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Apoptotic inducers activate the release of D-aspartate through a hypotonic stimulus-triggered mechanism in PC12 cells

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ABSTRACT

We have characterized release of D-aspartate (D-Asp), a regulator of hormone synthesis and secretion, via a volume-sensitive organic anion channel (VSOC) in PC12 cells by studying its response to apoptotic stimuli. PC12 cells have been demonstrated to endogenously synthesize D-Asp. Apoptotic inducers, including staurosporin (STS), tumor necrosis factor (TNF)-α, H_2O_2, and C2-ceramide, activate the release of D-Asp through a hypotonic stimulus-triggered mechanism. Putative blockers of the anion channel, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and 4,4′-diisothiocyanostilbene-2,2′-sulphonic acid (DIDS), significantly inhibited stress-induced D-Asp release under hypotonic conditions following the application of apoptotic inducers. Hypotonic conditions are essential for activation by apoptotic inducers. Phorbol 12-mirystate 13-acetate and the Ca^{2+} ionophore A23187 increased D-Asp efflux via the VSOC, implying the involvement of intracellular Ca^{2+} in the activation of the D-Asp efflux. However, hypotonic stress and STS had no effect on the concentration of intracellular Ca^{2+} in PC12 cells. Furthermore, an unknown EGTA-sensitive factor(s), other than Ca^{2+}, and peroxynitrite may play pivotal roles in STS-enhanced D-Asp release.

Introduction

D-Aspartate (D-Asp), an enantiomer of L-Asp, is a unique amino acid and is now known to be widely distributed in various mammalian tissues, such as the pineal gland, pituitary gland, adrenal gland and testis [1–4]. Immunohistochemical and quantitative studies have revealed that the localization and levels of D-Asp in these tissues change dramatically during development [5–7]. D-Asp has been shown to suppress melatonin secretion in the pineal gland [8,9], stimulate prolactin release in the anterior pituitary gland [10–12], modulate oxytocin and vasopressin production in the posterior pituitary gland [13], and increase testosterone synthesis in Leydig cells by stimulating the expression of the Steroidogenic Acute Regulatory protein (StAR) [14–16]. These observations indicate that D-Asp may play important roles in the regulation of hormone synthesis and secretion in these neuroendocrine and endocrine tissues.

In the rat testis, it was demonstrated that D-Asp is primarily localized in germ cells, particularly in the region rich in elongate spermatids, the most mature of the germ cells, located in the lumen of the seminiferous tubules [17]. We propose that D-Asp is secreted from the tubules into the interstitial space where it stimulates Leydig cells to increase testosterone production by stimulating StAR gene expression. Subsequently, testosterone, produced in the interstitial space, can be incorporated back into the tubules to promote germ cell development in a positive feedback mechanism. However, details on the molecular mechanism of the synthesis and efflux of D-Asp remain to be elucidated.

We have been investigating the dynamics of D-Asp homeostasis in mammalian cells with regard to its synthesis [18], degradation [19,20], and transport [21–23]. Previously, we demonstrated, for the first time, that D-Asp is synthesized endogenously in cultured mammalian cells such as pheochromocytoma (PC)12, MPT1, and GH3 cells [12,18,24], although its precise synthetic pathway is still unknown. We also demonstrated that endogenous D-Asp is localized in the cytoplasm of PC12 cells and is released through at least two distinct mechanisms [23]. One mechanism is a spontaneous and continuous release pathway that does not require any extracellular stimulus as a trigger. Since the properties of this type of release are different from vesicular exocytotic dopamine release, this pathway does not appear to involve the exocytotic efflux of D-Asp of vesicular origin. The other pathway is a hypotonic stimulus-induced release pathway, which is further activated by acetylcholine stimulation. Our previous study demonstrated that this pathway is mediated by a volume-sensitive organic anion channel (VSOC) and is inhibited by the putative anion channel blockers, 5-nitro-2-

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(3-phenylpropylamino)benzoic acid (NPPB)[1] and 4,4′-diisothiocyanostilbene-2,2′-sulphonic acid (DIDS) [23].

The VSOC is presumably involved in regulating the cell volume upon its perturbation by hyposmolarity, although its structural properties remain undefined [25,26]. Thus cell-swelling, induced by extracellular hypotonic fluids, activates the VSOC, resulting in the efflux of Cl^- anions and small organic anions, together with the release of K^+ ions through a specific K^+ channel. The efflux of these ions, along with a concomitant loss of water, allows swollen cells to return to their normal volumes. Recently, it has been demonstrated that apoptotic stimuli activate Cl^- ion efflux and the attendant release of K^+ ions, and the resulting net efflux of water leads to a decrease in cell volume under normotonic conditions [27]. It is suggested that this "apoptotic cell volume decrease" is an early prerequisite for apoptotic cell death, and the activation of the VSOC is presumably involved in this apoptotic cell shrinkage [26–29].

In the mammalian testis, spontaneous germ cell apoptosis occurs during spermatogenesis in the seminiferous tubules [30–32]. As mentioned above, D-Asp is localized in germ cells in the seminiferous tubules of the testis, and therefore it is possible that apoptotic stimuli induce D-Asp efflux from the germ cells into the interstitial space to stimulate testosterone production.

In this study, we have addressed the possibility of an increase in D-Asp efflux in mammalian cells (PC 12 cells) caused by apoptotic stimuli. We investigated the effects of various apoptotic inducers on D-Asp release in PC12 cells under isotonic and hypotonic conditions. We found that the inducers, such as staurosporin (STS), tumor necrosis factor (TNF)-α, H_2O_2, and C2-ceramide, enhanced hypotonic stress-induced D-Asp release, presumably via the VSOC-mediated pathway. The signal mechanism involved in the apoptosis-stimulated D-Asp release was also investigated.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM), heat-inactivated horse serum, and fetal bovine serum employed for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). The culture plates and dishes were produced by Asahi Techno Glass Corporation (Tokyo, Japan). The reverse-phase column (TSKgel ODS-80Ts [250 × 4.6 mm, 5 μm]) and the Pirkle-type chiral column (Sumichiral OA3200 [250 × 4.6 mm]) were obtained from the Tosoh Corporation (Tokyo, Japan) and Sumika Chemical Analysis Service (Osaka, Japan), respectively. C2-Ceramide, TNF-α, Proteinase K, and dihydrorhodamine 123 (DHR 123) were purchased from Sigma (St. Louis, MO, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and Fura-2 AM were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. STS was prepared as described in a previous report [33]. Other chemicals used were of the highest grade commercially available.

Cell culture

PC12 cells were cultured at 37 °C, 5% CO_2 and 100% humidity in Type I collagen coated-plates with DMEM containing 10% horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were passaged with 0.1% trypsin in Hank's balanced salt solution every three days.

Cell viability assay

Cell viability was determined by the MTT assay as described previously [22]. Briefly, PC12 cells (1×10^5 cells/well), suspended in 100 μL of culture medium, were inoculated into 24-well plates and cultured for 24 h. Various concentrations of STS and/or the channel blockers NPPB or DIDS were added subsequently, and the cells were cultured for another 4 h. STS was added rapidly after the addition of the blockers. MTT was then added at a final concentration of 0.5 mg/mL, and the plates were incubated for 4 h at 37 °C. The cells were then disrupted by the addition of 100 μL lysis solution (50% *N*,*N*-dimethylformamide, 20% SDS, 2% acetic acid, 1.7 mM HCl), followed by an additional incubation for 4 h. Cell viability was determined by measuring the absorbance at 570 nm.

Ladder detection assay

PC12 cells (2×10^6 cells/well) were inoculated into 6-well plates and cultured for 24 h. Following the addition of various concentrations of STS and the channel blockers NPPB or DIDS, cells were cultured for another 4 h, and the cells (both attached and detached) were then transferred into a 1.5 mL tube and collected by centrifugation at 600*g* for 5 min. DNA was isolated and separated on a 1.0% agarose gel to identify the formation of a DNA ladder following the procedure reported previously [34], with minor modifications. Briefly, the cells were washed once with 0.5 mL PBS, incubated with 100 μL of lysis buffer (10 mM Tris, pH 7.5, 10 mM EDTA, 10% Triton X-100) for 10 min at 4 °C, and then centrifuged at 23,000*g* at 4 °C for 20 min. The supernatant was incubated with RNase A (0.4 mg/mL) for 1 h at 37 °C and then with proteinase K (0.4 mg/mL) for 1 h at 37 °C. After the addition of 20 μL of 5 M NaCl and 120 μL of isopropanol, DNA was precipitated overnight at 20 °C and centrifuged at 23,000*g* at 4 °C for 15 min. After removal of the supernatant, the pellet was recentrifuged at 4 °C for 15 min and any residual liquid was removed. The DNA pellet was dissolved in 20 μL of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and separated on a 1.0% agarose gel. Following electrophoresis, the gel was stained with 0.5 μg/mL ethidium bromide and ladder formation of oligonucleosomal DNA was detected under ultraviolet light.

Measurement of caspase activity

Caspase activity was detected using the Caspase-Glo® 3/7 assay kit (Promega Corporation, Madison, WI, USA). Briefly, PC12 cells (1×10^5 cells/well) were inoculated into a 24-well luminometer plate (Corning, Lowell, MA, USA) and incubated for 24 h at 37 °C. After the addition of various concentrations of STS and the channel blockers NPPB or DIDS, the cells were cultured for another 2 h. One hundred microliters of caspase 3/7 reagents were added to each well and the cells were incubated for 2 h. Luminescence was measured for each well using a Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan).

Measurement of amino acid release

PC12 cells (2×10^6 cells/well) were inoculated into 6-well plates and cultured for 24 h. After washing with isotonic solution (90 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes, 10 mM glucose, and 130 mM Mannitol, pH 7.4), the cells were incubated for 20 min in either isotonic or hypotonic solution (90 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes, 10 mM glucose and 15 mM Mannitol, pH 7.4) containing the apoptotic inducers A23187, or phorbol 12-myristate 13-acetate (PMA), and/or

[1] *Abbreviations used:* VSOC, volume-sensitive organic anion channel; STS, staurosporin; TNF, tumor necrosis factor; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-sulphonic acid; UA, uric acid; PC12, pheochromocytoma 12; RVD, regulatory volume decrease; AVD, apoptotic volume decrease; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; DHR123, dihydrorhodamine 123; PMA, phorbol 12-myristate 13-acetate.