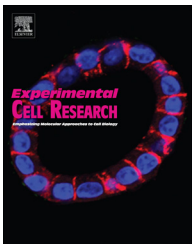
EXPERIMENTAL CELL RESEARCH 323 (2014) 337–345

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/yexcr



Research Article

Molecular mechanism of 2-APB-induced Ca^{2+} influx in external acidification in PC12

*Kenji Takahashi, Minae Yokota, Toshio Ohta**

Department of Veterinary Pharmacology, Faculty of Agriculture, Tottori University, 4-101, Koyama-minami, Tottori 680-8553, Japan

ARTICLE INFORMATION

Article Chronology:
Received 10 January 2014
Received in revised form
27 February 2014
Accepted 1 March 2014
Available online 11 March 2014

Keywords:
2-APB
Dopamine secretion
Orai 3
Proton
PC12

ABSTRACT

2-Aminoethoxydiphenyl borate (2-APB) is used as a pharmacological tool because it antagonizes inositol 1,4,5-trisphosphate receptors and store-operated Ca^{2+} (SOC) channels, and activates some TRP channels. Recently, we reported that 2-APB enhanced the increase in cytotoxic $[Ca^{2+}]_i$, resulting in cell death under external acidic conditions in rat pheochromocytoma cell line PC12. However, the molecular mechanism and functional role of the 2-APB-induced Ca^{2+} influx in PC12 have not been clarified. In this study, to identify the possible target for the action of 2-APB we examined the pharmacological and molecular properties of $[Ca^{2+}]_i$ and secretory responses to 2-APB under extracellular low pH conditions. 2-APB dose-dependently induced a $[Ca^{2+}]_i$ increase and dopamine release, which were greatly enhanced by the external acidification (pH 6.5). $[Ca^{2+}]_i$ and secretory responses to 2-APB at pH 6.5 were inhibited by the removal of extracellular Ca^{2+} and SOC channel blockers such as SK&F96365, La^{3+} and Gd^{3+}. PC12 expressed all SOC channel molecules, Orai 1, Orai 2 and Orai 3. When we used an siRNA system, downregulation of Orai 3, but not Orai 1 and Orai 2, attenuated both $[Ca^{2+}]_i$ and secretory responses to 2-APB. These results suggest that 2-APB evokes external acid-dependent increases of $[Ca^{2+}]_i$ and dopamine release in PC12 through the activation of Orai 3. The present results indicate that 2-APB may be a useful pharmacological tool for Orai channel-related signaling.

Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; BTP2, N-{4-[3, 5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4-methyl-1, 2, 3-thiadiazole-5-carboxamide; DA, dopamine; $[Ca^{2+}]_i$, intracellular calcium concentration; DMSO, dimethyl sulfoxide; ECD, electrochemical detection; ER, endoplasmic reticulum; IP3R, inositol 1, 4, 5 trisphosphate receptor; NMDG, N-methyl D-glucamine; PC12, rat pheochromocytoma 12; PCA, perchloric acid; RT-PCR, reverse transcription-polymerase chain reaction; SK&F96365, 1-(b-[3-(4-methoxyphenyl) propoxy]-4- methoxyphenethyl)-1H-imidazole hydrochloride; siRNA, small interfering RNA; SOC, store-operated Ca^{2+}; STIM, stromal interacting molecule; Tris, tris(hydroxymethyl)aminomethane; TRPM7, transient receptor potential melastatin 7; TRPV, transient receptor potential vanilloid

**Corresponding author.* Fax: +81 857 31 5427.
E-mail address: tohta@muses.tottori-u.ac.jp (T. Ohta).

http://dx.doi.org/10.1016/j.yexcr.2014.03.001
0014-4827

Introduction

2-Aminoethoxydiphenyl borate (2-APB) is known as an antagonist of the inositol 1,4,5-trisphosphate receptor (IP_3R) [1]. This agent also inhibits the store-operated Ca^{2+} (SOC) channel [2] and sarco/endoplasmic reticulum Ca^{2+}-ATPase pump [3]. Moreover, Hu et al. reported that 2-APB activated transient receptor potential (TRP) V1, V2 and V3 [4]. Thus 2-APB has been widely used as a pharmacological tool to assess intracellular Ca^{2+} signaling. It is known that SOC channels operate to mobilize extracellular Ca^{2+} to intracellular stores when depletion of these stores occurs. A sensor molecule, stromal interacting molecule (STIM), recognizes the depletion of stores, forms a complex with Orai, a part of the SOC channel on the plasma membrane, and then promotes SOC entry [5]. Mammals have two STIM proteins, STIM 1 and 2, and three Orai proteins, Orai 1, 2 and 3 [6]. Unique pharmacological characteristics of 2-APB upon Orai channel activation have been reported [7].

Recently, we demonstrated that 2-APB evoked cell death of rat pheochromocytoma cell line PC12 under low extracellular pH conditions [8]. PC12 is frequently used for exocytosis research because it stores neurotransmitters, dopamine (DA) and can secrete them [9–12]. Since exocytosis is closely related to intracellular Ca^{2+} signaling [13], PC12 cells are suitable as a neural model. Therefore, in this study, to determine whether 2-APB is capable of exocytotic response, we investigated the effects of 2-APB on the increase of $[Ca^{2+}]_i$ and DA release under acidic conditions in PC12. Furthermore, we clarified a possible target for 2-APB at low pH by pharmacological and molecular approaches using a variety of blockers related to Ca^{2+} signaling and an siRNA system.

Materials and methods

Chemicals

The following chemicals were used (concentrations for stock solution): 2-APB (1 M), 1-(β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride (SK&F96365) (0.03 M), U73122 hydrate (0.01 M), capsazepine (0.01 M) and thapsigargin (1 mM) were purchased from SIGMA-ALDRICH (Tokyo, Japan). N-{4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl] phenyl}-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP2) (0.05 M) was obtained from Merck Millipore (Darmstadt, Germany). These chemicals were dissolved in dimethyl sulfoxide (DMSO) and diluted more than 1000-folds with HEPES-buffered solution (in mM; 134 NaCl, 6 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 10 glucose, pH 7.4). As a vehicle, we used 0.1–0.2% DMSO, which did not show any effects.

Cell culture

The PC12 cell line was purchased from RIKEN (Tsukuba, Japan). Cells were cultured in Dulbecco's modified Eagle's medium (D6171, SIGMA) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies, Tokyo, Japan), 10% heat inactivated horse serum (Life Technologies), 0.584 g/L L-glutamine (SIGMA), 100 μg/ml streptomycin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 100 U/ml penicillin (Meiji Seika Pharma Co., Ltd.).

Measurement of $[Ca^{2+}]_i$

The intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in individual cells were measured with the fluorescent Ca^{2+} indicator fura-2 by dual excitation using a fluorescent imaging system controlling illumination and acquisition (Aqua Cosmos, Hamamatsu Photonics, Hamamatsu, Japan) as described previously [14]. To load fura-2, cells were incubated for 40 min at 37 °C with 10 μM fura-2 AM (Life Technologies) in HEPES-buffered solution. A coverslip with fura-2-loaded cells was placed in an experimental chamber mounted on the stage of an inverted microscope (Olympus IX71, Japan) equipped with an image acquisition and analysis system. Cells were illuminated every 5 s with lights at 340 and 380 nm and the respective fluorescence signals of 500 nm were detected. Emitted fluorescence was projected onto a charge-coupled device camera (ORCA-ER, Hamamatsu Photonics) and the ratios of fluorescent signals (F_{340}/F_{380}) for $[Ca^{2+}]_i$ were stored on the hard disk of a computer (Endeavor Pro2500, Epson, Suwa, Japan).

Dopamine-release experiments

PC12 cells (1×10^5) were seeded in 24-well plates and cultured for 48 h. The medium was then removed, and the cells were washed with HEPES-buffered solution twice and preincubated in HEPES-buffered solution at 37 °C for 20 min. Then they were washed and treated with or without 2-APB in HEPES-buffered solution under each pH condition. Incubation was performed at room temperature for 2 min and then was stopped on ice. Sample solution was collected in a 1.5 mL tube and centrifuged. The supernatant was collected and prepared as supernatant assay solution containing 0.4 N perchloric acid (PCA) (Sup sol.). The cells remaining on the dish were extracted with 0.4 N PCA and prepared as a cell assay solution (Cell sol.). These preparations were performed on ice.

Measurement of DA was carried out using an HPLC system (HTEC-500, Eicom Co., Kyoto, Japan) equipped with an electrochemical detection (ECD) system. Samples (10 μL) were injected into the HPLC system. The flow rate was 0.5 mL/min and electrodetection was performed at 0.75 V. As the mobile phase, we used 0.1 M acetate–citrate buffer containing 17% methanol, 190 mg/L sodium 1-octanesulfonate and 5 mg/L EDTA-2Na. The percent secretion was calculated by the DA level in Sup sol. divided by that in total contents (Cell sol. plus Sup sol.).

Reverse transcription-polymerase chain reaction

Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described in a previous report [15]. Briefly, the oligonucleotides used for the specific amplification of Orai protein cDNAs were based on sequences registered in GenBank for each of the rat molecules studied. The nucleotide sequence and the length of the expected PCR product for each primer pair were Orai 1; 5'-CCAAGCTCAAAGCCTCCA-3' (sense), 5'-CGATGACGGTGGAGAAGG-3' (antisense) for 297 bp. Orai 2; 5'-CCGTGAGCAACATCCACA-3' (sense), 5'-CAGCCAGGAAAAGCAGGA-3' (antisense) for 120 bp. Orai 3; 5'-CCACCCGTCACTGTCTCC-3' (sense), 5'-GGCGACTGAGTTCCTCCA-3' (antisense) for 268 bp. β-actin (NM031144); 5'-AGCCATGTACGTAGCCATCC-3' (sense),