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Correspondence: Ricardo P. Garay, 46bis, rue du Marechal Gallieni, 91360 Villemoisson-sur-Orge, France.
E-mail: ricardo.garay@orange.fr

Research Paper

Protein kinase A signalling is involved in the relaxant responses to the selective β-oestrogen receptor agonist diarylpropionitrile in rat aortic smooth muscle *in vitro*

Marta S. Valero[a], Desiree Pereboom[a], Silvia Barcelo-Batllory[a], Laia Brines[a], Ricardo P. Garay[b] and José O. Alda[a]

[a]Department of Physiology and Pharmacology, School of Medicine, Zaragoza, Spain and [b]INSERM U999, Université Paris-Sud and Hôpital Marie Lannelongue, Le Plessis-Robinson, France

Abstract

Objectives The oestrogen receptor β (ERβ) selective agonist diarylpropionitrile (DPN) relaxes endothelium-denuded rat aorta, but the signalling mechanism is unknown. The aim of this study was to assess whether protein kinase A (PKA) signalling is involved in DPN action.
Methods cAMP was measured by radioimmunoassay, HSP20 phosphorylation by 2D gel electrophoresis with immunoblotting, and membrane potential and free cytosolic calcium by flow cytometry.
Key findings DPN increased cAMP content and hyperpolarised cell membranes over the same range of concentrations as it relaxed phenylephrine-precontracted aortic rings (10–300 μM). DPN-induced vasorelaxation was largely reduced by the PKA inhibitors Rp-8-Br-cAMPS (8-bromoadenosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer) and H-89 (*N*-(2-bromocynnamyl(amino)ethyl)-5-isoquinoline sulfonamide HCl) (–73%) and by the adenylate cyclase inhibitor MDL12330A (*cis*-*N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine)) (–65.5%). Conversely, the PKG inhibitor Rp-8-Br-cGMP was inactive against DPN vasorelaxation. In aortic smooth muscle segments, DPN increased PKA-dependent HSP20 phosphorylation, an effect reversed by H-89. Relaxant responses to DPN were modestly antagonised (–23 to –48% reduction; $n = 12$ per compound) by the potassium channel inhibitors iberiotoxin, PNU-37883A, 4-aminopyridine, or $BaCl_2$. All four potassium channel inhibitors together reduced DPN relaxation by 86 ± 9% ($n = 12$) and fully blocked DPN hyperpolarisation.
Conclusions ERβ-dependent relaxation of rat aortic smooth muscle evokes an adenylate cyclase/cAMP/PKA signalling pathway, likely activating the cystic fibrosis transmembrane conductance regulator chloride channel and at least four potassium channels.
Keywords diarylpropionitrile; oestrogen receptors; protein kinase A; rat aorta; smooth muscle

Introduction

Non-genomic vasorelaxation seems to be one important mechanism explaining the cardio-protective properties of oestrogens.[1] Oestrogen receptors α (ERα) and β (ERβ) are both expressed in vascular smooth muscle.[2–5] In isolated rat aorta, the selective ERα agonist propyl pyrazole triol and the selective ERβ agonist diarylpropionitrile (DPN) both evoke endothelium-independent, non-genomic relaxant effects.[6,7] ERα relaxation is mediated by protein kinase G (PKG) signalling,[7] but the signalling pathway of ERβ is unknown.

Interest in ERβ vasorelaxation was raised by the observations that ERβ-deficient mice develop sustained hypertension as they age,[8] and the antihypertensive potency of the ERβ agonist 8β-VE2 in spontaneously hypertensive rats is superior to that of 17β-oestradiol or the ERα agonist 16α-LE2.[9] Therefore, we decided to investigate the DPN signalling pathway in isolated rat aorta.

The cGMP and the cAMP pathways are major regulators of smooth muscle contractility and most vasorelaxant agonists act via PKG or protein kinase A (PKA).[10–12] In isolated rat aorta, DPN relaxation is largely endothelium independent,[6] and in preliminary experiments with endothelium denuded rat aortic rings, we found that the PKG inhibitor Rp-8-Br-cGMP

did not reverse DPN relaxation. We therefore decided to investigate PKA signalling. DPN was tested for its ability to increase both cyclic AMP (cAMP) content and PKA-dependent phosphorylation of small heat shock-related protein (HSP20). Moreover, selective inhibitors of PKA signalling (acting on adenylate cyclase, PKA or PKA-operated ion channels) were tested against DPN-induced membrane hyperpolarisation, calcium signal and vasorelaxation.

Materials and Methods

The investigation was performed according to the European Community Guidelines for Animal Ethical Care and the Guide for Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised 1985). The study was approved by the ethical committee of the Department of Physiology and Pharmacology (School of Medicine, University of Zaragoza, Spain).

Compounds

DPN was obtained from Tocris Cookson Inc (Ellisville, MO, USA). Other compounds were from Sigma Chemical Co. (St Louis, MO, USA). Concentrated solutions of compounds in dimethylsulfoxide were prepared on the day of the experiment and diluted in incubation media. In control experiments, we verified that the final dimethylsulfoxide concentration by itself had no effect on rat aortic contraction and other measured parameters.

Isolation of rat aorta

Male Wistar rats, 250–300 g, were deprived of food on the evening and the morning before the experiment. Similar to other investigators,[6,13–17] we used male rats due to their lower interindividual variability in oestrogen non-genomic responses. Anaesthesia was induced with pentobarbital sodium (60 mg/kg, i.p.). After cervical dislocation, the thoracic aortas were immediately removed and carefully cleaned.

Experiments with aortic smooth muscle segments

cAMP content in aortic smooth muscle and HSP20 phosphorylation were both measured in smooth muscle segments. Aortic smooth muscle segments were prepared as follows. The endothelium of the thoracic aorta was removed by gently rubbing the intima surface with a small wooden stick. Endothelium-denuded aorta was separated from the adventitia, cut into segments and then placed in flasks containing Krebs medium (pH 7.4) with 5% CO_2/95% O_2 as the gas phase.

cAMP content

Flasks containing different smooth muscle segments were incubated with DPN (0–300 μM) for 15 min at 37°C, with continuous shaking and gassing every time they were opened. The aortic segments were frozen in liquid nitrogen and then homogenised in 100 mM HCl. Samples were centrifuged (10 000*g*) and the cAMP content was determined by radioimmunoassay (Sigma Chemical Co.). Proteins were determined by the Bradford method.[18]

HSP20 phosphorylation

HSP20 phosphorylation was assessed in aortic smooth muscle segments by 2D gel electrophoresis with immunoblotting. We used a previously described method,[19] with slight modifications. Proteins were extracted from each sample with a UDC buffer containing: 8 M urea, 2 M thiourea, 30 mM Tris, 4% CHAPS, 65 mM DTT and phosphatase inhibitors (1.8 mg/ml Na_3VO_4, 13.3 mg/ml sodium pyrophosphate, 4.2 mg/ml sodium fluoride) pH 8.5. For 2D gel electrophoresis, passive rehydration was done in a UDC buffer containing: 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, 0.5% IPG buffer and traces of bromphenol blue. The first SDS equilibration buffer contained: 6 M urea, 375 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS and 130 mM DTT. The second SDS equilibration buffer contained: 6 M urea, 375 mM Tris-HCl pH 8.8, 20% (v/v) glycerol, 2% (w/v) SDS, 135 mM iodoacetamide and traces of bromphenol blue. For the immunoblotting, proteins from the 2D gel electrophoresis were transferred to Immobilon membrane (Millipore Corp., Barcelona, Spain) for 1 h at a constant voltage of 100 V. Finally, membranes incubated with β-actin were used as controls.

Experiments with isolated aortic smooth muscle cells

Endothelium-denuded aortic segments were separated from the adventitia. The muscular layer was dissociated and digested with collagenase type II in phosphate buffered saline for 8 min at 37°C. Then, samples were washed three times with Krebs medium supplemented with fetal calf serum, filtered through a 50-μ mesh and finally suspended in Krebs medium with 10 mM HEPES (pH 7.4).

Membrane potential

Membrane potential in isolated aortic smooth muscle cells was measured by flow cytometry using a previously published method.[20] Briefly, cells were loaded for 15 min with 1 μM bis-oxonol sensitive probe DIBAC2(3) (Invitrogen, Carlsbad, CA, USA). Fluorescence quantification was performed by using flow cytometry (Epics Elite, Coulter Hielary, FL, USA) with a 70 μM flow cell at 37°C. Cells were excited with an argon laser at 488 nm and the emitted fluorescence was 525 $\pm$ 25 nm. Fluorescence readings were expressed in arbitrary units.

Cytosolic calcium

Free cytosolic calcium in isolated aortic smooth muscle cells was measured by flow cytometry using a previously published method.[21] Briefly, cells were loaded for 15 min with Fluo 3-AM (Invitrogen). Fluorescence quantification was performed by using flow cytometry (Epics Elite) with a 70 μM flow cell at 37°C. Cells were excited with an argon laser at 488 nm and the emitted fluorescence was 525 $\pm$ 25 nm. Fluorescence readings were expressed in arbitrary units.

DPN relaxation of rat aortic smooth muscle

DPN relaxant responses in phenylephrine-precontracted rat aortic rings were investigated by using a previously described method.[7] Briefly, endothelium-denuded, thoracic aorta was cut into rings (3 mm long). The rings were individually placed

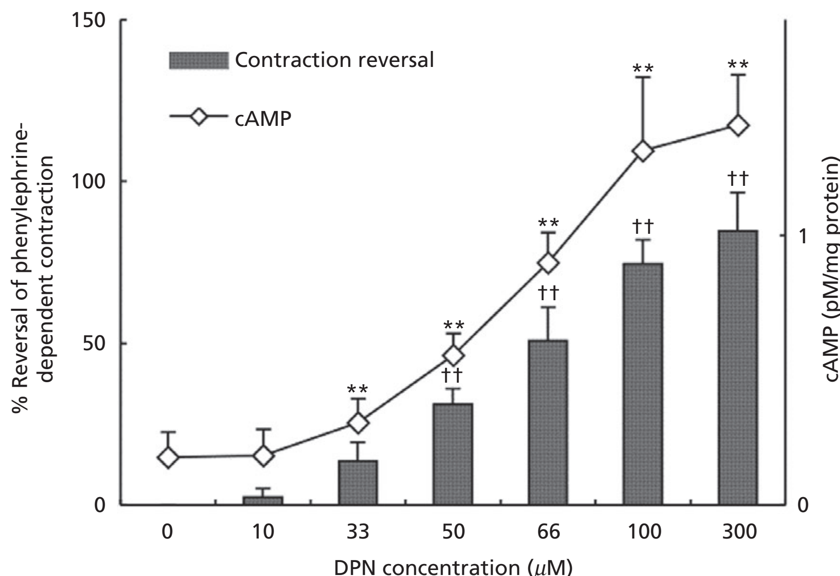

Figure 1 Diarylpropionitrile vasorelaxation and stimulation of cAMP levels in rat aortic smooth muscle. DPN, diarylpropionitrile. Values are given as mean ± SD ($n = 8$ for experiments with aortic rings and $n = 3$ for experiments with cAMP). $^{**}P < 0.01$, significantly different compared with control values (analysis of variance multiple measurement comparison, followed by the Bonferroni–Dunn test). $^{††}P < 0.01$, significantly different compared with control values (Kruskal–Wallis test, followed by unilateral Mann–Whitney test).

between platinum hooks in 5 ml Krebs solution maintained at 37°C and gassed with 95% O_2/5% CO_2. The Krebs medium contained (in mmol/l): NaCl 118, $NaHCO_3$ 25, $CaCl_2$ 1.25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.5. To suppress basal tone, 1 μM isoproterenol was added to the incubation media for 10 min. An initial load of 1 g was applied to the preparations. Then, the preparations were washed three times and maintained throughout a 40-min equilibration period during which the incubation medium was renewed every 20 min. Tension was recorded on a MacLab (Analog Digital Instruments, Castle Hill, Australia) via Dynamometers Pioden Control UF1. Then, aortic rings were contracted with 1 μM phenylephrine. Phenylephrine 1 μM evoked maximal contractile responses. After 15 min, DPN, with or without potential antagonists, or vehicle were added to the bath over 15 min. The concentration of the tested compounds was selected in control experiments on the basis of: (1) having no effect by itself on vascular tone; and (2) being active on the desired target as reported in the literature. An absence of functional endothelium was indicated by the failure of the preparation precontracted with a submaximum concentration of phenylephrine to relax in response to 1 μM acetylcholine. Finally, we verified that 1 μM cromakalim-dependent relaxation was fully inhibited by PNU-37883A with an IC50 (the concentration of compound antagonising 50% of the maximal responses to vasorelaxant agonists) of 0.27 ± 0.07 μM ($n = 5$) and by $BaCl_2$ with an IC50 of 100 μM.

Statistical analysis

Values are given as the mean ± SD. Concentration–response curves of compounds were fitted by linear regression analysis of the straight portions of the vasorelaxant responses. The IC50 was calculated for each experiment. Statistical differences between mean values were determined by using an unpaired Student's *t*-test. Multiple measurement comparison was performed by using an analysis of variance program followed by a Bonferroni–Dunn test when the sample size was greater than 5. When this condition was not fulfilled, we used a non-parametric multiple comparisons Kruskal–Wallis test, followed by unilateral Mann–Whitney test.

Results

DPN-induced relaxation is associated with increases in cAMP content

The ERβ agonist DPN was tested for its ability to relax isolated endothelium-denuded rat aortic rings precontracted with 1 μM phenylephrine ($n = 8$) using concentration–response curves. DPN relaxed aortic smooth muscle in the micromolar range of concentrations (Figure 1). The IC50 calculated from the concentration–response curves was 67.0 ± 12.2 μM.

DPN was also tested in rat aortic smooth muscle segments for its effect on cAMP content ($n = 3$). DPN greatly increased cAMP levels, with a 6–7-fold cAMP increase at a DPN concentration of 300 μM (Figure 1). 4-OH-tamoxifen (1 μM) fully suppressed the action of DPN on cAMP content (Table 1). The concentration–response curve for DPN-induced cAMP increase superimposed with its vasorelaxant action is shown in Figure 1.

DPN relaxation is reduced by PKA signalling inhibitors

Table 2 shows the effect of different compounds on the vasorelaxant responses to 50 μM DPN ($n = 6$–12 for each condition). The PKG inhibitor Rp-8-Br-cGMP was inactive in this test. Conversely, the PKA inhibitors Rp-8-Br-cAMPS

Table 1 cAMP increase by diarylpropionitrile in aortic smooth muscle

Compound	cAMP content (pM/mg protein)
Control	0.58 ± 0.04
Diarylpropionitrile (100 μM)	1.79 ± 0.12*
Diarylpropionitrile (100 μM) + 4-OH-tamoxifen (1 μM)	0.60 ± 0.19

Antagonism by 4-OH-tamoxifen. Values are given as mean ± SD, $n = 4$. *$P < 0.05$, significantly different compared with control (non-parametric Kruskal–Wallis test, followed by unilateral Mann–Whitney test).

Table 2 Effects of compounds on the vasorelaxant responses to 50 μM diarylpropionitrile

Compound	% of relaxant reduction
Rp-8-Br-cGMP (10 μM)	0.5 ± 12.2 ($n = 6$)
Rp-8-Br-cAMPS (100 μM)	73.3 ± 11.2 ($n = 10$)**
H-89 (200 nM)	73.0 ± 13.8 ($n = 10$)**
MDL12330A (20 μM)	65.5 ± 21.2 ($n = 10$)**
Diphenylamine-2-carboxylic acid (100 μM)	76.4 ± 14.4 ($n = 12$)**
Glibenclamide (100 μM)	80.3 ± 9.9 ($n = 12$)**
Iberiotoxin (30 nM)	22.9 ± 11.4 ($n = 12$)**
PNU-37883A (5 μM)	33.9 ± 12.7 ($n = 12$)**
4-Aminopyridine (1 mM)	36.9 ± 10.0 ($n = 12$)**
$BaCl_2$ (30 μM)	42.6 ± 11.0 ($n = 12$)**
Clotrimazole (100 μM)	0.1 ± 6.7 ($n = 6$)
TRAM-34 (1 μM)	8.6 ± 15.1 ($n = 6$)
Glibenclamide (10 μM)	63.1 ± 5.6 ($n = 12$)**
Iberiotoxin (30 nM) + PNU-37883A (5 μM) + 4-aminopyridine (1 mM) + $BaCl_2$ (30 μM)	85.6 ± 9.3 ($n = 12$)††
Glibenclamide (100 μM) + H-89 (200 nM)	88.4 ± 7.2 ($n = 12$)††
Glibenclamide (100 μM) + iberiotoxin (30 nM)	89.7 ± 3.0 ($n = 12$)††

Values are given as mean ± SD. The number of experiments is indicated in parentheses. **$P < 0.01$ and $^{††}P < 0.01$, significantly different compared with control and single ion channel inhibitor, respectively (one-way analysis of variance, followed by Bonferroni–Dunn test).

(8-bromoadenosine-3′, 5′-cyclic monophosphorothioate, Rp-isomer) and H-89 (*N*-(2-bromocynnamyl(amino)ethyl)-5-isoquinoline sulfonamide HCl) greatly reduced DPN-induced vasorelaxation (–73%; Table 2). Vasorelaxant responses to DPN were also greatly reduced by the adenylate cyclase inhibitor MDL12330A (*cis*-*N*-(2-phenylcyclopentyl)-azacyclotridec-1-en-2-amine)) (–65.5%).

DPN-induced calcium signal is reversed by PKA inhibition

Free cytosolic calcium in isolated aortic smooth muscle cells was measured. Phenylephrine (2 μM) induced a 20.3 ± 2.5% increase in the Fluo 3-AM fluorescence signal in cells treated with vehicle, and 2.2 ± 1.8% in the presence of 100 μM DPN. The PKA inhibitor Rp-8-Br-cAMPS (100 μM) partially reversed (–61.3%, $n = 4$) the antagonistic action of DPN on the phenylephrine-induced calcium signal.

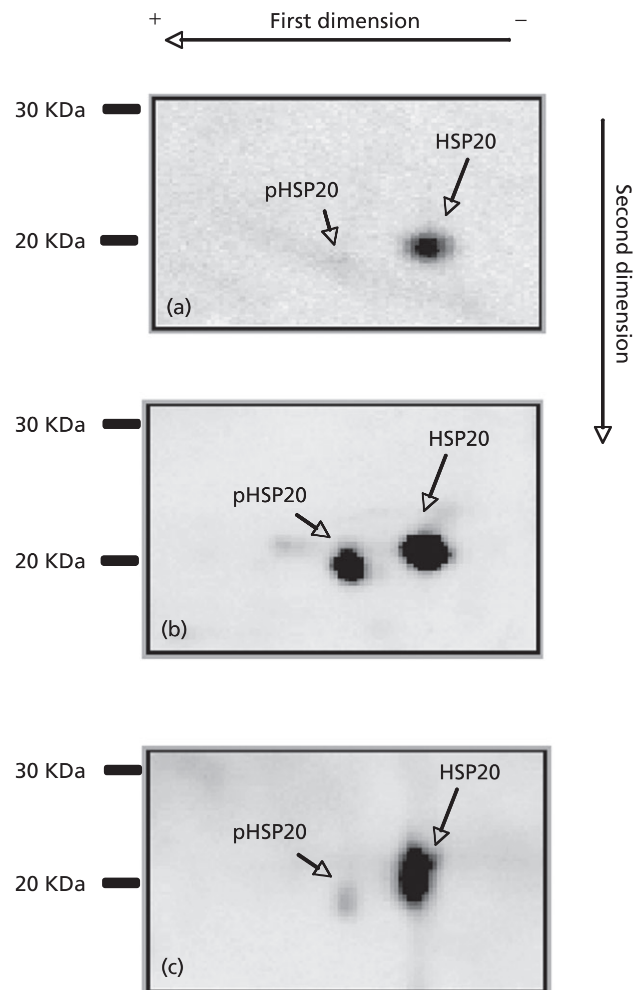

Figure 2 Phosphorylation of HSP20 with diarylpropionitrile. Rat aortic smooth muscle segments were treated with: (a) vehicle alone; (b) diarylpropionitrile; or (c) diarylpropionitrile + H89 ($n = 6$). The segments were homogenised, separated by 2D gel electrophoresis and analysed by Western blotting. The Figure shows a typical experiment where treatment with diarylpropionitrile led to increases in the phosphorylation of HSP20 (pHSP20), an effect reversed by the PKA inhibitor H89.

Targets of PKA signalling

DPN increases PKA-dependent HSP20 phosphorylation

Rat aortic smooth muscle segments (approximately 10 mg) were incubated for 15 min at 37°C with DPN, DPN with the PKA inhibitor H-89 (*N*-(2-bromocynnamyl(amino)ethyl)-5-isoquinoline sulfonamide) or vehicle, and the amount of pHSP20 was determined using 2D gel electrophoresis and immunoblotting ($n = 6$). Vehicle-treated segments and segments treated with DPN + H-89 showed no phosphorylation of HSP20, whereas those treated with DPN alone showed an increase in HSP20 phosphorylation (Figure 2).

Membrane ion channels

Table 2 shows that vasorelaxant responses to DPN were greatly reduced by: (1) the cystic fibrosis transmembrane con-

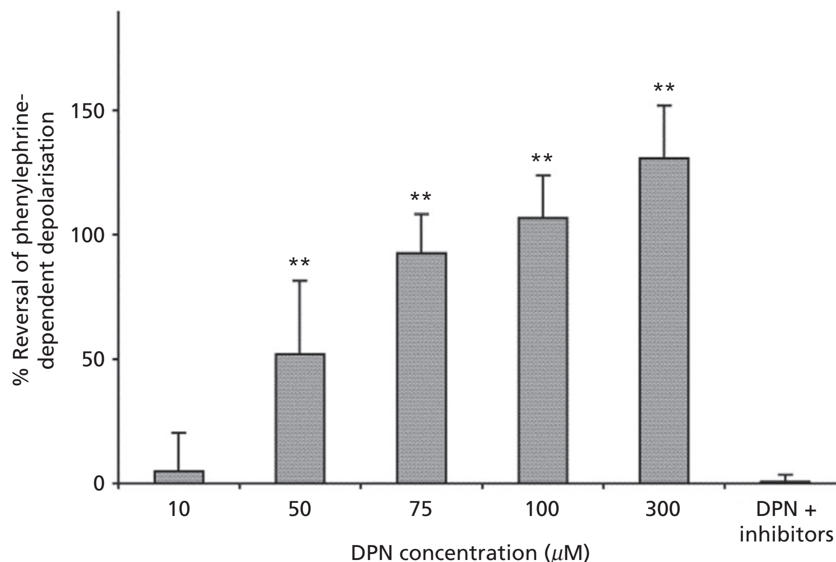

Figure 3 Antagonism by diarylpropionitrile of phenylephrine-induced membrane depolarisation in isolated rat aortic smooth muscle cells. Phenylephrine (2 μM) induced a 33 + 10% ($n = 7$) increase in the fluorescence signal of the bis-oxonol sensitive probe DIBAC2(3), which was significantly reversed by diarylpropionitrile (DPN). Values are given as mean ± SD ($n = 4$). $^{**}P < 0.01$, significantly different compared with control values (Kruskal–Wallis test, followed by unilateral Mann–Whitney test). The hyperpolarising effect of DPN (75 μM) was fully inhibited by a combination of the potassium channel inhibitors iberiotoxin (30 nM), PNU-37883A (5 μM), 4-aminopyridine (1 mM) and $BaCl_2$ (30 μM) (DPN + inhibitors).

ductance regulator (CFTR) inhibitor DPC (diphenylamine-2-carboxylic acid) (–76.4%; the selective CFTR inhibitor Inh-172 reduced by 60–70% DPN vasorelaxation at the non-saturating dose of 0.8 μM, but at higher doses it relaxed the preparation by itself); (2) 100 μM glibenclamide (a concentration able to inhibit both CFTR and K_{ATP} channels) (–80.3%). Glibenclamide concentrations able to inhibit K_{ATP} channels (10 μM) antagonised DPN relaxation to a lesser extent (–63.1%; Table 1).

The vasorelaxant responses to DPN (50 μM) were modestly antagonised (–23 to –48% reduction) by iberiotoxin (IbTX), a selective BK_{Ca} inhibitor, by PNU-37883A (4-morpholinecarboxamidine-*N*-1-adamantyl-*N′*-cyclohexyl-hydrochloride), a selective inhibitor of ATP-sensitive potassium channels (K_{ATP}), by 4-aminopyridine (4-AP) an inhibitor of the voltage-gated potassium channels (K_v), and by $BaCl_2$, an inhibitor of the inward rectifier potassium channel (K_{IR}) (Table 2). Conversely, clotrimazole (1-(*o*-chlorotrityl) imidazole) and TRAM-34 (1-((2-chlorophenyl) diphenylmethyl)-1H-pyrazole), two inhibitors of calcium-activated potassium channels of intermediate conductance, were unable to inhibit DPN-dependent vasorelaxation (Table 2).

The four potassium channel inhibitors that modestly inhibited DPN vasorelaxation (IbTX, PNU-37883A, 4-AP and $BaCl_2$) were combined to investigate potential additive effects. The most interesting result was that the simultaneous addition of these four potassium channel inhibitors inhibited DPN vasorelaxation significantly more (–86%) than any one of them added separately (Table 2).

Several ion channel inhibitors were tested on the H-89-resistant component of DPN vasorelaxation (PKA-independent pathway). The most interesting result was that glibenclamide (100 μM) inhibited by about half the H-89-resistant DPN vasorelaxation. Glibenclamide (100 μM) also had additive effects with IbTX (the latter had no additive effects with H-89, data not shown) (Table 2).

Membrane potential

In isolated rat aortic smooth muscle cells, phenylephrine (2 μM) induced a 33 + 10% ($n = 7$) increase in the fluorescence signal of the bis-oxonol sensitive probe DIBAC2(3), indicating membrane depolarisation. DPN fully abolished phenylephrine depolarisation, and even reversed it at high concentrations (Figure 3). The concentration–response curve for DPN antagonism of membrane depolarisation (Figure 3) superposed with those for cAMP increase and vasorelaxant action (Figure 1). Finally, the hyperpolarising effect of DPN in isolated aortic smooth muscle cells was also fully inhibited by the combination of IbTX, PNU-37883A, 4-AP and BaCl2 (Figure 3).

Receptors of DPN signalling

Several authors assumed that DPN relaxes vascular smooth muscle by acting on ERβ.[4,6,17,22] Therefore, we attempted to verify this point in our preparation. The DPN interaction with ERs was investigated using the ER antagonists tamoxifen, 4-OH-tamoxifen and ICI 182 780. Unfortunately, these antagonists, particularly tamoxifen and ICI 182 780, had vasorelaxant effects by themselves at active antagonistic concentrations (for relaxant actions of ER antagonists see Austin[23]). A maximal, non-relaxant 4-OH-tamoxifen concentration of 100 nM antagonised DPN relaxation by 73.7 ± 6.6% ($n = 7$). By contrast, the selective ERα antagonist methyl-piperidinopyrazole[24] was inactive against the relaxant responses to 100 μM DPN (66.5 ± 10.0 vs 66.8 ± 5.9% relaxation in the absence and presence of 1 μM methyl-piperidinopyrazole, respectively). Methyl-piperidinopyrazole

was used at 1 μM because this concentration was previously found to selectively block ERα stimulation of pS2 mRNA in MCF-7 breast cancer cells.[24]

Discussion

The ERβ agonist DPN increased cAMP levels in rat aortic smooth muscle over the same range of concentrations as it antagonised phenylephrine depolarisation and phenylephrine contraction. Moreover, DPN vasorelaxation was largely reduced by the adenylate cyclase inhibitor MDL12330A and the PKA inhibitors H-89 and Rp-cAMP. Finally, DPN increased PKA-dependent HSP20 phosphorylation. These results clearly show that ERβ signalling evokes a PKA-operated pathway.

In vascular smooth muscle, non-genomic actions of oestrogen can be evoked by three different membrane receptors, ERα, ERβ and the G-protein coupled receptor GPR30.[2,5,7,25,26] DPN has high ERβ/ERα selectivity, it does not stimulate GPR30-mediated actions, and several authors assumed that it relaxes vascular smooth muscle by acting on ERβ receptors.[4,6,17,22,25,26] These relaxant effects are often seen at high (micromolar) agonist concentrations, whereas binding affinities are frequently seen in the nanomolar range. Moreover, we previously found that the selective ERα agonist propyl pirazole relaxes rat aortic smooth muscle.[7] Therefore, we verified that DPN relaxation of rat aortic smooth muscle was mediated by ERβ stimulation, that is DPN relaxation was insensitive to the selective ERα antagonist methyl-piperidinopyrazole, whereas it was largely reduced by the antagonist 4-OH-tamoxifen.

It is well established that vasodilators that induce relaxation by elevating intracellular cAMP concentrations and activating PKA in vascular myocytes cause hyperpolarisation of membrane potential by increasing K^+ channel open probability.[27,28] Accordingly, phenylephrine depolarisation in aortic smooth muscle cells was fully abolished and even reversed by DPN, over the same range of concentrations that it increased cAMP content and relaxed aortic smooth muscle. Moreover, the use of selective inhibitors suggested that four potassium channels participate in membrane hyperpolarisation by DPN, namely BK_{Ca}, K_{ATP}, K_v and K_{IR} channels.

Wang *et al.*[29] suggested that DPN can interact directly with the BK_{Ca} channel to affect potassium currents. Such direct action of DPN can be excluded because DPN vasorelaxation was inhibited by the selective PKA inhibitor Rp-8-CPT-cAMPS, which has little or no significant effect on BK_{Ca},[30] and by the adenylate cyclase inhibitor MDL12330A.

In the experiments with $BaCl_2$, we used a concentration sufficiently high (30 μM) to inhibit K_{IR} channels,[31] but lower than that reported to inhibit K_{ATP} channel current in several cell types.[32–34]

Our results suggest that DPN signalling in rat aortic smooth muscle cells involves membrane hyperpolarisation by four potassium channels: BK_{Ca}, K_{ATP}, K_v and K_{IR} channels. Other vasodilators acting via cAMP were also found to activate one or more of these potassium channels. β-Adrenoceptor stimulation activates K_v currents in rabbit portal vein myocytes, via a transduction pathway involving adenylyl cyclase and PKA.[35,36] Both BK_{Ca} and K_v channels were found to be involved in PGI_2 relaxation of the rabbit middle cerebral artery[14] and also in isoprenaline relaxation of rat aortic rings.[37] Hypoxia augments K_{IR} currents in rabbit coronary arterial smooth muscle cells via cAMP- and PKA-dependent signalling cascades, which might, at least in part, explain hypoxia-induced coronary vasodilation.[38] Finally, PKA-operated, ATP-sensitive potassium (K_{ATP}) channels were found to be activated by calcitonin gene-related peptide in isolated smooth muscle cells from pig coronary arteries,[39] rabbit mesenteric arteries[40] and gallbladder,[41] and by adenosine in isolated smooth muscle cells from rabbit mesenteric arteries[42] (however, see Martinez *et al.*,[15] Satake *et al.*[37] and Omar *et al.*[43]).

PKA can directly phosphorylate and activate the BK_{Ca} channel itself[12,44] and Zhou *et al.*[12] identified one isoform (BK_C, cloned from tracheal smooth muscle) that was exclusively stimulated by PKA. Conversely, we found no evidence in the literature of direct PKA-dependent phosphorylation of K_{ATP}, K_{IR} or K_v channels.

Robert *et al.*[45] reported that the PKA-operated, CFTR chloride channel is a target for vasodilators that increase cAMP. Accordingly, we found that DPN vasorelaxation was greatly reduced by the CFTR chloride channel inhibitors DPC, glibenclamide (100 μM) and Inh-172 (glibenclamide inhibits CFTR currents with an IC50 = 20 μM[46]). Moreover, IbTX exhibited additive effects with glibenclamide (100 μM). Therefore, a PKA-operated CFTR can participate as an intermediate element in K_{ATP}, K_{IR} or K_v activation by DPN.

In the rat pulmonary artery, DPN decreased pulmonary artery vasoconstriction by a nitric oxide-dependent mechanism.[47] Therefore, ERβ stimulation can activate different signalling pathways in different vascular territories.

Bolego *et al.*[22] pointed out that DPN vasorelaxation was observed at pharmacological (i.e. micromolar) concentrations, thereby questioning the possible relevance of ERβ vasorelaxation to the biology of the vascular wall. However, DPN is not a natural substance to directly extrapolate biological relevant conclusions from its active concentration range. Moreover, rat aorta relaxation by the non-selective ER agonist diethylstilbestrol is partially antagonised by the PKA inhibitor Rp-cAMPS and by the K_{ATP} channel inhibitor glibenclamide (1–10 μM), strongly suggesting the involvement of PKA-operated K_{ATP} channels[15] in ER signalling. On the other hand, Zhu *et al.*[48] found that ERβ-deficient mice develop sustained hypertension as they age. Therefore, the Erβ signalling pathway may become important when the NO/cGMP is impaired (e.g. by ageing of cardiovascular disease).

Two minor aspects of our investigation deserve comment. First, H-89 exhibited additive effects with glibenclamide (100 μM), suggesting that CFTR and/or K_{ATP}, can mediate, at least in part, a PKA-independent pathway of DPN vasorelaxation. Second, the soy phytoestrogen genistein, which possesses 20-fold higher relative binding affinity for ERβ than for ERα[49] and is a potent endothelium-independent vasorelaxant,[50,51] can act by a similar mechanism as DPN.

Conclusions

ERβ-dependent relaxation of rat aortic smooth muscle evokes an adenylate cyclase/cAMP/PKA signalling pathway, likely

activating the CFTR chloride channel and at least four potassium channels. The establishment of a causal link between the adenylate cyclase/cAMP/PKA pathway and the activation of the above-mentioned channels requires more direct electrophysiological experiments (channel activation by DNP and antagonism by modulators of the adenylate cyclase/cAMP/PKA pathway).

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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