Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 487 (2004) 167–173
www.elsevier.com/locate/ejphar

Protein kinase A increases electrical stimulation-induced neuronal nitric oxide release in rat mesenteric artery

Mercedes Ferrer[a], Margarita Sánchez[a], Maria del Carmen Martín[a], Iván Márquez-Rodas[a], Maria Jesús Alonso[b], Mercedes Salaices[b], Gloria Balfagón[a,*]

[a] *Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma, C/Arzobispo Morcillo, 4, Madrid 28029, Spain*
[b] *Departamento de Farmacologia y Terapéutica, Facultad de Medicina, Universidad Autónoma, C/Arzobispo Morcillo, 4, Madrid 28029, Spain*

Received 21 October 2003; received in revised form 5 January 2004; accepted 21 January 2004

Abstract

The aim of this study was to analyse the possible influence of cyclic AMP–protein kinase A (cAMP–PKA) activation on neuronal nitric oxide (NO) release induced by electrical field stimulation in mesenteric arteries from Wistar Kyoto (WKY) rats. Western blot experiments demonstrated the expression of neuronal NO synthase (nNOS) in mesenteric artery from WKY rats; however, electrical field stimulation alone did not induce detectable NO release. Preincubation with forskolin allowed NO release induced by electrical field stimulation, which was abolished by: the neuronal toxine tetrodotoxin, the nNOS inhibitors 7-nitroindazole or N^{ω}-propil-L-arginine (NPLA), and the PKA inhibitors *N*-(2-(*p*-Bromocinnamylamino) ethyl 5-isoquinolinesulfonamide hydrochloride (H-89) or (9*R*,10*S*,12*S*)-2,3,9,10,11, 12-Hexahydro-10-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-*fg*:3,2,1*k*)pyrrolo(3,4-*l*)(1,6) benzodiazocine-10-carboxylic acid hexyl ester (KT-5720). Preincubation with prostacyclin also allowed the NO release induced by electrical field stimulation which was significantly decreased by: the neuronal toxine tetrodotoxin, the nNOS inhibitors 7-nitroindazole or NPLA, and the PKA inhibitors H-89 or KT-5720. The NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) did not modify the vasoconstrictor response induced by electrical field stimulation. However, in the presence of forskolin or prostacyclin, L-NAME increased the vasoconstrictor response to electrical field stimulation. These results indicate that forskolin and prostacyclin allow neuronal NO release induced by electrical field stimulation through a mechanism involving cAMP–PKA activation in rat mesenteric arteries.

Keywords: Nitrergic innervation; Mesenteric artery; cAMP; PKA (protein kinase A); Forskolin

1. Introduction

Nitric oxide (NO) is an important neurotransmitter in both the peripheral (Marín and Balfagón, 1998) and central nervous (Bredt et al., 1992) system. Biosynthetic regulation of NO is very important because NO cannot be stored in nitrergic endings. Neuronal nitric oxide synthase (nNOS), the regulator of NO in neurons, is regulated by multiple kinases (Dinerman et al., 1994). Several studies have reported that protein kinase A (PKA) could phosphorylate nNOS in vivo and in vitro (Brune and Lapetina, 1991; Bredt et al., 1992; Dinerman et al., 1994; Yu et al., 2002) in the central nervous system.

Perivascular nitrergic innervation has been described in several vascular beds such as the cerebral (Estrada et al., 1993) and mesenteric arteries (Marín and Balfagón, 1998). We have demonstrated that β_2-adrenoceptor activation increases neuronal NO release in mesenteric arteries from different rat strains (Marín and Balfagón, 1998; Ferrer et al., 2003). On the other hand, it has been widely described that β-adrenoceptors agonist activation increases cAMP formation, with the subsequent PKA activation (Taylor et al., 1990; Zhang and Hintze, 2001). As stated above, PKA activation regulates NO release in the central nervous system; however, little is yet known about a possible effect of PKA on neuronal NO release in vascular beds.

Prostacyclin is an endogenous prostanoid with important vascular effects, whose mechanism of action is through adenylyl cyclase activation and subsequent PKA activation (Narumiya et al., 1999). With this in mind, it is reasonable to hypothesize that prostacyclin can modulate the neuronal NO release in a manner like already described for endothelial NO (Shimokawa et al., 1988). Therefore, the aim of this

* Corresponding author. Tel.: 34-91-4975450, Fax: 34-91-4975353.
E-mail address: gloria.balfagón@uam.es (G. Balfagón).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.01.030

work was to study the possible role of the cAMP–PKA pathway, in the neuronal NO release in rat mesenteric artery, using forskolin and prostacyclin.

2. Materials and methods

2.1. Tissue preparation

Male 6-month-old WKY rats (250–300 g) were used. They were sacrificed by CO_2 inhalation. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the USA National Institutes of Health (NIH publication No. 85.23 revised 1985). The first branch of the mesenteric artery was carefully dissected out, cleaned of connective tissue and placed in Krebs–Henseleit solution (KHS) at 4 °C. In the present work, we used endothelium-denuded segments to eliminate this source of vascular NO and avoid the possible actions by different study drugs on the endothelial cells that could lead to misinterpretation of results. The absence of vascular endothelium was tested by the inability of acetylcholine to relax segments precontracted with noradrenaline.

2.2. Nitric oxide release

Denuded rat mesenteric artery segments (24.4 ± 2.6 mg) were immersed for 30 min in 10 ml of KHS at 37 °C continuously gassed with a 95% O_2–5% CO_2 mixture (stabilization period). Afterwards, the arteries were transferred to a 500-μl chamber containing two parallel platinum electrodes, 0.5 cm apart, connected to a stimulator (Grass, model S44) for electrical field stimulation. After two washout periods of 6.5 min, the medium was collected to measure the basal release of NO, which was estimated as nitrites. Once the chambers had been refilled, cumulative electrical field stimulation periods of 30 s at 1, 2, 4, 8 and 16 Hz at 1 min intervals were applied, and then the medium was taken from the bath to measure the concentration of nitrites, according to the colorimetric method based on the Griess reaction (Griess, 1979). To eliminate the possibility that electrical field stimulation induced nitrites production in the incubation medium, experiments were also performed in the absence of tissue.

To study the effect of forskolin (0.1 and 0.5 nM) and prostacyclin (10 and 50 nM) in NO release, the arteries were incubated with one of these substances during 30 min before being placed in the electrical field stimulation chamber. To determine the neuronal NO origin, in another set of experiments either neuronal toxin tetrodotoxin (0.1 μM), or nNOS inhibitors 7-nitroindazole (10 μM) or N^{ω}-propil-L-arginine (NPLA, 0.1 μM) was added to the bath 10 min before 0.5 nM forskolin or 50 nM prostacyclin. To analyse the possible participation of PKA in the forskolin effect on NO release, a PKA inhibitor, either *N*-(2-(*p*-Bromocinnamylamino) ethyl 5-isoquinolinesulfonamide hydrochloride (H-89, 1 μM), or (9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo(1,2,3-*fg*:3,2,1*k*)pyrrolo(3,4-*l*)(1,6)benzodiazocine-10-carboxylic acid hexyl ester (KT-5720, 0.1 and 1 μM), was added to the bath 10 min before forskolin. To analyse the possible participation of PKA in the prostacyclin effect on NO release, H-89 (1 μM), or KT-5720 (1 μM), was added to the bath 10 min before prostacyclin. The amount of nitrites released was expressed in pmol/mg tissue.

2.3. Western blot analysis of neuronal NOS expression

For Western Blot analysis of nNOS protein expression, superior mesenteric arteries were homogenized in a boiling buffer composed of 10 mM (pH = 7.4), 1% sodium lauryl sulphate (SDS) and the protease inhibitor sodium metavanadate (1 mM). Homogenates containing 15 μg protein were electrophoretically separated on a 7.5% SDS-polyacrylamide gel (SDS-PAGE) and then transferred to polyvinyl difluoride membranes overnight, using a Bio-Rad Trans-Blot Cell system (Bio-Rad Laboratories, Hercules, CA, USA) containing 25 mM Tris, 190 mM glycine, 20% methanol and 0.05% SDS. Prestained SDS-PAGE standards (Bio-Rad Laboratories) were used as molecular mass standards. The membrane was blocked for 60 min at room temperature in Tris-buffered solution (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% non-fat powdered milk before being incubated for 1 h at room temperature with mouse monoclonal antibody for nNOS (1:2500 dilution), all purchased from Transduction Laboratories (Lexington, UK). After washing, the membrane was incubated with a 1:2000 dilution of antimouse Immunoglobulin G antibody conjugated to horseradish peroxidase (Transduction Laboratories). The membrane was thoroughly washed and the immunocomplexes were detected using an enhanced horseradish peroxidase/luminol chemiluminiscence system (ECL Plus, Amersham International, Little Chalfont, UK) and subjected to autoradiography (Hyperfilm ECL, Amersham International). Signals on the immunoblot were quantified using a computer program (NIH Image V1.56). The same membrane was used to determine α-actin expression, and the content of the latter was used to correct NOS expression in each sample, by means of a monoclonal antibody anti α-actin (1:30000 dilution, Boehringer Mannheim, Mannheim, Germany). Rat pituitary (from Transduction Laboratories) was used as positive control for nNOS; in addition, homogenates from rat caudal arteries, with a known absence of nNOS protein expression (Rossoni et al., 2002) was used as negative control.

2.4. Vascular reactivity

The method used for isometric tension recording has been described elsewhere (Marín and Balfagón, 1998). Experiments were performed in endothelium-denuded segments to eliminate the main source of vasoactive substances

so as to avoid any action by different drugs on the endothelial cells that could lead to misinterpretation of results. The segments were subjected to a tension of 0.5 g that was readjusted every 15 min during a 90 min equilibration period before drug administration. After this, the vessels were exposed to 75 mM K^+ to check their functional integrity. The absence of vascular endothelium was proven by the inability of 10 μM acetylcholine to relax segments precontracted with 1 μM noradrenaline.

Two frequency–response curves to electrical field stimulation (1, 2, 4, 8 and 16 Hz) were performed in a consecutive manner. The parameters used for electrical field stimulation were 200 mA, 0.3 ms, for 30 s with an interval of 1 min between each stimulus, the time required to recover basal tone. A rest period of at least 1 h was necessary to avoid desensitization between consecutive curves.

To determine the participation of NO on electrical field stimulation-induced responses in control segments and segments pretreated with prostacyclin (50 nM) or forskolin (0.5 nM), 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) or 10 μM 7-nitroindazole was added to the bath 30 min before a second frequency–response curve.

The possible effect of L-NAME and of 7-nitroindazole on noradrenaline–response curves was studied by examining the response to exogenous noradrenaline (10 nM–10 μM) in the presence of the drug.

The possible effect of L-NAME and 7-nitroindazole on the vasodilator response to sodium nitroprusside (0.1 nM–10 μM) was analysed in noradrenaline precontracted segments (1016 ± 89 mg).

2.5. Solutions and drugs

The composition of KHS was as follows (mM): NaCl 115, CaCl2 2.5, KCl 4.6, KH_2PO_4 1.2, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, glucose 11.1, Na_2 EDTA 0.03 (to prevent the oxidation of unstable substances). Drugs used were: N^{ω}-propil-L-arginine (Tocris Cookson, Bristol, UK (NPLA), N^{ω}-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside, tetrodotoxin, 7-nitroindazole, *N*-(2-(*p*-Bromocinnamylamino) ethyl 5-isoquinolinesulfonamide hydrochloride (H-89), (9*R*,10*S*,12*S*)-2,3,9,10,11,12-Hexahydro-10-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo (1,2,3-*fg*:3,2,1*k*)pyrrolo(3,4-*l*)(1,6)benzodiazocine-10-carboxylic acid hexyl ester (KT-5720), HEPES, leupeptin, pepstatin A, bestatin, chymostatin, PMSF, soybean trypsin inhibitor, calmodulin, EDTA, BH_4, FAD, FMN, NADPH, L-valine, L-citrulline, glycine, sodium metavanadate and dithiothreitol (Sigma, St. Louis, MO, USA); gentamycin sulphate (Biological Industries, Kibbutz Beit-Haemek, Israel), Tween 20, Tris, SDS and acrylamide (Bio-Rad, Laboratories, Hercules, CA, USA) and methanol and sucrose (Merck, Darmstadt, Germany). Stock solutions (10 mM) of drugs were made in distilled water, except for 7-nitroindazole, which was dissolved in ethanol and KT-5720 in DMSO. These solutions were kept at −20 °C and appropriate dilutions were made in KHS on the day of the experiment.

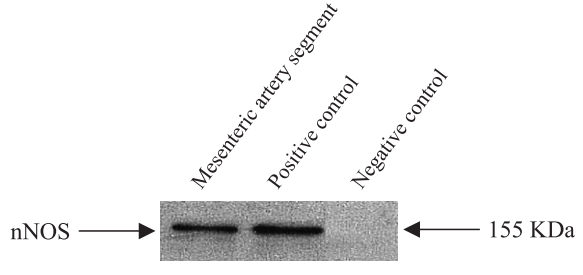

Fig. 1. Representative Western blot of four distinct experiments for nNOS protein expression in intact mesenteric artery segments from WKY rats ($n = 4$). Rat pituitary and caudal arteries from WKY rats were also used as positive and negative controls, respectively. Arterial homogenates were subjected to SDS-PAGE followed by immunoblot analysis using anti-nNOS antibody (see Materials and methods for more details).

Table 1
Effect of forskolin, tetrodotoxin plus forskolin, 7-nitroindazole plus forskolin, NPLA plus forskolin, H-89 plus forskolin or KT-5720 plus forskolin on nitrite release induced by electrical field stimulation in mesenteric arteries from WKY rats

	(pmol/mg)
Control	undetected
0.1 nM forskolin	undetected
0.5 nM forskolin	59.4 ± 9
0.1 μM tetrodotoxin + 0.5 nM forskolin	undetected
10 μM 7-nitroindazole + 0.5 nM forskolin	undetected
0.1 μM NPLA + 0.5 nM forskolin	undetected
1 μM H-89 + 0.5 nM forskolin	undetected
0.1 μM KT-5720 + 0.5 nM forskolin	47.6 ± 5.7
1 μM KT-5720 + 0.5 nM forskolin	undetected

Results (mean ± S.E.M.) are expressed in pmol/mg tissue. $n = 5–7$.

2.6. Statistical analysis

Results are given as mean ± S.E.M. of the number of rats indicated in each case. The results of NOS expression were expressed as the ratio between signals on the immunoblot corresponding to nNOS and α-actin. Statistical analysis was

Table 2
Effect of prostacyclin, tetrodotoxin plus prostacyclin, 7-nitroindazole plus prostacyclin, NPLA plus prostacyclin, H-89 plus prostacyclin or KT-5720 plus prostacyclin on nitrite release induced by electrical field stimulation in mesenteric arteries from WKY rats

	(pmol/mg)
Control	undetected
10 nM prostacyclin	undetected
50 nM prostacyclin	9.8 ± 0.7
0.1 μM tetrodotoxin + 50 nM prostacyclin	undetected
10 μM 7-nitroindazole + 50 nM prostacyclin	undetected
0.1 μM NPLA + 50 nM prostacyclin	undetected
1 μM H-89 + 50 nM prostacyclin	undetected
1 μM KT-5720 + 50 nM prostacyclin	undetected

Results (mean ± S.E.M.) are expressed in pmol/mg tissue. $n = 5–7$.

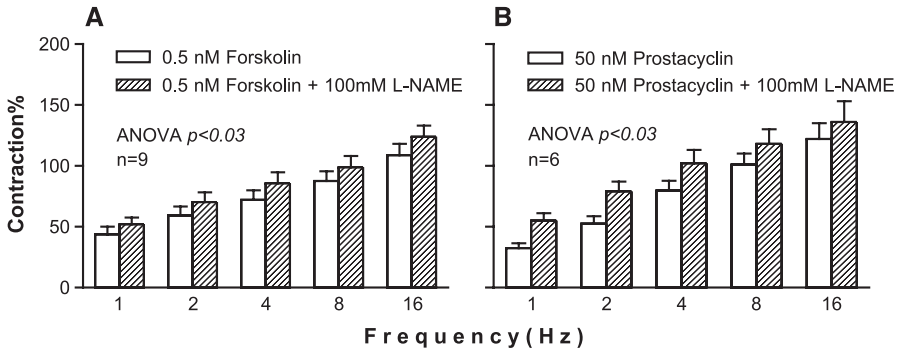

Fig. 2. Effect of 100 μM L-NAME on the frequency–response Curves in mesenteric segments from WKY rats preincubated with 0.5 nM forskolin (A) or with 50 nM prostacyclin (B). Results (mean ± S.E.M.) are expressed as a percentage of a previous tone with 75 mM K^+ (1021 ± 107 mg); *n*, number of animals.

done by comparing the modifications induced by some drugs in electrical field stimulation-induced nitrites release. A Student's *t*-test for unpaired experiments was used. A *P* value of less than 0.05 was considered significant.

3. Results

nNOS protein expression was detected in homogenates from fresh rat superior mesenteric arteries (Fig. 1).

Basal nitrites formation was not detected in the medium in the presence or absence of forskolin (data not shown). Electrical field stimulation did not induce a detectable nitrites formation in control segments or segments pretreated with 0.1 nM forskolin, but in segments pretreated with 0.5 nM forskolin, electrical field stimulation induced a significant increase. This release was significantly decreased by the neuronal toxin tetrodotoxin (0.1 μM), the nNOS inhibitors 7-nitroindazole (10 μM) and NPLA (0.1 μM). Electrical field stimulation-induced nitrites formation was also significantly decreased by the PKA inhibitor H-89 (1 μM) and KT-5720 (1 μM). However, a lower concentrations of KT-5720 (0.1 μM) did not modify the nitrites formation induced by electrical field stimulation in the presence of forskolin (Table 1).

Basal nitrites formation was not detected in the medium in the presence or absence of prostacyclin (data not shown). Electrical field stimulation did not induce any detectable nitrites formation in segments pretreated with 10 nM prostacyclin but, in segments pretreated with prostacyclin (50 nM), electrical field stimulation induced a significant increase. This release was significantly decreased by the neuronal toxin tetrodotoxin (0.1 μM), the nNOS inhibitors 7-nitroindazole (10 μM) or NPLA (0.1 μM), and the PKA inhibitors H-89 or KT-5720 (1 μM) (Table 2).

In a previous work we have reported that the NOS inhibitor, L-NAME, did not modify the vasoconstrictor response induced by electrical field stimulation in endothelium-denuded segments (Marín et al, 2000). However, L-NAME (100 μM) increased the vasoconstrictor response induced by electrical field stimulation in arteries pretreated with forskolin (0.5 nM) or prostacyclin (50 nM) (Fig. 2A and B), whereas 7-nitroindazole (10 μM) did not modify this response. (Fig. 3A and B).

The contraction induced by exogenous noradrenaline (1 nM–10 μM) remained unmodified by the pretreatment with L-NAME although it was decreased by 7-nitroindazole (Fig. 4).

In segments precontracted with noradrenaline, sodium nitroprusside (0.1 nM–10 μM) induced a concentration-

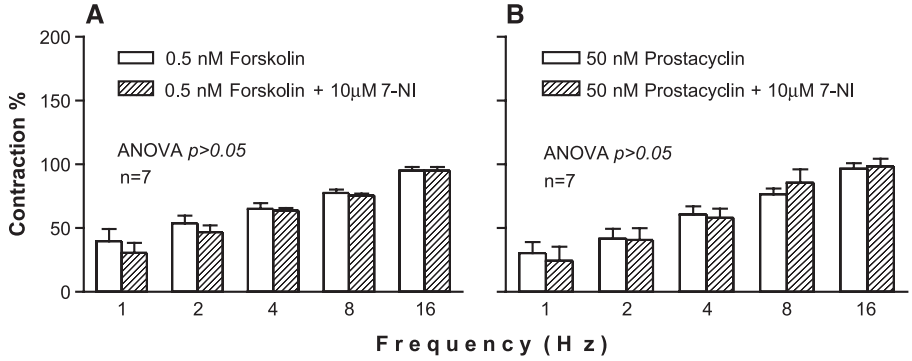

Fig. 3. Effect of 10 μM 7-nitroindazole (7-NI) on the frequency–response curves in mesenteric segments from WKY rats preincubated with 0.5 nM forskolin (A) or with 50 nM prostacyclin (B). Results (mean ± S.E.M.) are expressed as a percentage of a previous tone with 75 mM K^+ (975 ± 117 mg); *n*, number of animals.

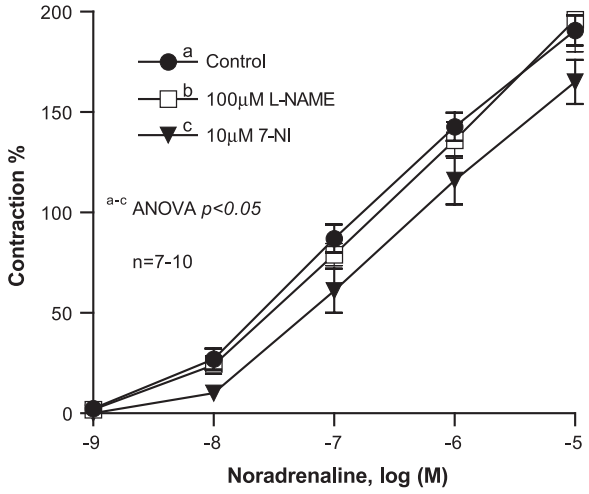

Fig. 4. Effect of 100 μM L-NAME or 10 μM 7-nitroindazole (7-NI) on the concentration–response curves to noradrenaline in mesenteric segments from WKY rats. Results (mean ± S.E.M.) are expressed as a percentage of a previous tone with 75 mM K^+ (934 ± 43 mg); *n*, number of animals.

dependent relaxation that was not modified by L-NAME or 7-nitroindazole (data not shown).

4. Discussion

The findings shown in this work suggest that PKA activation, probably through a cAMP-dependent pathway, increases the neuronal NO release induced by electrical field stimulation in rat mesenteric arteries.

In a previous work we observed the functional role of the neuronal NO released by electrical field stimulation in mesenteric arteries from SHR rats (Marín et al., 2000), and we have recently quantified this neuronal NO (Ferrer et al., 2003). However, in mesenteric segments from WKY rats, the neuronal NO does not seem to participate in the vasomotor response induced by electrical field stimulation (Marín et al., 2000). Therefore, our first objective was to quantify the neuronal NO release induced by electrical field stimulation. The results obtained in the present work confirm that electrical field stimulation-stimulated NO release could not be detected in WKY rat mesenteric arteries, which suggests that nNOS would be unexpressed in normotension. However, Western blot analysis showed nNOS protein expression in these arteries, indicating a possible non-activation of nNOS in our experimental conditions.

PKA regulation of NOS has been described in several tissues. In the central nervous system, nNOS is regulated in multiple ways, one of which is cAMP-dependent PKA (Brune and Lapetina, 1991; Bredt et al., 1992; Dinerman et al., 1994). On the other hand, it is known that β-adrenoceptor agonists act through an increase in cAMP production with subsequent PKA activation (Zhang and Hintze, 2001). Similarly, β-adrenoceptor agonists has been reported to increase endothelial NO production (Graves and Poston, 1993), and we also have demonstrated that, in rat mesenteric arteries, β-adrenoceptor activation increases neuronal NO release (Marín and Balfagón, 1998; Ferrer et al., 2003). Therefore, we studied if forskolin, which directly activates adenylate cyclase and thereby increase the concentration of cAMP (Seamon et al., 1981), modulated NO release induced by electrical field stimulation in segments from WKY rats. The results obtained indicate that forskolin increased stimulated NO release. The fact that tetrodotoxin significantly decreased the nitrite release induced by electrical field stimulation in the presence of forskolin, indicates that the NO came from nerve endings. Additionally, we studied the actions of the specific nNOS inhibitors 7-nitroindazole (Kalisch et al., 1996) and NPLA (Ralevic, 2002) on electrical field stimulation-induced neuronal NO release in pre-incubated arteries. The results showed that both inhibitors prevented the enhancement induced by forskolin in the electrical field stimulation-induced NO release. These results strongly support the thesis that electrical field stimulation induces NO release in the presence of forskolin, similarly to what has been observed in coronary arteries where this drug increased endothelial NO release (Zhang and Hintze, 2001). It is important to note the low concentration of forskolin required to induce its facilitatory effect, in contrast to the higher concentrations reported in other cells (Kimura et al., 1998; Zhang and Hintze, 2001).

Since forskolin activates PKA indirectly through adenylate cyclase, the next task undertaken was to elucidate whether PKA was involved in the mechanism that increased the neuronal NO release observed in the presence of forskolin. For this purpose, we used the PKA inhibitor H-89 (Kimura et al., 1998). This drug prevented the enhancement of neuronal NO release induced by electrical field stimulation in the presence of forskolin, suggesting that this release was mediated by PKA activation. To support this suggestion, we also used the PKA inhibitor KT-5720 (Kimura et al., 1998; Del Olmo et al., 2003). The concentrations habitually reported to block PKA activation oscillate between 0.1 and 1 μM (Garber and Heiman, 2002; Del Olmo et al., 2003). In the present work, 0.1 μM KT-5720 did not modify the neuronal NO increase induced by electrical field stimulation in the presence of forskolin, while 1 μM KT-5720 abolished that increase. These results suggest that the facilitatory effect of forskolin on neuronal NO release is mediated through PKA activation.

Released in the vascular wall the prostanoid prostacyclin has important vascular effects (Narumiya et al., 1999). Since prostacyclin also activates adenylyl cyclase by interaction with its specific receptor (Narumiya et al., 1999), it seems possible that prostacyclin could activate nNOS and increase the neuronal NO release induced by electrical field stimulation, as was observed with forskolin. Preincubation with prostacyclin induced NO release by electrical field stimulation, but failed to increase basal NO release. Additionally, preincubation with the neurotoxin tetrodotoxin or the nNOS inhibitors 7-nitroindazole (Kalisch et al., 1996)

and NPLA (Ralevic, 2002), blocked the effect of prostacyclin. These results supported the hypothesis that prostacyclin increases electrical field stimulation-induced neuronal NO release by activating nNOS, in contrast to observations in endothelial cells, where a reciprocal inhibition of NO and prostacyclin synthesis has been described (Bolz and Pohl, 1997; Marcelín-Jiménez and Escalante, 2001). To elucidate whether PKA participated in the increase of neuronal NO release observed in the presence of prostacyclin, we performed another set of experiments in the presence of one of the PKA inhibitors, either H-89 or KT-5720. The results obtained indicate that, similarly to our observations in the presence of forskolin, H-89 and KT-5720 prevented the enhancement of NO release induced by electrical field stimulation in the presence of prostacyclin again, suggesting that this release is also mediated by PKA activation.

These results indicate that nNOS is activated by a cAMP-dependent PKA mechanism and suggest that substances acting through this signal pathway could increase neuronal NO release in rat mesenteric arteries. This possibility is especially relevant since many substances are produced in the vascular wall, and response to these substances is mediated through this second messenger (Kikkawa et al., 1986; Schubert et al., 1997; Zhu et al., 2002). In addition the release of these substances can be altered in pathological conditions, and in this case neurogenic NO release would also be altered, thereby affecting vascular tone (Narumiya et al., 1999; Esler et al., 2003). The fact that protein kinase C activation also increased neuronal NO release induced by electrical field stimulation in rat mesenteric arteries from diabetic (Ferrer et al., 2000) and SHR (Marín et al., 2000) rats shows that nNOS, located in perivascular nitreregic endings, could be regulated by multiple protein kinases, as has been widely reported in the central nervous system (Bredt et al., 1992; Dinerman et al., 1994).

Electrical field stimulation is widely used to study the influence of nerve ending-released neurotransmitters on vasomotor response (Marín and Balfagón, 1998). To study the possible effect of neuronal NO release induced by forskolin or prostacyclin, we analysed the effect of L-NAME and 7-nitroindazole on the vasomotor response induced by electrical field stimulation in segments pretreated with these drugs. The results show that the vasoconstrictor response remained unmodified in the presence of 7-nitroindazole. However, 7-nitroindazole decreased the response to noradrenaline as has been described (Allawi et al., 1994), but did not modify the response to sodium nitroprusside which would explain the non-effect on electrical field stimulation-induced vasoconstrictor response. The fact that L-NAME did not modify the response to exogenous noradrenaline and sodium nitroprusside but did increase the vasoconstrictor response to electrical field stimulation in segments pretreated with forskolin or prostacyclin confirms our hypothesis that the increase in neuronal NO release induced by forskolin and prostacyclin has a functional role.

In conclusion, the present results show that forskolin and prostacyclin allow the neuronal NO release induced by electrical field stimulation through a mechanism involving cAMP–PKA activation in rat mesenteric arteries.

Acknowledgements

We thank the veterinarian Dr. Ma. del Carmen Fernández-Criado for the care of the animals, Ms. Rocio Baena and Marta Miguel for their technical assistance and Ms. Esther Martínez for her assistance in typing. Supportedd by grants from DGCYT (BFI2001-1324 and BBX2000-0153) and FIS (PI020335 and C03/01).

References

Allawi, H.S., Wallace, P., Pitcher, A., Galfen, Z., Bland-Ward, P.A., Moore, P.K., 1994. Effect of 7-nitroindazoletro indazole on neurotransmission in the rat vas deferens: mechanisms unrelated to inhibition of nitric oxide synthase. Br. J. Pharmacol. 113, 282–288.

Bolz, S.S., Pohl, U., 1997. Indomethacin enhances endothelial NO release—evidence for a role of PGI_2 in the autocrine control of calcium-dependent autacoid production. Cardiovasc. Res. 36, 437–444.

Bredt, D.S., Ferris, C.D., Snyder, S.H., 1992. Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kisase, protein kinase C, and calcium/calmodulin protein kinase: identification of flavin and calmodulin binding sites. J. Biol. Chem. 247, 10976–10981.

Brune, B., Lapetina, E.G., 1991. Phosphorylation of nitric oxide synthase by protein kinase A. Biochem. Biophys. Res. Commun. 181, 921–926.

Del Olmo, N., Handler, A., Alvarez, L., Bustamante, J., Martín del Río, R., Solís, J.M., 2003. Taurine-induced synaptic potentiation and the late phase of long-term potentiation are related mechanistically. Neuropharmacology 44, 26–39.

Dinerman, J.L., Steiner, J.P., Dawson, T.M., Dawson, V., Snyder, S.H., 1994. Cyclic nucleotide dependent phosphorylation of neuronal nitric oxide synthase inhibits catalytic activity. Neuropharmacology 33, 1245–1251.

Esler, M., Lambert, G., Brunner-La Rocca, H.P., Vaddadi, G., Kaye, D., 2003. Sympathetic nerve activity and neurotransmitter release in humans: translation from pathophysiology into clinical practice. Acta Physiol. Scand. 177, 275–284.

Estrada, C., Mengual, E., González, C., 1993. Local NADPH-diaphorase neurons innervate pial arteries and lie close or project to intracerebral blood vessels: a possible role for nitric oxide in the regulation of cerebral blood flow. J. Cereb. Blood Flow Metab. 13, 978–984.

Ferrer, M., Marín, J., Balfagón, G., 2000. Diabetes alters neuronal nitric oxide release from rat mesenteric arteries. Role of protein kinase C. Life Sci. 66, 337–345.

Ferrer, M., Sánchez, M., Minoves, N., Salaices, M., Balfagón, G., 2003. Aging increases neuronal nitric oxide release and superoxide anion generation in mesenteric arteries from spontaneously hypertensive rats. J. Vasc. Res. 40, 509–519.

Garber, M.M., Heiman, A.S., 2002. The in vitro effects of Pb acetate on NO production by C6 glial cells. Toxicol. In Vitro 16, 499–508.

Graves, J., Poston, L., 1993. β-Adrenoceptor agonist mediated relaxation of rat isolated resistance arteries: a role for the endothelium and nitric oxide. Br. J. Pharmacol. 108, 631–637.

Griess, J.P., 1979. Bemerkungen zu der abhandlung der HH. Weselsky und Benedikt "Ueber einigre Azoverhindungen". Ber. Dtsch. Chem. Ges. 12, 426–428.

Kalisch, B.E., Connop, B.P., Jhamandas, K., Beninger, R.J., Boegman, R.J., 1996. Differential action of 7-nitroindazole on rat brain nitric oxide synthase. Neurosci. Lett. 219, 75–78.

Kikkawa, F., Furuta, T., Ishikawa, N., Shigei, T., 1986. Different types of relationship between beta-adrenergic relaxation and activation of cyclic AMP-dependent protein kinase in canine saphenous and portal veins. Eur. J. Pharmacol. 128, 187–194.

Kimura, S., Uchiyama, S., Takahashi, H.E., Shibuki, K., 1998. cAMP-dependent long-term potentiation of nitric oxide release from cerebellar parallel fibers in rats. J. Neurosci. 18, 8551–8558.

Marcelín-Jiménez, G., Escalante, B., 2001. Functional and cellular interactions between nitric oxide and prostacyclin. Comp. Biochem. Physiol., Part C Pharmacol. Toxicol. 129, 349–359.

Marín, J., Balfagón, G., 1998. Effect of clenbuterol on non-endothelial nitric oxide release in rat mesenteric arteries and the involvement of β-adrenoceptors. Br. J. Pharmacol. 124, 473–478.

Marín, J., Ferrer, M., Balfagón, G., 2000. Role of protein kinase C in electrical-stimulation-induced neuronal nitric oxide release in mesenteric arteries from hypertensive rats. Clin. Sci. 99, 277–283.

Narumiya, S., Sugimoto, Y., Ushikubi, F., 1999. Prostanoid receptors: structures, properties, and functions. Physiol. Rev. 79, 1193–1226.

Ralevic, V., 2002. Endothelial nitric oxide modulates perivascular sensory neurotransmission in the rat isolated mesenteric arterial bed. Br. J. Pharmacol. 137, 19–28.

Rossoni, L.V., Salaices, M., Miguel, M., Briones, A.M., Barker, L.A., Vassallo, D.V., Alonso, M.J., 2002. Ouabain-induced hypertension is accompanied by increases in endothelial vasodilator factors. Am. J. Physiol. 283, H2110–H2118.

Schubert, R., Serebryakov, V.N., Mewes, H., Hopp, H.H., 1997. Iloprost dilates rat small arteries: role of K(ATP)- and(Ca)-channel activation by cAMP-dependent protein kinase. Am. J. Physiol. 272, H1147–H1156.

Seamon, K.B., Padgett, W., Daly, J.W., 1981. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc. Natl. Acad. Sci. U. S. A. 78, 3363–3367.

Shimokawa, H., Flavahan, N.A., Lorenz, R.R., Vanhoutte, P.M., 1988. Prostacyclin releases endothelium-derived relaxing factor and potentiates its action in coronary arteries of the pig. Br. J. Pharmacol. 95, 1197–1203.

Taylor, S.S., Buechler, J.A., Yonemoto, W., 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annu. Rev. Biochem. 59, 971–1005.

Yu, J., Yu, L., Chen, Z., Zheng, L., Chen, X., Wang, X., Ren, D., Zhao, S., 2002. Protein inhibitor of neuronal nitric oxide synthase interacts with protein kinase A inhibitors. Mol. Brain Res. 99, 145–149.

Zhang, X., Hintze, T.H., 2001. cAMP signal transduction cascade, a novel pathway for the regulation of endothelial nitric oxide production in coronary blood vessels. Arterioscler. Thromb. Vasc. Biol. 21, 797–803.

Zhu, S., Han, G., White, R.E., 2002. PGE2 action in human coronary artery smooth muscle: role of potassium channels and signaling cross-talk. J. Vasc. Res. 39, 477–488.