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Two conventional PKC isoforms, α and βI, are involved in the ATP-induced regulation of VRAC and glutamate release in cultured astrocytes

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Abstract

Volume-regulated anion channels (VRACs) are activated by cell swelling and are permeable to inorganic and small organic anions, including the excitatory amino acids glutamate and aspartate. In astrocytes, ATP potently enhances VRAC activity and glutamate release via a P2Y receptor-dependent mechanism. Our previous pharmacological study identified protein kinase C (PKC) as a major signaling enzyme in VRAC regulation by ATP. However, conflicting results obtained with potent PKC blockers prompted us to re-evaluate PKC involvement in regulation of astrocytic VRAC using siRNA and pharmacological inhibitors that selectively target individual PKC isoforms. In primary rat astrocyte cultures, application of hypoosmotic medium (30% reduction in osmolarity) and 20 μM ATP synergistically increased the release of excitatory amino acids, measured with non-metabolized analogue of L-glutamate, D-[^{3}H]aspartate. Both Go6976, the selective inhibitor of Ca^{2+}-sensitive PKCα, βI/II, and γ, and MP-20-28, a cell permeable pseudosubstrate inhibitory peptide of PKCα and βI/II, reduced the effects of ATP on D-[^{3}H]aspartate release by ~45-55%. Similar results were obtained with a mixture of siRNAs targeting rat PKCα and βI. Surprisingly, downregulation of individual α and βI PKC isozymes by siRNA was completely ineffective. These data suggest that ATP regulates VRAC activity and volume-sensitive excitatory amino acid release via cooperative activation of PKCα and βI.

Keywords

osmotic cell swelling; volume-regulated anion channel; protein kinase C; siRNA

INTRODUCTION

Volume-regulated anion channels (VRACs), also termed volume-sensitive outwardly rectifying (VSOR) Cl^- channels or volume-sensitive organic osmolyte and anion channels (VSOAC), are evolutionarily conserved Cl^- channels that are ubiquitously expressed in mammalian cells (Strange et al. 1996;Okada 1997;Nilius et al. 1997). Although VRACs are well characterized biophysically, their molecular identity remains unknown (Nilius and Droogmans 2003; Okada 2006). The major physiological role for VRACs is cell volume homeostasis. Release of inorganic and organic osmolytes via VRAC, in conjunction with K^+

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release via swelling activated K^+ channels, mediates the reduction of intracellular osmolarity and regulatory cell volume decrease (Lang et al. 1998; Mongin and Orlov 2001; Nilius and Droogmans 2003). In addition to cell volume regulation, VRACs are also thought to participate in cell proliferation, apoptosis, modulation of vascular tone, and regulation of membrane potential in various cell types (Okada et al. 2001; Nilius and Droogmans 2003; Stutzin and Hoffmann 2006).

In the brain, VRACs likely play additional physiological roles. One important property of VRACs that may underlie their unique contributions to the CNS physiology is permeability to a number of small organic molecules, including the amino acids aspartate, glutamate, and taurine (Pasantes-Morales et al. 1990; Kimelberg et al. 1990; Banderali and Roy 1992; Jackson and Strange 1993; Pasantes-Morales et al. 1994; Nilius and Droogmans 2003). Glial taurine release via a VRAC-like channel regulates neuronal activity in the hypothalamo-neurohypophysial system (Hussy et al. 2000). Based on *in vitro* observations, several groups propose that swelling- and receptor-activated release of glutamate via a VRAC pathway may contribute to regulation of neuronal activity in other brain areas (Jeremic et al. 2001; Mongin and Kimelberg 2002; Takano et al. 2005; Mulligan and MacVicar 2006). Also of importance, VRACs have been implicated in pathological glutamate release in ischemia and other conditions, which are associated with pronounced astrocytic swelling (Phillis et al. 1997; Kimelberg and Mongin 1998; Seki et al. 1999; Feustel et al. 2004; Mongin 2007).

In spite of extensive research efforts, the intracellular mechanisms involved in VRAC activation remain poorly understood. Several groups discovered that ATP and agonists of several other G-protein coupled receptors (GPCR) strongly increase VRAC activity in swollen cells. GPCR agonists may also produce limited VRAC activation in non-swollen cells of both neural and non-neural origin (Tilly et al. 1994; Mongin and Kimelberg 2002; Loveday et al. 2003; Darby et al. 2003;Franco et al. 2004; Falktoft and Lambert 2004; Heacock et al. 2006; Cheema et al. 2007). Regulation of VRAC activity by several purinergic (ATP) receptors may be especially important in the brain. In the CNS, ATP is released by a variety of mechanisms, including co-release with other neurotransmitters during synaptic communication (Ralevic and Burnstock 1998;Fields and Burnstock 2006). Furthermore, ATP has been implicated in regulation of glial glutamate release and bidirectional astrocyte-neuron communication (Haydon 2001;Haydon and Carmignoto 2006;Fields and Burnstock 2006).

GPCR control of VRAC function involves increases in intracellular Ca^{2+} and activation of several Ca^{2+}-sensitive signaling cascades that may involve protein kinase C (PKC). PKC encompasses a family of closely related phospholipid-dependent serine-threonine kinases expressed in all eukaryotic cells. Twelve different PKC isoforms are described to date based on their gene homologies. PKCs are classified according to their regulatory properties into (i) classical or conventional PKC isoforms (PKCα, PKCβI, PKCβII, and PKCγ), which are activated by diacylglycerol (DAG) and increases in $[Ca^{2+}]_i$; (ii) novel isoforms (PKCδ, PKCε, PKCη, PKCθ) that are DAG-sensitive but Ca^{2+}-insensitive; and (iii) atypical isoforms (PKCζ, PKCι/λ and PKMζ), which are not regulated by either DAG or Ca^{2+} (Parker and Murray-Rust 2004).

The evidence for PKC involvement in VRAC regulation is based primarily on pharmacological data. In our previous work, we have found that although some of the PKC inhibitors (such as chelerythrine and bisindolylmaleimide) potently blocked ATP-dependent VRAC activation, others (such as Go6983 and Ro-32-0432) showed little efficacy (Mongin and Kimelberg 2005;Haskew-Layton et al. 2005). In other cell types, PKC has been reported to play either major or minor role in the GPCR-dependent regulation of organic osmolyte release via VRAC (Loveday et al. 2003; Falktoft and Lambert 2004; Cheema et al. 2007). In

the present study we used isoform-specific pharmacological inhibitors and siRNAs to explore the functional significance of individual PKC isozymes. Since previous findings revealed a strong $[Ca^{2+}]_i$ dependency of the ATP effect on VRAC activity (Mongin and Kimelberg 2002; Mongin and Kimelberg 2005), we focused our efforts on conventional PKCs.

MATERIALS AND METHODS

Cell culture preparation

Confluent primary astrocyte cultures were prepared from the cerebral cortex of newborn Sprague-Dawley rats as previously described (Mongin and Kimelberg 2002), according to the animal procedure approved by the institutional animal care and use committee. Briefly, newborn Sprague-Dawley rats were euthanized by rapid decapitation, the cerebral cortices were separated from the meninges and basal ganglia, and tissue was dissociated using solution of the neutral protease Dispase II (Roche Applied Science, Indianapolis, IN) supplemented with DNAse I (Sigma). Dissociated cells were seeded at low density on poly-D-lysine coated 18×18 mm glass coverslips (Caroline Biological Supply Co, Burlington, NC) for efflux experiments, or grown in poly-D-lysine coated 60-mm plastic Petri dishes for transfection experiments. Cell cultures were grown for 3-4 weeks in Minimal Essential Medium (MEM) supplemented with 10% heat inactivated horse serum (HIHS), 50 U/ml penicillin and 50 μg/ml streptomycin (all cell culture reagents were purchased from Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO2/95% air atmosphere. Culture medium was replaced twice a week. After two weeks of cultivation, penicillin and streptomycin were removed from the culture medium. Control immunocytochemistry experiments showed ≥95% of the cells stained positively for the astrocytic marker glial fibrillary acid protein (data not shown).

Glutamate (D-[^{3}H]aspartate) efflux assay

Excitatory amino acid efflux measurements were performed as previously described (Mongin and Kimelberg 2002). Astrocytes grown on glass coverslips were loaded overnight with D-[^{3}H]aspartate (4 μCi/ml) in 2.5 ml of MEM containing 10% HIHS at 37°C in a CO_2 incubator. The cells were then washed free of extracellular isotope and serum-containing medium in HEPES-buffered solution. The isoosmotic HEPES-buffered medium contained (in mM) 135 NaCl, 3.8 KCl, 1.2 $MgSO_4$, 1.3 $CaCl_2$, 1.2 KH_2PO_4, 10 D-glucose, and 10 HEPES (pH =7.4). The coverslips were inserted into a Lucite perfusion chamber that had a space above the coverslip of ~150-200 μm in height. The cells were superfused at a constant flow rate of 1.2 ml/min with HEPES-buffered media in an incubator set at 37°C. In hypoosmotic medium the NaCl concentration was reduced to 85 mM. The osmolarities of all buffers were checked with a freezing point osmometer (μOmette 5004, Precision Systems, Natick, MA) and were 287±2 and 198±2 for isoosmotic and hypoosmotic media, respectively. Superfusate fractions were collected at 1-min intervals. At the end of each experiment, the isotope remaining in the cells was extracted with a solution containing 2% sodium dodecylsulfate plus 8 mM EDTA. Four milliliters of Ecoscint scintillation cocktail (National Diagnostics, Atlanta, GA) was added, and each fraction was counted for ^{3}H in a Packard Tri-Carb 1900TR liquid scintillation analyzer (PerkinElmer, Downers Grove, IL). Percent fractional isotope release for each time point was determined by dividing radioactivity released in each 1-min interval by the radioactivity left in the cells at this time point, which has been calculated using custom computer program. Such calculation allows for measuring the fractional release independent of gradual decreases in cellular isotope content during perfusion. In a typical experiment, cells retained not less than 60% of initially loaded D-[^{3}H]aspartate.

siRNA transfections

Small interfering RNAs (siRNAs) targeting rat PKCα and βI were purchased from Qiagen (Valencia, CA). The validated rat genome specific siRNA sequences for PKC isozymes were as follows. **PKCα:** Qiagen Rn_Prkca_2_HP targeting rPKCα sequence GTC CTT CAC GTT CAA ATT AAA; and Rn_Prkca_4_HP targeting PKCα sequence ATG AAC TGT TTC AGT CTA TAA. **PKCβI:** Qiagen Rn_Prkcb1_1_HP targeting PKCβI sequence ACG GAG CAA ACA CAA GTT TAA; and Rn_Prkcb1_3_HP targeting PKCβI sequence TTC GGG TAC TTA GGC GTT CAA. AllStars Negative Control labeled with AlexaFluor-488, a scrambled siRNA, was used as a negative control for transfection and for monitoring the efficacy of transfection.

Confluent cultured primary cortical astrocytes were detached from the substrate using recombinant TrypLE Express protease (Invitrogen) and plated on poly-D lysine-coated coverslips for glutamate efflux assays, or on 60 mm plastic Petri dishes for the Western blotting, and left to recover overnight. Cells were 50-70% confluent, when transfected. A combination of individual siRNAs were mixed with X-tremeGENE siRNA transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer instructions and applied to the cells. Efficiency of siRNA transfection was assessed by monitoring the uptake of scrambled siRNA labeled with Alexa-488, and was always 95% or higher. After 72 h incubation with siRNA, cells were used in D-[^{3}H]aspartate efflux assays and for Western blot analysis.

Western blot analysis

The efficacy of PKC downregulation was assessed in each experiment by the Western blot analysis using monoclonal antibody raised against PKCα (Millipore, Temecula, CA; cat# 05-154), monoclonal PKCβI antibody (Santa Cruz Biotechnology, Santa Cruz, CA, cat# sc-8049), and polyclonal PKCε antibody (Millipore, Temecula, CA; cat# 06-991). After 24-72 h of incubation with siRNA, cells were lysed with 2% SDS plus 8 mM EDTA. The total protein content of the lysates was determined using a colorimetric BCA assay kit (Pierce Biotechnology, Rockford, IL). Whole cell lysates were diluted with a reducing Laemmli buffer. Proteins were separated on 10% polyacrylamide gel followed by transfer onto an Immun-Blot™ PDVF membrane (Bio-Rad, Hercules, CA). The membranes were blocked overnight at 4°C with 5% nonfat milk in PBS buffer, containing 0.05% Tween 20. They were further incubated for 2 h at room temperature with either monoclonal PKCα antibody (1:500 dilution), monoclonal PKCβI antibody (1:500 dilution), or polyclonal PKCε antibody (1:500 dilution). After five washes for 5 min with PBS-Tween buffer (PBS-T), membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare/Amersham Biosciences, Piscataway, NJ; 1:10,000 dilution), followed by four PBS-T washes. The horseradish peroxidase signal was detected using a chemiluminescence ECLplus reagent (GE Healthcare/Amersham Biosciences) and CL-Xposure film (Pierce). The optical densities of the immunoreactive bands were analyzed using Molecular Dynamics Personal Densitometer SI and Image Quant 5.0 software (Molecular Dynamics). Equal amounts of protein of 10 μg per lane were loaded for each Western blotting assay. In addition, PDVF membranes were stripped and re-probed with monoclonal anti-actin antibody (EMD, cat# CP01) to assure equal levels of loading.

Total RNA isolation and quantitative RT-PCR

Total RNA isolation from siRNA-treated astrocyte cultures was performed using RNAqueous®-4PCR kit (Ambion, Austin, TX) according to the manufacturer's instructions. Concentration of RNA in samples was determined using a NanoDrop® spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE). 900 ng of total RNA was utilized to produce cDNA using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's

instructions. One μl of each RT product was used for quantitative PCR using iTaq SYBRGreen Supermix (Bio-Rad) and an iCycler thermocycler (Bio-Rad). Relative cDNA levels for PKC isozymes were determined with QuantiTect® primers optimized for quantitative PCR for rat PKCα (Rn_Prkca_2_SG) and PKCβI (Rn_Prkcb1_1_SG), and normalized to GAPDH (Rn_Gapdh_1_SG) in the same samples. All primers were from Qiagen. qPCR data were analyzed using iCycler iQ software (Bio-Rad).

Statistical analyses

The statistical significance of the data was determined using one-way ANOVA, followed by post-hoc Bonferroni correction for multiple comparisons, when appropriate. In D-[^{3}H]aspartate release experiments, we separately analyzed maximal release values under hypoosmotic conditions (results of analyses presented in the text and figures), as well as integral release values over 10-min exposure to hypoosmotic media (presented in the text only). Origin 6.0 (OriginLab, Northampton, MA) and Prism 5 (GraphPad, San Diego, CA) were used for statistical analysis.

RESULTS

Effects of the conventional PKC inhibitor Go6976 and the PKCα/β inhibitory peptide on swelling-activated D-[^{3}H]aspartate release

In our previous work, we found that ATP strongly potentiates excitatory amino acid release from swollen astrocytes via a mechanism sensitive to PKC inhibitors (Mongin and Kimelberg 2005). Since the effect of ATP on VRAC activity is Ca^{2+}-dependent (Mongin and Kimelberg 2005), we hypothesized that positive modulation of VRAC activity and glutamate release is mediated by one or several conventional PKC isoforms. To test the involvement of conventional PKCs, we initially used Go6976 (EMD, San Diego, CA), which, at a concentration of 1 μM, selectively blocks PKC isoforms α, β, and γ (IC_{50} ≤6 nM) (Martiny-Baron et al. 1993). Consistent with our previous findings, 20 μM ATP potently increased hypoosmotic glutamate release by ~5-fold (Fig. 1A, $p<0.001$ for both maximal release values and the integral release under hypoosmotic conditions). Go6976 strongly reduced swelling activated D-[^{3}H]aspartate release in the presence of ATP (Fig. 1A, $p<0.001$ for maximal release, $p=0.02$ for integral release). The ATP-induced increment in the maximal release values (measured as a difference between hypoosmotic release values in the presence and absence of ATP) was diminished by ~55%. Under control hypoosmotic conditions (in the absence of ATP), Go6976 had no effect.

To verify the involvement of conventional PKCs in the ATP-induced excitatory amino acid release, we used the cell permeable pseudosubstrate inhibitory peptide 20-28 (MP20-28, EMD), which, in cell assays, blocks PKCα and β with an IC_{50} of 8-20 μM (Eichholtz et al. 1993). Because of strong cytotoxicity at high concentrations, we were unable to use MP20-28 at the concentrations exceeding 10 μM (data not shown). Cultured astrocytes were preincubated with 10 μM MP20-28 for 30 min, followed by the D-[^{3}H]aspartate efflux assay. The peptide was not present in the release assay media. As seen in Fig. 1B, pretreatment with MP20-28 produced weak inhibition (~20%) of D-[^{3}H]aspartate release in the presence of ATP ($p=0.05$ for maximal release, $p=0.087$ for integral release), while the release in the absence of ATP was somewhat enhanced (~30%, $p=0.02$ for maximal release, $p=0.085$ for integral release). The effect of the peptide on the release under hypoosmotic conditions was likely due to toxicity, and was more evident at higher peptide concentrations (data not shown). Although the effect of MP-20-28 on the absolute release values in the presence of ATP was borderline significant, a difference between hypoosmotic release values in the presence and absence of ATP was reduced by 42%. This was similar to the effect found with Go6976.

Effect of PKC downregulation by PDBu on VRAC activity

To test if the Go6976- and MP20-28-insensitive component of ATP signaling involves non-conventional PKC isoforms, we decided to downregulate PKC isozymes using 24-hr preincubation with the phorbol ester PDBu. Such a treatment produces long-term PKC activation, followed by the proteolytic degradation of all PKC isozymes of the conventional and novel subfamilies (Young et al. 1987). As seen in Fig. 2A, 24-hr incubation of astrocytes with 500 nM PDBu strongly downregulated PKCα and βI (conventional) and PKCε (novel). Monoclonal PKCβI antibody recognized two immunoreactive bands of ~79-80 and ~ 60 kD. The upper band corresponds to the predicted molecular weight of PKCβI (79 kD); the lower band likely represents the product of proteolytic degradation of PKCβI, as its intensity was strongly upregulated in the PDBu-treated cells. In four different culture preparations, the downregulation of PKCα, βI, and ε, always exceeded 95% of the protein levels in control sister cultures plated and processed at the same time.

We next tested the effect of global PKC downregulation on VRAC activity. To increase the efficacy of the PDBu delivery, we preincubated the astrocytes with PDBu in a serum-free opti-MEM medium (Invitrogen). Opti-MEM pretreatment itself strongly increased the hypoosmotic D-[^{3}H]aspartate release, when compared to hypoosmotic control values in all other experiments, in which cells were maintained in serum-containing media (compare control HYPO values in Fig. 2B to those in Figs. 1 and 4B). The reasons for this are not clear. Nevertheless, in the serum-starved cells, ATP stimulated the D-[^{3}H]aspartate release by 100% ($p=0.011$ for maximal release, $p=0.035$ for integral release values) and this effect was strongly attenuated by long-term PDBu pretreatment. The ATP effect on D-[^{3}H]aspartate release, measured as a difference between hypoosmotic release values in the presence and absence of ATP, was reduced by 63%. This inhibition was quantitatively similar to the effect of Go6976 in the preceding experiments.

Functional effects of siRNA-mediated downregulation of the PKCα and βI

We further used a highly selective siRNA approach to explore the role of conventional PKC isoforms in mediating ATP-induced VRAC regulation. Cultured astrocytes express numerous PKC isoforms from all three PKC subfamilies (Slepko et al. 1999;Berdiev et al. 2002). Among conventional isoforms, expression of α, βI, βII, but not γ has been detected (Slepko et al. 1999). Since two previous studies implicated PKCα in VRAC regulation (Roman et al. 1998; Hermoso et al. 2004), we initially used a commercially available mammalian plasmid pKD-PKCα-v4 expressing shRNA that targets PKCα (Millipore). In these experiments, PKCα protein levels were reduced by ~70%, while the ATP-induced regulation of D-[^{3}H] aspartate release was completely unaffected (Supplemental figure 1, $n=5$, $p>0.9$ for both maximal and integral release values).

We then decided to target PKCα and βI simultaneously in order to eliminate the possible redundant activity of the complementary PKC isoform after downregulation of its functional homologue. We focused on PKCβI, rather than on βII, (i) because of the lack of validated siRNAs targeting the βII isoform, and (ii) based on a previous report on the critical role of PKCβI rather than βII in protecting cultured cortical astrocytes against ischemic injury (Wang et al. 2004). To increase the efficacy of protein knockdown, we used a mix of two different siRNAs targeting rat PKCα and two siRNAs targeting rat PKCβI (50 nM each, 200 nM total). Scrambled siRNA labeled with Alexa-488 was utilized as a control for non-specific effects, and to monitor the transfection efficacy. In all preparations we found that >95% of the cells incorporated the Alexa-488 signal, although a majority of the fluorescence resided in punctuate perinuclear structures, possibly representing endosomal and/or lysosomal compartments (Fig. 3). Nevertheless, as seen in Fig. 4A, a combination of four siRNAs, produced a time-dependent decrease in protein levels (immunoreactivity) of PKCα

and PKCβI, as compared to cells treated with scrambled siRNA, without affecting the levels of actin. At 72 hrs, the average decrease in protein levels was highest and reached 85.2±4.7% for PKCα (mean ±SE, Fig. 5C, n=8, $p<0.001$) and 83.3±3.4% for PKCβI (mean ±SE, Fig. 5D, n=4, $p<0.001$). Therefore, we used a 72-hr treatment in all the subsequent experiments.

When the same α/βI siRNA mix was tested in the $_D$-[^{3}H] aspartate efflux experiments, we found that the siRNA treatment significantly attenuated hypoosmotic $_D$-[^{3}H]aspartate release in the presence of ATP (Fig. 4B, $p<0.001$ for maximal release values, $p=0.005$ for the integral releases). Importantly, the release under control hypoosmotic conditions (i.e., in the absence of ATP) was not affected by siRNA treatment (Fig. 4B, $p>0.8$ for both maximal and integral release values). The ATP-induced increment in maximal $_D$-[^{3}H] aspartate release was reduced by 50%. Protein downregulation was verified in the sister cultures for each of the three independent transfections (Fig. 4B, and 5C and D for protein quantification).

To determine the contribution of individual PKC isoforms, we performed separate siRNAα and βI transfections. Neither downregulation of PKCα nor of PKCβI affected the ATP-stimulated release of $_D$-[^{3}H] aspartate from swollen astrocytes (Fig. 5A). As expected, in Western blotting assays, siRNA-PKCα strongly reduced immunoreactivity of PKCα (Fig. 5C, 68.4±3.1% reduction, mean ±SE, n=8, $p<0.001$). Surprisingly, we found that PKCβI immunoreactivity was also strongly reduced by siRNA-PKCα (Fig. 5D, 66.9±5.3%, n=4, $p=0.001$). When we tested siRNA-PKCβI, it predictably produced no effect on PKCα (Fig. 5C, 3.4±7.6% reduction, $p=0.667$). However, it also only marginally affected PKCβI immunoreactivity (Fig. 5D, 22.1±12% reduction, $p=0.162$). This was in striking contrast to the effect obtained with siRNA-PKCα/βI mix (Fig. 5D, PKCβI immunoreactivity reduction of 83.3±3.4%, $p<0.001$). Such paradoxical results were contradictory to the selective nature of siRNAs verified by the BLAST analysis. One explanation for this inconsistency is cross-reactivity of the PKCβI antibody for PKCα and PKCβI isoforms.

Selectivity of the PKCβI antibody has been tested using lysates of cultured rat smooth muscle cells, which do not express PKCβI/II (Singer 1996). We found strong “PKCβI” immunoreactivity in the smooth muscle cell lysates, similar to that seen in cultured astrocytes (Supplemental figure 2). We next performed quantitative RT-PCR analysis of PKC mRNA levels in cells treated with various siRNAs for 24 hrs. Shorter siRNA treatment for the mRNA assay was chosen because decreases in mRNA levels typically precede a decrease in protein levels. Consistent with the specific nature of the siRNA effects, PKCα and PKCβI were downregulated in an isoform-specific fashion (Fig. 5E). siRNA-PKCα downregulated the PKCα mRNA levels by 64±3% by (n=6, $p<0.001$) but did not affect PKCβI mRNA levels (9±15% reduction, $p>0.9$). Vice versa, siRNA-PKCβI reduced the levels of PKCβI mRNA by 81±2% (n=6, $p<0.001$), but was only marginally effective against PKCα (25±6% reduction, n=6, $p<0.05$). Importantly, combination of siRNA-PKCα/βI did not produce any additive effects on the mRNA levels of individual PKCs (Fig. 5E). Therefore, the results of efflux experiments—in which only siRNA mix was effective, while individual siRNAs targeting PKCα and βI were not—should be interpreted as evidence for the involvement of ***both*** PKCα and βI in the purinergic regulation of the VRAC activity.

DISCUSSION

In the present study, we established that, in primary cortical astrocytes, cooperative activation of two conventional PKC isoforms, α and βI, is largely responsible for ATP receptor-dependent regulation of excitatory amino acid release via anionic channel VRAC. In contrast to the ATP-stimulated release, release of excitatory amino acids under hypoosmotic conditions was insensitive to inhibition or downregulation of conventional

PKCs. Therefore, cell swelling and ATP regulate VRAC via two separate signaling mechanisms.

Although the identity of the swelling-activated D-[^{3}H]aspartate release pathway has not been addressed in this study, the involvement of VRAC in astrocytic excitatory amino acid release in the absence or presence of ATP was previously verified by using several pharmacological inhibitors including the selective VRAC blocker DCPIB (Mongin and Kimelberg 2002;Haskew-Layton et al. 2005;Abdullaev et al. 2006). DCPIB potently and selectively blocks the VRAC activity measured electrophysiologically or as swelling-activated release of D-[^{3}H]aspartate release (Decher et al. 2001; Abdullaev et al. 2006).

Conventional PKC isoforms are major contributors to VRAC regulation by ATP

Cell swelling serves as a primary signal for VRAC activation. However, a number of laboratories have demonstrated that stimulation of GPCRs, including several metabotropic P2Y receptors for ATP and ADP, produces limited activation of a VRAC-like pathway in non-swollen cells and greatly increases its activity in cells exposed to hypoosmotic medium (Jeremic et al. 2001;Mongin and Kimelberg 2002;Mongin and Kimelberg 2003;Loveday et al. 2003; Darby et al. 2003;Franco et al. 2004). Depending on the experimental system and the GPCR involved, PKCs have been found to play major or minor roles in the regulation of VRAC activity by GPCR agonists (Loveday et al. 2003; Falktoft and Lambert 2004; Mongin and Kimelberg 2005; Heacock et al. 2006; Cheema et al. 2007; Ramos-Mandujano et al. 2007). In HeLa cells, HTC rat hepatoma cells, and Mz-ChA-1 human cholangiocarcinoma cells, PKCα activity was found to be critical for hypotonic (*i.e.* non-GPCR-related) activation of VRAC (Roman et al. 1998; Hermoso et al. 2004). The study by A. Stutzin and colleagues (Hermoso et al. 2004) employed the dominant-negative PKCα expression. The authors found that DN-PKCα blocks swelling activated Cl^- conductance and suppresses the process of regulatory volume decrease under hypoosmotic conditions. In contrast to all the aforementioned studies that showed positive modulation of VRAC activity by PKC, PKCε has been found to inhibit VRAC in pulmonary arterial smooth muscle cells (Zhong et al. 2002).

The evidence for PKC involvement in the effects of ATP and other GPCR agonists is largely based on the effects of pharmacological inhibitors, which may possess a variety of off-target effects (Davies et al. 2000; Bain et al. 2003). We have previously found that although the PKC inhibitors chelerythrine and bisindolylmaleimide potently suppress the effect of ATP on VRAC activity, two other selective PKC blockers, Go6983 and Ro-32-0432, are marginally effective (Mongin and Kimelberg 2005; Haskew-Layton et al. 2005). Our present work unequivocally demonstrates that Ca^{2+}-dependent conventional PKCs are major contributors to the P2Y receptor-dependent VRAC regulation in astrocytes. However, all treatments employed in this study inhibited the ATP-induced potentiation of VRAC activity by ≤60-65%. Thus, additional signaling pathway is also involved. In our previous work, we identified Ca^{2+}/calmodulin kinase II as a complementary signaling enzyme (Mongin and Kimelberg 2005).

Unlike ATP-dependent regulation, VRAC activation by hypoosmotic media in astrocytes is PKC-independent and, thus, drastically different in terms of its cellular mechanisms. This is in agreement with a number of previous findings (Du and Sorota 1999; Falktoft and Lambert 2004; Mongin and Kimelberg 2005; Heacock et al. 2006;Cheema et al. 2007). Additional work is needed to establish if aforementioned PKC dependence of hypoosmotic VRAC activation in HeLa, HTC and Mz-ChA-1 cells (Roman et al. 1998;Hermoso et al. 2004) is restricted to a few cell types or is more universal phenomenon.

Interestingly, since only double PKCα/βI siRNA transfections reduced the effect of ATP on organic osmolyte release, our findings imply that, not one, but two conventional PKC isoforms are involved in the VRAC modulation by ATP. Although peculiar, such a finding is not unique. A cooperative role of PKCs α and β in LDL-induced Akt phosphorylation has recently been found in the THP-1 monocyte cell line (Preiss et al. 2007). In glioma cell lines, PKCβI and βII act cooperatively to inhibit the activity of the proton-sensitive Na^+ channel ANaC2 (ASIC1) (Berdiev et al. 2002). Two interpretations of our data may be considered: (i) PKCα and PKCβI act as functional homologues, replacing each other after downregulation of their counterpart; (ii) these PKCs work in sequence, and activation of one isoform precedes activation of the second isoform. Sequential activation of PKCβI and PKCα has been found to govern the targeted translocation of PKCα to cell-cell contacts in response to hormonal stimulation in pituitary GH3B6 cells (Collazos et al. 2006). However, since in our experiments downregulation of either of the individual isoforms was ineffective; sequential activation of PKC isoforms in astrocytes seems unlikely to play a role in regulation of VRAC activity.

Functional significance for GPCR control of VRAC function in the brain

Analysis of the currently available data suggests that GPCRs are not required for VRAC opening in swollen cells and, therefore, do not constitute an obligatory part of cell volume regulatory mechanism. An early hypothesis that cell swelling activates VRACs indirectly, via an autocrine release of ATP and subsequent activation of P2Y receptors (Wang et al. 1996), was later rejected based on contradicting experimental observations in numerous cell lines (see Hazama et al. 1999; Mongin and Kimelberg 2002; Mongin and Kimelberg 2003, and references therein). Nonetheless, GPCR-related signaling increases VRAC sensitivity to cell swelling and accelerates the process of cell volume regulation (Dezaki et al. 2000; Mongin and Kimelberg 2002; Loveday et al. 2003; Franco et al. 2004), thus potentially aiding in cell volume restoration under normal and pathological conditions.

VRACs are also functionally important for cell proliferation and apoptosis. In the case of proliferation, augmented VRAC activity is generally associated with progression via different stages of cell cycle, while in apoptosis VRACs are paradoxically activated in non-swollen cells and play a part in apoptotic cell shrinkage (reviewed in Lang et al. 2000; Okada et al. 2001; Stutzin and Hoffmann 2006). In astrocytes, ATP stimulates proliferation via the mechanism involving P2Y receptors and PKC (Neary et al. 1999). Furthermore, changes in the activity of individual PKC isoforms differentially affect cell proliferation and differentiation in glioma cells (Brodie et al. 1998; Mandil et al. 2001). It would be interesting to know if GPCRs can modulate the processes of apoptosis and cell proliferation via a VRAC-related mechanism.

ATP and a number of other GPCR agonists produce limited VRAC activation in non-swollen and moderately swollen cells (see for example Mongin and Kimelberg 2002; Darby et al. 2003; Takano et al. 2005; Cheema et al. 2007). This may be important in the context of intercellular signaling in the brain, since VRAC opening results in the release of amino acid neurotransmitters. Glial taurine release via a VRAC-like pathway tonically regulates neuronal activity by acting at the glycine receptors in the hypothalamo-neurohypophysial system (Hussy et al. 2000), and such release is modulated by GPCRs (Rosso et al. 2004). Similarly, VRAC-mediated glutamate release from astrocytes may regulate neuronal activity in other brain areas (Mongin and Kimelberg 2002; Takano et al. 2005; Mulligan and MacVicar 2006). In astrocytes, activation of several GPCRs triggers Ca^{2+}-dependent glutamate release via a putative exocytotic pathway and mediates neuron-astrocyte communication (reviewed in Haydon 2001; Haydon and Carmignoto 2006). Functional involvement of VRAC in such communication has not been experimentally tested.

In conclusion, complementary activation of two Ca^{2+}-sensitive conventional PKC isoforms, α and βI, plays a major role in the ATP receptor-dependent modulation of astrocytic excitatory amino acid release via anionic channel VRAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCE LIST

Abdullaev IF, Rudkouskaya A, Schools GP, Kimelberg HK, Mongin AA. Pharmacological comparison of swelling-activated excitatory amino acid release and Cl^- currents in rat cultured astrocytes. J Physiol. 2006; 572:677–689. [PubMed: 16527858]

Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. Biochem J. 2003; 371:199–204. [PubMed: 12534346]

Banderali U, Roy G. Anion channels for amino-acids in Mdck cells. Am J Physiol. 1992; 263:C1200–C1207. [PubMed: 1282298]

Berdiev BK, Xia J, Jovov B, Markert JM, Mapstone TB, Gillespie GY, Fuller CM, Bubien JK, Benos DJ. Protein kinase C isoform antagonism controls BNaC2 (ASIC1) function. J Biol Chem. 2002; 277:45734–45740. [PubMed: 12244121]

Brodie C, Kuperstein I, Acs P, Blumberg PM. Differential role of specific PKC isoforms in the proliferation of glial cells and the expression of the astrocytic markers GFAP and glutamine synthetase. Brain Res Mol Brain Res. 1998; 56:108–117. [PubMed: 9602083]

Cheema TA, Pettigrew VA, Fisher SK. Receptor regulation of the volume-sensitive efflux of taurine and iodide from human SH-SY5Y neuroblastoma cells: Differential requirements for Ca^{2+} and protein kinase C. J Pharmacol Exp Ther. 2007; 320:1068–1077. [PubMed: 17148779]

Collazos A, Diouf B, Guerineau NC, Quittau-Prevostel C, Peter M, Coudane F, Hollande F, Joubert D. A spatiotemporally coordinated cascade of protein kinase C activation controls isoform-selective translocation. Mol Cell Biol. 2006; 26:2247–2261. [PubMed: 16508001]

Darby M, Kuzmiski JB, Panenka W, Feighan D, MacVicar BA. ATP released from astrocytes during swelling activates chloride channels. J Neurophysiol. 2003; 89:1870–1877. [PubMed: 12686569]

Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J. 2000; 351:95–105. [PubMed: 10998351]

Decher N, Lang HJ, Nilius B, Bruggemann A, Busch AE, Steinmeyer K. DCPIB is a novel selective blocker of $I_{Cl,swell}$ and prevents swelling- induced shortening of guinea-pig atrial action potential duration. Br J Pharmacol. 2001; 134:1467–1479. [PubMed: 11724753]

Dezaki K, Tsumura T, Maeno E, Okada Y. Receptor-mediated facilitation of cell volume regulation by swelling-induced ATP release in human epithelial cells. Jpn J Physiol. 2000; 50:235–241. [PubMed: 10880880]

Du XY, Sorota S. Protein kinase C stimulates swelling-induced chloride current in canine atrial cells. Pflugers Arch. 1999; 437:227–234. [PubMed: 9929563]

Eichholtz T, de Bont DB, de Widt J, Liskamp RM, Ploegh HL. A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor. J Biol Chem. 1993; 268:1982–1986. [PubMed: 8420972]

Falktoft B, Lambert IH. Ca^{2+}-mediated potentiation of the swelling-induced taurine efflux from HeLa cells: On the role of calmodulin and novel protein kinase C isoforms. J Membr Biol. 2004; 201:59–75. [PubMed: 15630544]

Feustel PJ, Jin Y, Kimelberg HK. Volume-regulated anion channels are the predominant contributors to release of excitatory amino acids in the ischemic cortical penumbra. Stroke. 2004; 35:1164–1168. [PubMed: 15017010]

Fields RD, Burnstock G. Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci. 2006; 7:423–436. [PubMed: 16715052]

Franco R, Rodriguez R, Pasantes-Morales H. Mechanisms of the ATP potentiation of hyposmotic taurine release in Swiss 3T3 fibroblasts. Pflugers Arch. 2004; 449:159–169. [PubMed: 15322850]

Haskew-Layton RE, Mongin AA, Kimelberg HK. Hydrogen peroxide potentiates volume-sensitive excitatory amino acid release via a mechanism involving Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem. 2005; 280:3548–3554. [PubMed: 15569671]

Haydon PG. Glia: listening and talking to the synapse. Nat Rev Neurosci. 2001; 2:185–193. [PubMed: 11256079]

Haydon PG, Carmignoto G. Astrocyte control of synaptic transmission and neurovascular coupling. Physiol Rev. 2006; 86:1009–1031. [PubMed: 16816144]

Hazama A, Shimizu T, Ando-Akatsuka Y, Hayashi S, Tanaka S, Maeno E, Okada Y. Swelling-induced, CFTR-independent ATP release from a human epithelial cell line: lack of correlation with volume-sensitive Cl^- channels. J Gen Physiol. 1999; 114:525–533. [PubMed: 10498671]

Heacock AM, Dodd MS, Fisher SK. Regulation of volume-sensitive osmolyte efflux from human SH-SY5Y neuroblastoma cells following activation of lysophospholipid receptors. J Pharmacol Exp Ther. 2006; 317:685–693. [PubMed: 16415087]

Hermoso M, Olivero P, Torres R, Riveros A, Quest AFG, Stutzin A. Cell volume regulation in response to hypotonicity is impaired in HeLa cells expressing a protein kinase C alpha mutant lacking kinase activity. J Biol Chem. 2004; 279:17681–17689. [PubMed: 14960580]

Hussy N, Deleuze C, Desarmenien MG, Moos FC. Osmotic regulation of neuronal activity: a new role for taurine and glial cells in a hypothalamic neuroendocrine structure. Prog Neurobiol. 2000; 62:113–134. [PubMed: 10828380]

Jackson PS, Strange K. Volume-sensitive anion channels mediate swelling-activated inositol and taurine efflux. Am J Physiol. 1993; 265:C1489–C1500. [PubMed: 8279513]

Jeremic A, Jeftinija K, Stevanovic J, Glavaski A, Jeftinija S. ATP stimulates calcium-dependent glutamate release from cultured astrocytes. J Neurochem. 2001; 77:664–675. [PubMed: 11299329]

Kimelberg HK, Goderie SK, Higman S, Pang S, Waniewski RA. Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. J Neurosci. 1990; 10:1583–1591. [PubMed: 1970603]

Kimelberg HK, Mongin AA. Swelling-activated release of excitatory amino acids in the brain: Relevance for pathophysiology. Contrib Nephrol. 1998; 123:240–257. [PubMed: 9761972]

Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D. Functional significance of cell volume regulatory mechanisms. Physiol Rev. 1998; 78:247–306. [PubMed: 9457175]

Lang F, Ritter M, Gamper N, Huber S, Fillon S, Tanneur V, Lepple-Wienhues A, Szabo I, Bulbins E. Cell volume in the regulation of cell proliferation and apoptotic cell death. Cell Physiol Biochem. 2000; 10:417–428. [PubMed: 11125224]

Loveday D, Heacock AM, Fisher SK. Activation of muscarinic cholinergic receptors enhances the volume-sensitive efflux of myo-inositol from SH-SY5Y neuroblastoma cells. J Neurochem. 2003; 87:476–486. [PubMed: 14511125]

Mandil R, Ashkenazi E, Blass M, Kronfeld I, Kazimirsky G, Rosenthal G, Umansky F, Lorenzo PS, Blumberg PM, Brodie C. Protein kinase Calpha and protein kinase Cdelta play opposite roles in the proliferation and apoptosis of glioma cells. Cancer Res. 2001; 61:4612–4619. [PubMed: 11389098]

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem. 1993; 268:9194–9197. [PubMed: 8486620]

Mongin AA. Disruption of ionic and cell volume homeostasis in cerebral ischemia: The perfect storm. Pathophysiology. 2007; 14:183–193. [PubMed: 17961999]

Mongin AA, Kimelberg HK. ATP potently modulates anion channel-mediated excitatory amino acid release from cultured astrocytes. Am J Physiol Cell Physiol. 2002; 283:C569–C578. [PubMed: 12107067]

Mongin AA, Kimelberg HK. Is autocrine ATP release required for activation of volume-sensitive chloride channels? J Neurophysiol. 2003; 90:2791–2792. [PubMed: 14534282]

Mongin AA, Kimelberg HK. ATP regulates anion channel-mediated organic osmolyte release from cultured rat astrocytes via multiple Ca^{2+}-sensitive mechanisms. Am J Physiol Cell Physiol. 2005; 288:C204–C213. [PubMed: 15371260]

Mongin AA, Orlov SN. Mechanisms of cell volume regulation and possible nature of the cell volume sensor. Pathophysiology. 2001; 8:77–88. [PubMed: 11720802]

Mulligan SJ, MacVicar BA. VRACs CARVe a Path for Novel Mechanisms of Communication in the CNS. Sci STKE. 2006; 2006:e42.

Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM. Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. J Neurosci. 1999; 19:4211–4220. [PubMed: 10341225]

Nilius B, Droogmans G. Amazing chloride channels: an overview. Acta Physiol Scand. 2003; 177:119–147. [PubMed: 12558550]

Nilius B, Eggermont J, Voets T, Buyse G, Manolopoulos V, Droogmans G. Properties of volume-regulated anion channels in mammalian cells. Prog Biophys Mol Biol. 1997; 68:69–119. [PubMed: 9481145]

Okada Y. Volume expansion-sensing outward-rectifier Cl^- channel: fresh start to the molecular identity and volume sensor. Am J Physiol Cell Physiol. 1997; 273:C755–C789.

Okada Y. Cell volume-sensitive chloride channels: phenotypic properties and molecular identity. Contrib Nephrol. 2006; 152:9–24. [PubMed: 17065805]

Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S. Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). J Physiol. 2001; 532:3–16. [PubMed: 11283221]

Parker PJ, Murray-Rust J. PKC at a glance. J Cell Sci. 2004; 117:131–132. [PubMed: 14676268]

Pasantes-Morales H, Moran J, Schousboe A. Volume-sensitive release of taurine from cultured astrocytes: properties and mechanism. Glia. 1990; 3:427–432. [PubMed: 2146228]

Pasantes-Morales H, Murray RA, Sanchez-Olea R, Moran J. Regulatory volume decrease in cultured astrocytes. II. Permeability pathway to amino acids and polyols. Am J Physiol. 1994; 266:C172–C178. [PubMed: 8304414]

Phillis JW, Song D, O'Regan MH. Inhibition by anion channel blockers of ischemia-evoked release of excitotoxic and other amino acids from rat cerebral cortex. Brain Res. 1997; 758:9–16. [PubMed: 9203527]

Preiss S, Namgaladze D, Brune B. Critical role for classical PKC in activating Akt by phospholipase A_2-modified LDL in monocytic cells. Cardiovasc Res. 2007; 73:833–840. [PubMed: 17261275]

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998; 50:413–492. [PubMed: 9755289]

Ramos-Mandujano G, Vazquez-Juarez E, Hernandez-Benitez R, Pasantes-Morales H. Thrombin potently enhances swelling-sensitive glutamate efflux from cultured astrocytes. Glia. 2007; 55:917–925. [PubMed: 17437307]

Roman RM, Bodily KO, Wang Y, Raymond JR, Fitz JG. Activation of protein kinase Calpha couples cell volume to membrane Cl^- permeability in HTC hepatoma and Mz-ChA-1 cholangiocarcinoma cells. Hepatology. 1998; 28:1073–1080. [PubMed: 9755245]

Rosso L, Peteri-Brunback B, Poujeol P, Hussy N, Mienville JM. Vasopressin-induced taurine efflux from rat pituicytes: a potential negative feedback for hormone secretion. J Physiol. 2004; 554:731–742. [PubMed: 14617676]

Seki Y, Feustel PJ, Keller RW Jr. Tranmer BI, Kimelberg HK. Inhibition of ischemia-induced glutamate release in rat striatum by dihydrokinate and an anion channel blocker. Stroke. 1999; 30:433–440. [PubMed: 9933284]

Singer, HA. Protein kinase C. In: Barany, M., editor. Biochemistry of Smooth Muscle Contraction. Academic Press; San Diego: 1996. p. 155-165.

Slepko N, Patrizio M, Levi G. Expression and translocation of protein kinase C isoforms in rat microglial and astroglial cultures. J Neurosci Res. 1999; 57:33–38. [PubMed: 10397633]

Strange K, Emma F, Jackson PS. Cellular and molecular physiology of volume-sensitive anion channels. Am J Physiol. 1996; 270:C711–C730. [PubMed: 8638650]

Stutzin A, Hoffmann EK. Swelling-activated ion channels: functional regulation in cell-swelling, proliferation and apoptosis. Acta Physiol (Oxf). 2006; 187:27–42. [PubMed: 16734740]

Takano T, Kang J, Jaiswal JK, Simon SM, Lin JH, Yu Y, Li Y, Yang J, Dienel G, Zielke HR, Nedergaard M. Receptor-mediated glutamate release from volume sensitive channels in astrocytes. Proc Natl Acad Sci U S A. 2005; 102:16466–16471. [PubMed: 16254051]

Tilly BC, Edixhoven MJ, van den BN, Bot AG, De Jonge HR. Ca^{2+}-mobilizing hormones potentiate hypotonicity-induced activation of ionic conductances in Intestine 407 cells. Am J Physiol. 1994; 267:C1271–C1278. [PubMed: 7977690]

Wang J, Bright R, Mochly-Rosen D, Giffard RG. Cell-specific role for epsilon- and betaI-protein kinase C isozymes in protecting cortical neurons and astrocytes from ischemia-like injury. Neuropharmacology. 2004; 47:136–145. [PubMed: 15165841]

Wang Y, Roman R, Lidofsky SD, Fitz JG. Autocrine signaling through ATP release represents a novel mechanism for cell volume regulation. Proc Natl Acad Sci U S A. 1996; 93:12020–12025. [PubMed: 8876255]

Young S, Parker PJ, Ullrich A, Stabel S. Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem J. 1987; 244:775–779. [PubMed: 3446191]

Zhong J, Wang GX, Hatton WJ, Yamboliev IA, Walsh MP, Hume JR. Regulation of volume-sensitive outwardly rectifying anion channels in pulmonary arterial smooth muscle cells by PKC. Am J Physiol Cell Physiol. 2002; 283:C1627–C1636. [PubMed: 12388117]

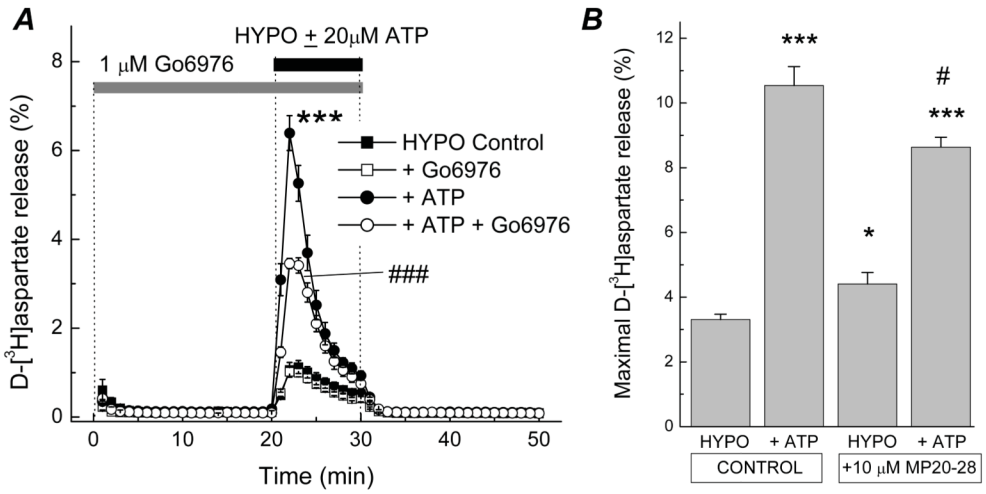

Fig. 1. The conventional PKC inhibitor Go6976 and the membrane-permeable PKCα/β inhibitory peptide (MP20-28) reduce the ATP-induced stimulation of the excitatory amino acid release from swollen rat astrocytes

A, Primary astrocyte cultures were loaded with D-[^{3}H]aspartate overnight, washed from the extracellular isotope, and then superfused with isoosmotic or hypoosmotic media as indicated. Go6976 (1 μM) was added 20 min before and during application of hypoosmotic medium. 20 μM ATP was added to hypoosmotic medium only. Data are the mean values ±SE of 5-6 experiments in each group. ***, $p<0.001$, ATP vs. all other groups; ###, $p<0.001$ ATP+Go6976 vs. all other groups. ***B***, Similar experiment performed with the inhibitory peptide MP20-28. For clarity, only maximal release values under hypoosmotic conditions are presented. Cells were pretreated with 10 μM MP20-28 for 30 min before the efflux experiment. The peptide was not present in the release assay media. Data are means ±SE of 5-6 experiments in each group. *$p<0.05$, ***$p<0.001$, vs. hypoosmotic control. #$p=0.05$, ATP vs. ATP+MP20-28.

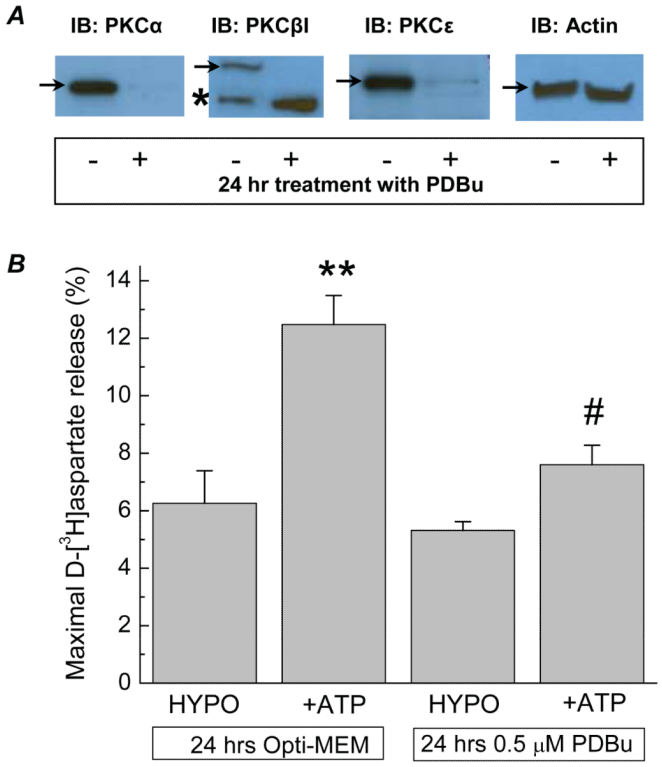

Fig. 2. Phorbol ester-induced PKC downregulation strongly inhibits the effect of ATP on swelling-activated D-[^{3}H]aspartate release

A, Representative Western blots showing downregulation of conventional PKCs α and βI, and novel PKCε in astrocyte cultures treated with the phorbol ester PDBu (500 nM) for 24 hrs in serum-free opti-MEM. Actin immunoreactivity in the same cell lysate is shown as a protein loading control. Arrows indicate positions of the predicted molecular weights for PKCα (~82 kD), PKC βI (~79kD), PKCε (~95 kD) and actin (~42 kD). * marks likely product of proteolytic degradation of PKC βI. ***B***, Maximal D-[^{3}H]aspartate release values in cultured astrocytes exposed to hypoosmotic medium in the presence/absence of 20 μM ATP. Cells were maintained in opti-MEM for 24 hrs in the presence/absence of 500 nM PDBu as indicated. Data are the mean values ±SE of 5 experiments in each group. **p=0.01 vs. hypoosmotic control, $^{\#}$p<0.05 vs. hypoosmotic control in the PDBu-treated cells.

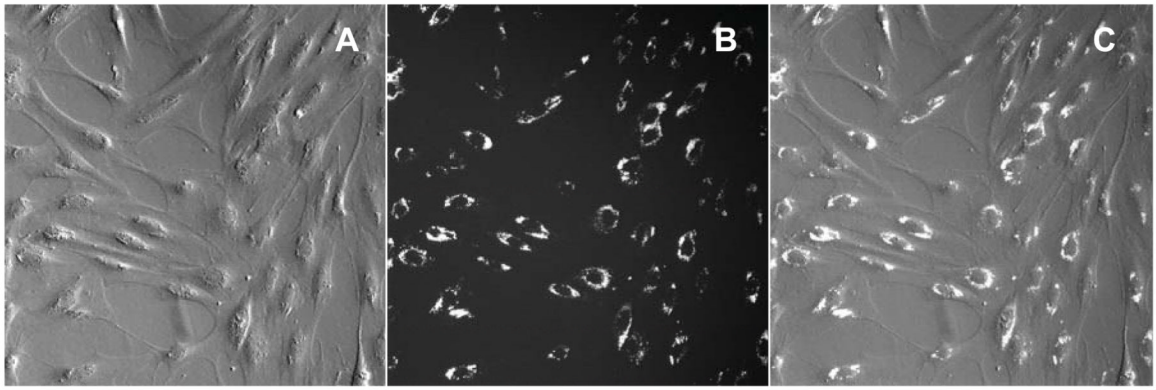

Fig. 3. Representative micrographs showing primary astrocyte cultures transfected with scrambled siRNA labeled with Alexa-488

A, Nomarski DIC optic image of siRNA-transfected cultured astrocytes, 72 hrs after transfection. ***B***, The same optical field showing Alexa-488 fluorescence captured with FITC set of filters. Florescent signal was largely restricted to puncta surrounding nuclei; dim fluorescent signal in cytosol is not seen because of the difference in the signal intensities. ***C***, Overlap of DIC and fluorescence images. All images were captured on Olympus IX-71 fluorescence microscope at 16× magnification.

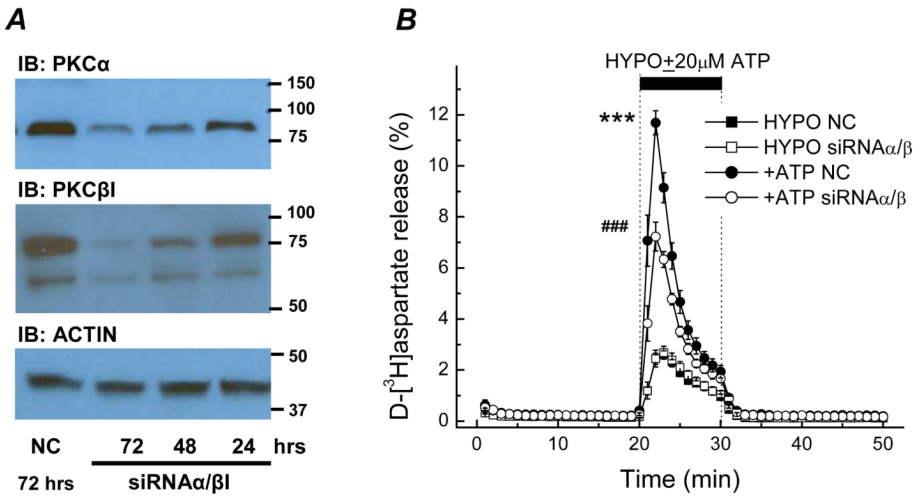

Fig. 4. siRNA-induced downregulation of conventional PKC isoforms α and βI attenuates the effect of ATP on swelling-activated excitatory amino acid release

A, Representative Western blots showing the effect of the siRNA mix targeting PKCα and βI on the PKCα and PKCβI protein levels at 24-72 hrs post-transfection. **NC**, negative control, cells treated with the scrambled siRNA for 72 hrs. Actin staining in a stripped blot is shown as a loading control. Bars and numbers on the right show positions of molecular weight markers. ***B***, The effect of PKCα and βI downregulation on the ATP-induced D-[^{3}H]aspartate release from swollen astrocytes. Cells were treated with siRNA mix for 72 hrs. Scrambled siRNA was used as a negative control (**NC**). Three independent siRNA transfections were performed. Data represent the mean values ±SE of 5-6 experiments in each group. *** $p<0.001$, ATP NC vs. HYPO NC. ### $p<0.01$, siRNA treatment significantly decreased D-[^{3}H]aspartate release in the presence of ATP.

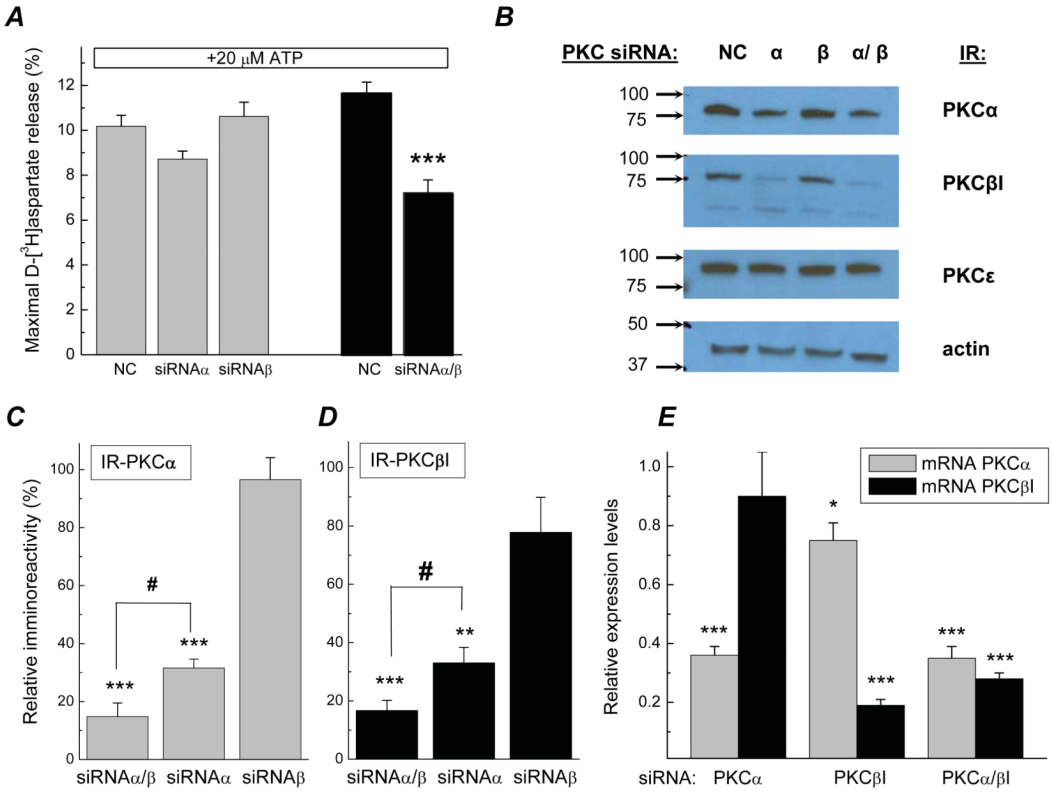

Fig. 5. Independent downregulation of PKCα or βI does not affect the ATP-induced D-[^{3}H]aspartate release from swollen cells

A, Effect of siRNA transfections targeting PKCα or βI on the swelling-activated D-[^{3}H]aspartate release in the presence of ATP. The results were always compared to the matching negative controls (**NC**) treated with scrambled siRNA on the same day. The experimental design was identical to that presented in Fig. 4B. For clarity, only the maximal release values are presented. Data are the mean values ±SE of 5 experiments in each group. For comparison, the effect of α/βI siRNA mix is shown on the right (the data are taken from the experiments presented in Fig. 4B). ***p<0.001 vs. NC. ***B***, Representative Western Blots showing changes in the PKC isozyme levels 72 hrs after transfection of astrocytes with siRNA-PKCα (α), siRNA-PKC βI (β), or their combination (α/β). Immunostaining of PKCε and actin was used to control for the specificity of the siRNA effects and protein loading. Arrows and numbers on the left show positions of molecular weight markers. ***C,*** Quantified changes in the PKCα immunoreactivity in cells treated with siRNA targeting PKCα, βI, or α +βI. Protein lysates from cultures treated with siRNA for 72 hrs were subjected to Western blot analysis using a monoclonal anti-PKCα antibody. The integral density of an immunoreactive band of ~82 kD was compared to the PKC band in protein lysates from cells treated with scrambled siRNA. Mean normalized optical densities ±SE of 8 independent protein preparations are shown. ***p<0.001 vs. scrambled siRNA, #p<0.05 siRNAα vs. siRNAα/βI. ***D***, Quantified changes in the PKCβI immunoreactivity in cells treated with siRNA targeting PKCα, βI, or α+βI. Protein lysates were analyzed using monoclonal anti-PKCβI antibody recognizing the band of ~80 kD. Mean normalized optical densities ±SE of 4 independent protein preparations are shown. ***p<0.05, **p<0.01 vs. scrambled siRNA. *p<0.05 vs. scrambled siRNA. #p<0.01 siRNAα vs. siRNAα/βI. ***E***, Quantitative RT-PCR analysis of the mRNA levels for PKCα and PKCβI in astrocyte cultures treated for 24 hrs with siRNA targeting PKCα, PKCβI, or their combination. All message levels were normalized to the levels of GAPDH in the same samples. Data are the

mean values of 3 independent qRT-PCR analyses performed for two independent siRNA transfections (n=6). *p<0.05, ***p<0.001 vs. scrambled siRNA control.