ELSEVIER

Biochemical Pharmacology 61 (2001) 1169–1175

Biochemical Pharmacology

Role of G_i-proteins in norepinephrine-mediated vasoconstriction in rat tail artery smooth muscle

Marie-Anne Petitcolin[a,b], Esther Spitzbarth-Régrigny[b], Jean-Luc Bueb[a], Christine Capdeville-Atkinson[b], Eric Tschirhart[a,*]

[a]*Laboratoire de Neuroimmunologie et Inflammation, Centre de Recherche Public-Santé, L-1150 Luxembourg, Luxembourg*
[b]*Laboratoire de Pharmacologie Cardiovasculaire, UPRES EA 3116, Faculté de Pharmacie, UHP-Nancy 1, 54000 Nancy, France*

Received 4 September 2000; accepted 12 December 2000

Abstract

We showed, in rat de-endothelialised tail artery, that pertussis toxin (PTX) (1 μg/mL, 2 hr) attenuated norepinephrine (NE)-induced vasoconstriction without modifying intracellular calcium concentration $[Ca^{2+}]_i$ mobilisation. We suggested the existence of two NE-induced intracellular pathways: a first, which would be insensitive to PTX and lead to $[Ca^{2+}]_i$ mobilisation, and a second sensitive to PTX and involved in the $[Ca^{2+}]_i$ sensitivity of NE-induced contraction. The aim of this study was to demonstrate the existence of the second intracellular pathway. PTX-sensitive $G_{i/o}$-proteins in rat tail artery SMC membrane were identified by immunoblot and ADP-ribosylation. [^{32}P]ADP-ribosylation of $\alpha_{i/o}$-subunits was demonstrated *in situ* by perfusing rat de-endothelialised tail artery segments with PTX (1 μg/mL, 2 hr), which suggested that $G_{i/o}$-protein inactivation was involved in the reduction by PTX of the $[Ca^{2+}]_i$ sensitivity of NE-induced contraction. Coupling between $G_{i/o}$-proteins and NE receptors was confirmed by the NE-induced increase in $G_{i/o}$-specific GTPase activity (24.1 $\pm$ 1.9 vs 8.8 $\pm$ 0.4 pmol P_i/mg protein at 5 min; $P < 0.05$ vs basal). [^{3}H]Prazosin-binding data showed the presence of a heterogeneous α_1-AR population in rat tail artery smooth muscle cells. We demonstrated the *in vitro* coupling between α_{1A}-AR subtype and α_i-subunits. In conclusion, we identified, in rat de-endothelialised tail artery, a PTX-sensitive $G_{i/o}$-protein-modulated pathway that is coupled to NE receptors via α_{1A}-AR. We suggest that NE stimulates two α_1-AR-mediated intracellular pathways: a first, which is mediated by a G_q-protein and leads to $[Ca^{2+}]_i$ mobilisation and contraction, and a second, which is mediated by a G_i-protein and is involved in the amplification of the $[Ca^{2+}]_i$ sensitivity of NE-induced tension.

Keywords: Smooth muscle; Norepinephrine; Pertussis toxin; $G_{i/o}$-proteins; Vasoconstriction; Tail artery

1. Introduction

PTX catalyses the ADP-ribosylation of the α-subunit of G_i- and G_o-proteins, resulting in the uncoupling of $G_{i/o}$-proteins from their receptors [1] and thus from their intracellular signalisation pathways. In vascular SMC, pressor responses induced by agonists, such as α-AR agonists, are attenuated by *ex vivo* [2] or *in vitro* treatment with PTX [3,4]. In a small diameter muscular artery, such as the rat tail artery, PTX pretreatment produced a marked inhibition of the α_2-AR-mediated pressor response. In contrast, PTX treatment had only a slight effect on α_1-AR-mediated contraction [5]. Furthermore, in previous studies performed in perfused de-endothelialised tail artery segments, we showed that the vasoconstrictor response induced by NE, a mixed α-AR agonist, was decreased by PTX with no modification of $[Ca^{2+}]_i$ mobilisation [6,7]. Thus, PTX attenuates the $[Ca^{2+}]_i$ sensitivity of tension of rat tail artery SMC following stimulation with NE. All the above results suggest that, in rat tail artery SMC, NE stimulates two α_1-AR-mediated intracellular transduction signals: a first, which is related to $[Ca^{2+}]_i$ mobilisation and is insensitive to PTX, and a second, which is sensitive to PTX and is involved in the amplification of the $[Ca^{2+}]_i$ sensitivity of agonist-induced tension.

We hypothesised that NE stimulates these two transduction signals by coupling to two different types of G-protein: G_q, which is insensitive to PTX and leads to $[Ca^{2+}]_i$ mo-

* Corresponding author. Tel.: +352-45-19-03-31; fax: +352-45-19-03-50.

E-mail address: eric.tschirhart@crp-sante.lu (E. Tschirhart).

Abbreviations: α-AR, alpha-adrenoceptor; $[Ca^{2+}]_i$, intracellular calcium concentration; DTT, DL-dithiothreitol; NE, norepinephrine; PSS, physiological salt solution; PTX, pertussis toxin; and SMC, smooth muscle cells.

0006-2952/01/$ – see front matter
PII: S0006-2952(01)00589-5

bilisation, and $G_{i/o}$, which are sensitive to PTX and increase the $[Ca^{2+}]_i$ sensitivity of NE-induced tension. The aim of this study was to demonstrate, in rat tail artery SMC, the existence of the second transduction pathway.

2. Materials and methods

2.1. Materials

Tris(hydroxymethyl)aminomethane was purchased from Merck-Eurolab, while benzamidine, DTT, leupeptin, NAD, NE, PTX, phenylmethylsulphonyl fluoride, and SDS were from Sigma Chemical Co. Materials used for immunoblotting were obtained from Bio-Rad. Fura 2 acetoxymethyl ester was purchased from Molecular Probes. [α-^{32}P]NAD (30 Ci/mmol), [γ-^{32}P]GTP (30 Ci/mmol), and [^{3}H]prazosin (77 Ci/mmol) were purchased from New England Nuclear Life Science Products (NEN). PTX (1 μg/mL) was activated extemporaneously by incubation with DTT (25 mM, 1 hr, 37°). To avoid *in vitro* effects of DTT, solutions were then dialysed (Biotech dispodialyser Spectra/Por®, molecular weight cut-off = 8 kDa, Spectrum Laboratories Inc.) for 3 hr against PSS (containing in mM: 140 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 6 glucose, pH 7.4) with a change of bath at 15, 30, 60, and 120 min.

2.2. Animals

Adult, male, outbred Wistar rats (Harlan Netherlands B.V.) were given food and water *ad lib*. The procedures followed in the care and euthanasia of the animals were in accordance with the legislation of the European Community (Directive 86/609/EEC).

2.3. Simultaneous measurement of $[Ca^{2+}]_i$ mobilisation and vasomotion in rat de-endothelialised tail artery segments

The experimental protocol is described in detail elsewhere [7,8]. Tail arteries were dissected out from anesthetised rats. A 1-cm segment of the tail artery was cannulated and mounted in a perfusion/cuvette system placed in a dual-wavelength spectrofluorometer (Fluorolog F1 T11, Instruments SA Jobin Yvon SPEX) and perfused at a constant rate with PSS (1.5 mL/min, 100% O_2, 37°). Experiments were performed in the absence of endothelium, which was removed by perfusing the segments with air (0.4 mL/min) plus PSS for 10 min [9]. Following this procedure, carbachol (10 μM) failed to relax segments precontracted with NE (3 μM) (results not shown). Histological evidence of the removal of endothelium has already been published [9]. Arteries were loaded with Fura 2 acetoxymethyl ester (5 μM, 90 min) and activated PTX (1 μg/mL, 2 hr) or DTT (25 mM) dialysed in PSS (control). They were then stimulated with either modified PSS containing 80 mM KCl (composition in mM: 65 NaCl, 80 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 6 glucose, pH 7.4; 2 min) or NE (1 μM; 2 min). KCl- or NE-evoked increases in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$, arbitrary units, a.u.) and perfusion pressure (ΔP, mmHg) were measured. The $[Ca^{2+}]_i$ sensitivity of tension was estimated as: $\Delta P/\Delta[Ca^{2+}]_i$ (mmHg/a.u.). Values are means ± SEM.

2.4. Isolation of the membrane fraction from rat de-endothelialised tail artery

Rats were killed by cervical dislocation and the tail arteries excised. Endothelium was removed by rubbing the arterial intimal surface with a stainless wire. De-endothelialised tail arteries were homogenised in ice-cold buffer (in mM: 15 tris(hydroxymethyl)aminomethane (pH 7.4), 1 ethylenediaminetetraacetate, 0.1 phenylmethylsulphonyl fluoride, 0.1 benzamidine, and 0.001 leupeptin) with a glass Dounce homogeniser (Kontes) [10]. Following differential centrifugation (900 × *g* for 10 min, 10,000 × *g* for 10 min, and 60,000 × *g* for 45 min at 4°), the pelleted membrane fraction was resuspended in the same buffer and stored at −80° until use. Protein concentration was determined by the method of Lowry *et al*. [11].

2.5. Identification of PTX-sensitive $G_{i/o}$-proteins

2.5.1. Immunoblotting

The membrane fractions diluted in Laemmli buffer were resolved by 16.5% SDS–PAGE and transferred to a 0.4-μm thick nitrocellulose membrane (Bio-Rad). Ten micrograms of proteins from the membrane fraction was loaded on each lane. Blotting was performed using polyclonal rabbit antibody directed against G_i α-subunits 1–2 (1:1000 dilution, Calbiochem), polyclonal rabbit antibody directed against G_i α-subunit 3 (1:1000 dilution, Calbiochem), or monoclonal mouse antibody directed against G_o α-subunits (1:3000 dilution, Chemicon). Molecular weight markers (Bio-Rad) were run in parallel with the samples. Immunocomplexes were detected using alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a substrate (Bio-Rad). Positive control was a mixture of bovine brain purified G-protein subunits (Calbiochem).

2.5.2. ADP-ribosylation experiment

PTX-catalysed transfer of ADP-ribose from [α-^{32}P]NAD to α-subunits was performed in the membrane fraction. An ADP-ribosylation mixture (in mM: 200 KH_2PO_4 (pH 7.6), 5 $MgCl_2$, 2 ATP, 2 ethylenediaminetetraacetate, 20 thymidine, 2 DTT, 2 GTP, 0.02 NAD [10 μCi/60 μL of assay]) and activated PTX (1 μg/mL) were added to the membrane fraction (70 μg) [12]. Control was the membrane fraction (70 μg) incubated with the ADP-ribosylation mixture and DTT (25 mM) dialysed in PSS or activated PTX (1 μg/mL). Following incubation for 1 hr at 30°, samples were resolved by 10% SDS–PAGE and blotted as described above. La-

belled blots were dried and exposed to an X-ray film (Fuji Medical).

The same technique was also applied to perfused tail artery segments using the protocol described for the simultaneous measurement of $[Ca^{2+}]_i$ mobilisation and vasomotion. Segments were perfused *in situ*, for 2 hr at 37°, with activated PTX (1 μg/mL) and the ADP-ribosylation mixture containing 0.02 mM NAD (10 μCi/500 μL of assay). Control segments were perfused *in situ*, for 2 hr at 37°, with the ADP-ribosylation mixture and DTT (25 mM) dialysed in PSS or activated PTX (1 μg/mL). The membrane fraction was prepared, resolved by 10% SDS–PAGE and blotted as described above. Labelled blots were dried and exposed to an X-ray film.

Densitometric analysis was performed using the NIH Image® program (v.1.58, Wayne Rasband, National Institutes of Health) and results are expressed as pixels.

2.6. Coupling of NE receptors to $G_{i/o}$-proteins

2.6.1. Measurement of $G_{i/o}$-specific GTPase activity following stimulation with NE

The method for the measurement of GTPase activity was based upon the release of $^{32}P_i$ from [γ-^{32}P]GTP. The assay was performed in a buffer (containing in mM: 100 NaCl, 50 HEPES (pH 8.0), 1 ethylenediaminetetraacetate, 2 $MgCl_2$, 1 DTT) containing NE (1 μM) and [γ-^{32}P]GTP (0.1 μM, 30 Ci/mmol). The membrane fractions were pre-incubated for 1 hr at 30° with activated PTX (1 μg/mL) or DTT (25 mM) dialysed in PSS. Ten micrograms of the membrane fraction was added to start the reaction at 30°. Different incubation times were utilised (30 sec–30 min). The reaction was stopped by addition of 5% ice-cold activated charcoal in 50 mM KH_2PO_4 (pH 7.4). Tubes were then centrifuged (600 $\times$ *g* for 10 min at 4°) to separate $^{32}P_i$ from nucleotide-bound phosphate. An aliquot of the supernatant (0.4 mL) was counted by liquid scintillation in Aquasol-2 (NEN) with a Beckman LS6000 Counter (Beckman Instruments Inc.). Results are expressed as pmol P_i/mg proteins. GTPase activity was calculated by subtracting non-enzymatic hydrolysis (determined in the absence of the membrane fraction) and contamination of the substrate with $^{32}P_i$ (determined by zero-time blank). Non-enzymatic $^{32}P_i$ release and $^{32}P_i$ contamination of the substrate during the assay were lower than 2% of the total amount of radioactivity. GTPase activity specifically produced by $G_{i/o}$-proteins was defined as the difference in GTP hydrolysis between untreated and PTX-treated membrane fractions. All assays were performed in triplicate and results are expressed as means ± SEM.

2.6.2. Detection of α_1-ARs by [^{3}H]prazosin binding

Total binding was determined by incubation of the membrane fraction (10 μg) with [^{3}H]prazosin (3–30,000 pM) in an assay buffer (in mM: 50 tris(hydroxymethyl)aminomethane (pH 7.4), 200 NaCl, 0.5 ethylenediaminetetraacetate) containing 0.1% (w/v) ascorbic acid. After 1 hr at room temperature, assay mixtures were filtered under vacuum on GF/C filters (Whatman) presoaked with 0.3% (v/v) polyethylenimine using a cell harvester (Biomedical Research and Development Laboratories Inc.). Filters were washed 3 times with ice-cold tris(hydroxymethyl)aminomethane buffer (50 mM, pH 7.4) and dried. The filter-bound radioactivity was determined by liquid scintillation counting in Aquasol-2 (NEN) using a Beckman LS6000 Counter (Beckman Instruments Inc.). Non-specific [^{3}H]prazosin binding was defined as the binding in the presence of NE (0.1 mM). Assays were carried out in triplicate and results are means ± SEM of three separate experiments. Binding data were analysed with the Radlig® program (v. 4, Biosoft) [13].

2.6.3. Coupling of G-protein α-subunits to α_1-AR subtypes

The membrane fractions (400 μg) were solubilised as described by Gurdal *et al*. [14] and incubated at 4° for 3 hr with polyclonal goat antibody directed against α_{1A}-, α_{1B}-, or α_{1D}-AR subtype (1:250 dilution, Santa Cruz Biotechnology). Non-immune serum at the same dilution was used as a control. Following incubation of the membrane fraction with anti-α_1-AR antibody, 100 μL of a 10% suspension of protein A from *Staphylococcus aureus* cells (Pansorbin® cells, Calbiochem) were added and incubation was continued for a further hour at 4°. Samples were centrifuged (3000 $\times$ *g* for 5 min at 4°), washed 2–3 times, and resuspended in Laemmli buffer. Proteins were separated by 10% SDS–PAGE and immunoblotted with a polyclonal goat antibody directed against G_q α-subunit (1:500 dilution, Santa Cruz Biotechnology), a monoclonal mouse antibody directed against G_o α-subunits (1:3000 dilution, Chemicon), or two polyclonal rabbit antibodies directed against G_i α-subunits 1–2 and 3 (1:1000 dilution, Calbiochem). Blots were then incubated for 1 hr at room temperature with corresponding species-specific horseradish peroxidase-labelled secondary antibody (Santa Cruz Biotechnology and Amersham Pharmacia Biotech) and exposed to an X-ray film with enhanced chemiluminescent reagents (SuperSignal West Pico Chemiluminescent substrate, Pierce).

The selectivity of the antibody directed against α_{1A}-, α_{1B}-, or α_{1D}-AR was tested. Rat tail artery membrane fractions were solubilised and subjected to immunoprecipitation with antibody directed against α_{1A}-, α_{1B}-, or α_{1D}-AR. Solubilised immunocomplexes were separated on 10% SDS–polyacrylamide gels, transferred onto nitrocellulose membranes, and immunoblotted with specific anti-α_{1A}-, α_{1B}-, or α_{1D}-AR. A single band was observed with each subtype of α_1-AR indicating that there was no cross-reactivity among the three antibodies tested (results not shown).

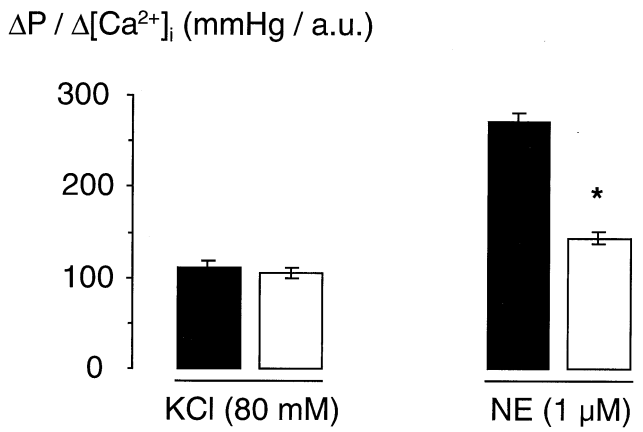

Fig. 1. $[Ca^{2+}]_i$ sensitivity of tension induced by KCl (80 mM) and NE (1 μM) in rat de-endothelialised tail artery segments. $[Ca^{2+}]_i$ sensitivity of tension was estimated as the ratio $\Delta P/\Delta[Ca^{2+}]_i$ (mmHg/arbitrary units [a.u.]). Segments were perfused for 2 hr at 37° in the absence (full columns, N = 9) or presence of PTX (1 μg/mL; open columns, N = 6). Data are means ± SEM. Significant differences (*: $P < 0.05$ PTX vs control) were determined by ANOVA plus Bonferroni tests.

2.7. Statistical analysis

Data are given as means ± SEM; N = number of experiments. Means were compared using ANOVA plus Bonferroni tests or ANOVA plus Scheffe tests, as indicated in the legends. The level of significance was $P < 0.05$.

3. Results

3.1. Effect of PTX on $[Ca^{2+}]_i$ mobilisation and vasomotion in rat de-endothelialised tail artery segments

In perfused segments of the rat de-endothelialised tail artery loaded with Fura 2, NE (1 μM) induced a slight increase in $[Ca^{2+}]_i$ and a pronounced vasoconstriction. The ratio "vasoconstriction/$[Ca^{2+}]_i$ mobilisation" was high (Fig. 1, right panel). KCl (80 mM) led to the opposite effects and also to a smaller ratio (Fig. 1, left panel). Treatment with PTX had no effect on baseline perfusion pressure or on fluorescence, and did not modify the form of the vasoconstrictor response (results not shown) [7]. PTX (1 μg/mL, 2 hr, 37°) lowered the vasoconstrictor response produced by NE with no modification of $[Ca^{2+}]_i$ mobilisation. PTX modified neither the vasoconstrictor response nor $[Ca^{2+}]_i$ mobilisation induced by KCl. Thus, PTX reduces $[Ca^{2+}]_i$ sensitivity of tension induced by NE but not that induced by KCl (Fig. 1).

3.2. Identification of PTX-sensitive $G_{i/o}$-proteins in the membrane fraction isolated from rat de-endothelialised tail artery

PTX-sensitive $G_{i/o}$-proteins were first identified by Western blot (results not shown) [7]. Immunoblotting of G_i-proteins, using a combination of two antibodies directed against G_i α-subunits 1–2 and 3, revealed a doublet band at 40–41 kDa in rat tail artery SMC membrane fraction. Immunodetection was repeated using the two antibodies separately to confirm the presence of the different α_i-subunits. A slight band around 40 kDa was observed in rat tail artery SMC membrane fraction incubated with an antibody directed against G_o α-subunits.

To further identify PTX-sensitive $G_{i/o}$-proteins in rat tail artery SMC, PTX-catalysed transfer of ADP-ribose from [α-^{32}P]NAD to membrane components was assessed *in vitro* on membrane fraction (Fig. 2A). A noticeable band (12,084 pixels) was observed in PTX (1 μg/mL, 1 hr, 30°)-treated membrane fraction (lane 1), the molecular weight of which was consistent with that of $\alpha_{i/o}$-subunits. A slight band (3,830 pixels) was present in lane 2, corresponding to intrinsic ribosylation activity or non-specific binding. These results indicate that membrane fraction from rat tail artery SMC contains a substantial amount of $G_{i/o}$ α-subunits that are [^{32}P]ADP-ribosylated by PTX.

3.3. Demonstration of a PTX-sensitive intracellular pathway in rat de-endothelialised tail artery

PTX-catalysed transfer of ADP-ribose from [α-^{32}P]NAD to $\alpha_{i/o}$-subunits was performed in rat tail artery segments perfused *in situ* with [α-^{32}P]NAD and PTX (1 μg/mL, 2 hr, 37°). A band was present in the PTX-treated preparation

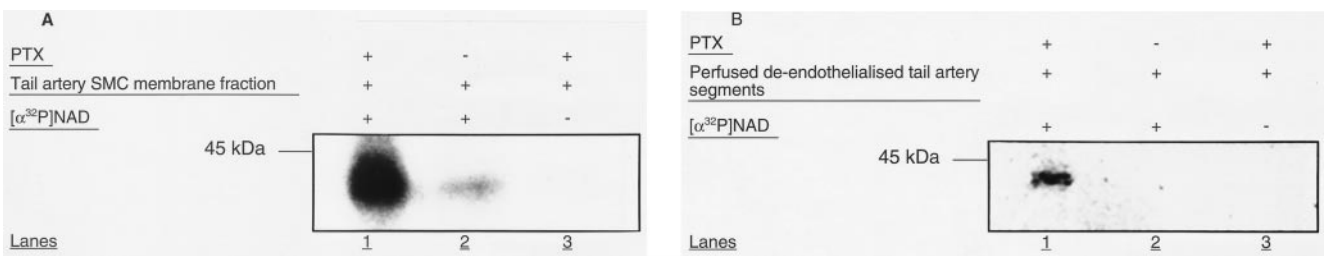

Fig. 2. Identification of a PTX-sensitive $G_{i/o}$-protein-modulated pathway. PTX-catalysed transfer of ADP-ribose from [α-^{32}P]NAD to $\alpha_{i/o}$-subunits was assessed *in vitro* in the membrane fraction (70 μg) from rat de-endothelialised tail artery (A) and *in situ* in rat perfused de-endothelialised tail artery segments (B). A 1-hr incubation of the membrane fraction or a 2-hr perfusion of the segments was performed with NAD (0.02 mM, 30 Ci/mmol, 10 μCi) and activated PTX (1 μg/mL) (lanes 1), NAD and DTT (25 mM) dialysed in PSS (lanes 2), or activated PTX (lanes 3).

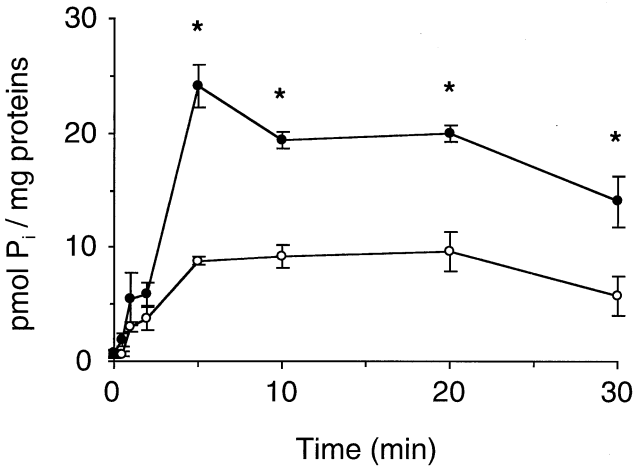

Fig. 3. $G_{i/o}$-specific GTPase activity measured in rat tail artery SMC membrane fraction. Ten micrograms of the membrane fraction was incubated in the presence (full circles, N = 3) or absence (open circles, N = 3) of NE (1 μM). $G_{i/o}$-specific GTPase activity (pmol P_i/mg proteins) was defined as the difference in GTP hydrolysis between untreated and PTX (1 μg/mL)-treated membrane fractions. Data are means ± SEM. Significant differences (*: $P < 0.05$ stimulated vs basal) were determined by ANOVA plus Scheffe tests.

(Fig. 2B, lane 1, 5,119 pixels), the molecular weight of which was consistent with that of $G_{i/o}$ α-subunits. No radioactivity was detected in the controls. Thus, an *in situ* perfusion with PTX induces the ADP-ribosylation of $\alpha_{i/o}$-subunits in rat tail artery segment.

3.4. Coupling of NE receptors to $G_{i/o}$-proteins

3.4.1. Measurement of $G_{i/o}$-specific GTPase activity following stimulation with NE

Total GTPase activity, corresponding to all G-proteins present in rat tail artery SMC membrane fraction, was shown to be a time-dependent process and was enhanced by NE (1 μM) (total GTPase activity at t = 5 min: 61.0 ± 3.2 vs 45.0 ± 6.1 pmol P_i/mg proteins, $P < 0.05$ stimulated vs basal). Following treatment with PTX, both basal and NE-stimulated GTPase activities, corresponding to all G-proteins, were reduced.

The $G_{i/o}$-specific GTPase activity was also a time-dependent process (Fig. 3). NE (1 μM) was found to stimulate the basal level of $G_{i/o}$-specific GTPase activity in rat tail artery SMC membrane fraction (Fig. 3).

3.4.2. Detection of α_1-ARs by [^{3}H]prazosin binding

[^{3}H]Prazosin binding in rat tail artery SMC membrane fraction was saturable and of high affinity. The specific binding represented approximately 60% of the total binding at a [^{3}H]prazosin concentration close to its K_d. Non-linear regression analysis of the saturation data was consistent with the presence of two populations of sites: one with a high affinity ($pK_{d\,high} = 9.9 \pm 0.2$ and $B_{max\,high} = 20 \pm 1$ fmol/mg proteins) and a second one with a low affinity ($pK_{d\,low} = 7.8 \pm 0.4$ and $B_{max\,low} = 323 \pm 87$ fmol/mg proteins).

3.4.3. Coupling of G-protein α-subunits to α_1-AR subtypes

Blots presented in Fig. 4A demonstrated that the three α_1-AR subtypes were present in rat tail artery SMC membrane fraction and that each subtype interacted with the α_q-subunit. None of the α_1-AR subtypes was coupled to a G_o-protein, as no band was observed in Fig. 4B. Only the α_{1A}-AR subtype co-immunoprecipitated with α_i-subunits in rat tail artery SMC membrane fraction (Fig. 4C).

4. Discussion

In a previous work, we demonstrated that, in rat de-endothelialised tail artery, $[Ca^{2+}]_i$ sensitivity of tension was higher following stimulation with NE than with a depolarisation [7]. We also demonstrated that PTX reduces $[Ca^{2+}]_i$ sensitivity of tension induced by NE, but not that induced by a depolarisation. These results suggest the existence of a PTX-sensitive intracellular transduction pathway and its involvement in the amplification of $[Ca^{2+}]_i$ sensitivity of tension induced by NE. The present study was aimed at investigating the potentiel involvement of PTX-sensitive $G_{i/o}$-proteins in this PTX-sensitive intracellular transduction pathway in rat tail artery SMC.

In rat de-endothelialised tail artery membrane, PTX-sensitive $G_{i/o}$-proteins were identified *in vitro* by both immunoblotting and ADP-ribosylation experiments. The latter was reproduced by perfusing rat de-endothelialised tail artery segments *in situ* with [α-^{32}P]NAD and PTX. There was a clear demonstration that PTX ADP-ribosylated $G_{i/o}$-proteins *in situ*. Thus, when the rat tail artery segments are perfused with PTX, the decrease in NE-induced vasoconstriction is associated with the ADP-ribosylation of $G_{i/o}$-proteins. These results suggest that PTX lowers $[Ca^{2+}]_i$ sensitivity of NE-induced tension by uncoupling $G_{i/o}$-proteins from NE receptors.

We investigated the existence of a coupling between NE receptors and $G_{i/o}$-proteins by measuring the $G_{i/o}$-specific GTPase activity in rat tail artery SMC. An NE-induced increase in $G_{i/o}$-specific GTPase activity can be taken as an index of effective coupling between $G_{i/o}$-proteins and NE receptors [15]. Weber and MacLeod have previously shown that in rat de-endothelialised tail artery, GTPase activity produced by all G-proteins was stimulated by NE, predominantly via α_1-ARs [16]. In the same vessel, *in vitro* studies have confirmed that NE-induced vasoconstrictor response was mediated predominantly by α_1-ARs in comparison to α_2-ARs [17,18]. Furthermore, α_1-AR-induced pressor responses are partially diminished by PTX [5]. According to these results, we hypothesised that $G_{i/o}$-proteins were coupled to α_1-ARs.

α_1-ARs were assayed in rat tail artery SMC by prazosin

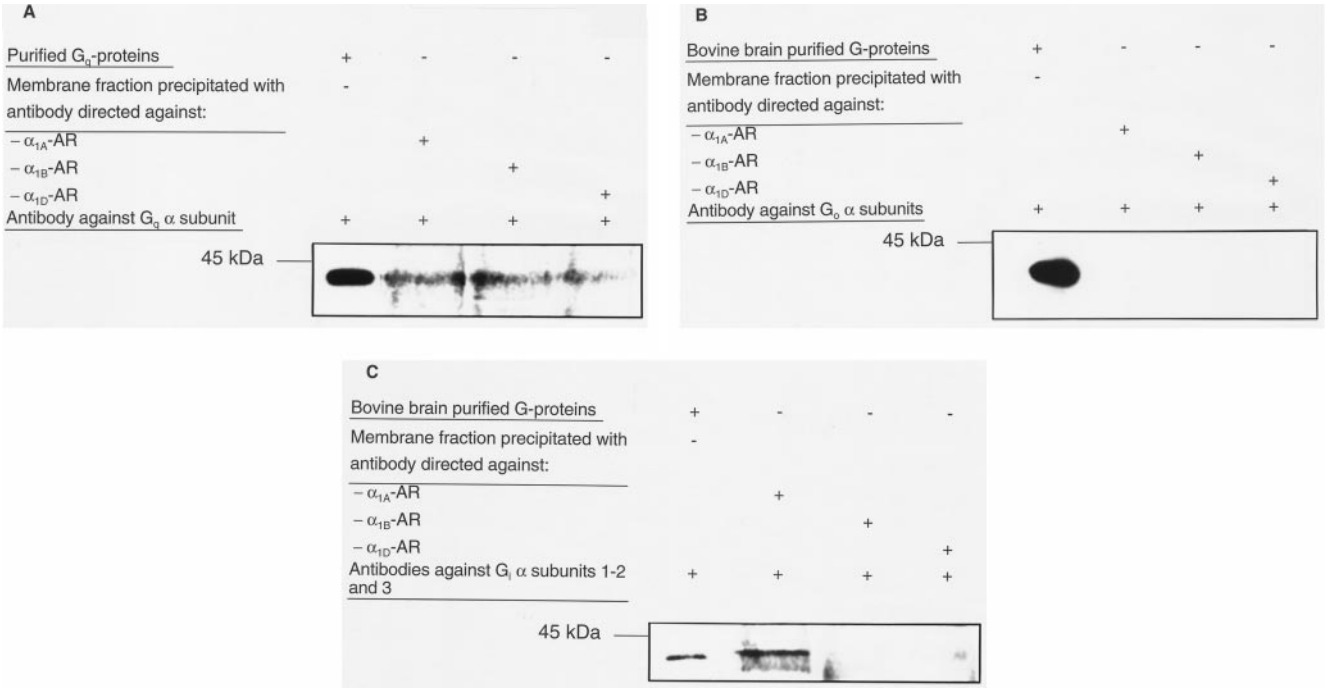

Fig. 4. Coupling of G-protein α-subunits to α_1-AR subtypes. Rat tail artery SMC membrane fraction (400 μg) was precipitated with antibody directed against α_{1A}-, α_{1B}-, or α_{1D}-AR. The three immunocomplexes were separated by SDS–PAGE and immunoblotted with antibody directed against G_q (A), G_o (B), or G_i (C) α-subunits.

binding, a selective α_1-AR antagonist in the concentration range used [19]. Results suggested the presence of different α_1-AR sites [20] in accordance with Medgett and Langer [17], who showed that NE mediates contraction of rat tail artery by activation of more than one α_1-AR subtype. Furthermore, our results confirm that α_1-AR subtypes are discriminated by prazosin as prazosin high- and low-affinity sites [20]. According to the α_{1H}, α_{1L}, and α_{1N} subclassification [21], these sites may correspond to the α_{1H}- and α_{1L}-AR subtypes, respectively. In rat tail artery, Lachnit *et al*. have characterised the prazosin high-affinity site as the α_{1A}-AR subtype [22].

Three different α_1-AR subtypes have been cloned and corresponding mRNAs detected in rat tail artery: α_{1A}-, α_{1B}-, and α_{1D}-AR [23]. In this artery, α_{1A} is implicated in the NE-induced response [22], whereas α_{1D} does not appear to play any role in the contractile response induced by NE [24]. We investigated the coupling between α_1-ARs and G-proteins by co-immunoprecipitation. We confirmed that the three α_1-AR subtypes are coupled to G_q-protein [25], but we also demonstrated that only the α_{1A}-AR subtype is coupled *in vitro* to two different families of G-proteins: G_q- and G_i-proteins. These results add further weight to our hypothesis (see Introduction) stating that NE mediates two intracellular transduction signals: a first, which is mediated by a G_q-protein and leads to $[Ca^{2+}]_i$ mobilisation and contraction, and a second, which is mediated by a G_i-protein and is involved in the amplification of $[Ca^{2+}]_i$ sensitivity of NE-induced tension. Our *in vitro* experiments suggest that, in rat de-endothelialised tail artery, NE stimulates these two intracellular transduction signals through the α_{1A}-AR subtype. The existence of two intracellular transduction signals corroborates the study performed by Spitzbarth-Régrigny *et al*., which showed that PTX reduces NE-induced vasoconstrictor responses without modifying $[Ca^{2+}]_i$ mobilisation induced by the agonist [7].

In addition, this study substantiates the relationship found between the presence of sympathetic nerves and the distribution of α_1-AR subtypes with the effect of PTX [26]. In densely innervated rat vessels, such as the mesenteric and tail arteries, vasoconstriction is predominantly mediated by the α_{1A}-AR subtype and PTX attenuates this response (this work) [7,27]. In a poorly innervated vessel, such as the aorta, contractile responses are mediated predominantly by the α_{1D}-AR subtype and are resistant to PTX [28,29].

In conclusion, we have identified, in rat de-endothelialised tail artery, a PTX-sensitive $G_{i/o}$-protein-modulated intracellular pathway that is coupled to NE receptors via the α_{1A}-AR subtype. We suggest that NE stimulates two α_1-AR-mediated intracellular pathways: a first, which is mediated by a G_q-protein and leads to $[Ca^{2+}]_i$ mobilisation and contraction, and a second, which is mediated by a G_i-protein and is involved in the amplification of the $[Ca^{2+}]_i$ sensitivity of NE-induced tension.

Acknowledgments

This study was supported by Grant 95/01 from the Centre de Recherche Public-Santé and by the Weicker Founda-

tion of Luxembourg. M.A.P. was supported by a doctoral grant (BFR 96/076) from the Luxembourg Education and Research Ministry and by the Fondation pour la Recherche Médicale of France. The Nancy group (E.S.R., C.C.A.) was supported by a grant from the French Ministry (EA 3116).

References

[1] Clark MA, Conway TM, Bennett CF, Crooke ST, Stadel JM. Islet-activating protein inhibits leukotriene D_4- and leukotriene C_4- but not bradykinin- or calcium ionophore-induced prostacyclin synthesis in bovine endothelial cells. Proc Natl Acad Sci USA 1986;83:7320–4.

[2] Nichols AJ, Motley ED, Ruffolo RR. Effect of pertussis toxin treatment on postjunctional alpha-1 and alpha-2 adrenoceptor function in the cardiovascular system of the pithed rat. J Pharmacol Exp Ther 1989;249:203–9.

[3] Boonen HC, De Mey JG. G-proteins are involved in contractile responses of isolated mesenteric resistance arteries to agonists. Naunyn Schmiedebergs Arch Pharmacol 1990;342:462–8.

[4] Abebe W, Edwards J, Agrawal D. G-proteins in rat blood vessels. 2. Assessment of functional involvement. Gen Pharmacol 1995;26:75–83.

[5] Li XF, Triggle CR. Effects of pertussis and cholera toxins on α-adrenoceptor function in rat tail artery: differences in hypertension. Can J Physiol Pharmacol 1993;71:791–9.

[6] Robert A, Tran NN, Giummelly P, Atkinson J, Capdeville-Atkinson C. Sensitivity of norepinephrine-evoked vasoconstriction to pertussis toxin in the old rat. Am J Physiol 1998;274:R1604–12.

[7] Spitzbarth-Régrigny E, Petitcolin MA, Bueb JL, Tschirhart EJ, Atkinson J, Capdeville-Atkinson C. Pertussis toxin-sensitive G_i-proteins and intracellular calcium sensitivity of vasoconstriction in the intact rat tail artery. Br J Pharmacol 2000;131:1337–44.

[8] Capdeville-Atkinson C, Oster L, Thorin-Trescases N, Robert A, Boutinet S, Atkinson J. Intracellular free Ca^{2+} and vasoconstriction determined simultaneously in the perfused rat tail artery. Am J Physiol 1993;265:C1689–702.

[9] Tran NN, Spitzbarth E, Robert A, Giummelly P, Atkinson J, Capdeville-Atkinson C. Nitric oxide lowers the calcium sensitivity of tension in the rat tail artery. J Physiol 1998;507:163–74.

[10] Abebe W, Edwards J, Agrawal D. G-proteins in rat blood vessels. 1. Identification. Gen Pharmacol 1995;26:65–73.

[11] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–75.

[12] Ribeiro-Neto FAP, Mattera R, Hildebrandt JD, Codina J, Field JB, Birnbaumer L, Sekura RD. ADP-ribosylation of membrane components by pertussis and cholera toxin. Methods Enzymol 1985;109:566–72.

[13] McPherson GA. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J Pharmacol Methods 1985;14:213–28.

[14] Gurdal H, Seasholtz T, Wang HY, Brown R, Johnson M, Friedman E. Role of $G_{\alpha q}$ or $G_{\alpha o}$ proteins in α_1-adrenoceptor subtype-mediated responses in Fischer 344 rat aorta. Mol Pharmacol 1997;52:1064–70.

[15] Milligan G. Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. Biochem J 1988;255:1–13.

[16] Weber L, MacLeod K. Noradrenaline stimulation of high-affinity GTPase activity in membranes from rat aorta and caudal artery. Biochem Pharmacol 1996;52:677–84.

[17] Medgett I, Langer S. Heterogeneity of smooth muscle alpha adrenoceptors in rat tail artery *in vitro*. J Pharmacol Exp Ther 1984;229:823–30.

[18] Atkinson J, Trescases N, Benedek C, Boillat N, Fouda A, Krause F, Pitton M, Rafizadeh C, de Rivaz J, Sautel M, Sonnay M. Alpha-1 and alpha-2 adrenoceptor agonists induce vasoconstriction of the normotensive rat caudal artery *in vitro* by stimulation of a heterogeneous population of alpha-1 adrenoceptors. Naunyn Schmiedebergs Arch Pharmacol 1988;338:529–35.

[19] Michel M, Insel P. Comparison of cloned and pharmacologically defined rat tissue alpha 1-adrenoceptor subtypes. Naunyn Schmiedebergs Arch Pharmacol 1994;350:136–42.

[20] Oshita M, Kigoshi S, Muramatsu I. Pharmacological characterization of two distinct alpha 1-adrenoceptor subtypes in rabbit thoracic aorta. Br J Pharmacol 1993;108:1071–6.

[21] Muramatsu I, Murata S, Isaka M, Piao HL, Zhu J, Suzuki F, Miyamoto S, Oshita M, Watanabe Y, Taniguchi T. Alpha1-adrenoceptor subtypes and two receptor systems in vascular tissues. Life Sci 1998;62:1461–5.

[22] Lachnit W, Tran A, Clarke D, Ford A. Pharmacological characterisation of an α_{1A}-adrenoceptor mediating contractile responses to noradrenaline in isolated caudal artery of rat. Br J Pharmacol 1997;120:819–26.

[23] Piascik MT, Smith MS, Soltis EE, Perez DM. Identification of the mRNA for the novel α_{1D}-adrenoceptor and two other α_1-adrenoceptors in vascular smooth muscle. Mol Pharmacol 1994;46:30–40.

[24] Piascik MT, Guarino RD, Smith MS, Soltis EE, Saussy DL, Perez DM. The specific contribution of the novel α_{1D} adrenoceptor to the contraction of vascular smooth muscle. J Pharmacol Exp Ther 1995;275:1583–9.

[25] Zhong H, Minneman KP. Alpha1-adrenoceptor subtypes. Eur J Pharmacol 1999;375:261–76.

[26] Stassen FR, Maas RG, Schiffers PM, Janssen GM, De Mey JG. A positive and reversible relationship between adrenergic nerves and α_{1A} adrenoceptors in rat arteries. J Pharmacol Exp Ther 1998;284:399–405.

[27] Daniel EE, Brown RD, Wang YF, Low AM, Lu-Chao H, Kwan CY. Alpha-adrenoceptors in canine mesenteric artery are predominantly 1A subtype: pharmacological and immunochemical evidence. J Pharmacol Exp Ther 1999;291:671–9.

[28] Lyles GA, Birrell C, Banchelli G, Pirisino R. Amplification of α_{1D}-adrenoceptor mediated contractions in rat aortic rings partially depolarised with KCl. Pharmacol Res 1998;37:437–54.

[29] Petitcolin MA, Vandeputte C, Spitzbarth-Régrigny E, Bueb JL, Capdeville-Atkinson C, Tschirhart EJ. Lack of involvement of pertussis toxin-sensitive G-proteins in norepinephrine-induced vasoconstriction of rat aorta smooth muscle. Biochem Pharmacol 2001;61:485–91.