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SUBNANOMOLAR CONCENTRATIONS OF THROMBIN ENHANCE THE VOLUME-SENSITIVE EFFLUX OF TAURINE FROM HUMAN 1321N1 ASTROCYTOMA CELLS

Tooba A. Cheema, Caroline E. Ward and Stephen K. Fisher

Molecular and Behavioral Neuroscience Institute (TAC,CEW,SKF) and Department of Pharmacology (TAC,SKF), University of Michigan, Ann Arbor, Michigan.

Running Title: Thrombin receptors regulate osmolyte efflux

Corresponding author: Dr. Stephen K. Fisher, University of Michigan, Molecular and Behavioral Neuroscience Institute laboratories at MSRB II, 1150 West Medical Center Drive, C560, MSRB II, Ann Arbor, MI 48109-0669, e-mail: skfisher@umich.edu. Telephone: (734)-763-4376; Fax: (734)-936-2690

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Non-standard abbreviations: Ca^{2+}_i, cytoplasmic calcium; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester; DDF, 1,9-dideoxyforskolin; DIDS, 4,4'diisothiocyanatostilbene-2,2'-disulfonic acid; DMEM, Dulbecco's modified Eagle's medium; HEPES, N-[2 hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; PAR, protease-activated receptor; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate, SITS: 4-acetamido-4'-isothiocyanatostilbene-2, 2'-disulfonic acid; VSOAC, volume-sensitive organic osmolyte anion channel; RVD, regulatory volume decrease; PPACK, D-Phe-Pro-Arg Chloromethyl Ketone; mAChR, muscarinic cholinergic receptor; Ca^{2+} CaMKII, calcium calmodulin-dependent protein kinase II; PD 98059, 2'-amino-3'-methoxyflavone; KN-93,{2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4- chlorocinnamyl)-N-(methylbenzylamine)}.

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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 hypoosmotic stress. Incubation of human 1321N1 astrocytoma cells with hypoosmolar buffers (320-227 mOsM) resulted in a time-dependent release of taurine. Inclusion of thrombin (EC_{50}=60 pM), resulted in a marked increase in taurine efflux which, 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 Ca^{2+} 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 chelerythrine, resulted in a 30-50% inhibition of thrombin-stimulated taurine efflux. Under conditions in which intracellular Ca^{2+} 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 Ca^{2+} and PKC activity.

Introduction

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; Ossovaskaya and Bunnett, 2004; Suo et al., 2004). Thrombin may also be produced in the brain following cerebral hemorrhage or disruption of the blood brain barrier. It mediates its cellular functions through a family of receptors known as Proteinase Activated Receptors (PARs). PARs belong to the superfamily of G-protein coupled receptors (GPCRs) which operate via G_q, G_i or $G_{12/13}$ families of G-proteins and currently four members of the group (PAR1, PAR2, PAR3 and PAR4) have been identified. PARs have a unique activation mechanism. They are activated by an irreversible proteolytic cleavage of the receptor's extracellular N-terminus, thus unmasking a new N-terminus that functions as a "tethered peptide ligand," which then binds intra-molecularly to the receptors and initiates intracellular signal transduction (Macfarlane at al., 2001; Hollenberg and Compton, 2002; Trejo, 2003; Wang and Reiser, 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; Striggow 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 anti-apoptotic 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 mechanism(s) underlying neuroprotection remain to be established.

A neuroprotective mechanism utilized 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 followed by a recovery process of regulatory volume decrease (RVD), during which intracellular osmolytes (K^+, Cl^- and organic osmolytes) are extruded and cell volume normalized following the exit of intracellular water (McManus et al.,1995). Of the organic osmolytes utilized by cells, taurine is ideally suited because of its abundance, water solubility and metabolic inertness (Lambert, 2004). Swelling of neural cells due to fluctuations in osmolarity is very common in elderly, infants and during pregnancy. It can be derived from excessive water intake, such as occurs in athletes and psychotic polydipsia, or alternatively in conditions such as glucocorticoid deficiency, hypothyroidism, use of thiazide diuretics, and renal or hepatic failure (Kimelberg, 2000; Pasantes-Morales et al., 2000, 2002).

The principal cell type involved in volume regulation within the central nervous system is the astrocyte, since these cells comprise up to 90% of cell number within the brain. The importance of taurine release in RVD is indicated from studies in which taurine-deficient astrocytes were shown to exhibit a less efficient volume recovery in comparison to control cells (Moran et al., 1994). The extrusion of taurine occurs predominantly via a channel known as Volume Sensitive Organic Osmolyte Anion Channel (VSOAC). VSOAC is a chloride channel, impermeable to cations (for reviews, see Nilius et al.,1997; Lang et al.,1998; Nilius and Droogmans, 2003). Taurine efflux, as well as RVD, can be blocked by classical non-selective Cl^- channel inhibitors, such as 1,9-dideoxyforskolin (DDF), 5-nitro-2-(3-phenypropylamine)benzoic acid (NPPB), 4,4' diisothiocyano-2-2' disulfonic stilbene (DIDS) and 4-acetamido-4 isothiocyano-2-2' disulfonic stilbene (SITS).

In the present study, we demonstrate that subnanomolar concentrations of thrombin, operating primarily via a PAR-1 receptor, facilitate the volume-sensitive efflux of taurine from human astrocytoma cells. Thrombin-stimulated taurine efflux is mediated via a VSOAC channel and intracellular Ca^{2+} and protein kinase C (PKC) are implicated in the mechanism of osmolyte release. A preliminary account of part of this study has previously been reported (Cheema et al., 2005).

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Methods

Materials. [1,2-^{3}H]Taurine (1.15 TBq/ml) was obtained from Amersham Biosciences (Piscataway, NJ). Thrombin, DIDS, NPPB, SITS and 4α-phorbol 12,13-didecanoate were purchased from Sigma-Aldrich (St. Louis, MO). 1,9-Dideoxyforskolin, PMA, ionomycin, KN-93, PD 98059 and wortmannin were obtained from Calbiochem (San Diego, CA). Pertussis toxin was obtained from List Biological Laboratories (Campbell, CA). Thrombin receptor activating peptides, SFFLRN, TFLLRN, TFRGAP, GYPGKF were obtained from BaChem (Torrance, CA). Guanidinethyl sulfonate was obtained from Toronto Chemicals (Toronto, ON). Dulbecco's modified Eagle medium (DMEM) and 50x penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA). Fetal calf serum was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Tissue culture supplies were obtained from Corning Glassworks (Corning, NY), Starstedt (Newton, NC) and BD BioSciences (Franklin Lakes, NJ). Universol was obtained from ICN biomedical (Urora, OH). Dowex-1 resin 100-200 mesh; x 8 formate) was obtained from Bio-Rad (Hercules, CA).

Cell culture conditions.

1321N1 astrocytoma cells (passages 5-21) were grown in tissue culture flasks (75 cm^2/250 ml) in 20 ml of DMEM supplemented with 10% (v/v) of fetal calf serum with 1% penicillin/streptomycin. The osmolarity of the medium was 330 - 340mOsM. Cells were grown at 37°C in a humidified atmosphere containing 10% CO_2. The medium was aspirated and the cells detached from the flask with a tryspin-versene mixture

(Biowhittaker, MD). Cells were then resuspended in DMEM/10% fetal calf serum with penicillin/streptomycin and subcultured into 35 mm, six-well culture plates for 3-5 days. Experiments were routinely conducted on cells that had reached 50–90% confluency.

Preparation of primary astrocyte cultures

Neonatal cultures of rat astrocytes were prepared from 2-day-old rats (Sprague Dawley) essentially according to the method previously described (Jiang et al., 2002).

Measurement of taurine efflux.

Osmolyte efflux from 1321N1 astrocytoma cells was monitored essentially as previously described (Loveday et al. 2003, Heacock et al., 2004). In brief, cells were prelabeled overnight with 18.5 KBq/ml of [^{3}H]taurine at 37^{o}C. Under these conditions, approximately 10-20% of the added radiolabel was taken up into the cells. Uptake of radiolabel into 1321N1 cells was time-dependent ($t_{1/2}$ ~7h), temperature sensitive (inhibited >98% by lowering the temperature to 4^{o}C) and was inhibited by 75-80% by inclusion of a 500 µM guanidinethyl sulfonate, an inhibitor of the taurine uptake transporter (Lambert, 2004). After prelabeling, the cells were washed with 2 x 2 ml of isotonic Buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1mM $MgCl_2$ and 30 mM HEPES, pH 7.4, 1 mg/ml D-glucose; approx. 340 mOsM). Cells were then allowed to incubate in 2ml of hypotonic buffer A (320-200 mOsM; rendered hypotonic by a reduction in NaCl concentration) in the absence or presence of thrombin. In some experiments, buffer A was made hypertonic (370 mOsM) by the addition of NaCl. Osmolarities of buffer A were monitored by means of an Osmette precision

osmometer (PS Precision Systems, Sudbury, MA). At times indicated, aliquots (200 µl) of the extracellular medium were removed and radioactivity determined after the addition of 5ml Universol scintillation fluid. The reactions were terminated by rapid aspiration of the buffer and cells lysed by the addition of 2 × 1 ml of ice-cold 6% (wt/vol) trichloroacetic acid. Taurine efflux was calculated as a fractional release, i.e., the radioactivity released in the extracellular media as a percentage of the total radioactivity present initially in the cells. The latter was calculated as the sum of radioactivity recovered in the extracellular medium and that remaining in the lysate at the end of the assay (Novak et al., 1999). “Basal” release of taurine is defined as that which occurs at a specified osmolarity in the absence of thrombin.

Measurement of Phosphoinositide Turnover.

To monitor phosphoinositide turnover, 1321N1 cells that had been prelabeled with 148 KBq/ml of [^{3}H]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 the Ca^{2+} indicator, fura-2 AM (Molecular Probes, OR), as previously described (Fisher et al., 1989). The fluorometer

used was a Shimadzu RF-5301PC spectrofluorometer (Shimadzu Scientific Instruments, Columbia, MD).

Data analysis.

Experiments were performed in triplicate and repeated at least three times. Values quoted are given as means ± SEM for the number (n) of independent experiments indicated. A two-tailed Student's t-test (paired) was used to evaluate differences between two experimental groups (level of significance, $p<0.05$). One-way or repeated measures Analysis of Variance (ANOVA) followed by Dunnett's multiple comparisons test was used for statistical significance of differences between multiple groups. EC_{50} values were obtained using Prism 3.03 (GraphPad Software, Inc. San Diego, CA).

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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 [^{3}H]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 μM D-Phe-Pro-Arg Chloromethyl Ketone (PPACK), a protease inhibitor. Although PPACK had no effect on basal taurine 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 Proteinase-Activated Receptor (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- 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 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 vs. 40-64% inhibition) at concentrations of 100 µM and 500 µM, respectively. Higher concentrations of the latter agents could not be tested as they resulted in detachment of cells from the dishes.

Thrombin-stimulated taurine release is unaffected by pertussis toxin or inclusion of inhibitors of phophatidylinositol 3-kinase, mitogen-activated protein kinase or

Ca^{2+} calmodulin dependent protein kinase II signaling pathways. Since the PAR-1 subtype is known to couple to the pertussis toxin-sensitive G_i subfamily of hetrotrimeric G-proteins (Coughlin, 2000), we tested the ability of pertussis toxin to inhibit thrombin-stimulated taurine release. Overnight pretreatment of the cells with pertussis toxin (60 ng/ml) resulted in a small reduction in basal release of taurine (68 ± 9% of control, n=6), whereas thrombin-stimulated taurine efflux was unaffected (387 ± 54% vs. 509 ± 29% of basal for untreated- and pertussis-toxin-treated cells, respectively, n=6).

To examine the possibility that other known mediators of PAR-1 activation were involved in the thrombin-induced taurine release, cells were incubated with wortmannin (100 nM), a phosphatidylinositol 3-kinase inhibitor or PD 98059 (50 μM), mitogen activated protein (MAP) kinase inhibitor (Wang et al., 2002), and KN-93 (10 μM), a Ca^{2+} CaMK II inhibitor. No significant effect on either basal or thrombin-stimulated taurine release was observed for any of these inhibitors (data not shown).

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_q, with a concomitant rise in the concentration of cytoplasmic calcium, $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ (592 ± 78 nM vs 196 ± 23 nM for basal, n=13), which was markedly attenuated when extracellular Ca^{2+} was omitted (69 ± 4% inhibition, n=14; Fig. 6B). In the absence of extracellular Ca^{2+}, the depletion of intracellular Ca^{2+} stores with 5 μM thapsigargin further reduced the ability of thrombin to increase $[Ca^{2+}]_i$ (89 ± 2% inhibition, n = 8).

A rise in $[Ca^{2+}]_i$, mediated by the addition of 1 μM ionomycin (which facilitates both the influx of extracellular Ca^{2+} and the release of Ca^{2+} 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^{2+} in basal- and thrombin-stimulated taurine efflux, taurine release was monitored under conditions in which extracellular- and/or intracellular Ca^{2+} had been depleted. Removal of extracellular Ca^{2+} had little or no effect on either basal- or thrombin-stimulated taurine efflux (Fig. 8A). In contrast, chelation of intracellular Ca^{2+} 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^{2+} in osmolyte release, the cells were preincubated with 5 μM thapsigargin (in the absence 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 µM BIM or 10 µM chelerythrine. Although BIM slightly decreased the basal taurine efflux, both BIM and chelerytherine 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% vs control cells, n=3). The combination of inhibition of PKC with 10 µM chelerythrine, along with depletion of intracellular Ca^{2+} with 5 µM 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 astrocytoma cells following a mild hypoosmotic insult. The ability of thrombin to enhance taurine release, which is dependent on its protease activity, occurs rapidly (within 1-2 min of addition) and can be observed regardless of whether thrombin is added prior to or after the initiation of taurine release (Figs. 1A and B). The concentrations of thrombin required 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 SFLLRN and TFLLRN to fully mimic the ability of thrombin to enhance taurine release (and the relative ineffectiveness of PAR-3- and PAR-4-specific peptides), it appears that the PAR-1 receptor subtype is primarily responsible for thrombin-stimulated osmolyte release (Fig. 3B). Although an astrocytoma cell line was primarily utilized 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 cells 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 reported to be between 5.4 and 8.0 Å (McManus et al., 1995; Nilius et al., 1997), osmolytes such as glutamate, D-aspartate or taurine will readily exit the cells, whereas *myo*-inositol, whose molecular dimensions are close to the pore diameter, is 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 5-20% – 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 Ca^{2+} 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 Ca^{2+} ionophore, ionomycin. Second, the mobilization of an intracellular pool of Ca^{2+} appears to be required since either chelation of intracellular Ca^{2+} with BAPTA, or discharge of the pool with thapsigargin, resulted in a 52-56% inhibition of thrombin-stimulated taurine efflux. In contrast, removal of extracellular Ca^{2+} had no effect on the magnitude of efflux. The reliance of thrombin-stimulated taurine release on intracellular (rather than

extracellular) stores of Ca^{2+} contrasts with the Ca^{2+} signals generated in fura-2 loaded cells upon thrombin addition, which depend on both sources of Ca^{2+} (Fig. 6B). This result suggests that (1) thrombin-stimulated taurine release may require a rise in $[Ca^{2+}]_i$, (2) the magnitude of osmolyte release is not directly proportional to that of the Ca^{2+} signal. Moreover, it appears that a significant fraction of thrombin-stimulated osmolyte release can still occur in the absence of Ca^{2+} (see Fig. 8B). Although thrombin addition results in an activation of PLC (Fig. 6A) and a rise in $[Ca^{2+}]_i$ 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 Ca^{2+} is depleted and PKC inhibited, thrombin's ability to stimulate osmolyte release was inhibited by 87%. In contrast to the Ca^{2+}- 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 Ca^{2+} and PKC in receptor-regulated osmolyte release is emerging as a general characteristic (Loveday et al., 2003; Mongin and Kimelberg, 2005). However, the source of Ca^{2+} may differ depending upon the receptor and/or cell type (Loveday et

al., 2003). Both PKC and Ca^{2+} CaMKII are potential downstream targets for Ca^{2+} activation. However, the inability of KN-93 to inhibit thrombin-stimulated taurine efflux suggests that Ca^{2+} CaMKII 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, M_3-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. While this possibility has previously been raised for ATP modulation of glutamate release from astrocytes under isotonic conditions (Jeremic et al., 2001), the present data raises 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 emphasizes 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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Footnotes

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b) Address correspondence to: Dr. Stephen K. Fisher, Molecular and Behavioral Neuroscience Institute Laboratories at MSRB ll, University of Michigan, 1150 West Medical Center Drive, C560 MSRBll, Ann Arbor, MI 48109-0669. E-mail: skfisher@umich.edu

Legends for Figures

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 [^{3}H]taurine were washed twice with 2 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 zero, as indicated by the arrow). Reactions were terminated at the times indicated and taurine efflux measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for triplicate replicates. Data shown are representative of 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).

Fig. 2. Concentration-response relationship for thrombin-stimulated taurine efflux. Cells that had been prelabeled with [^{3}H]taurine were washed with isotonic buffer A and then incubated in 270 mOsM buffer in the presence of thrombin at the concentrations indicated. Reactions were terminated after 20 min and taurine efflux was monitored. Results are expressed as percentage of maximum agonist response (obtained at 5 nM thrombin) and are the means ± S.E.M. for three independent experiments. The calculated EC_{50} value for stimulated taurine efflux was 0.06 nM.

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 [^{3}H]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. In (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 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 4 to 6 independent experiments. **, different from basal control, $p<0.01$ (by repeated measures ANOVA followed by Dunnett's multiple comparisons test). In (C) primary cultures of rat astrocytes were prelabeled overnight with [^{3}H]taurine, washed in isotonic buffer A and then incubated for 20 min in 270 mOsM buffer A in the presence or absence of 1.25nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M for 7 independent experiments. **, different from basal control, $p < 0.01$ (by paired student's t-test).

Fig. 4. Basal- and thrombin-stimulated release of taurine as a function of osmolarity. Cells prelabeled with [^{3}H]taurine were first washed in isotonic buffer A and then incubated for 20 min in buffer A at the osmolarities indicated in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 4 to 8 independent experiments, each performed in triplicate. #, different from taurine release observed in cells incubated in isotonic buffer A (340 mOsM), $p<0.01$ (by one-way ANOVA followed by Dunnett's multiple comparison test). **, different from basal release

(in the absence of thrombin-open bars), $p<0.01$ (by paired student's t-test). *Inset*- thrombin-stimulated efflux calculated as a percentage of basal, at each osmolarity.

Fig. 5. Inhibition of basal- and thrombin-stimulated taurine release by anion channel blockers. Cells that had been prelabeled with [^{3}H]taurine were washed in isotonic buffer A and then incubated in hypotonic buffer A (270 mOsM) with 100 µM dideoxyforskolin (DDF), 100 µM DIDS, 500 µM SITS or 100 µM NPPB in the absence (open bars) or presence (filled bars) of 1.25 nM thrombin. Reactions were terminated after 20 min and efflux of taurine monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. of 6-8 independent experiments, each performed in triplicate. * and **, different from control basal, $p<0.05$ and $p<0.01$ respectively. #, different from thrombin-stimulated efflux under control conditions, $p<0.01$ (by one-way ANOVA followed by Dunnett's multiple comparisons test).

Fig. 6. Thrombin elicits an increase in phosphoinositide turnover and in cytoplasmic free calcium. (A) Cells that had been prelabeled for 96 h with [^{3}H]inositol were washed in isotonic Buffer A and then incubated for either 5 or 10 min in hypotonic buffer A (270 mOsM) in the presence or absence of thrombin (1.25 nM). Reactions were terminated by the addition of trichloroacetic acid and the accumulation of radiolabeled inositol phosphates was monitored as an index of stimulated phosphoinositide turnover. Results are expressed as inositol phosphate release/total soluble radioactivity and are the means ± S.E.M. for five (5 min incubation) or four (10

minute incubation) independent experiments. **, different from basal release, $p< 0.001$ (by paired Student's *t* test). (B) Fura-2 loaded cells were resuspended in 270 mOsM buffer A with (●) or without (○) extracellular Ca^{2+} or (△) pretreated 5 min with 5 μM thapsigargin in the absence of extracellular Ca^{2+}. Ca^{2+} signals were monitored after the addition of thrombin (1.25 nM) at 120 sec (indicated by the arrow). Traces shown are representative of n= 8-14 experiments obtained with 4-7 separate cell preparations.

Fig. 7. Activation of either PKC, or a rise in intracellular Ca^{2+}, facilitates taurine efflux. (A) Cells that had been prelabeled with [^{3}H]taurine were washed with isotonic buffer A and then incubated in hypotonic buffer A (270 mOsM) with or without PMA (100 nM), ionomycin (1 μM) or both. Reactions were terminated after 20 min and taurine efflux monitored. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 6 independent experiments, each performed in triplicate. **, different from control, $p<0.01$ (by repeated measures ANOVA followed by Dunnett's multiple comparisons test). (B) Cells were treated as described in (A) but incubated with thrombin (1.25 nM), in the absence or presence of PMA or ionomycin. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for four independent experiments. Thrombin-stimulated taurine efflux was not increased by the presence of either PMA or ionomycin.

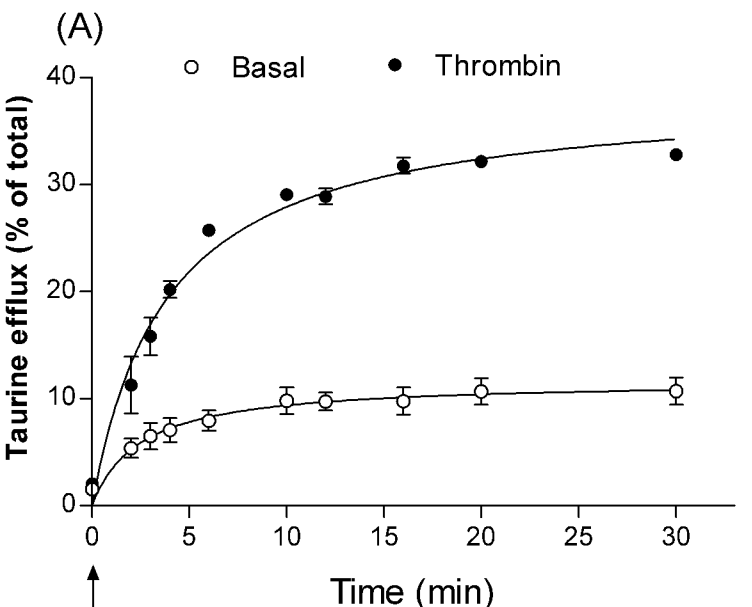
Fig. 8. The role of extracellular- and intracellular calcium in thrombin-stimulated taurine efflux. (A) Cells that had been prelabeled with [^{3}H]taurine were washed in isotonic buffer A and then incubated for 20 min in hypotonic buffer A (270 mOsM) in the

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 BAPTA-AM prior to the measurement of efflux (in the presence of Ca^{2+}). Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 3 to 6 independent experiments. Basal- (open bars) and thrombin-stimulated taurine release (filled bars) was monitored under all three conditions. ***, different from control basal, $p<0.001$, #, different from thrombin-stimulated efflux under control conditions, $p<0.05$ (by one-way ANOVA followed by Dunnett's multiple comparisons test). (B) Cells were preincubated for 5 min in 270 mOsM buffer A (Ca^{2+} omitted and 50 µM EGTA added) in either the absence (control) or presence of 5 µM thapsigargin. Thrombin (final concentration 1.25 nM) or buffer A was then added and incubations allowed to proceed for an additional 10 min. Results are expressed as taurine efflux (percent of total soluble radioactivity) and are the means ± S.E.M. for 5 independent experiments. **, different from control basal, $p<0.01$; #, different from thrombin-stimulated efflux under control conditions, $p<0.01$ (by 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 Ca^{2+}. (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 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, $p<0.05$ and #, 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 Ca^{2+} containing (control) or with Ca^{2+} 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 measured. Results are expressed as taurine efflux (percentage of total soluble radioactivity) and are the means ± S.E.M. for 6 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).

Figure 1



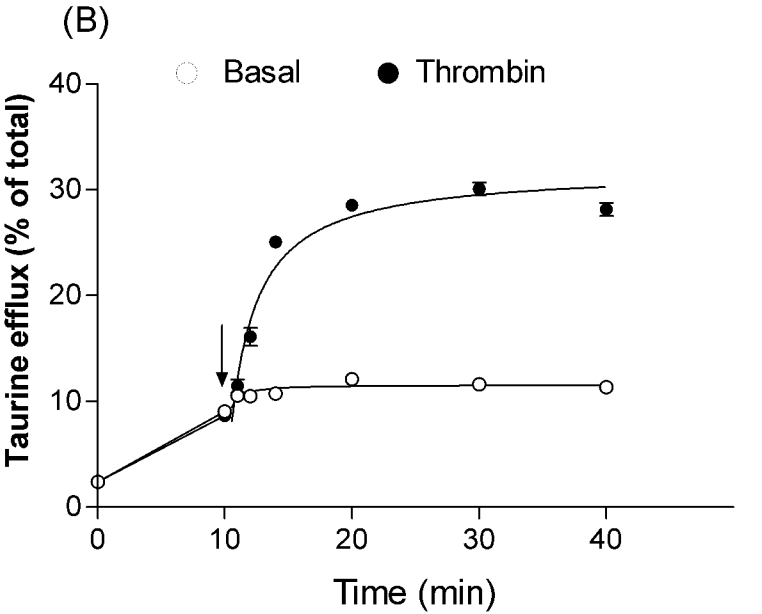


Figure 2

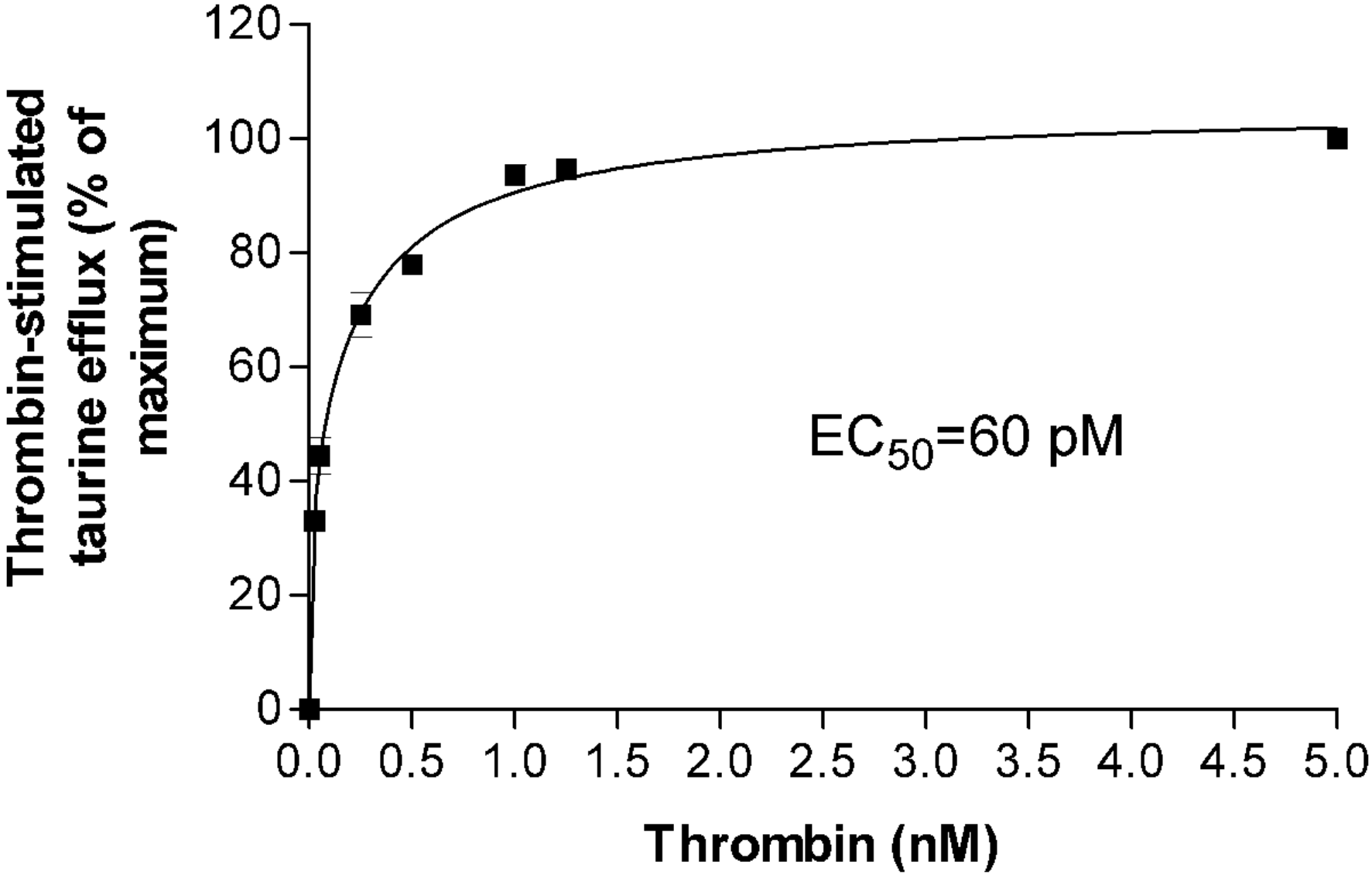

Thrombin-stimulated taurine efflux (% of maximum)
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
Thrombin (nM)
EC50=60 pM

Figure 3

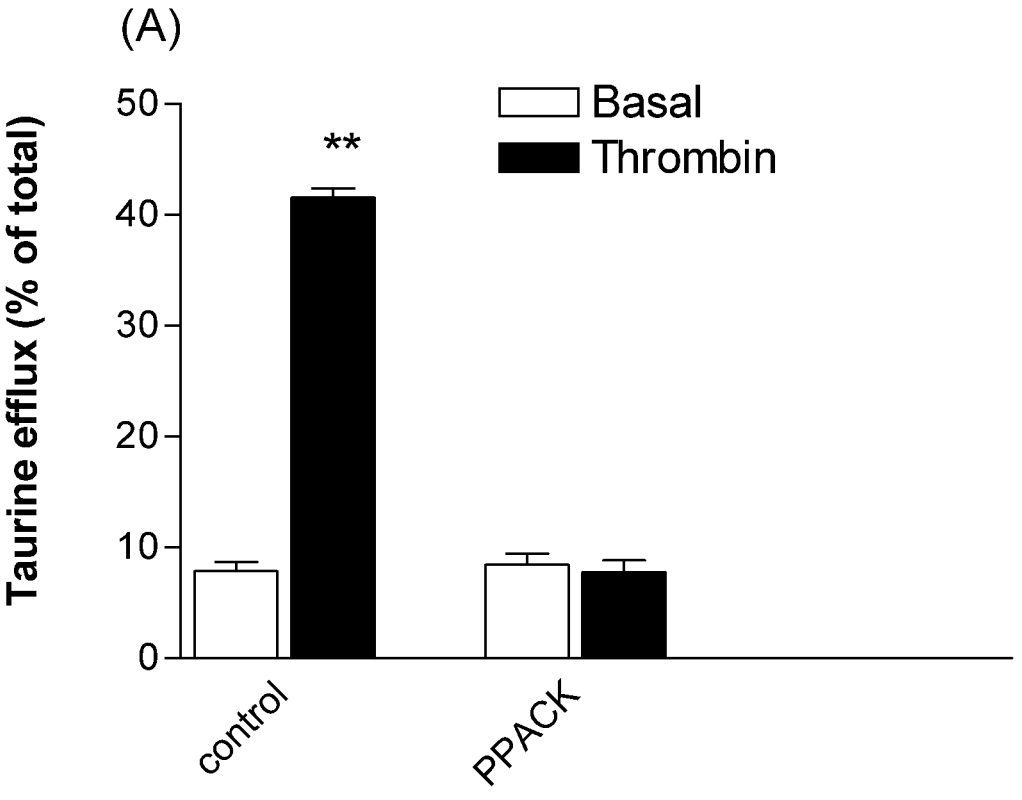

(A)
Basal
Thrombin
**
Taurine efflux (% of total)
50
40
30
20
10
0
control
PPACK

Figure 3

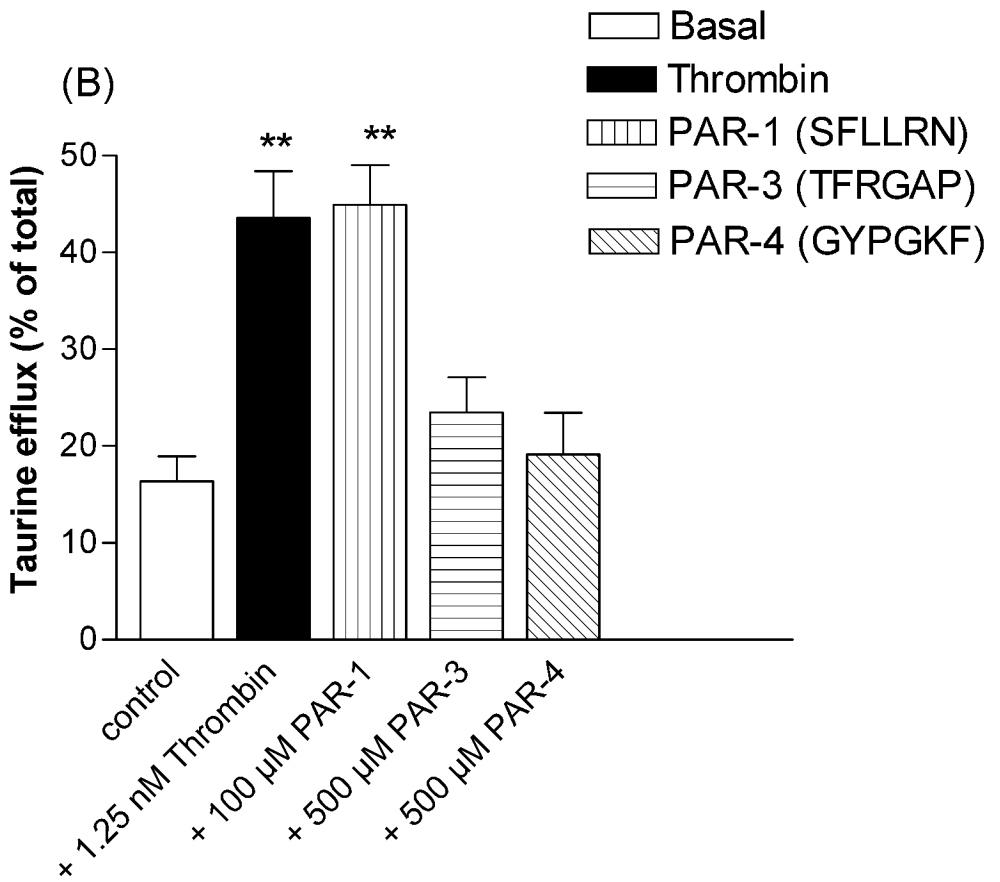

(B)
Basal
Thrombin
PAR-1 (SFLLRN)
PAR-3 (TFRGAP)
PAR-4 (GYPGKF)
Taurine efflux (% of total)
0
10
20
30
40
50
**
**
control
+ 1.25 nM Thrombin
+ 100 µM PAR-1
+ 500 µM PAR-3
+ 500 µM PAR-4

Figure 3

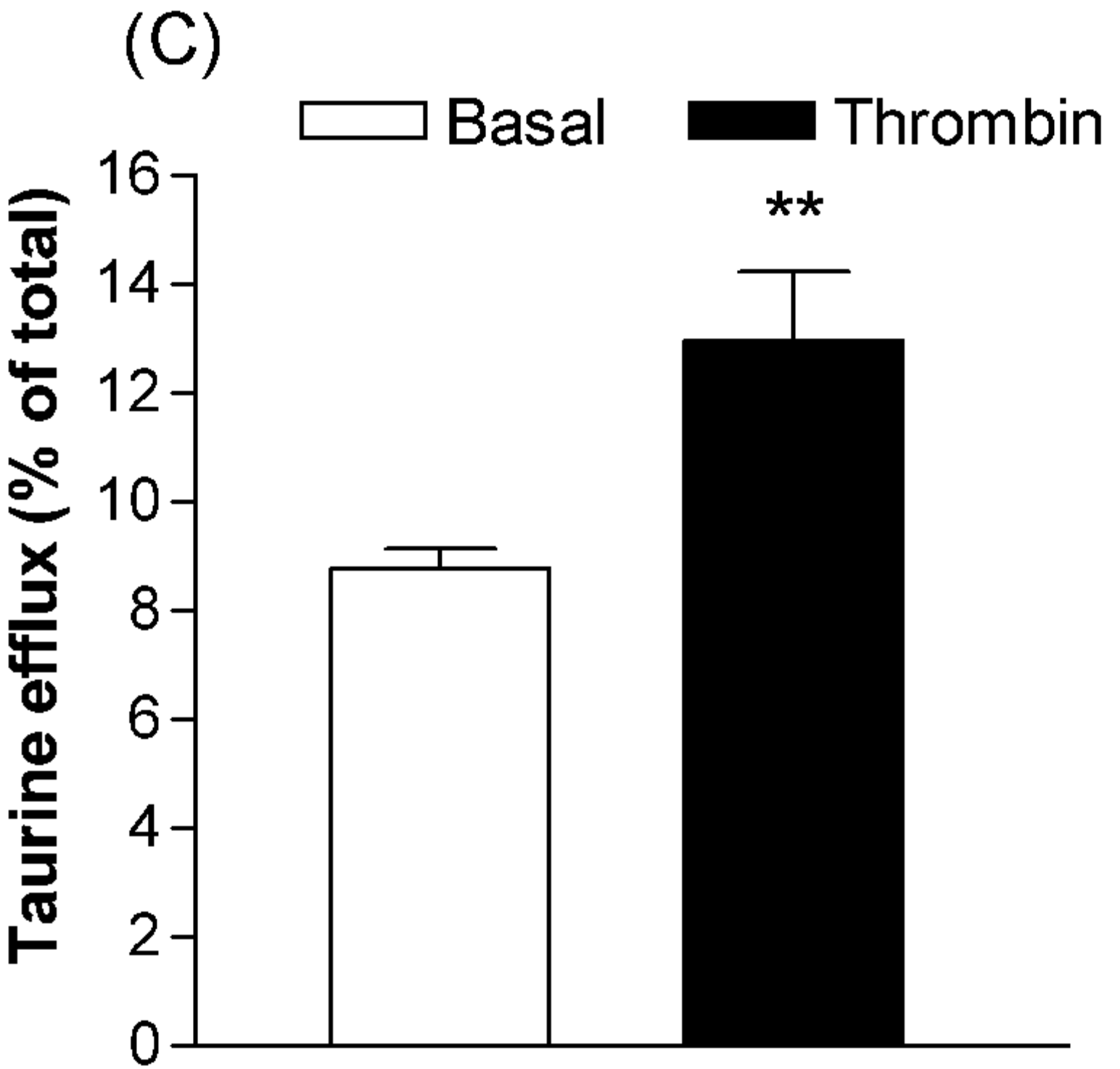


Figure 4

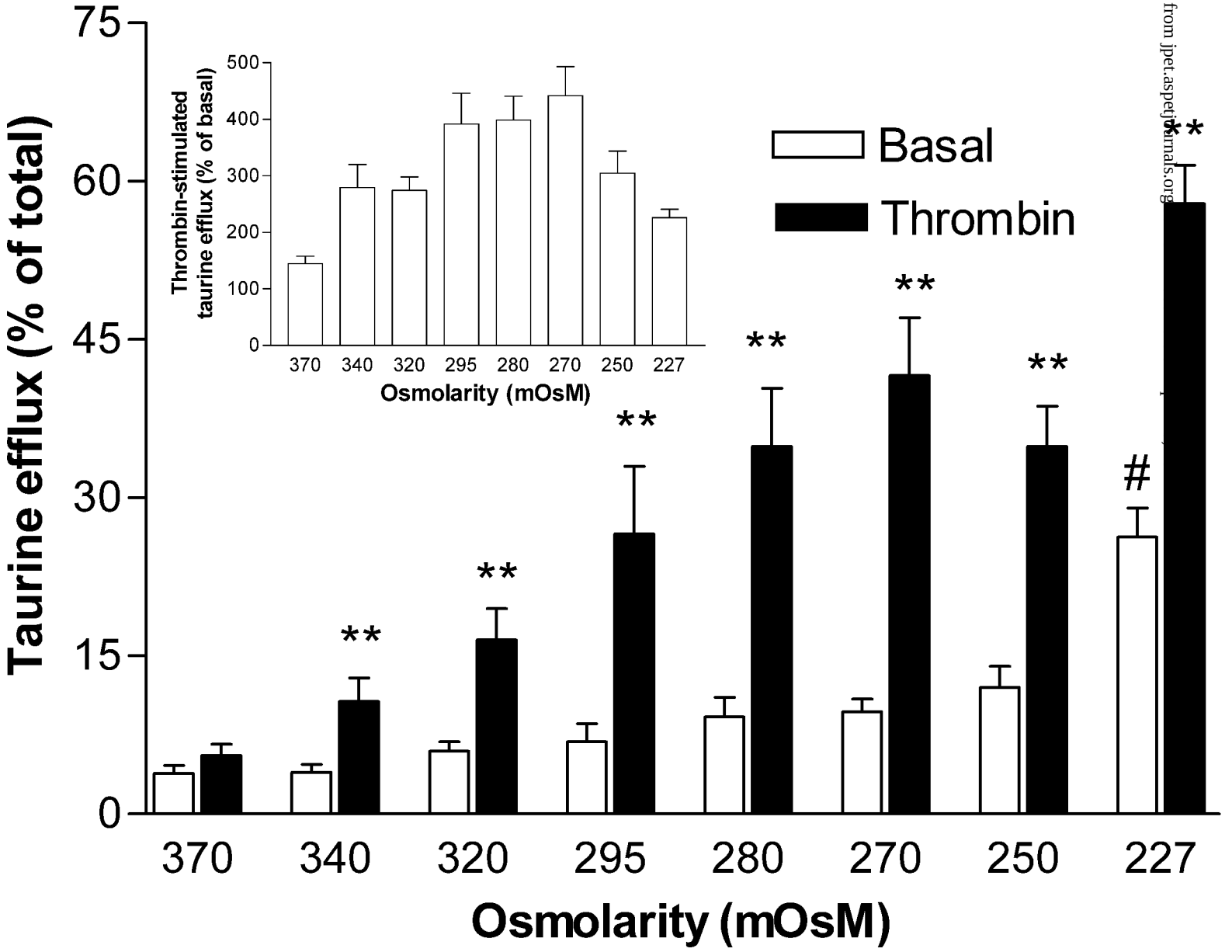


Figure 5

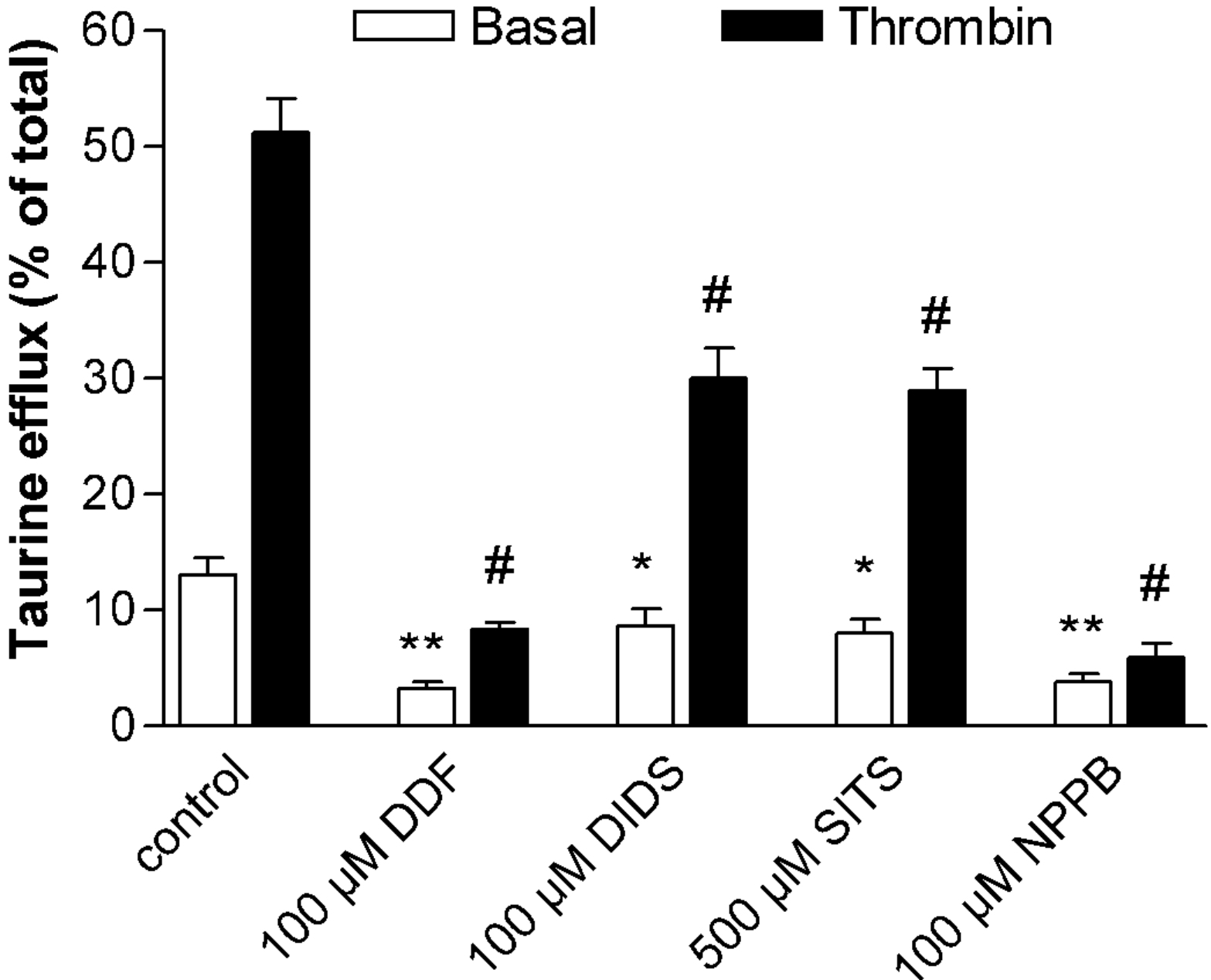

Basal
Thrombin
Taurine efflux (% of total)
60
50
40
30
20
10
0
**
#
*
#
*
#
**
#
control
100 μM DDF
100 μM DIDS
500 μM SITS
100 μM NPPB

Figure 6

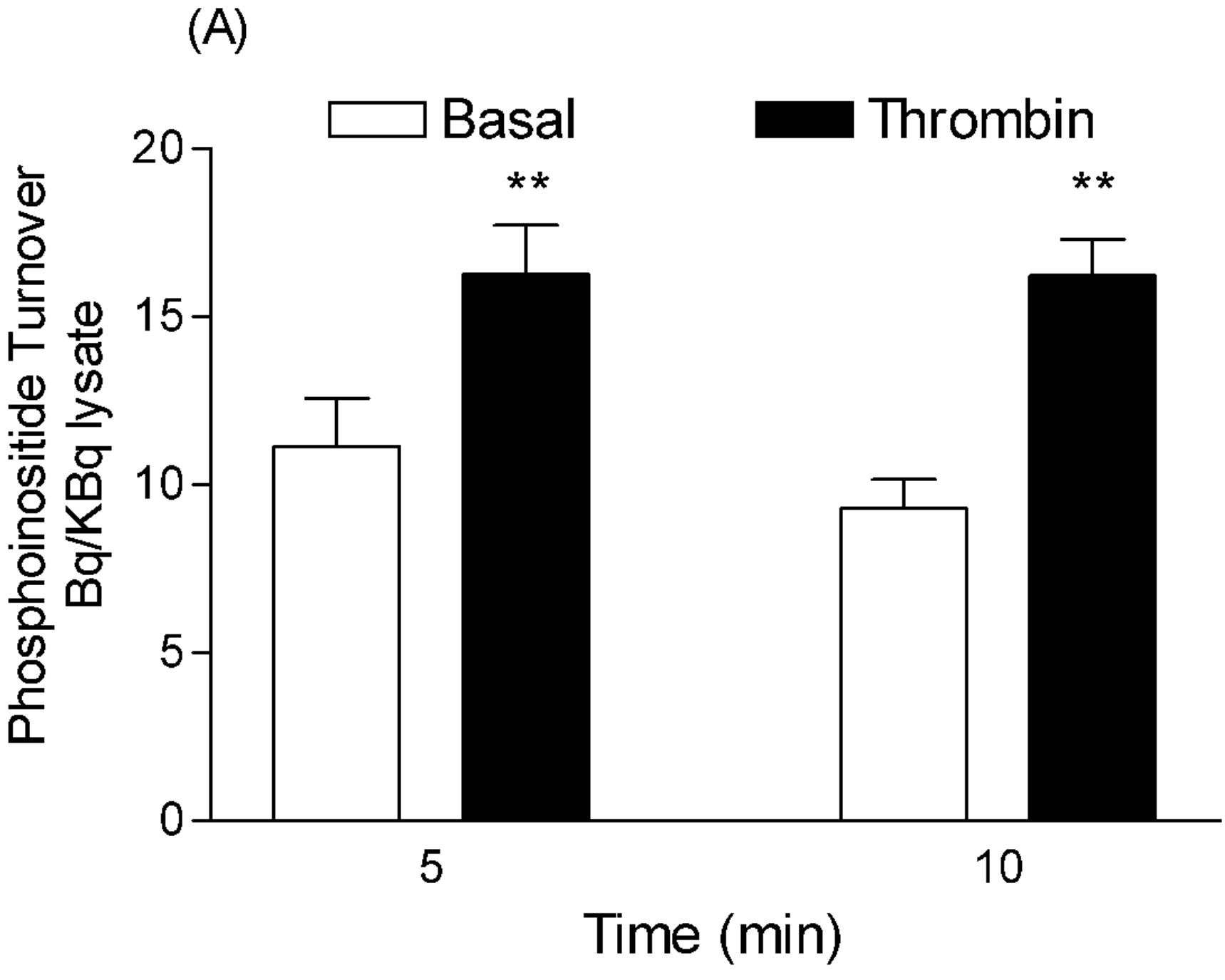

(A)
Basal
Thrombin
20
15
10
5
0
**
**
Phosphoinositide Turnover
Bq/KBq lysate
5
10
Time (min)

Figure 6

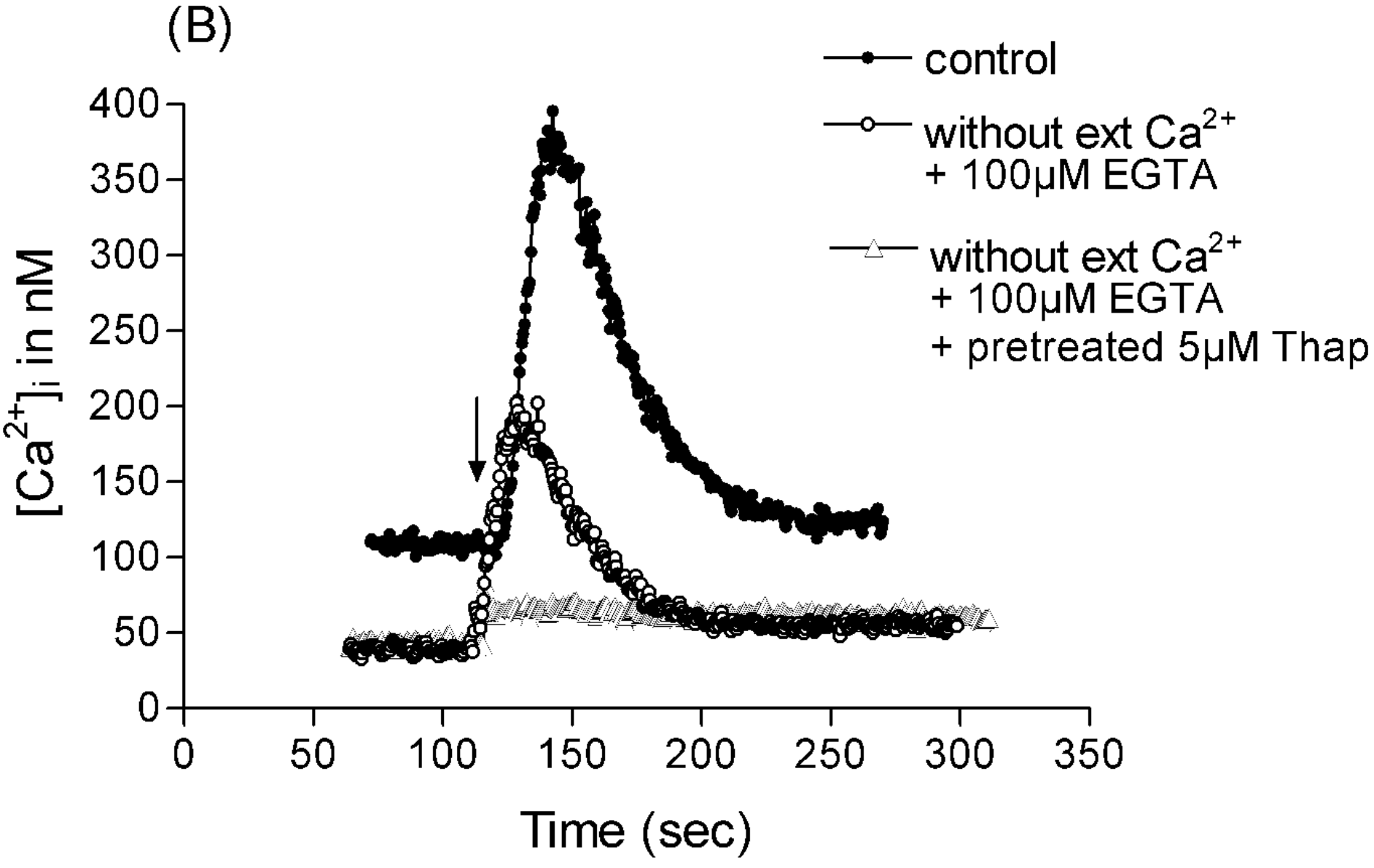
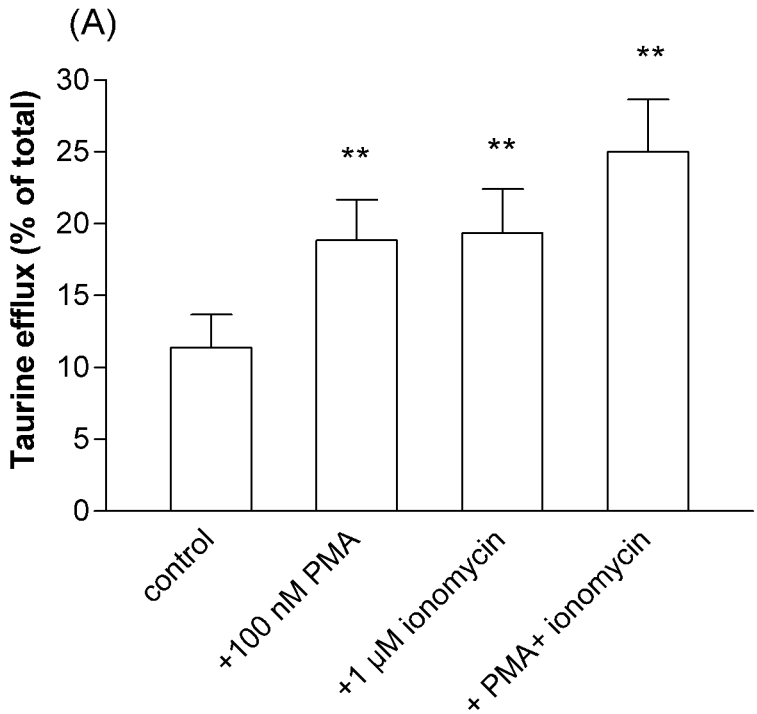

(B)
control
without ext Ca2+
+ 100μM EGTA
without ext Ca2+
+ 100μM EGTA
+ pretreated 5μM Thap
[Ca2+]i in nM
400
350
300
250
200
150
100
50
0
0
50
100
150
200
250
300
350
Time (sec)

Figure 7


(A)
Taurine efflux (% of total)
30
25
20
15
10
5
0
**
**
**
control
+100 nM PMA
+1 μM ionomycin
+ PMA+ ionomycin

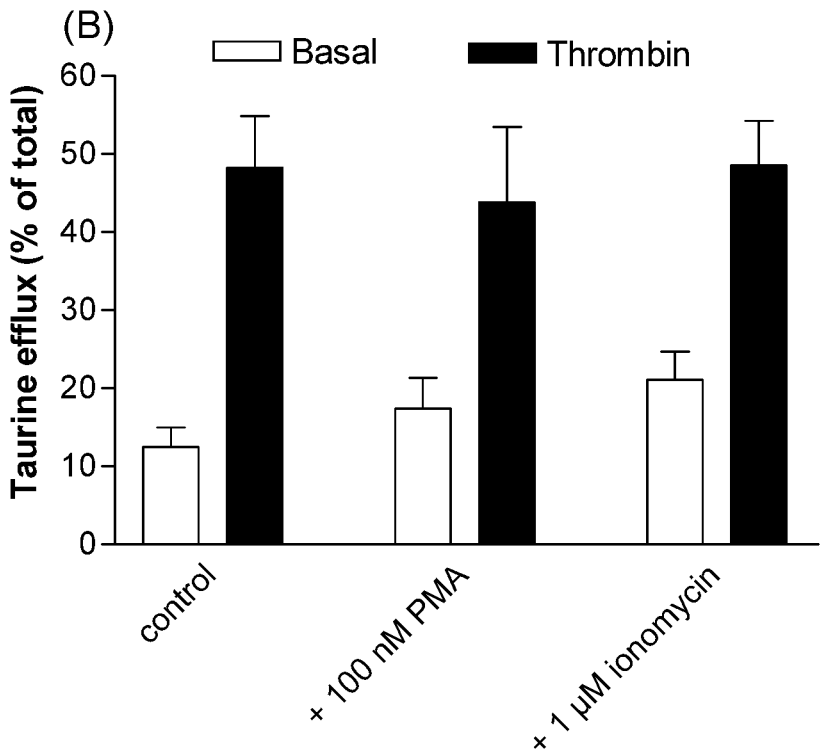
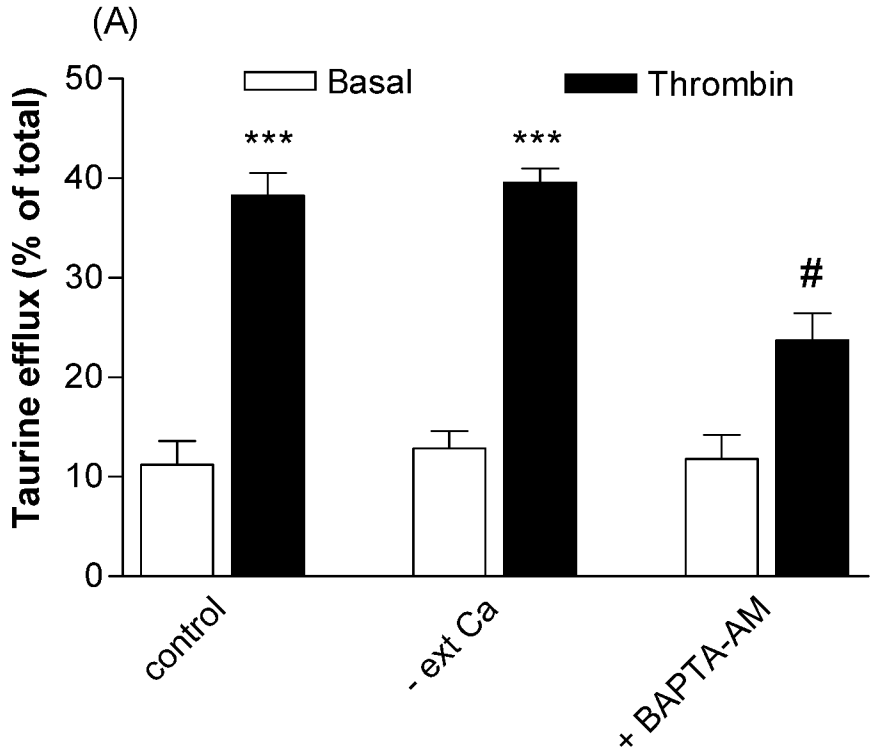

(B)
Basal
Thrombin
Taurine efflux (% of total)
60
50
40
30
20
10
0
control
+ 100 nM PMA
+ 1 μM ionomycin

Figure 8


(A)
Basal
Thrombin
Taurine efflux (% of total)
50
40
30
20
10
0
***
***
#
control
- ext Ca
+ BAPTA-AM

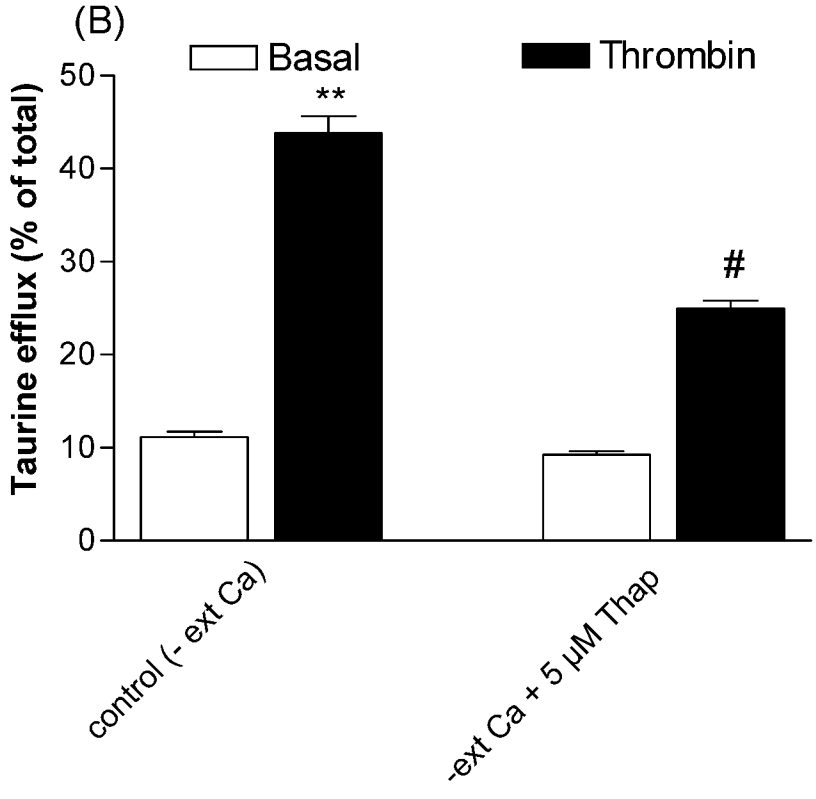

(B)
Basal
Thrombin
Taurine efflux (% of total)
50
40
30
20
10
0
**
#
control (- ext Ca)
-ext Ca + 5 µM Thap

Figure 9