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Regulation of NO-dependent acetylcholine relaxation by K^+ channels and the Na^+–K^+ ATPase pump in porcine internal mammary artery

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ABSTRACT

This study was designed to determine whether K^+ channels play a role in nitric oxide (NO)-dependent acetylcholine relaxation in porcine internal mammary artery (IMA). IMA segments were isolated and mounted in organ baths to record isometric tension. Acetylcholine-elicited vasodilation was abolished by muscarinic receptor blockade with atropine (10^{-6} M). Incubation with indomethacin (3×10^{-6} M), superoxide dismutase (150 U/ml) and bosentan (10^{-5} M) did not modify the acetylcholine response ruling out the participation of cyclooxygenase-derivates, reactive oxygen species or endothelin. The relaxation response to acetylcholine was strongly diminished by NO synthase- or soluble guanylyl cyclase-inhibition using L-NOArg (10^{-4} M) or ODQ (3×10^{-6} M), respectively. The vasodilation induced by acetylcholine and a NO donor ($NaNO_2$) was reduced when rings were contracted with an enriched K^+ solution (30 mM), by voltage-dependent K^+ (K_v) channel blockade with 4-amynopiridine (4-AP; 10^{-4} M), by Ca^{2+}-activated K^+ (K_{Ca}) channel blockade with tetraethylammonium (TEA; 10^{-3} M), and by apamin (5×10^{-7} M) plus charybdotoxin (ChTx; 10^{-7} M) but not when these were added alone. In contrast, large conductance K_{Ca} (BK_{Ca}), ATP-sensitive K^+ (K_{ATP}) and inwardly rectifying K^+ (K_{ir}) channel blockade with iberiotoxin (IbTx; 10^{-7} M), glibenclamide (10^{-6} M) and $BaCl_2$ (3×10^{-5} M), respectively, did not alter the concentration–response curves to acetylcholine and $NaNO_2$. Na^+-K^+ ATPase pump inhibition with ouabain (10^{-5} M) practically abolished acetylcholine and $NaNO_2$ relaxations. Our findings suggest that acetylcholine-induced relaxation is largely mediated through the NO-cGMP pathway, involving apamin plus ChTx-sensitive K^+ and K_v channels, and Na^+-K^+-ATPase pump activation.

1. Introduction

The internal mammary artery (IMA) is the vessel of choice for coronary artery bypass grafting due to improved long-term patency rates compared with other arteries and saphenous vein (González Santos et al., 2005). However, arterial segments are inherently prone to vasospasm, which can lead to perioperative graft failure (Ding et al., 2008). Progress has been made in understanding the primary mechanism of the myogenic response in human IMA and other arterial segments, with increasing attention paid to the endothelium (Liu et al., 2002; Wei et al., 2007).

The balance between endothelial-derived contractile and relaxant factors determines the tone and the physical state of vascular smooth muscle. Endothelium-dependent vascular relaxation is mediated by nitric oxide (NO), prostacyclin and endothelial-derived hyperpolarizing factor (EDHF). The actions of these endothelium-derived relaxing substances often involve membrane hyperpolarization of vascular smooth muscle, and plasma membrane K^+ channels are key molecules for producing membrane electrical events (Busse et al., 2002). Studies over the past 20 years have identified at least four different classes of K^+ channels expressed by arterial smooth muscle cells. These include inward rectifier K^+ (K_{IR}), ATP-sensitive K^+ (K_{ATP}), Ca^{2+}-activated K^+ (K_{Ca}), and voltage-dependent K^+ (K_V) channels (Busse et al., 2002; Feletou and VanHoutte, 2006).

Several studies have tried to determine whether the higher patency of IMA as a bypass graft may be explained by a difference in its ability to release NO and EDHF. Thus, Hamilton et al. (1999) observed that endothelium-dependent relaxation in response to bradykinin in human IMA was strictly NO-dependent. Conversely, the group of He (He and Liu, 2001; Liu et al., 2000) reported the ability of IMA to release both NO and EDHF. More recently, Archer et al. (2003) identified the EDHF involved in human IMA and reported that acetylcholine and bradykinin induce the release of the cytochrome epoxygenase-derived 11,12-EET promoting hyperpolarization and relaxation through the activation of large conductance K_{Ca} channels (BK_{Ca}) located on smooth muscle cells.

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Endothelium-dependent vasodilation can be assessed using receptor-operated stimuli such as acetylcholine. This approach is commonly considered a marker of endothelial function. The differences revealed by the above-mentioned reports could be the outcome of variable IMA behaviour depending on the cardiovascular risk factors of the patients included in these studies. Cardiovascular risk factors such as diabetes, hypercholesterolemia, hypertension, obesity or smoking may be linked to endothelial dysfunction, atherosclerosis or coronary heart disease (Smith, 2007). Further, surgical manipulation or vasodilatory drugs administered during coronary artery bypass grafting may also affect the endothelial responses of human IMA (Gao et al., 2003; González Santos et al., 2005). Accordingly, data obtained using isolated human IMA segments may differ to those observed in healthy vessels. Thus, further investigations on the IMA require the use of an experimental model such as the pig (Pagán et al., 2009; Pesic et al., 1999) to eliminate confounding cardiovascular risk factors present in patients undergoing bypass surgery. Indeed, knowledge of endothelial function in this vessel could be essential for understanding the good long-term patency of the IMA.

This study was designed to examine whether K^+ channels and Na^+–K^+ ATPase pump activation play important roles in NO-dependent acetylcholine relaxation in porcine IMA.

2. Materials and methods

2.1. Tissue preparation, dissection and mounting

IMA segments from 37 cross-breed male pigs (weight 35–45 kg) were obtained from the Experimental Surgery Department of the Hospital Universitario Ramón y Cajal (Madrid, Spain) shortly after the animals were euthanized. Animals were handled according to European Union regulation (*86/609/EEC*) and the Spanish Normative for the Care and Use of Laboratory Animals (*RD 1201/2005*). The study protocol was approved by the Ethics Committee for Animal Welfare of the Hospital Universitario Ramón y Cajal.

After dissection, the tissue was transported to the laboratory in cooled (4 °C) physiological saline solution (PSS). Arterial segments were cleaned from adhering connective tissue and cut into 2 mm length rings. Vessel rings were transferred to 5 ml organ baths containing PSS at 37 °C and gassed with a mixture of 95% O_2 and 5% CO_2 to maintain the pH at 7.4. Rings (external diameter = 3.18 ± 0.06 mm and internal diameter = 2.08 ± 0.04 mm; $n = 37$) were mounted between two parallel L-shaped stainless steel wires. One wire was fixed to a displacement unit allowing fine adjustment of tension while the other was attached to a force transducer (Grass FT03C). Special care was taken to avoid damage to the endothelium. The isometric tension of the vessel wall was displayed and recorded using a PowerLab data acquisition system and Chart v5.5 software.

2.2. Experimental procedure

Each ring was stretched in a stepwise manner to its optimal resting tension (≈20 mN). This tension was determined previously in length-active tension relationship experiments. The contractile capacity of the preparation was tested by exposing the arterial rings to 124 mM K^+ (K-PSS, 17.4 ± 0.3 mN, $n = 37$). Concentration–response curves for acetylcholine and a nitric oxide donor ($NaNO_2$) were constructed by adding increasing concentrations of the agonist into the organ bath. Acetylcholine- and $NaNO_2$-induced relaxations were examined in preparations contracted with noradrenaline (10^{-7}–3×10^{-7} M). These concentrations of noradrenaline produced a stable contraction, corresponding to 60–70% of the response induced by K-PSS and of sufficient duration to permit the analysis of agonist responses.

Previous experiments showed that two consecutive acetylcholine or $NaNO_2$ concentration–response curves were not reproducible. Thus, the following experiments were conducted using consecutive segments from the same animal, with one segment acting as the control of the other. This meant that only one acetylcholine or $NaNO_2$ concentration–response curve per arterial ring could be obtained.

We assessed the involvement of muscarinic receptors, NO-cGMP or cyclooxygenase pathways, K^+ channels, Na^+–K^+ ATPase pump, reactive oxygen species and endothelin receptors in the acetylcholine- or $NaNO_2$-responses by adding to the organ bath atropine, L-NOArg, ODQ, indomethacin, different K^+ channels blockers (4-aminopyridine (4-AP), apamin, barium chloride ($BaCl_2$), charybdotoxin (ChTx), glibenclamide, iberiotoxin (IbTx), tetraethylammonium (TEA)), ouabain, superoxide dismutase (SOD) or bosentan, respectively, 30 min before the construction of the concentration–response curve.

To test the participation of a hyperpolarizing component in acetylcholine-evoked relaxation, concentration–response curves for acetylcholine and $NaNO_2$ were constructed on contractions elicited by a high concentration K^+ solution (30 mM) and compared to those produced by noradrenaline. The stable contractions induced by 30 mM KCl (14.3 ± 2.6 mN; $n = 16$) and noradrenaline were effectively very similar (14.2 ± 1.7 mN; $n = 16$).

2.3. Drugs and solutions

The following drugs were used: acetylcholine, 4-aminopyridine, apamin, atropine, barium chloride, charybdotoxin, glibenclamide, iberiotoxin, indomethacin, N^{ω}-nitro-L-arginine (L-NOArg), noradrenaline, ouabain, 1H-[1,2,4] oxadiazol [4,3,-α]quinaxolin-1-one (ODQ), sodium nitrite, superoxide dismutase, tetraethylammonium (all from Sigma-Aldrich, St. Louis, MO, U.S.A.) and bosentan (a gift from Hoffmann-La Roche, Inc., USA).

All drugs were dissolved in distilled water except: indomethacin, which was prepared in ethanol (96%), glibenclamide and ODQ, which were dissolved in dimethylsulphoxide, and $NaNO_2$, which required an acidified solution. In prior experiments, these solvents had no effect on the preparations. The concentrations of agents are expressed as their final concentration in the organ bath.

The composition of PSS was (mM); NaCl 119, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, KH_2PO_4 1.2 and ethylenediaminetetraacetic acid (EDTA) 0.027. K-PSS was identical to PSS except that NaCl was replaced on an equimolar basis. We also prepared a high K^+ concentration solution using normal PSS in which 30 mM NaCl was exchanged isotonically with KCl.

2.4. Statistical analysis

Relaxing responses to acetylcholine and $NaNO_2$ observed in contracted arteries were expressed as the percentage inhibition of the vascular contraction induced by noradrenaline. E_{max} refers to the maximum response achieved. The effects of different blockers on basal tension were expressed as percentages of K-PSS contraction.

For each concentration–response curve, the agonist concentration eliciting the half-maximal response (EC_{50}) was estimated by computerized nonlinear regression analysis (GraphPad Software, U.S.A.). The sensitivity of the drugs is expressed in terms of their pD_2, which is defined as the negative logarithm of the EC_{50} for the agonist used ($pD_2 = -\log EC_{50}$).

Results were expressed as the mean ± standard error of the mean (S.E.M.) of n animals. Statistical determinations were performed using the Student's t-test for unpaired data. A P value of less than 5% was taken to denote a significant difference.

3. Results

Acetylcholine (10^{-9}–10^{-5} M) caused the concentration-dependent relaxation of noradrenaline-contracted arterial rings ($pD_2 = 7.29 \pm 0.09$ and $E_{max} = 75.4 \pm 3.1\%$; $n = 37$) (Fig. 1A). This relaxant effect was