Reproductive Toxicology 65 (2016) 95–103

Contents lists available at ScienceDirect

Reproductive Toxicology

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

Roles of ion channels in regulation of acetylcholine-mediated vasoconstrictions in umbilical cords of rabbit/rats

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ARTICLE INFO

Article history:
Received 31 March 2016
Received in revised form 31 May 2016
Accepted 8 July 2016
Available online 13 July 2016

Keywords:
Acetylcholine
Umbilical cord
Calcium channel
Potassium channel

ABSTRACT

We recently demonstrated that acetylcholine (ACh) produced reliable vasoconstrictions in the umbilical cords. This study investigated the possible mechanisms with different antagonists. ACh-mediated vasoconstrictions were decreased by voltage-operated calcium (Ca^{2+}) channels antagonist nifedipine or inositol-1,4,5-trisphosphate-mediated Ca^{2+} release antagonist 2-aminoethyl diphenylborinate, indicating that both extracellular and intracellular calcium modulated the ACh-stimulated umbilical contraction. Intracellular Ca^{2+} concentrations were increased simultaneously with vasoconstrictions by ACh in the umbilical vessels. Inhibiting large-conductance calcium-dependent potassium (BK) channels enhanced ACh-mediated contraction, whereas inhibiting voltage dependent potassium (K^+), inward rectifier K^+ and ATP-sensitive K^+ channels had no effects. Incubation with specific K^+ channel inhibitors showed that ACh suppressed BK currents rather than 4-aminopyridine-sensitive K^+ channels currents. The results suggested that blood vessels in umbilical cords had special characteristics in response to cholinergic signals. ACh-stimulated umbilical vasoconstrictions were mediated *via* muscarinic receptor subtype 1/3–protein kinase C/cyclooxygenase-BK channel pathways.

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1. Introduction

The umbilical cord is vital to fetal growth and development. Lacking autonomic innervation, regulation of umbilical vascular tone mainly depends on release of vasoactive substances [1]. Acetylcholine (ACh) is the most common cholinergic chemical in the organs of the body, including the placenta and umbilical vessels [2,3] as well as in other smooth muscle cells [4–6]. Based on others' and our studies on human umbilical arteries and veins, as well as our recent findings in rats and sheep [7], this study tested rabbit and rat umbilical cords in order to determine ACh's special actions on umbilical vessels.

It is well known that ACh mediates vasorelaxation in intact and healthy blood vessels in almost all organs of the body [8]. Our recent work [7] demonstrated that ACh induced reliable vasoconstrictions in human, sheep, and rats. Notably, vascular diameters can control blood and oxygen supply *via* umbilical vessels to fetuses [9]. *In utero* hypoxia has been demonstrated to cause poor development of fetuses as well as low birth weight babies [10,11]. Thus, vigorous vasoconstrictions induced by cholinergic chemicals may induce *in utero* hypoxia and cause toxic consequences to fetuses. The present study determined possible underlying cellular mechanisms involved in the ACh-stimulated umbilical vasoconstrictions.

Responding to the signals transferred from muscarinic receptor (MR) cascades, cytoplasmic calcium concentration ($[Ca^{2+}]_i$) is fundamental in the regulation of vascular tone [12]. ACh induces contraction through vascular L-type voltage-dependent Ca^{2+} channel and intracellular calcium release *via* inositol 1,4,5-triphosphate (IP_3) receptors [13]. In addition, potassium (K^+) channels play an important role in the regulation of vascular tone and membrane potential [14], MR agonists could transiently increase the large-conductance calcium-dependent potassium (BK) channel currents followed by persistent inhibition in different smooth muscle cell types [15,16]. However, it was largely unknown how smooth muscle cells as well as the ion channels reacted to ACh in umbilical blood vessels. Thus, the present study focused on those signaling pathways and determined whether phospholipase C (PLC) or protein kinase C (PKC) [17], as well as calcium and potassium channels

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http://dx.doi.org/10.1016/j.reprotox.2016.07.003
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might be involved in ACh-mediated umbilical vasoconstrictions. The new information gained should advance further understanding vascular regulations in the umbilical cord-fetal circulation, which is critical to fetal development *in utero,* and benefit for prevention of possible fetal hypoxia induced by toxic effects by cholinergic chemicals.

2. Methods

2.1. Animals

Pregnant Sprague–Dawley rats and pregnant New Zealand white rabbits were purchased from the Animal Center of Soochow University. At gestational day (GD) 21, rats were anesthetized with a mixture of ketamine (75 mg/kg) and xylazine (10 mg/kg; Hengrui Medicine, Jiangsu, China) intraperitoneally [18] and at GD 26, rabbits were anesthetized intravenously with ketamine (30 mg/kg) [19] through an ear vein followed with uterine-incision delivery. Umbilical cords were collected and kept in Ca^{2+}-free physiological salt solution. All procedures were approved by the Institutional Animal Care Committee and conformed to the National Guide for the Care and Use of Laboratory Animals.

2.2. Testing on large blood vessels

Rabbit umbilical vein and carotid artery were isolated, and immediately mounted in an organ bath containing modified Krebs solution (in mM: 115.0 NaCl, 25.0 $NaHCO_3$, 4.6 KCl, 2.5 $CaCl_2$, 1.2 Na_2HPO_4, 1.2 $MgCl_2$, and 10 D-glucose; pH 7.4), then gassed continuously with a mixture of 95% O_2 and 5% CO_2. Vascular tension was measured with JZ101 isometric force transducer (Xinhangxingye Technology, Beijing, China). After 60 min of equilibration, each vessel ring was stretched for the optimal resting tension determined by the tension developed in response to 60 mM KCl. Vascular responses to addition of phenylphrine or ACh was monitored and recorded.

2.3. Testing on small blood vessels

Rat umbilical veins were separated and cut into segments. Segments (~3 mm) were mounted on a M series Myograph System (Radnoti Glass Technology, Inc. USA) in a chamber containing Krebs solution with 5% CO_2 in O_2 at 37 °C [20], followed by a 30-min equilibration period, vessels were exposed to an external solution containing 60 mM KCl to assess vessel functional capability. To determine possible signaling pathways mediated the contractile responses to ACh, several inhibitors, including muscarinic receptor antagonist atropine (10^{-6} M), muscarinic receptor subtype 1 (MR1) antagonist pirenzepine (10^{-6} M), MR2 antagonist methoctramine (10^{-5} M), MR3 antagonist pFFHsiD (10^{-6} M) [21], phospholipase C (PLC) inhibitor U73122 (5×10^{-5} M), cyclooxygenase inhibitor indomethacin (10^{-5} M), and protein kinase C (PKC) inhibitor GF109203X (10^{-6} M)were added into the chambers before application of ACh.

To assess possible involvement of extracellular or/and intracellular calcium in ACh-induced vessel contractions, L-type Ca^{2+} inhibitor nifedipine (10^{-5} M) and antagonist 2-aminoethyl diphenylborinate (2-APB,10^{-7} M) [22] for IP_3 were used followed by ACh.

To determine the role of potassium channels in ACh-induced contraction in rat umbilical veins, ACh concentration-response curves were recorded in the presence or absence of the following inhibitors: 4-aminopyridine (4-AP,3×10^{-3} M), charybdotoxin(CTX,10^{-7} M), barium chloride ($BaCl_2$,10^{-5} M), or glibenclamide (10^{-5} M) [23]. Vascular tone was normalized by the maximum contraction elicited with 60 mM KCl. Signals were recorded continuously using Chart 7 with PowerLab system (AD Instruments, Bella Vista, NSW Australia).

2.4. Measurement of $[Ca^{2+}]_i$ in rat umbilical veins

The venous segments were mounted in organ chambers (Living Systems, Burlington, USA) as described [24]. Vascular intracellular calcium was monitored and measured using Ca^{2+} indicator fura-2 AM by a Bio-Rad Radiance 2100 confocal system (Zeiss, Oberkochen, Germany) as reported [25]. In brief, the cannulated umbilical venous rings were incubated with 10 μmol/L fura-2 AM-PSS solution for 4 h at room temperature (22–25 °C), and gassed continuously with 95% O2–5% CO2. Then rings were incubated in PSS solution for 30 min at 37 °C. Ca^{2+} concentration was calculated qualitatively by fluorescence ratio of fura-2AM at 340 and 380 nm wave lengths (Rf340/380) [26]. KCl($6*10^{-2}$ M) or ACh(10^{-6} M, 10^{-5} M) were added to the chamber respectively. Each venous segment was used for only one drug.

2.5. Electrophysiology

Umbilical veins in rats were placed in ice-cold physiological salt solution (PSS) containing (in mM) 137 NaCl, 5.6 KCl, 1 $MgCl_2$, 0.42 Na_2HPO_4, 0.44 NaH_2PO_4, 4.2 $NaHCO_3$, and 10 HEPES (pH 7.4).

3. Isolation of rat umbilical vein myocytes

Umbilical veins were dissected gently and cut into small fragments (about 1 mm) and placed for 22 min at 37 °C in PSS containing 1 mg/ml collagenase II, 2 mg/ml BSA, and 2 mg/ml DTT. They were then transferred to PSS containing 3 mg/ml papain, 2 mg/ml BSA, and 2 mg/ml DTT for 20 min. Single cells were obtained by gentle trituration with a wide-bore glass pipette, stored at 4 °C and used within 6 h.

4. Whole-cell K^+ current recording

The isolated myocytes were continuously superfused with a bath solution containing (in mM) 137 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 Glucose; pH 7.4. Patch pipettes (tip resistance, 3–5 M) were pulled by P-97 and filled with pipettes solution containing (in mM) 143 KCl, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES, and 3 Na_2ATP, pH 7.35. Na_2ATP (3 mM) was included to inhibit ATP-sensitive K^+ currents and provided a substrate for energy-dependent processes [27]. Seal resistances ranged from 1 to 10 M after seal formation. Protocols were applied using an Axo patch 700 B amplifier with Pclamp 10.2 software. Whole-cell K^+ currents were evoked by voltage steps delivered from a holding potential of −70 mV to potentials ranging from −60 to +60 mV, in 10 mV increments. Whole cell K^+ currents were normalized to cell capacitance and expressed as picoampere per picofarad (pA pF-1). All experiments were performed at room temperature (22–25 °C). Whole-cell K^+ currents were monitored before or after adding ACh (10^{-5} M), iberiotoxin (IBTX, 10^{-7} M), 4-AP (3×10^{-3} M), and atropine (10^{-7} M). BK channel currents were tested with 0.1 μmol/L IBTX.

All agonists and antagonists used were purchased from Sigma-Aldrich unless otherwise stated. Drugs were prepared freshly.

4.1. Data analysis

Data were expressed as means ± SEM, where n equals the number of maternal rats from which offspring were taken. A value of $P < 0.05$ was considered to indicate statistical significance, and

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