release of the organic osmolyte, taurine, in response to hypoxic stress. Incubation of human 1321N1 astrocytoma cells with hypo-osmolar buffers (320–227 mOsM) resulted in a time-dependent release of taurine. Incubation of thrombin (EC50 = 60 pM) resulted in a marked increase in taurine efflux that, although evident under isotonic conditions (340 mOsM), was maximal at an osmolarity of 270 mOsM (3–4-fold stimulation). Thrombin-stimulated taurine efflux was dependent upon its protease activity and could be mimicked by addition of the peptide SFLLRN, a proteinase activated receptor-1 (PAR-1) subtype-specific ligand. Inclusion of anion channel blockers known to inhibit the volume-sensitive organic osmolyte anion channel attenuated thrombin-stimulated taurine release. Depletion of intracellular Ca2+ with either 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid (BAPTA) or thapsigargin, or alternatively, inhibition of protein kinase C (PKC) with bisindolylmaleimide or chelethrine resulted in a 30 to 50% inhibition of thrombin-stimulated taurine efflux. Under conditions in which intracellular Ca2+ was depleted and PKC activity inhibited, thrombin-stimulated taurine efflux was reduced by >85%. The results indicate that activation of PAR-1 receptors by thrombin facilitates the ability of 1321N1 astrocytoma cells to release osmolytes in response to a reduction in osmolarity via a mechanism that is dependent on intracellular Ca2+ and PKC activity.

Recent evidence indicates that in addition to its role in platelet aggregation and tissue repair, thrombin, a serine protease generated from prothrombin, regulates numerous physiological and pathological responses including development, inflammation, atherogenesis, stroke, and Alzheimer’s disease (Gingrich and Traynelis, 2000; Xi et al., 2003; Ossovskaya and Bunnett, 2004). Thrombin may also be produced in the brain following cerebral hemorrhage (Hollenberg and Compton, 2002; Trejo, 2003; Wang and Rei- ser, 2003). All four PARs are expressed abundantly in the central nervous system (Striggow et al., 2001); however, their function is still unclear. Previous studies indicate biphasic and
dose-dependent actions of thrombin on astrocytes. Low concentrations of thrombin (50 pM to 100 nM) mediate neuroprotection against ischemia and environmental insults such as oxidative stress, hypoglycemia, hypoxia, and growth supplement deprivation. High concentrations of thrombin however, can cause degeneration and cell death (Vaughan et al., 1995; Strigov et al., 2000; Jiang et al., 2002). In vivo, pretreatment of the brain with a low dose of thrombin attenuates brain injury induced by cerebral hemorrhage or trauma, although high-dose thrombin infusion can cause astrogliosis (Xi et al., 1999; Masada et al., 2000). It has also been demonstrated that PAR1 and PAR3 receptors are up-regulated in various regions of the brain after environmental insults (Xi et al., 2003). Recently, PAR1 and PAR3 were shown to mediate antiapoptotic signaling by activated protein C in neurons (Guo et al., 2004). Although these studies indicate the potential importance of low concentration of thrombin for the development of new therapeutic strategies to treat neurodegenerative disorders, the molecular mechanisms(s) underlying neuroprotection remain to be established.

A neuroprotective mechanism used by both neurons and glia is that of volume regulation following exposure of the cells to osmotic insult. In response to hypotonic stress, cells swell with a magnitude proportional to the reduction in osmolarity. This rapid increase in volume is transient and is regulated in various regions of the brain after environmental insults (Xi et al., 2003). Recently, PAR1 and PAR3 were shown to mediate antiapoptotic signaling by activated protein C in neurons (Guo et al., 2004). Although these studies indicate the potential importance of low concentration of thrombin for the development of new therapeutic strategies to treat neurodegenerative disorders, the molecular mechanisms(s) underlying neuroprotection remain to be established.

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Basal release of taurine is defined as that which occurs at a specified osmolarity in the absence of thrombin.

Measurement of Phosphoinositide Turnover. To monitor phosphoinositide turnover, 1321N1 cells that had been prelabeled with $148 \text{ KBq/ml}$ $[^3H]$inositol for 96 h were incubated in hypotonic buffer A (270 mOsM) that contained 5 mM LiCl. The accumulation of radiolabeled inositol phosphates present in the trichloroacetic acid cell lysates was determined as previously described (Thompson and Fisher, 1990).

Measurement of Cytoplasmic Calcium Concentration. Cytoplasmic free calcium concentrations ($[Ca^{2+}]_i$) were determined in suspensions of 1321N1 astrocytoma cells after preloading cells with $[^48]$ KBq/ml $[^3H]$taurine were exposed to hypotonic buffer (270 mOsM), there was a time-dependent release of the radiolabeled amino acid from the cells (Fig. 1A). The initial rate of release monitored over the first 5 min was greater than that observed following prolonged incubations. Inclusion of thrombin (0.25U/ml equivalent to 1.25 nM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of response by approximately 3-fold over basal (basal release being that monitored in the absence of thrombin; Fig. 1A). If 1321N1 cells were first exposed to hypotonic buffer for 10 min and then thrombin added (i.e., after the initial rapid phase of taurine efflux), a significant stimulation of taurine release was still observed (Fig. 1B). As a result of these observations, taurine efflux was routinely monitored after a 20-min incubation in subsequent experiments. The addition of thrombin resulted in a concentration-dependent stimulation of taurine efflux with a maximum effect observed at 1.25 nM (Fig. 2). The EC$_{50}$ value for thrombin-stimulated taurine release from 1321N1 astrocytoma was 0.06 nM.

Thrombin Enhances Osmosensitive Taurine Release via the PAR-1 Receptor. To determine whether the ability of thrombin to enhance taurine release was mediated via its protease action at a receptor, two series of experiments were performed. In the first, thrombin was pretreated with 4 $\mu$M $\alpha$-Phe-Pro-Arg chloromethyl ketone (PPACK), a protease inhibitor. Although PPACK had no effect on basal taurine

**Results**

Osmosensitive Efflux of Taurine from 1321N1 Astrocytoma Cells Is Enhanced by the Addition of Thrombin. When 1321N1 astrocytoma cells that had been prelabeled with $[^3H]$taurine were exposed to hypotonic buffer (270 mOsM), there was a time-dependent release of the radiolabeled amino acid from the cells (Fig. 1A). The initial rate of release monitored over the first 5 min was greater than that observed following prolonged incubations. Inclusion of thrombin (0.25U/ml equivalent to 1.25 nM) significantly enhanced the rate of release of taurine at all time points examined and increased the magnitude of response by approximately 3-fold over basal (basal release being that monitored in the absence of thrombin; Fig. 1A). If 1321N1 cells were

Fig. 1. Kinetics of basal- and thrombin-stimulated taurine efflux from human 1321N1 astrocytoma cells. A, 1321N1 human astrocytoma cells that had been prelabeled in the presence of $[^3H]$taurine were washed twice with $2 \text{ ml}$ of isotonic buffer A before incubation in 270 mOsM buffer A in the presence or absence of 1.25 nM thrombin (added at time 0, as indicated by the arrow). Reactions were terminated at the times indicated, and taurine efflux was measured. Results are expressed as percentage of total soluble radioactivity and are the means $\pm$ S.E.M. for triplicate replicates. Data shown represent three experiments. B, cells were treated as described in A with the exception that cells were allowed to incubate for 10 min in hypotonic buffer A (270 mOsM) prior to the addition of thrombin (indicated by arrow).
release, it essentially abolished the ability of thrombin to enhance taurine release (Fig. 3A). In a second series of experiments, the ability of three synthetic PAR peptides to enhance taurine release was evaluated. Addition of 100 μM SFFLRN, a synthetic peptide specific for PAR-1 subtype, significantly increased taurine release over basal (275% of basal; Fig. 3B). This enhancement, which was also observed with 100 μM TFFLRN, an additional PAR-1-specific peptide, was similar in magnitude to that observed for thrombin. Although inclusion of 500 μM PAR-3-specific peptide (TFRGAP) also increased taurine release, the effect was not significant and was markedly less than that of the PAR-1 agonist. In contrast, the addition of the PAR-4 peptide, GYPGKF, did not significantly increase taurine release over basal. Taken collectively, these data indicate that the ability of thrombin to enhance taurine release in 1321N1 astrocytoma is mediated primarily by the PAR-1 receptor subtype.

The ability of thrombin to stimulate taurine release under hypotonic conditions was also observed for primary cultures of rat astrocytes, although the effect was less marked (148% of basal; Fig. 3C) than that observed for 1321N1 astrocytoma cells.

**Thrombin Enhances the Volume-Sensitive Efflux of Taurine from 1321N1 Astrocytoma Cells as Osmolarity Decreases.** The ability of thrombin to potentiate the release of taurine at different osmolarities was examined. Both basal- and thrombin-stimulated release of taurine was monitored under conditions of isotonicity (340 mOsM; defined by the osmolarity of the DMEM/fetal calf serum medium in which the cells are grown), mild to severe hypotonicity (320–227 mOsM), or mild hypertonicity (370 mOsM). In the series of experiments conducted, the basal release of taurine was not significantly enhanced until the osmolarity of the buffer had been reduced to 227 mOsM. In contrast, the addition of thrombin resulted in a significant increase in taurine efflux (279% of basal) even under isotonic conditions (Fig. 4). Moreover, as the osmolarity of the buffer was reduced, the ability of thrombin to enhance taurine efflux over the basal component was further increased. The maximum enhancement of taurine efflux was observed at an osmolarity of 270 mOsM (442% of basal). In contrast, when cells were exposed to mildly hypertonic buffer A (370 mOsM), the addition of thrombin did not significantly enhance taurine release. As a result of these findings, an osmolarity of 270 mOsM was chosen for all subsequent experiments.

**Taurine Efflux from 1321N1 Astrocytoma Is Mediated via a VSOAC.** To determine whether thrombin-stimulated taurine release occurred via a VSOAC, the ability of four anion channel inhibitors, all of which are putative blockers of the VSOAC channel, to inhibit the basal and thrombin-stimulated efflux of taurine was examined. All four anion channel inhibitors resulted in a significant inhibition of both basal- and thrombin-stimulated efflux of taurine was examined. All four anion channel inhibitors resulted in a significant inhibition of both basal- and thrombin-stimulated taurine release (Fig. 5). At a concentration of 100 μM, dideoxyforskolin and NPPB were more effective at inhibiting taurine release than either of the stilbene derivatives, DIDS and SITS (67–94% inhibition ver-

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**Fig. 3.** Thrombin enhances taurine efflux from 1321N1 astrocytoma through its protease activity and via a proteinase-activated receptor-1 subtype. A, cells that had been prelabeled with [3H]taurine were washed in isotonic buffer A and incubated for 20 min in 270 mOsM buffer A in the presence or absence of 1.25 nM thrombin. In some experiments, thrombin was pretreated with 4 μM PPACK, a protease inhibitor. B, cells were incubated for 20 min in the presence of thrombin or synthetic peptides specific for PAR-1, PAR-3, and PAR-4 subtypes, and efflux was monitored. Maximally effective concentrations of the synthetic peptides were used. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for four to six independent experiments. ***, different from basal control, p < 0.01 (by repeated measures ANOVA followed by Dunnett’s multiple comparisons test). C, primary cultures of rat astrocytes were prelabeled overnight with [3H]taurine, washed in isotonic buffer A, and then incubated for 20 min in 270 mOsM buffer A in the presence or absence of 1.25 nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for seven independent experiments. ***, different from basal control, p < 0.01 (by paired Student’s t test).
Thrombin receptors regulate osmolyte efflux

Taurine Release from 1321N1 Astrocytoma Cells Is Enhanced following a Rise in the Concentration of Intracellular Calcium or Activation of Protein Kinase C. Activation of thrombin receptors on 1321N1 astrocytoma cell has also been reported to elicit an increase in the activity of phospholipase C (PLC) mediated via G<sub>q</sub> with a concomitant rise in the concentration of cytoplasmic calcium ([Ca<sup>2+</sup>]<sub>i</sub>) and activation of PKC (Jones et al., 1989). In agreement with these previous observations, the addition of thrombin to 1321N1 cells (incubated in hypotonic buffer A) resulted in a small, but significant, increase in the release of inositol phosphates (146 and 174% of basal after 5 or 10 min, respectively; Fig. 6A). Thrombin addition also elicited a rise in [Ca<sup>2+</sup>]<sub>i</sub>, mediated by the addition of 1 μM ionomycin (which facilitates both the influx of extracellular Ca<sup>2+</sup> and the release of Ca<sup>2+</sup> from intracellular stores), partially mimicked the ability of thrombin to enhance taurine efflux from the astrocytoma cells (165% of basal; Fig. 7A). A similar significant increase in taurine release was observed following the addition of PMA, a PKC agonist (170% of basal; Fig. 7A), whereas its inactive analog, 4-α-phorbol 12,13-didecanoate, was without effect (data not shown). When both PMA and ionomycin were added to the hypotonically treated cells, their effect on taurine release was additive (219% of basal). However, thrombin-stimulated taurine efflux was not further enhanced by the presence of either PMA or ionomycin (Fig. 7B).

To determine the role, if any, played by Ca<sup>2+</sup> in basal- and thrombin-stimulated taurine efflux, taurine release was monitored under conditions in which extracellular and/or intracellular Ca<sup>2+</sup> had been depleted. Removal of extracellular Ca<sup>2+</sup> had little or no effect on either basal- or thrombin-stimulated taurine efflux (Fig. 8A). In contrast, chelation of intracellular Ca<sup>2+</sup> with BAPTA-AM significantly reduced the extent of the thrombin-stimulated taurine efflux (56% inhibition), whereas basal efflux was unaffected. To further examine the role of intracellular Ca<sup>2+</sup> in osmolyte release, the cells were preincubated with 5 μM thapsigargin (in the ab-
sence of extracellular Ca\(^{2+}\) to discharge the intracellular Ca\(^{2+}\) pools. Under these conditions, the ability of thrombin to stimulate taurine efflux was reduced by 52% (Fig. 8B).

To test the involvement of PKC in thrombin-stimulated taurine efflux, cells were preincubated with either 1 \(\mu\)M BIM or 10 \(\mu\)M chelerythrine. Although BIM slightly decreased the basal taurine efflux, both BIM and chelerythrine significantly attenuated thrombin-stimulated taurine release (30% inhibition; Fig. 9A). Down-regulation of PKC following overnight incubation of the cells with 100 nM PMA also resulted in reduction in an inhibition of thrombin-stimulated taurine efflux (54 ± 6% versus control cells, \(n = 3\)). The combination of inhibition of PKC with 10 \(\mu\)M chelerythrine, along with depletion of extracellular Ca\(^{2+}\) with 5 \(\mu\)M thapsigargin, resulted in an 87% inhibition of thrombin-stimulated taurine release (Fig. 9B).

**Discussion**

Subnanomolar concentrations of thrombin have been demonstrated to protect both neurons and astrocytes against metabolic insults such as hypoglycemia, ischemia, or oxidative stress (Vaughan et al., 1995; Striggow et al., 2000). In the present study, we demonstrate that similarly low concentrations of thrombin also markedly increase the release of taurine, an organic osmolyte, from human 1321N1 astrocy-
The role of extracellular and intracellular calcium in thrombin-stimulated taurine efflux. A, cells that had been prelabeled with [3H]taurine were washed in isotonic buffer A and then incubated for 20 min in hypotonic buffer A (270 mOsM) in the absence (−ext Ca, Ca\(^{2+}\) was omitted from buffer and 100 μM EGTA added) or presence of extracellular Ca\(^{2+}\) and with the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. In some experiments, cells were pretreated for 15 min in isotonic buffer A in the presence of 50 μM EGTA added prior to the measurement of efflux (in the presence of Ca\(^{2+}\)). The concentrations of thrombin required to enhance taurine release (EC\(_{50}\) = 60 pM) are similar to those previously demonstrated to provide neuroprotection (Fig. 2). Based upon the ability of the receptor-specific peptides SFLRN and TFLRN to fully mimic the ability of thrombin to enhance taurine release (and the relative ineffectiveness of PAR-3- and PAR-4-specific peptides), it seems that the PAR-1 receptor subtype is primarily responsible for thrombin-stimulated osmolyte release (Fig. 3B). Although an astrocytoma cell line was primarily used for the present study, we observed that the addition of thrombin to primary cultures of rat brain astrocytes incubated under hypoosmotic conditions also resulted in a stimulation of taurine release above basal (Fig. 3C). Although the ability of thrombin to stimulate osmolyte release has previously been reported for myoblasts (Manolopoulos et al., 1997), to the best of our knowledge, the present results are the first to indicate a similar role for thrombin in neural tissues and to identify the receptor subtype involved. Thus, one of the in vivo functions of thrombin in the brain, whether synthesized within the CNS or resulting from cerebral hemorrhage or a compromised blood-brain barrier, may be that of osmoregulation.

The pharmacological profile of inhibition of both basal- and thrombin-stimulated taurine efflux from 1321N1 astrocytoma cells by anion channel inhibitors is consistent with the involvement of a VSOAC in osmolyte release. Thus, taurine efflux was significantly inhibited by DIDS, SITS, NPPB, and DDF, all of which are purported to be inhibitors of volume-sensitive anion channels (Nilius et al., 1997). However, as previously observed for the volume-dependent efflux of taurine from SH-SY5Y neuroblastoma cells (Heacock et al., 2004), DDF and NPPB are markedly more potent inhibitors of osmolyte release from the astrocytoma cells than either of the two stilbene derivatives, DIDS and SITS.

One notable feature of thrombin-stimulated taurine efflux is that the protease is able to substantially enhance osmolyte release (279% of basal) even under isotonic conditions (340 mOsM; Fig. 4). This result suggests that even though the VSOAC primarily responds to a reduction of osmolarity, the channel is partially open under conditions of isotonicity but not under hypertonic conditions (370 mOsM). The ability of receptor activation to enhance osmolyte release under isotonic conditions has been observed previously. For example, ATP-induced D-aspartate release from astrocytes and muscarinic cholinergic receptor (mAChR)-mediated taurine efflux from neuroblastoma can be observed under isotonic conditions (Mongin and Kimelberg, 2002; Heacock et al., 2004). In contrast, the mAChR-stimulated release of myo-inositol, another quantitatively major organic osmolyte, was not observed under isotonic conditions (Loveday et al., 2003). In this context, it should be noted that the ability of organic osmolytes to permeate VSOAC is dependent upon their molecular dimensions. Since the minimum diameter of the pore channel of the VSOAC is dependent upon their molecular dimensions, the ability of DAsp to enhance taurine release (EC\(_{50}\) = 60 pM) is similar to those previously demonstrated to provide neuroprotection (Fig. 2). Based upon the ability of the receptor-specific peptides SFLRN and TFLRN to fully mimic the ability of thrombin to enhance taurine release (and the relative ineffectiveness of PAR-3- and PAR-4-specific peptides), it seems that the PAR-1 receptor subtype is primarily responsible for thrombin-stimulated osmolyte release (Fig. 3B). Although an astrocytoma cell line was primarily used for the present study, we observed that the addition of thrombin to primary cultures of rat brain astrocytes incubated under hypoosmotic conditions also resulted in a stimulation of taurine release above basal (Fig. 3C). Although the ability of thrombin to stimulate osmolyte release has previously been reported for myoblasts (Manolopoulos et al., 1997), to the best of our knowledge, the present results are the first to indicate a similar role for thrombin in neural tissues and to identify the receptor subtype involved. Thus, one of the in vivo functions of thrombin in the brain, whether synthesized within the CNS or resulting from cerebral hemorrhage or a compromised blood-brain barrier, may be that of osmoregulation.

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followed by Dunnett's multiple comparisons test). B, cells were first preincubated for 15 min in the absence (control) or presence of 10 μM chelerythrine (chel) in isotonic buffer A. The medium was then aspirated and replaced with 270 mOsm buffer A, either containing Ca2+ (control) or with Ca2+ omitted and 50 μM EGTA, 5 μM thapsigargin, and 10 μM chelerythrine added. Cells were preincubated for 5 min prior to the addition of thrombin (final concentration 1.25 nM) or buffer A, prepared in the same media. After an additional 10 min, reactions were terminated, and taurine efflux was measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for six independent experiments. * different from control basal release, p < 0.01; #, different from thrombin-stimulated efflux under control conditions (repeated measures ANOVA followed by Dunnett's multiple comparisons test).

Fig. 9. Inhibition of thrombin-stimulated taurine efflux by PKC inhibitors in the presence or absence of Ca2+. A, cells were pretreated with 10 μM chelerythrine or 1 μM BIM in isotonic buffer A for 15 min before incubation of cells in hypotonic buffer A (270 mOsm) in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 20 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 three independent experiments, each performed in triplicate. * different from basal control, p < 0.05; #, different from thrombin-stimulated efflux under control conditions, p < 0.01 (by repeated measures ANOVA followed by Dunnett's multiple comparisons test). B, cells were first preincubated for 15 min in the absence (control) or presence of 10 μM chelerythrine (chel) in isotonic buffer A. The medium was then aspirated and replaced with 270 mOsm buffer A, either containing Ca2+ (control) or with Ca2+ omitted and 50 μM EGTA, 5 μM thapsigargin, and 10 μM chelerythrine added. Cells were preincubated for 5 min prior to the addition of thrombin (final concentration 1.25 nM) or buffer A, prepared in the same media. After an additional 10 min, reactions were terminated, and taurine efflux was measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for six independent experiments. * different from control basal release, p < 0.01; #, different from thrombin-stimulated efflux under control conditions (repeated measures ANOVA followed by Dunnett's multiple comparisons test).

released less readily. Although activation of PAR-1 receptors on astrocytoma cells can enhance taurine release under isotonic conditions, its effects become even more pronounced when the osmolarity is reduced by 5 to 20%, conditions under which the basal release of taurine is only minimally increased (Fig. 4). These results are consistent with the concept that PAR-1 activation facilitates the ability of the cells to release osmolytes (and, by inference, to regulate their volume) under conditions of very limited reductions in osmolarity, i.e., those that might be expected to pertain to physiological or pathological conditions in vivo.

Although PAR-1 receptors are pleiotropic and can couple to multiple GTP-binding proteins, thereby activating a diverse array of signaling pathways (Coughlin, 2000; Trejo, 2003), our data suggest that Ca2+ and PKC play the major roles in facilitation of taurine release. The evidence for this is as follows. First, the ability of thrombin to enhance taurine release could be mimicked, in part at least, by the addition of the Ca2+ ionophore, ionomycin. Second, the mobilization of an intracellular pool of Ca2+ seems to be required since either chelation of intracellular Ca2+ with BAPTA or discharge of the pool with thapsigargin resulted in a 52 to 56% inhibition of thrombin-stimulated taurine efflux. In contrast, removal of extracellular Ca2+ had no effect on the magnitude of efflux. The reliance of thrombin-stimulated taurine release on intracellular (rather than extracellular) stores of Ca2+ contrasts with the Ca2+ signals generated in fura-2 loaded cells upon thrombin addition, which depend on both sources of Ca2+ (Fig. 6B). This result suggests that thrombin-stimulated taurine release may require a rise in [Ca2+]i, and the magnitude of osmolyte release is not directly proportional to that of the Ca2+ signal. Moreover, it seems that a significant fraction of thrombin-stimulated osmolyte release can still occur in the absence of Ca2+ (see Fig. 8B). Although thrombin addition results in an activation of PLC (Fig. 6A) and a rise in [Ca2+], in these cells, an obligatory link between this pathway and osmolyte release is yet to be established because of the absence of a specific inhibitor of PLC (see Loveday et al., 2003).

A role for PKC is indicated from the ability of PMA, when added alone, to stimulate taurine efflux and from the observation that either BIM or chelerythrine, two inhibitors of PKC, can inhibit thrombin-stimulated taurine release. Further indication of the involvement of PKC was obtained from experiments in which down-regulation of the enzyme, following an overnight incubation of the cells with PMA, resulted in an attenuation of thrombin-stimulated taurine efflux. Under conditions in which both intracellular Ca2+ is depleted and PKC inhibited, thrombin's ability to stimulate osmolyte release was inhibited by 87%. In contrast to the Ca2+ and PKC dependence of thrombin-stimulated taurine efflux, the basal release of taurine is not dependent on either parameter. This suggests that the ability of thrombin to activate VSOAC involves signaling pathways that are distinct from those elicited by hypotonicity alone. A requirement for Ca2+ and PKC in receptor-regulated osmolyte release is emerging as a general characteristic (Loveday et al., 2003). However, the source of Ca2+ may differ depending upon the receptor and/or cell type (Loveday et al., 2003). Both PKC and Ca2+ calmodulin-dependent protein kinase II are potential downstream targets for Ca2+ activation. However, the inability of KN-93 to inhibit thrombin-stimulated taurine efflux suggests that Ca2+ calmod-
ulin-dependent protein kinase II is not involved in VSOAC regulation.

Although the general characteristics of swelling-induced osmolyte release from neural cells have been extensively examined, only recently has evidence emerged that certain pharmacologically distinct receptors such as the P2Y purinergic, M3-mACHr and now PAR-1, are able to positively regulate osmolyte efflux. There are two major implications of these findings. The first is that the ability of receptors to stimulate osmolyte release suggests that the process in vivo whereby hypoosmotically stressed cells restore their volume may be more dynamic than previously considered since these cells are also likely to be continuously subjected to neurohumoral regulation. The second is that the ability of these receptors to facilitate osmolyte release under isotonic conditions via VSOAC raises the possibility that receptor-mediated release of another quantitatively major osmolyte in the brain, namely glutamate, which also functions as a neurotransmitter, may constitute a means for intercellular signaling between glia and neurons. Although this possibility has previously been raised for ATP modulation of glutamate release from astrocytes under isotonic conditions (Jeremic et al., 2001), the present data raise the possibility that additional receptors may also share this property. In addition, it should be noted that taurine itself has agonist properties at both GABA_A and glycine receptors (Hussy et al., 1997; Hilgier et al., 2005).

In summary, the present data emphasize the importance of receptor regulation of osmolyte release through a VSOAC. Control of neural cell swelling is of particular importance to the CNS due to the spatial restrictions of the skull. In this case, thrombin via its PAR-1 receptor plays a role in protection of neural cells from osmotic insults and regulates volume via a mechanism dependent upon intracellular Ca^{2+} and PKC. This role for thrombin in osmoregulation within the brain adds to the growing list of functions attributed to this protease in CNS physiology and pathology.

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References


Jones LG, McDonough PM, and Brown HJ (1989) Thrombin and trypsin act at the same site to stimulate phosphoinositide hydrolysis and calcium mobilization. Mol Pharmacol 36:142–149.


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