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Involvement of IP_3-receptor activation in endothelin-1-induced Ca^{2+} influx in rat pulmonary small artery

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ABSTRACT

We examined the endothelin-1 (ET-1)-induced increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura-2-loaded rat pulmonary small arteries. ET-1 (30 nM) elicited a long-lasting increase in $[Ca^{2+}]_i$ in physiological salt solution (PSS). In subsequent experiments, arteries were pretreated with BQ-788, an ET_B-specific blocker, to allow us to focus on responses mediated via the ET_A receptor, the existence of which was confirmed by immunohistochemistry. In Ca^{2+}-free PSS, ET-1 evoked a small transient increase in $[Ca^{2+}]_i$, indicating Ca^{2+} release from the SR (sarcoplasmic reticulum). After a switch to PSS (containing 2 mM $CaCl_2$), ET-1 elicited a long-lasting increase in $[Ca^{2+}]_i$ that was not inhibited by 1 μM nicardipine, an L-type Ca^{2+}-channel inhibitor, suggesting involvement of a Ca^{2+}-influx pathway independent of that channel. In arteries preincubated with 30 μM cyclopiazonic acid (CPA) or 2 μM thapsigargin (TG), the ET-1-induced Ca^{2+}-release was greatly reduced, and the induced Ca^{2+}-influx was attenuated. U-73122, a phospholipase C (PLC) inhibitor, had inhibitory effects similar to those of CPA and TG on the ET-1-induced Ca^{2+}-release and Ca^{2+}-influx, whereas U-73343, an inactive analogue of U-73122, had no such effects. Two putative membrane-permeable IP_3-receptor blockers, 2-aminoethoxydiphenyl borate (2APB, 50 μM) and Xestospongin C (20 μM), (a) almost completely inhibited the ET-1-induced Ca^{2+}-release and Ca^{2+}-influx, and (b) reduced the ET-1-induced contraction. These results indicate that in rat pulmonary small arteries, ET-1 induces receptor-operated Ca^{2+} influx via the ET_A receptor, and that this influx interacts with $InsP_3$-receptor activation.

1. Introduction

It is generally accepted that an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) serves as a key trigger for the elicitation of certain physiological functions. For instance, vasoconstriction occurs via an increase in $[Ca^{2+}]_i$ when the smooth muscle is stimulated by an agonist such as endothelin-1 (ET-1). Generally, agonist-induced increases in $[Ca^{2+}]_i$ occur via Ca^{2+} influx from the extracellular side or Ca^{2+} release from its intracellular stores [e.g., the sarcoplasmic reticulum (SR) within smooth muscle]. As a mediator of such Ca^{2+} influx, the L-type voltage-dependent Ca^{2+} channel (VOCC) has been well studied, and is recognized as one of the main pathways for Ca^{2+} influx. However, receptor-operated Ca^{2+} channels (ROCCs) and store-operated Ca^{2+} channels (SOCCs) have also attracted considerable attention as mediators of contractile responses (see reviews by Kuriyama et al., 1998; Minke and Cook, 2002; Albert et al., 2006). For instance, ET-1-induced contraction is only slightly inhibited by VOCC blockers in rat pulmonary artery (Leach et al., 1990; Kato et al., 1999, 2006 and review Shimoda et al., 2000). Depletion of the intracellular Ca^{2+} stores activates SOCCs which, like ROCCs, contribute to the elicitation of cell functions in many smooth muscle cells (see review Albert et al., 2006). Therefore, it seems likely that ROCCs and SOCCs are important in the induction of various functions in many cells, including smooth muscle cells.

Almost all ROCCs and SOCCs seem to be regulated by second messengers downstream of G-protein activation (review, Kuriyama et al., 1998), but the mechanisms of activation of these channels after receptor stimulation remain controversial. For instance, Chen et al. (2011) showed that inhibition of protein kinase A (PKA) and protein kinase G (PKG) activated SOCC in rat pulmonary artery, while SKF-96365 almost completely inhibited the SOCC currents. In our previous study on rat pulmonary small arteries, however, the increase in $[Ca^{2+}]_i$ and contraction induced by ET-1 were only partially inhibited by (±)-S-nitroso-N-acetylpenicillamine (SNAP), forskolin or SKF-96365 (Kato et al., 2006). To try to resolve this discrepancy in one and the same artery, we focused here on the detailed mechanisms underlying the ET-1-induced $[Ca^{2+}]_i$ increase in rat pulmonary small arteries, especially the ET-1-induced Ca^{2+}-influx pathway. The data, we obtained suggest that

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receptor-operated non-L-type Ca^{2+} influx is essential for the ET-1-induced response in this artery. In addition, IP_3-receptor activation seems to be crucial for activation of such ET-1-induced Ca^{2+} influx.

2. Materials and methods

2.1. Tissue isolation and measurement of intracellular free Ca^{2+} concentration

The procedures used were approved by the Animal Research Committee of Fukuoka Dental College. Male Wistar rats (250–350 g) were killed with an overdose of pentobarbitone (approx. 100 mg/kg body weight, i.p.). After the heart and lungs, together with the abdominal aorta, had been removed, the second to third branches of the intrapulmonary arteries were dissected free of the surrounding connective tissue and adventitia. Then, a glass capillary (i.d. 100–300 μm; length approx. 1.0 mm) was inserted into the dissected arteries to prevent any constriction during measurements of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). To prevent any effects mediated via the endothelium, we denuded the endothelium using the glass capillary or a thin surgical thread when we inserted it into the arteries (Hartley et al., 1998). The arteries were then incubated with 5 μM fura-2 acetoxymethyl ester (AM; 1 mM stock solution in DMSO) for 2 h at room temperature (23–25 °C). After loading, arteries were washed with PSS and mounted in the experimental bath by fixing both ends of the glass capillary. Additional arteries were stored in a refrigerator at 4 °C until needed.

$[Ca^{2+}]_i$ was measured by means of an image-analysis system (ARGUS-50/CA; Hamamatsu Photonics, Hamamatsu, Japan) using the fluorescent dye fura-2 (Grynkiewicz et al., 1985), as described previously (Furuya et al., 1994). The experimental bath (volume 1 ml) was maintained at 33 ± 1 °C. The physiological salt solution (PSS) contained (in mM): NaCl 135.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.2, $NaHCO_3$ 5, HEPES 10.0, glucose 5.0; pH adjusted to 7.4 with NaOH. The experimental bath was mounted on the stage of an inverted epifluorescence microscope (× 20; Diaphot-300; Nikon, Kawasaki, Japan) fitted with a xenon lamp. Fura-2 fluorescence was excited at 340 and 380 nm alternately through the microscope using a beam-splitter (DM-3000; SPEC Industries, Edison, USA). Emission images for both wavelengths were detected using an ICCD camera (C2400-87; Hamamatsu Photonics) and stored on a computer (PC/AT-compatible) at 3-s intervals after subtraction of both background (without excitation) and autofluorescence. A standard area (200 × 50 μm) of smooth muscle was selected in each artery for the measurement of $[Ca^{2+}]_i$ responses. The fluorescence ratios were converted to Ca^{2+} concentrations using a cell-free calibration method (Molecular Probes, Oregon, USA) (Furuya et al., 1994). Since our previous studies showed that 30 nM endothelin-1 (ET-1) induced a submaximal contractile response in rat pulmonary small artery (Kato et al., 1999, 2006), this concentration of ET-1 was used throughout the present study. Furthermore, the arteries used were pretreated with BQ-788, an ET_B-specific blocker, since both ET_A and ET_B receptors contribute to ET-1-induced responses in rat pulmonary small artery (Kato et al., 2006) and the aim was to focus on ET_A-induced responses. The blocking efficacy of 5 μM BQ-788 was confirmed in the same paper (Kato et al., 2006).

2.2. Immunofluorescence

For the immunocytochemistry, small blocks of lung (approx. 5 mm cube) were flash-frozen in liquid nitrogen and then embedded in SCEM compound (Leica Microsystems, Japan). The frozen blocks were sectioned at 8-μm thickness on a cryostat (MICROM HM 560; Zeiss, Germany), then fixed in acetone for 10 min. The sections were washed with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4, 1.47 mM KH2PO4), then incubated with 10% goat serum for 30 min at room temperature, followed by a 1-h incubation with rabbit anti-endothelin receptor A (AER-001; diluted 1:200; Alomone Labs, Jerusalem, Israel). Next, they were washed with PBS three times (5 min each), incubated for 1 h with anti-rabbit IgG antibody conjugated with Alexa Fluor 488 (1:800) (Molecular Probes Inc., OR, USA), and washed with PBS three times (5 min each). Nuclei were counterstained with DAPI (Molecular Probes Inc.). Fluorescence was observed using a confocal microscope (LSM710; Carl Zeiss MicroImaging GmbH, Germany), with ZEN 2011 Light Edition (Carl Zeiss) being used for image processing.

2.3. Tension measurements

Intrapulmonary arteries (i.d. 100–300 μm; length 1–2 mm) were dissected free of the surrounding connective tissue and adventitia. Then, two arteries were mounted on a Mulvany & Halpern-type Myograph (Mulvany and Halpern, 1977) (JP Trading, Aarhus, Denmark). The arterial endothelium was removed by rubbing the inner surface with thin surgical thread (o.d. about 0.1 μm) (Hartley et al., 1998). The experimental bath (volume 10 ml) was maintained at 37 ± 1 °C. Isometric muscle tension was sampled and analysed by a computer (Vaio, Sony, Japan) via a Myo-Interface (Model 500A; JP Trading, Aarhus, Denmark) and a LabChart 7 (AD Instruments Pty Ltd, Castle Hill, Australia). Pulmonary arteries were subjected to an initial tension of 0.1 mN. Prior to the experiments proper, arteries were constricted by high-K (50 mM) several times to verify that the tissue was viable and to allow for tissue equilibration. All drugs were applied directly to the bath solution.

2.4. Data analysis

All data are expressed as means ± S.E.M. $\Delta[Ca^{2+}]_i$ values were calculated using the following equation:

$$\Delta[Ca^{2+}]_i = \text{peak}[Ca^{2+}]_i \text{ during a given response} - [Ca^{2+}]_i \text{ at rest}$$

Resting $[Ca^{2+}]_i$ was obtained by averaging for 3 min before the application of any substances (in PSS or in Ca^{2+}-free PSS). Statistical significance was evaluated using ANOVA and Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

2.5. Chemicals

Endothelin-1 was purchased from Peptide Institute Inc. (Osaka, Japan), while BQ-788, cyclopiazonic acid (CPA), U-73122, and U-73343 were from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). Xestospongin C and nicardipine were purchased from Wako (Osaka, Japan), fura-2 from Dojindo (Kumamoto, Japan), 2-aminoethoxydiphenyl borate (2APB) from TOCRIS Inc. (Ellisville, MO, USA), and thapsigargin from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals mentioned above completely dissolved in all the buffers we used. We performed preliminary experiments to determine suitable concentrations and incubation times for the above chemicals.

3. Results

3.1. Immunohistochemistry for ET_A receptor in pulmonary arteries

To confirm the localization of ET_A receptor in rat pulmonary small artery, we first performed immunocytochemistry using confocal laser microscopy. Specific ET_A-receptor antibody recognized sites on