Ca²⁺ Nanodomain-Mediated Component of Swelling-Induced Volume-Sensitive Outwardly Rectifying Anion Current Triggered by Autocrine Action of ATP in Mouse Astrocytes

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Key Words

Volume-sensitive outwardly rectifying anion channel • Ca²⁺ nanodomain • Astrocyte • ATP • Autocrine signalling

Abstract

The volume-sensitive outwardly rectifying (VSOR) anion channel provides a major pathway for anion transport during cell volume regulation. It is typically activated in response to cell swelling, but how the channel senses the swelling remains unclear. Meanwhile, we recently found that in mouse astrocytes the channel is activated by an inflammatory chemical mediator, bradykinin, without cell swelling and that the activation is regulated via high concentration regions of intracellular Ca²⁺ ([Ca²⁺]_i) in the immediate vicinity of open Ca²⁺-permeable channels, so-called Ca²⁺ nanodomains. Here we investigated whether a similar mechanism is involved in the swelling-induced VSOR channel activation in the astrocytes. A hypotonic stimulus (25% reduction in osmolality) caused the [Ca²⁺]_i rises in the astrocytes, and the rises were abolished in the presence of an ATP-degrading enzyme, apyrase (10 U/ml). Application of ATP (100 μM) under isotonic conditions generated the current through VSOR channels via Ca²⁺ nanodomains, as bradykinin does. The current induced by the hypotonic stimulus was suppressed by ~40% in the Ca²⁺-depleted condition where the ATP-induced VSOR

current was totally prevented. Thus the swelling-induced VSOR channel activation in mouse astrocytes is partly regulated via Ca²⁺ nanodomains, whose generation is triggered by an autocrine action of ATP.

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Introduction

Cell volume regulation is essential for maintenance of cell integrity and functions. The regulation is attained by regulating the net influx or efflux of water and solutes across the plasma membrane. The volume-sensitive outwardly rectifying (VSOR) anion channel provides a major pathway for anion flux during the regulation in most types of animal cells. The VSOR channel is typically activated in response to an increase in cell volume beyond a threshold, and the channel activity is known to be enhanced linearly with increasing cell surface area [1]. How the channel senses cell swelling, however, is not elucidated yet despite the studies over many years [2]. One of the reasons would stem from the fact that the molecular identity of the VSOR channel is not yet confirmed, although many research groups had proposed many candidates, all of which were challenged or negated later [1-4].

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On the other hand, it has recently been confirmed that the VSOR channel may be activated even without cell swelling when certain types of stimulus are applied onto the cells. One of the signalling cascades known to be involved is the one inducing the generation of intracellular reactive oxygen species (ROS) by NADPH oxidases (NOX) [5-8]. Our group demonstrated for the first time that the VSOR channel activation mediated by the cascade causes the cell shrinkage prerequisite for apoptotic cell death [5, 9]. However, the ROS generation is not always accompanied by VSOR channel activation in some types of cell [10], and other unknown pathways without ROS may also induce the channel activation [5]. Several years ago, we reported that, in mouse astrocytes, an inflammatory chemical mediator, bradykinin, may elicit NOX-mediated ROS generation and VSOR channel activation [11]. Recently, we found that this VSOR channel activation is driven by intracellular Ca^{2+} release, store- or receptor-operated Ca^{2+} entry and activation of Ca^{2+} -dependent protein kinases $\text{C}\alpha$ and β , all of which are triggered by G_q protein-coupled bradykinin B2 receptor activation [12]. An important point is that all the Ca^{2+} -dependent processes in the VSOR channel activation are accomplished in the immediate vicinity of individual open Ca^{2+} -permeable channels, where a very high intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is generated, so-called “ Ca^{2+} nanodomains” [12]. Since volume-sensitive ion channels and transporters also contribute to cell shape changes, migration and division [4, 13, 14], the Ca^{2+} nanodomain-mediated mechanism should provide a firm basis for local activation of VSOR channels inducing cell shape changes even when only a minute amount of bradykinin released from surrounding tissues acts on a part of the cell.

It is well known that astrocytes have many types of G_q -coupled receptor for diverse agonists related not only to pathological conditions, but also to physiological ones, like ATP or glutamate, and the stimulation with some of them may indeed induce ROS generation [15]. Thus it is very likely that such an agonist may also induce the VSOR channel activation mediated by Ca^{2+} nanodomains as bradykinin does. Moreover, in many types of cells including astrocytes, cell swelling is usually accompanied by the release of ATP from themselves [16]. Our group previously demonstrated that ATP is released via Maxi-anion channels from the astrocytes upon cell swelling and that the amount of the release increases with increasing strength of hypotonicity [17]. Thus, the VSOR channel activation caused by the autocrine action of ATP is possible to contribute to the swelling-induced VSOR channel activity during a hypotonic stimulus, and this may explain,

at least partly, the dependence of the swelling-induced channel activity on cell size. Such a possibility was in fact previously suggested first in rat hepatoma cell [18] and later also in rat astrocytes [19], although the mechanism how the autocrine action augments the VSOR channel activity is still rather controversial [20-25]. Especially, whether Ca^{2+} -dependent processes may be involved or not in the swelling-induced VSOR channel activation has long been debated, but most reports have supported no essential contribution of intracellular Ca^{2+} [1, 3, 4]. However, because the Ca^{2+} in the nanodomains is unlikely to be captured even by a fast Ca^{2+} chelator BAPTA and because the $[\text{Ca}^{2+}]_i$ in the nanodomains is proportional to the single-channel Ca^{2+} current [26], not only adding Ca^{2+} chelators into the cells but also thoroughly removing Ca^{2+} both from the extracellular solution and from the intracellular Ca^{2+} stores are required for determining whether Ca^{2+} is involved even partly or not, although this point was not systematically examined in the previous reports.

Here we demonstrate that the swelling-induced intracellular Ca^{2+} signalling in mouse astrocytes depends greatly on the autocrine action of ATP and that the Ca^{2+} nanodomain-mediated VSOR channel activation is indeed involved partly in the swelling-induced activity of VSOR channels.

A preliminary report was published in abstract form [27].

Materials and Methods

Cell preparation

Astrocytes obtained from the cerebral cortices of 2 days-old ddY mouse pups were cultured and used as described previously [12]. The cells were cultivated until reaching confluence in flasks at 37°C in a humidified 5% CO_2 /95% air environment with the minimum essential medium supplemented with 10% fetal bovine serum, 40 U ml^{-1} penicillin and 100 $\mu\text{g ml}^{-1}$ streptomycin. Then the cells were trypsinized and plated on polyethyleneimine-coated glass coverslips at a density of 5×10^5 cells cm^{-2} , and used after overnight culture.

$[\text{Ca}^{2+}]_i$ measurements

Cells were loaded with the Ca^{2+} indicator, fura-2, in the culture medium containing 0.5 μM fura-2 AM (Biotium, Hayward, CA, USA) for 30 min at 37°C. The cells on the coverslips were placed in the recording chamber mounted on the stage of an Olympus IX81 inverted microscope (Olympus, Tokyo, Japan) and perfused at room temperature with the Ringer solution containing (in mM): 100 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 NaHEPES, 6 HEPES, 5 glucose and 70 mannitol (pH 7.4, 285 mosmol ($\text{kg H}_2\text{O}^{-1}$)). The hypotonic solution was made by

removing mannitol. When Ca^{2+} was removed from the bathing solution, the equimolar Mg^{2+} and 0.1 mM EGTA were added to the solution. The cytoplasmic fura-2 was excited alternatively with 340 nm light (26 nm bandwidth) and 380 nm light (11 nm bandwidth) from a mercury arc lamp (USH-1030L, USHIO, Tokyo, Japan), which was controlled with a Ludl MAC6000 filter controller (Ludl Electronic Products, Hawthorne, NY, USA). The emitted fluorescence was detected at 510 nm (84 nm bandwidth) through a 40x water immersion objective (UApo40XW340, NA1.15, Olympus Japan) with a cooled CCD camera (ORCA-NRG, Hamamatsu Photonics, Hamamatsu, Japan). The operations were made via AquaCosmos software (Hamamatsu Photonics). The image pairs obtained with 340 nm light and with 380 nm light were recorded at 2-s intervals. Image analysis was performed off-line with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The region of interest was set to include most of the cytoplasm for each cell. The ratios of the fluorescence intensities obtained with 340 nm light (F_{340}) to those with 380 nm light (F_{380}) in the same regions were calculated after background subtraction.

Quantitative RT-PCR

Quantitative real-time PCR was performed in an Applied Biosystems 7300 system (Life Technologies, Carlsbad, CA, USA) as described previously [12]. The total RNA in the cells was extracted with an RNeasy Mini kit (QIAGEN, Hilden, Germany), and reverse transcription with elimination of genomic DNA was made with a QuantiTect kit (QIAGEN) in a GeneAmp 9700 thermal cycler (Life Technologies). TaqMan Gene Expression Assay kits (Life Technologies) were used to detect the mRNAs of mouse P2Y_1 (assay ID: Mm00435471_m1), P2Y_2 (Mm00435472_m1), P2Y_4 (Mm00445136_s1) and β -actin (Mm00607939_s1) as an endogenous reference. No amplification signals after omission of reverse transcription were checked for all probes (data not shown). mRNA expression levels were analyzed by the comparative $\Delta\Delta C_T$ method, using the level of P2Y_1 mRNA as a calibrator. Measurements were repeated three times using the mRNA preparations isolated from different cultures.

Patch-clamp studies

The currents through VSOR channels were recorded under the whole-cell patch voltage-clamp mode with an EPC10 amplifier controlled via PATCHMASTER software (HEKA Elektronik, Lambrecht/Pfalz, Germany). The extracellular bathing solution used for hypotonic experiments contained (in mM): 105 N-methyl-D-glucamine (NMDG)Cl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 5 glucose and 0 or 65 mannitol (pH 7.4, 220 or 285 mosmol ($\text{kg H}_2\text{O}^{-1}$)). The patch pipette solution contained (in mM): 105 NMDGCl, 1 MgCl_2 , 3 Na_2ATP , 5 HEPES, 1 EGTA with 0.2 CaCl_2 (estimated free $[\text{Ca}^{2+}] \approx 15$ nM) and 55 mannitol (pH 7.4, 275 mosmol ($\text{kg H}_2\text{O}^{-1}$)). The extracellular isotonic solution for ATP application contained (in mM): 135 NMDGCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES and 5 glucose (pH 7.4, 280 mosmol ($\text{kg H}_2\text{O}^{-1}$)). The pipette solution for this purpose contained (in mM): 135 NMDGCl, 1 MgCl_2 , 3 Na_2ATP , 5 HEPES and 1 EGTA with 0.2 CaCl_2 (estimated free $[\text{Ca}^{2+}] \approx 15$ nM; pH 7.4, 275 mosmol ($\text{kg H}_2\text{O}^{-1}$)). When high concentrations of EGTA or BAPTA

were added to the pipette solution, the Cl^- concentration was reduced to keep the same osmolality, and CaCl_2 was added to maintain the same free $[\text{Ca}^{2+}]$. When Ca^{2+} was removed from the extracellular solution, the equimolar Mg^{2+} and 0.1 mM EGTA were added to the solution. Patch pipettes were made from borosilicate glass capillaries and the pipette resistance before patch formation was 2–3 M Ω . The current signals were filtered at 1 kHz and sampled at 5 kHz. Liquid junction potentials were calculated with JPCalc in pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA) and corrected on-line. The series resistance during the recording was kept at <10 M Ω and compensated for by 70%. Recording was performed at room temperature.

Cell volume measurements

Cell volume changes were measured as the changes in mean cell volume with a Coulter-type cell size analyzer (CDA-500, Sysmex, Kobe, Japan). Cells were first suspended in the isotonic Ringer solution containing (in mM): 135 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 5 NaHEPES, 6 HEPES and 5 glucose (pH 7.4, 285 mosmol ($\text{kg H}_2\text{O}^{-1}$)). Then the initial mean cell volume was measured before hypotonic shock or ATP application. The changes in mean cell volume after the stimulus were normalized to the initial value. The hypotonic shock was applied by adding an equal volume of the solution containing 65 mM NaCl and the unchanged concentrations of other solutes from those in the isotonic solution. This yielded the final 100 mM NaCl without a change in the other composition (pH 7.4, 215 mosmol ($\text{kg H}_2\text{O}^{-1}$)). For Ca^{2+} removal experiments, cells were preincubated in the culture medium containing 1 μM thapsigargin and 50 μM BAPTA-AM (Dojindo, Kumamoto, Japan) for 30 min, then the cells were suspended in the Ca^{2+} -free Ringer solution (supplemented with 0.1 mM EGTA and 2 mM MgCl_2) and used for the experiments.

Drugs

Thapsigargin and suramin were purchased from Wako Chemicals (Osaka, Japan). Apyrase (grade I, A6132), U73122, U73343 and 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid (DCPIB) were from Sigma-Aldrich (St. Louis, MO, USA). All drugs were first prepared as 100–2000x stock solutions and stored frozen. U73122 and U73343 were first dissolved in chloroform at 10 mM. Thapsigargin and DCPIB were in dimethyl sulfoxide (DMSO) as 1000x stock. Apyrase was in water at 1 kU/ml. Suramin was in the bathing solution as 100x stock. On the day of use, these were thawed and diluted with the bathing solution.

Statistics

Data in text and those with error bars in graphs are shown as mean $\pm$ SEM. Statistical multiple comparisons were made by Peritz's closed testing procedure with the F statistic after ANOVA tests. Differences were judged as significant when the P of making at least one incorrect assertion in one set of multiple comparisons, called the experimentwise error rate, was <0.05. Comparison between 2 groups was assessed by Student's unpaired t test.

Fig. 1. Effects of Ca^{2+} depletion, inhibition of ATP action, and an inhibitor of phospholipase C on hypotonicity-induced $[\text{Ca}^{2+}]_i$ rises in cultured mouse astrocytes. The rises are indicated as the changes in fura-2 ratio (F_{340}/F_{380}). The dashed line indicates the basal F_{340}/F_{380} level before the hypotonic stimulus. (Aa) Hypotonicity (75% osmolality)-induced $[\text{Ca}^{2+}]_i$ rise averaged over 227 cells as control. (Ab) The $[\text{Ca}^{2+}]_i$ rise in the absence of extracellular Ca^{2+} (0 Ca^{2+} , $n = 244$). 0.1 mM EGTA was added in the bathing solution. (Ac) The rise in the absence of extracellular Ca^{2+} observed after pretreatment with 1 μM thapsigargin (Thap.) for 30 min ($n = 247$). Thapsigargin was included in the fura-2 loading solution but not in the bathing solution. (B) Effects of 100 μM suramin (Ba, $n = 330$) and 10 U/ml apyrase (Bb, $n = 357$) on the $[\text{Ca}^{2+}]_i$ rise. The drugs were present both in the loading solution and in the bathing solution. (C) Effects of 5 μM U73343 (Ca, $n = 638$) and 5 μM U73122 (Cb, $n = 505$) on the $[\text{Ca}^{2+}]_i$ rise in the absence of extracellular Ca^{2+} . The drugs were present both in the loading solution and in the bathing solution.

Results

Swelling-induced $[\text{Ca}^{2+}]_i$ rises in mouse astrocytes are triggered by autocrine action of ATP

First we examined whether and how the swelling-induced $[\text{Ca}^{2+}]_i$ rise is generated in mouse astrocytes. Application of a hypotonic solution (25% reduction in osmolality) to cultured astrocytes elicited the $[\text{Ca}^{2+}]_i$ rise consisting of two rising phases (Fig. 1Aa). The first one is the initial large, rapidly rising phase, which dissipates within 1 min. The amplitude of the initial rise was 1.03 ± 0.038 in $\Delta F_{340}/F_{380}$ ($n = 227$) in our measurement system. The second one is the following small, slowly decaying phase, which continues over >20 min. This phase was evaluated as the time integral (area) of the net F_{340}/F_{380} rise from the basal level (indicated by dashed lines in Fig. 1) over 1–20 min after the beginning of hypotonic stimulation, which was 72.6 ± 10.6 in $(\Delta(F_{340}/F_{380}) \times \text{sec})$. Removal of extracellular Ca^{2+} suppressed the initial phase by half (0.51 ± 0.020 in $\Delta F_{340}/F_{380}$, $P < 0.05$ by multiple comparisons) and completely the second phase (0.19 ± 3.7 in $(\Delta(F_{340}/F_{380}) \times \text{sec})$, $P < 0.05$ by multiple comparisons; Fig. 1Ab). Pretreatment with a blocker of endoplasmic reticulum (ER) Ca^{2+} pumps,

Fig. 2. mRNA expression levels of G_q protein-coupled purinergic (P2Y₁, P2Y₂, P2Y₄) receptors in mouse astrocytes determined by quantitative RT-PCR. The expression levels are normalized to that of P2Y₁. Numbers indicate the mean values. P2Y₄ mRNA was not detected (ND). The results were obtained from total 30 pups born from 3 mother mice.

thapsigargin (1 μM), in addition to the Ca^{2+} removal eradicated the initial rise (0.072 ± 0.005 in $\Delta F_{340}/F_{380}$, $P < 0.05$ by multiple comparisons) without further changes in the second phase (1.28 ± 3.6 in $(\Delta(F_{340}/F_{380}) \times \text{sec})$, n.s. by multiple comparisons; Fig. 1Ac). These indicate that the initial phase would be caused by both intracellular Ca^{2+} release from ER Ca^{2+} stores and the Ca^{2+} entry at the plasma membrane, and that the second phase is exclusively caused by the Ca^{2+} entry.

Fig. 3. ATP-induced activation of VSOR anion current under isotonic conditions. In A-C, the time course of development and suppression of the whole-cell current was monitored by repetitively applying a pair of alternating step voltage pulses to ± 40 mV (0.5 s duration) from the holding potential of 0 mV every 5 s. ATP (100 μ M) and DCPIB (10 μ M) were applied through bath perfusion under isotonic conditions. The arrowhead indicates the 0 current level. To examine the changes in voltage dependency of the current, test voltage pulses were applied before application of ATP (open circles), at the maximum plateau of the current enhancement (open squares, diamonds and triangles), and after suppression to the minimum caused by DCPIB (filled symbols). The corresponding current responses are shown below on an expanded time scale. Test voltage pulses consisted of the pulses (0.5 s duration) ranging from -100 mV to $+100$ mV in 20 mV increments with fixed pre-pulses (0.1 s duration) to -100 mV and post-pulses (0.1 s duration) to -80 mV. (A) The current induced by ATP and its suppression by DCPIB. (B) ATP-induced current in the presence of intracellular EGTA at 10 mM and its suppression by DCPIB. EGTA was applied via patch pipettes. (C) ATP-induced current observed after 30 min pretreatment with 1 μ M thapsigargin and adding 10 mM BAPTA in the pipette solution and 0.1 mM EGTA in the Ca^{2+} -free bathing solution. The current suppression by DCPIB was to a negligible degree. Thapsigargin was not included during the current recording. (D) Current-voltage (I-V) relationships of the whole-cell currents under the conditions shown in A-C. The same symbols as in A-C are used to indicate the corresponding relationships. Each symbol represents the mean $\pm$ SEM of the peak current amplitude elicited by a test voltage pulse in current density (pA pF^{-1}). The peak amplitude was determined at 20 ms after the beginning of the pulse. (Da) I-V relationships under the conditions shown in A. The number of cells analyzed was 18. (Db) The relationships under the conditions shown in B. The number of cells analyzed was 15. (Dc) The relationships under the conditions shown in C. The number of cells analyzed was 17. (E) I-V relationships of the DCPIB-sensitive current components in Da-c. Symbols indicate the mean $\pm$ SEMs of the differences between the currents just before and after the DCPIB treatment in individual cells shown in Da (gray squares), Db (gray diamonds) and Dc (gray triangles), respectively. There was no significant difference between the DCPIB-sensitive current component in Da (29.4 ± 3.4 pA pF^{-1} at $+100$ mV, $n = 18$) and that in Db (32.4 ± 4.0 pA pF^{-1} , $n = 15$; n.s. by multiple comparisons). By contrast, the component in Dc (2.4 ± 0.8 pA pF^{-1} , $n = 17$) was evidently much smaller than those ($P < 0.001$ by ANOVA). The mean membrane capacitance of the cells used here was 38.4 ± 3.2 pF.

This pattern of the $[\text{Ca}^{2+}]_i$ rise was essentially the same as that of the bradykinin-induced $[\text{Ca}^{2+}]_i$ rise in our previous study [12]. Since this hypotonic stimulus should elicit the release of ATP from the cells in this preparation [17], we next examined whether the released ATP participates in the generation of the swelling-induced

$[\text{Ca}^{2+}]_i$ rise. In the presence of a non-specific antagonist of purinergic receptors, suramin (100 μ M), the initial phase of the $[\text{Ca}^{2+}]_i$ rise was greatly suppressed (0.15 ± 0.011 in $\Delta F_{340}/F_{380}$, $P < 0.05$ by multiple comparisons; Fig. 1Ba). The second phase was also basically suppressed greatly, although some irreversible $[\text{Ca}^{2+}]_i$ development occurred frequently >10 min after the hypotonic stimulus (Fig. 1Ba). When the hypotonic stimulus was applied in the presence of an enzyme degrading ATP and ADP, apyrase (10 U/ml), both phases of the $[\text{Ca}^{2+}]_i$ rise were abolished (the initial amplitude: 0.18 ± 0.015 in $\Delta F_{340}/F_{380}$, $P < 0.05$ by multiple comparisons; the area: 17.0 ± 2.9 in $\Delta(F_{340}/F_{380}) \times \text{sec.}$, $P < 0.05$ by multiple comparisons; Fig. 1Bb). Therefore, these indicate that the swelling-induced $[\text{Ca}^{2+}]_i$ rise in mouse astrocytes depends totally on the autocrine action of ATP released in response to the hypotonic stimulus.

Suppression of the initial Ca^{2+} release by inhibition of ATP action also implies that the Ca^{2+} release must be triggered by activation of G_q -coupled purinergic receptors inducing intracellular generation of inositol trisphosphate (IP_3). Indeed, the swelling-induced Ca^{2+} release elicited in the absence of extracellular Ca^{2+} was suppressed in the presence of an inhibitor of the IP_3 -generating phospholipase C, U73122 (5 μ M; 0.11 ± 0.009 in $\Delta F_{340}/F_{380}$; Fig. 1Cb), compared to its inactive analog, U73343 (5 μ M; 0.37 ± 0.013 in $\Delta F_{340}/F_{380}$, $P < 0.001$ by unpaired

Fig. 4. Effects of thorough removal of extracellular and intracellular Ca^{2+} on the VSOR current induced by application of a hypotonic solution (75% osmolality). Development and suppression of VSOR currents were monitored during application of voltage pulses as in Fig. 3. Note that the scale of the current is much larger than those in Fig. 3. (A) Hypotonicity-induced VSOR current in the normal Ca^{2+} -containing condition. (B) Hypotonicity-induced VSOR current in the presence of intracellular BAPTA at 10 mM and the absence of extracellular Ca^{2+} (supplemented with 0.1 mM EGTA) after 30 min pretreatment with 1 μM thapsigargin. Thapsigargin was not included during the current recording. Symbols in A and B indicate the responses to test voltage pulses as in Fig. 3. (C) I-V relationships of the currents before (open symbols) and after the DCPIB treatment (filled symbols) under the conditions shown in A (squares) and B (triangles). The amplitudes of the currents before the DCPIB treatment in both conditions were so large that the remaining components after the DCPIB treatment (11.9 ± 2.7 pA pF $^{-1}$ at +100 mV in A, $n = 15$, and 14.5 ± 2.8 pA pF $^{-1}$ in B, $n = 13$) were negligibly small. The asterisk indicates a significant difference in the amplitude at +100 mV at $P < 0.05$ by unpaired t test.

t test; Fig. 1Ca). As ATP or ADP-sensitive G_q -coupled receptors, P2Y_1 , P2Y_2 and P2Y_4 receptors are known in rodents [28, 29]. Our quantitative RT-PCR indicated that P2Y_1 and P2Y_2 receptors were expressed equally but the P2Y_4 receptor was absent in our astrocytes (Fig. 2). Therefore, the initial swelling-induced Ca^{2+} release must have been initiated by activation of P2Y_1 and/or P2Y_2 receptors, which is consistent with the findings in other reports [30, 31].

Application of ATP activates VSOR anion channels via “ Ca^{2+} nanodomains”

Since the bradykinin-induced $[\text{Ca}^{2+}]_i$ rise and VSOR channel activation are also initiated by activation of the G_q -coupled bradykinin B2 receptor [11], next we examined whether the application of ATP induces VSOR channel activation under isotonic conditions. We used 100 μM ATP for this purpose. At this concentration, almost all of the ATP-sensitive purinergic receptors would be activated [28, 32]. Furthermore, it is known that high concentrations of extracellular ATP at >1 mM levels may directly block VSOR channels in a voltage-dependent manner [33–35] but that this block is not elicited when ATP is bound to a divalent cation such as Mg^{2+} [1, 35]. Since 1 mM Mg^{2+} and 2 mM Ca^{2+} (3 mM Mg^{2+} when the extracellular Ca^{2+} was removed) were included in the

bathing solution in our condition (see Materials and Methods for details), virtually all ATP must be in the cation-bound form and its blocking effect on the channel would be negligible. Application of 100 μM ATP to the cells under the whole-cell voltage clamp condition in the isotonic bathing solution indeed induced development of an anion current showing mild outward rectification and weak inactivation at high voltages, which was greatly suppressed by a blocker of VSOR channels, DCPIB (10 μM ; Fig. 3A and 3Da). Thus ATP indeed induces VSOR channel activation, although the generation of a small DCPIB-insensitive inwardly rectifying current component in the negative voltage range frequently accompanied the activation (filled squares in Fig. 3A and 3Da, cf. Fig. 6 of [12]). The VSOR current development usually reached a plateau within 15–20 min, and the maximum amplitude of the current at +100 mV was 38.5 ± 4.2 pA pF $^{-1}$ ($n = 18$), which was comparable to the current induced by bradykinin [12].

The important characteristic of the bradykinin-induced mechanism is that Ca^{2+} -dependent processes are involved and accomplished within Ca^{2+} nanodomains [12]. The Ca^{2+} in the nanodomains is virtually not captured by a slow Ca^{2+} chelator EGTA [26]. Moreover, to prevent the generation of Ca^{2+} nanodomains as extensively as possible, it is important not only to introduce a high

Fig. 5. Effects of Ca^{2+} removal and apyrase on the cell volume of mouse astrocytes in hypotonic (75% osmolality) and isotonic conditions. (A) Changes in the relative mean cell volume induced by a hypotonic stimulus (Hypo.) as controls (squares), in the absence of extracellular and intracellular Ca^{2+} ($\text{Ca}^{2+}(-)$, open circles), and in the presence of 10 U/ml apyrase (diamonds), as well as those under isotonic conditions in the absence (Iso., filled circles) and presence of 10 U/ml apyrase (crosses), and the presence of 100 μM ATP (triangles). The initial relative values before stimulus (i.e. 1) are nominally plotted at 0 min. The results were obtained by 10 separate experiments for each condition. (B) Comparison of the initial mean cell volumes before stimulus (in femtoliters) in the absence and presence of apyrase and in the Ca^{2+} -depleted condition. Asterisk indicates a significant enlargement at $P < 0.05$ by multiple comparisons. (C) Comparison of the relative volumes measured 1 min (white bars) and 30 min (gray bars) after the beginning of the stimulus under each condition. Single asterisk (*) indicates a significant difference at $P < 0.05$ by unpaired t test, and double asterisks (**) indicate the difference at $P < 0.001$ by unpaired t test. NS indicates “not significant”.

concentration of a fast Ca^{2+} chelator BAPTA into the cells, but also to remove Ca^{2+} thoroughly from both the extracellular environment and the intracellular Ca^{2+} stores [12]. We checked these points on the ATP-induced VSOR channel activation. Introduction of EGTA at 10 mM into the cells did not affect the final maximum amplitude of the ATP-induced VSOR current at all (45.5 ± 4.8 pA pF^{-1} at +100 mV, $n = 15$, n.s. by multiple comparisons; Fig. 3B, Db and E). By contrast, when the cells were pretreated with thapsigargin and then ATP was applied in the absence of extracellular Ca^{2+} and the presence of intracellular BAPTA at 10 mM, the development of the DCPIB-sensitive VSOR current component was suppressed nearly completely (Fig. 3C, Dc and E; see legend for details). Therefore, these strongly support the idea that the ATP-induced VSOR channel activation is also regulated via Ca^{2+} nanodomains.

Swelling-induced VSOR current in mouse astrocytes contains a Ca^{2+} nanodomain-mediated component

The foregoing results indicate that the swelling-induced $[\text{Ca}^{2+}]_i$ rise in mouse astrocytes fully depends on the autocrine action of ATP and that ATP may induce VSOR current activation via Ca^{2+} nanodomains. Therefore, it is very likely that the swelling-induced VSOR current in mouse astrocytes contains a Ca^{2+} nanodomain-mediated component to some extent. This possibility was

examined by comparing the swelling-induced VSOR current generated in the normal Ca^{2+} -containing condition with the current generated in the Ca^{2+} -depleted condition where the ATP-induced VSOR current development was completely inhibited. Application of a hypotonic solution (25% reduction in osmolality) induced development of the DCPIB-sensitive typical VSOR current, whose amplitude (235.3 ± 34.2 pA pF^{-1} at +100 mV, $n = 15$; Fig. 4A and C) was much larger than that induced by 100 μM ATP (Fig. 3A and Da). When the hypotonic stimulus was applied in the absence of extracellular Ca^{2+} and the presence of intracellular BAPTA at 10 mM after pretreatment with thapsigargin, a substantially large amplitude of the current (142.4 ± 19.3 pA pF^{-1} at +100 mV, $n = 13$; Fig. 4B and C) was still generated, but it was significantly smaller than that in the Ca^{2+} -containing condition (the asterisk in Fig. 4C, $P < 0.05$ by unpaired t test). Thus, the swelling-induced VSOR current in mouse astrocytes indeed contains a Ca^{2+} nanodomain-mediated component that constitutes about 40% of the total current. Another 60% of the current, however, would be generated in a Ca^{2+} -independent manner.

Regulatory volume decrease in mouse astrocytes depends greatly on the autocrine action of ATP

We also examined how much the autocrine action of ATP contributes to the cell volume regulation in our astrocytes. We measured the cell volume changes

induced by the hypotonic stimulus with a Coulter counter, and the regulatory volume decrease (RVD) process after cell swelling was compared in the presence and absence of Ca^{2+} and in the presence of apyrase. The hypotonic stimulus (25% reduction in osmolality) rapidly increased the cell volume by 20% within 1 min, and the increase was alleviated to 10% within 30 min (squares in Fig. 5A and the leftmost pair of the bars in Fig. 5C), exhibiting RVD. When Ca^{2+} was removed thoroughly from both the extracellular and intracellular environments (see Materials and Methods for details), the initial volume before the hypotonic stimulus was not affected ($\text{Ca}^{2+}(-)$ in Fig. 5B) but a smaller swelling was induced just after the stimulus and RVD was slowed down, although a significant volume decrease still occurred (open circles in Fig. 5A and the 3rd pair of the bars from left in Fig. 5C). When the cells were treated with 10 U/ml apyrase, more drastic changes had happened: the initial volume before the hypotonic stimulus was slightly enlarged (the asterisk in Fig. 5B), and a larger swelling was induced just after the stimulus, and then RVD was completely suppressed (diamonds in Fig. 5A and the 2nd pair of the bars from left in Fig. 5C). Therefore, in mouse astrocytes, the RVD process relies totally on the autocrine action of ATP.

Even when no stimulus was applied, the cell volume significantly decreased gradually by 6% during the measurement for 30 min (filled circles in Fig. 5A and the 4th pair of the bars from left in Fig. 5C). This decrease was not altered further by application of 100 μM ATP under isotonic conditions (triangles in Fig. 5A and the 5th pair of the bars from left in Fig. 5C). However, the decrease disappeared in the presence of 10 U/ml apyrase (crosses in Fig. 5A and the rightmost pair of the bars in Fig. 5C). These imply that the maximally achievable action of ATP under isotonic conditions was already attained during the measurement and this action was inhibited by apyrase. This would be due to the inherent nature of the measurement system, because gentle mixing of cell suspension is unavoidable during sampling of the cells and this would have already facilitated the basal ATP release from the cells.

Discussion

This study demonstrated a component of the swelling-induced VSOR current, whose generation is triggered by the autocrine action of ATP and mediated by Ca^{2+} nanodomains in mouse astrocytes. First we showed

that the swelling-induced $[\text{Ca}^{2+}]_i$ rises in the astrocytes fully depend on the autocrine action of ATP, and this action is at least in part mediated by activation of G_q protein-coupled purinergic receptors, P2Y_1 and/or P2Y_2 . Next we demonstrated that exogenously applied ATP can induce VSOR channel activation via Ca^{2+} nanodomains under isotonic conditions, as bradykinin does. Then we showed that the swelling-induced VSOR current is significantly reduced in the Ca^{2+} -depleted condition where the ATP-induced VSOR current activation is totally prevented, although a substantially large fraction of the VSOR current is still generated presumably in a Ca^{2+} -independent manner. We also demonstrated that the autocrine action of ATP itself is essential for the RVD after cell swelling in mouse astrocytes.

The enhancement of VSOR current by autocrine action of ATP was initially reported in rat hepatoma cells [18] and in rat astrocytes [19]. However, these reports were questioned later [25, 36], because they did not consider the possible direct inhibitory effect of purinergic antagonists and high concentrations (>1 mM) of extracellular ATP on VSOR channels [33–35, 37, 38]. Our results of the VSOR current activation induced by the extracellular ATP at far less than 1 mM (0.1 mM) in the presence of plenty of divalent cations (Fig. 3), the total dependence of the swelling-induced $[\text{Ca}^{2+}]_i$ rise on the autocrine action of ATP (Fig. 1B) and the Ca^{2+} -dependent component of the swelling-induced VSOR current (Fig. 4) certainly indicate that the autocrine action of ATP helps promote the swelling-induced VSOR channel activation in our astrocytes. Therefore this study supports at least in part the initial reports. However, the considerably large fraction of the swelling-induced VSOR current remaining even after extensive Ca^{2+} removal (Fig. 4B and C) implies that the primary mechanism of the swelling-induced VSOR current must be independent of Ca^{2+} and ATP actions even in our astrocytes. The Ca^{2+} independence of the swelling-induced VSOR current has long been suggested in many types of cell, although some Ca^{2+} -related enzyme cascades may modulate the current in some cases (reviewed by [1, 3, 4]). In rat astrocytes, Mongin and Kimelberg [24] demonstrated that ~25% of the hypotonicity-induced aspartate efflux via VSOR channels is caused by the Ca^{2+} -dependent enzyme cascades, which can be augmented by exogenously applied ATP. Therefore, our results also confirm these preceding studies in that the autocrine action of ATP is a supplemental, not an essential, factor for the swelling-induced activation of VSOR current. How much ATP can promote the VSOR channel activation would vary considerably between cell

types. For example, our group previously demonstrated no facilitatory effect of ATP on VSOR channel activation in a human epithelial cell line [20, 22]. It must be stressed, nonetheless, that the present study revealed the Ca^{2+} nanodomain-mediated mechanism of the ATP-induced VSOR channel activation, which can be inhibited only when a high concentration of BAPTA is added to the cytosol and also the Ca^{2+} in the reservoirs is eliminated as thoroughly as possible (Fig. 3). This point therefore should be taken into account if one examines whether some Ca^{2+} - or ATP-dependent processes are involved in VSOR channel activation even partially or not in any type of cell.

The ATP action in our case is mediated at least partly by activation of G_q -coupled P2Y_1 and/or P2Y_2 receptors. However, other types of purinergic receptor, especially Ca^{2+} -permeable ionotropic P2X receptors, may also be involved. Several types of P2X receptor are reported to be expressed in rat astrocytes [31] and indeed they are expressed also in our astrocytes (unpublished data). Further studies are evidently needed for determining whether and how these P2X receptors are involved in the swelling-induced $[\text{Ca}^{2+}]_i$ rise and in VSOR channel activation.

Our group previously demonstrated that in HeLa cells the Ca^{2+} entry through the stretch-activated TRPM7 channel in the plasma membrane facilitates RVD [39]. This channel is well expressed also in the astrocytes [12]. However, at least during the inhibition of ATP action, there seems no noticeable contribution of the Ca^{2+} entry to the $[\text{Ca}^{2+}]_i$ rise (Fig. 1Bb). Nevertheless, it is still possible that TRPM7 channels might be activated in some way in an ATP-dependent manner and then contribute to the $[\text{Ca}^{2+}]_i$ rise.

Although the autocrine action of ATP contributes only partially to the swelling-induced VSOR channel activity, the action was found to be essential for the RVD process in our astrocytes. Evidently, the suppression of RVD is unlikely to be caused solely by the reduction in

VSOR channel activity. Since RVD requires not only Cl^- but also K^+ effluxes, the result suggests that the K^+ channel activation required for RVD would depend much more greatly on the autocrine action of ATP, as previously found in human epithelial Intestine 407 cells [22]. The K^+ channel activation seems to be caused both by Ca^{2+} -dependent and independent mechanisms, because a small but significant RVD still occurred even in the Ca^{2+} -depleted condition (open circles in Fig. 5A). Activation of both volume-regulating K^+ and Cl^- channels by ATP implies that a volume decrease should certainly be elicited by the action of ATP alone. This was indeed suggested in the volume records without stimuli (filled circles and crosses in Fig. 5A). ATP is known to be released from many cell types in response to a variety of stimuli, including those not necessarily accompanied by cell swelling [16]. The Ca^{2+} nanodomain-mediated mechanism of VSOR channel activation induced by ATP thus would provide a firm basis, in cooperation with the K^+ channel activation, for local cell volume regulation even when only a minute amount of ATP acts on a part of a cell, thereby promoting cell shape changes, migration etc. in response to the stimuli. On the other hand, the Ca^{2+} - and ATP-independent mechanism of VSOR channel activation during cell swelling would have a role in accelerating the volume regulation, in order to relieve a deleterious cell swelling as fast as possible.

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