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Pertussis toxin-sensitive G_i-proteins and intracellular calcium sensitivity of vasoconstriction in the intact rat tail artery

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1 We studied the involvement of pertussis toxin (PTX)-sensitive G-proteins in the sensitivity of arterial constriction to intracellular calcium ($[Ca^{2+}]_i$) mobilization.

2 Vasoconstriction was measured *in vitro* in perfused, de-endothelialized rat tail arteries loaed with the calcium-sensitive dye, fura-2 and treated or not with PTX (30–1000 ng ml^{-1}). Arteries were stimulated with noradrenaline (NA, 0.1–100 μM) or KCl (15–120 mM).

3 KCl elicited a smaller vasoconstrictor response ($E_{max} = 94 \pm 8$ mmHg) than NA ($E_{max} = 198 \pm 9$ mmHg) although $[Ca^{2+}]_i$ mobilization was similar ($E_{max} = 123 \pm 8$ and 135 ± 7 nM for KCl and NA, respectively). PTX (1000 ng ml^{-1}) had no effect on $[Ca^{2+}]_i$ mobilization but lowered NA- (but not KCl-) induced vasoconstriction ($E_{max} = 118 \pm 7$ mmHg).

4 $G_{i/o}$-proteins were revealed by immunoblotting with anti-$G_{i\alpha}$ and anti-$G_{o\alpha}$ antibodies in membranes prepared from de-endothelialized tail arteries. [α^{32}P]-ADP-ribosylation of G-proteins by PTX (1000 ng ml^{-1}) was demonstrated in the intact rat tail artery (pixels in the absence of PTX: 3150, presence: 25053).

5 In conclusion, we suggest that smooth muscle cells possess a PTX-sensitive G_i-protein-mediated intracellular pathway which amplifies $[Ca^{2+}]_i$ sensitivity of contraction in the presence of agonists such as NA.

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Abbreviations: AF, autofluorescence; $[Ca^{2+}]_c$, cytoplasmic contractile domain; $[Ca^{2+}]_i$, intracellular calcium; $[Ca^{2+}]_{pm}$, subplasmalemmal domain; c.p.s., counts per second; DTT, DL-dithiothreitol; E_{max}, maximal effect; F, fluorescence; NA, noradrenaline; MLC_{20}, myosin light chain; PSS, physiological saline solution; PTX, pertussis toxin

Introduction

In vascular smooth muscle cells, depolarization induced by a high potassium (KCl) solution evokes pronounced intracellular calcium ($[Ca^{2+}]_i$) mobilization but only a minor vasoconstrictor response. In contrast, agonists such as noradrenaline (NA) induce minor $[Ca^{2+}]_i$ mobilization but a pronounced vasoconstrictor response (Karaki *et al*., 1997). This suggests that the sensitivity of the smooth muscle cell contractile apparatus to $[Ca^{2+}]_i$ is higher in the presence of agonists. Myosin phosphorylation is the final determinant of smooth muscle contraction; $[Ca^{2+}]_i$ sensitivity of tension is correlated to $[Ca^{2+}]_i$ sensitivity of myosin light chain (MLC_{20}) phosphorylation (Ikebe *et al*., 1988; Rembold, 1990). Myosin phosphorylation is modulated by MLC_{20}-kinase and MLC_{20}-phosphatase which are under the control of G-proteins (Kitazawa *et al*., 1991; Kubota *et al*., 1992). Pertussis toxin (PTX), an inhibitor of $G_{i/o}$-proteins, reduces the vasoconstrictor response to α-adrenoceptor agonists in the rat tail artery (Li & Triggle, 1993).

Taken together these different observations suggest that a PTX-sensitive G-protein-modulated pathway increases the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$ in the presence of agonists but not in the presence of KCl. The object of this study was to test this working hypothesis.

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Experiments were performed in the perfused rat tail artery in which $[Ca^{2+}]_i$ was measured using the intracellular fluorescent calcium dye, fura-2, in parallel with measurement of perfusion pressure at a constant flow rate (as an indication of vasomotion) (Capdeville-Atkinson *et al*., 1993). The impact of PTX on NA- and KCl-induced changes in the $[Ca^{2+}]_i$ sensitivity of vasoconstriction was evaluated. To our knowledge this is the first study in which the effects of PTX are evaluated with simultaneous measurement of vasoconstriction and $[Ca^{2+}]_i$ in an intact perfused vessel thus allowing estimation of changes in $[Ca^{2+}]_i$-sensitivity of vasoconstriction.

In separate experiments using smooth muscle cells membranes prepared from de-endothelialized arteries, the presence of G_i and G_o was demonstrated by immunoblotting. We also demonstrated that PTX could induce [α^{32}P]-ADP-ribosylation of the intact tail artery segment.

Methods

Animals

Adult, male, outbred Wistar rats (610 ± 20 g, Iffa-Credo, L'Arbresle, France) were housed under standard conditions (temperature: 21 ± 1°C; humidity: 60 ± 10%; lights on: 0800 to 2000 h) and given a rodent diet (code number A04, UAR, Villemoisson-sur-Orge, France) and water *ad libitum* for 1 week before each experiment.

Measurement of $[Ca^{2+}]_i$ and of vasoconstriction in tail artery segments; treatment with PTX

The experimental protocol is described in detail elsewhere (Capdeville-Atkinson *et al*., 1993; 1995). Briefly, rats were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The tail artery was dissected out under constant pressure (40 mmHg) by perfusing a physiological salt solution (PSS, mM): NaCl 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid 10, and glucose 6, saturated with 100% O_2, at a rate of 1.5 ml min^{-1}.

A 1-cm segment was cannulated at both ends (polyethylene tubing, i.d. = 0.4 mm; o.d. = 0.8 mm, Portex S.A., Berck-sur-Mer, France) and mounted in a perfusion-cuvette system placed in a dual wavelength spectrofluorometer (Fluorolog F1 T11, Spex, Edison, NJ, U.S.A.). A longitudinal tension of 0.5 g was used to restore the segment to its *in situ* length, the degree of retraction was $20 \pm 1\%$ ($n = 46$) (Tran *et al*., 1998). Experiments were performed in the absence of endothelium which was removed by perfusing the segment with air (0.4 ml min^{-1}) plus PSS (1.5 ml min^{-1}) for 10 min (intraluminal pressure 40 ± 10 mmHg; $n = 46$) at the beginning of the equilibration period (Tran *et al*., 1998). Following this procedure carbachal (10 μM) failed to relax segments precontracted with NA (3 μM). Histological evidence of the removal of endothelium has already been published (Tran *et al*., 1998).

Arterial segments were perfused with oxygenated PSS (1.5 ml min^{-1}) for an initial equilibration period of 20 min; the cuvette was filled with oxygenated PSS. Baseline, NA- or KCl-evoked increases in perfusion pressure were measured with a pressure transducer (Baxter, Deerfield, IL, U.S.A.) placed between the peristaltic pump and the segment; the transducer was linked to a signal processing system (MacLab/MacBridge, World Precision Instruments Inc., Sarasota, FL, U.S.A.). All pressure valves were corrected by subtraction of the pressure generated by the resistance of the tubing (15 ± 1 mmHg).

Baseline perfusion pressure and tissue autofluorescence (AF_{360}, excitation wavelength 360 nm, emission wavelength 510 nm, global average $1.05 \pm 0.03 \times 10^6$ counts per second, c.p.s. $\times 10^6$, $n = 46$) were determined. The arterial segment was exposed to KCl (80 mM; three challenges, 2 min each) or NA (3 μM; one challenge, 2 min). When KCl preceded NA ($n = 6$), responses were: KCl: 38 ± 5 and NA: 110 ± 15 mmHg. When NA preceded KCl ($n = 6$), responses were: KCl: 41 ± 4 and NA: 107 ± 19 mmHg.

PSS was perfused for 5 min between challenges. If the vasoconstrictor responses were lower than 30 mmHg for KCl or 60 mmHg for NA the segment was discarded (exclusion criteria based on Tran *et al*. (1998). The exclusion rate was 2% (one of 46 experiments).

Segments were perfused with PSS containing fura-2-AM (5 μM, 90 min), Pluronic F 127 (0.06% w v^{-1}), and bovine serum albumin (0.01% w v^{-1}). Fura-2 loading was followed by washout with PSS (30 min). Fluorescence at the isobestic point (F_{360}) was measured in order to calculate loading efficiency ($= F_{360}/AF_{360}$).

The segment was illuminated at excitation wavelengths of 340 or 380 nm with a change from one to the other every second; emission fluorescence (510 nm) was measured with a photomultiplier. The ratio of fluorescence at the two excitation wavelengths ($R'_{340/380}$) was determined following background correction, at baseline and during perfusion of KCl or NA. $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz *et al*. (1985) as modified by Scanlon *et al*. (1987):

$$[Ca^{2+}]_i = K_d[(R'-R'_{min})/(R'_{max}-R')] \times \beta' \text{ (nM)}$$

where: $K_d = 224$ nM, $R' = R'_{340/380}$; R'_{max} = the ratio of background-corrected fluorescence in the presence of a saturating concentration of calcium ($CaCl_2$, 4 mM) and the calcium ionophore, ionomycin (10 μM, 5 min); R'_{min}, the ratio of background-corrected fluorescence in the presence of calcium-free PSS containing EGTA (10 mM) and ionomycin (10 μM, 7 min); β' = the ratio of F'_{380} at zero and saturating calcium concentration. The potential presence of $[Ca^{2+}]_i$-insensitive, fluorescent metabolites of fura-2 was checked by perfusing with calcium-free PSS containing manganese chloride (1 mM) and ionomycin (10 μM) at the end of each experiment for 3 min. Fluorescence values in presence of manganese (F_{Mn}), which were not significantly different from AF values (Table 1), were used as an estimation of background fluorescence. F_{Mn} can be used to determine possible fura-2-AM de-esterification and formation of $[Ca^{2+}]_i$-insensitive metabolites since manganese binds to fura-2 and so quenches fura-2-$[Ca^{2+}]_i$ fluorescence (Scanlon *et al*., 1987).

Following fura-2 loading, arteries were stimulated with KCl (15 – 120 mM, 2 min, perfusate and bath) followed by NA (0.1 – 100 μM, 2 min, perfusate only) with 5 min PSS between each challenge. The protocol lasted 80 min. In separate experiments, a change in the order (NA before KCl) and randomization of concentrations were investigated.

Increases in $[Ca^{2+}]_i$ ($\Delta([Ca^{2+}]_i$, nM) and perfusion pressure (ΔP, mmHg) were measured and $[Ca^{2+}]_i$ – sensitivity of vasoconstriction was estimated as: vasoconstriction/($[Ca^{2+}]_i$) mobilization (mmHg nM^{-1}).

In order to investigate the involvement of G_i-proteins in the modulation of $[Ca^{2+}]_i$ – sensitivity of vasoconstriction, activated PTX was added during the fura-2 loading period and to the washout solution.

PTX was activated by incubation with DL-dithiothreitol (20 mM in PSS, 37°C, 1 h; DTT) (Day *et al*., 1995; Mader *et al*., 1996). Solutions were dialysed (4 ml, Biotech dispodialy-

Table 1 Fluorescence (F) and autofluorescence (AF) values (c.p.s. $\times 10^6$), R'_{max}, R'_{min}, β'

PTX (ng ml^{-1})	n	*Before loading* AF_{340}	AF_{380}	*After loading* F_{340}	F_{380}	*Loading efficiency* F_{360}/AF_{360}	*Baseline fluorescence prior to calibration* F_{340}	F_{380}	R'_{max}	R'_{min}	β'
Time control	12	1.2 ± 0.2	1.2 ± 0.1	6.2 ± 0.4	4.2 ± 0.3	4.5 ± 0.5	5.8 ± 0.3	3.9 ± 0.3	8.5 ± 1.8	0.9 ± 0.3	2.8 ± 1.8
0	9	1.4 ± 0.2	1.2 ± 0.1	5.9 ± 0.3	4.2 ± 0.2	4.8 ± 0.7	5.4 ± 0.4	3.8 ± 0.3	8.9 ± 1.8	0.9 ± 0.1	3.5 ± 1.2
30	6	1.3 ± 0.2	1.3 ± 0.1	6.4 ± 0.3	5.2 ± 0.3	3.9 ± 0.4	5.9 ± 0.5	4.1 ± 0.2	8.7 ± 1.8	0.9 ± 0.1	2.8 ± 0.5
100	7	1.3 ± 0.2	1.1 ± 0.1	5.9 ± 0.4	4.5 ± 0.2	4.2 ± 0.5	5.5 ± 0.2	4.3 ± 0.4	8.7 ± 2.6	0.9 ± 0.1	3.0 ± 0.8
300	7	1.4 ± 0.3	1.3 ± 0.2	6.2 ± 0.2	4.8 ± 0.2	3.9 ± 0.4	5.9 ± 0.5	4.5 ± 0.2	9.1 ± 1.7	1.0 ± 0.1	2.8 ± 0.6
1000	5	1.2 ± 0.2	1.2 ± 0.2	6.1 ± 0.2	4.9 ± 0.2	4.3 ± 0.1	5.6 ± 0.2	4.6 ± 0.3	9.6 ± 1.3	0.9 ± 0.1	2.8 ± 0.4

zer Spectra/Por®, molecular weight cut-off = 8 kDa, Spectrum Laboratories, Laguna Hills, CA, U.S.A.) for 3 h against PSS (1 l, 22°C) with a change of bath at 15, 30, 60 and 120 min.

The concentrations of PTX used were 0 (dialysis control, $n=9$), 30 ($n=6$), 100 ($n=7$), 300 ($n=7$), and 1000 ng ml^{-1} ($n=5$). The stability of responses to KCl and NA over time was tested by repeated challenges with KCl (80 mM, 2 min, three challenges every 7 min) or NA (3 μM, 2 min, three challenges every 7 min) and *vice versa* over a 50 min period in fura-2-loaded arterial segments (time controls, $n=12$).

Membrane preparation from arteries

In order to demonstrate the existence of G_i- and G_o-proteins, the artery was dissected out and endothelium removed by rubbing the intimal surface with a stainless steel wire. Cell membrane proteins were isolated as described by Kwan *et al.* (1983). Briefly, arteries were pooled from five rats, cut into small segments and homogenized in ice-cold buffer (mM): Tris 15, EDTA 1, phenylmethylsulphonyl fluoride 0.1, benzamidine 0.1 and leupeptin 0.001, pH 7.4, with a glass Dounce Homogenizer (Kontes, Vineland, NJ, U.S.A.). The homogenate was then centrifuged at $900\times g$ for 10 min (4°C). The resulting supernatant was centrifuged at $10{,}000\times g$ for 10 min (4°C). After a last centrifugation ($60{,}000\times g$, 45 min, 4°C), the membrane pellet was resuspended in the same buffer plus 1% w v^{-1} of sodium cholate and stored at −80°C until assay. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Immunoblotting with anti-$G_{i\alpha}$ and anti-$G_{o\alpha}$ antibodies

Solutions containing membrane proteins were diluted 2.5 fold in buffer (Laemmli, 1970), heated for 3 min and then loaded onto a 16.5% SDS–PAGE (Bio-Rad, Hercules, CA, U.S.A.). Equal amounts (10 μg) of total proteins were loaded on each lane. After electrophoresis, proteins were electrotransferred onto a 0.4 μm thick nitrocellulose membrane (Bio-Rad; 100 V for 90 min at 4°C). Blotting was performed using polyclonal rabbit antibodies directed against $G_{i\alpha}$ subunits 1-2 and/or 3 (1 : 1000, Calbiochem, La Jolla, CA, U.S.A.) and monoclonal mouse antibody directed against $G_{o\alpha}$ subunits (1 : 3000, Chemicon International Inc., Temecula, CA, U.S.A.) ($n=3$). Detection was performed using a streptavidin-biotinylated alkaline phosphatase complex with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate (Bio-Rad). The molecular markers ovalbumin (45 kDa) and carbonic anhydrase (31 kDa) (Bio-Rad) were used. Densitometric analysis was performed using the NIH *Image*® program (version 1.58, Wayne Rasband, National Institutes of Health, Bethesda, MD, U.S.A.); results are expressed as pixels and compared with those obtained with purified bovine brain G-protein subunits (Calbiochem).

ADP-ribosylation of heterotrimeric G-proteins by PTX

De-endothelialized 12-cm tail artery segments were perfused as described in Methods with an ADP-ribosylation mixture (mM): KH_2PO_4 200, $MgCl\ 5_2$, ATP 2, EDTA 2, DTT 2, thymidine 20, GTP 1, NAD^+ 0.02, and [α^{32}P]-NAD^+ (30 Ci mmol^{-1}) (New England Nuclear, Zaventum, Belgium) containing or not activated PTX (1000 ng ml^{-1}; control dialysed DTT) for 2 h (37°C). Membrane proteins (70 μg) were prepared as described above.

Following incubation, samples ($n=3$ for each) were submitted to SDS–PAGE and blotted as described above. Labelled blots were dried and apposed on to X-ray film (Fuji Medical NIF, Tokyo, Japan) for 132 h. Densitometric analysis was performed using the NIH *Image*® program and results are expressed as pixels.

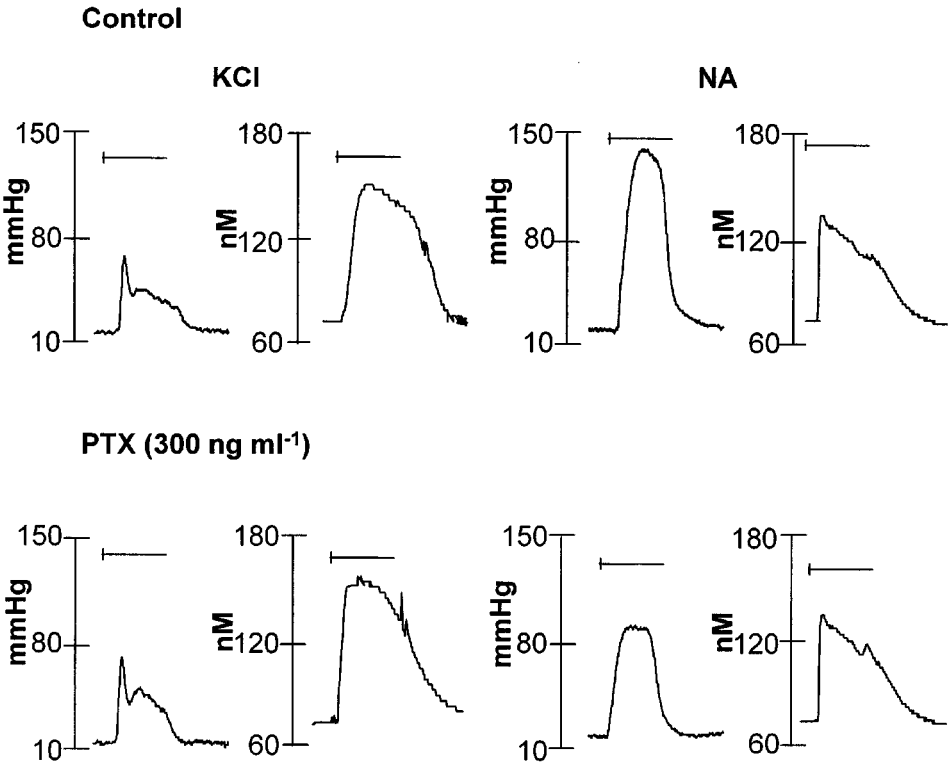


Figure 1 Typical recordings of changes in perfusion pressure (mmHg) and $[Ca^{2+}]_i$ (nM) in rat tail artery segments following KCl (80 mM, 2 min; left) or NA (3 μM, 2 min; right) in the absence (upper) or presence of PTX (300 ng ml^{-1}; lower). Bars represent perfusion with KCl or NA, respectively.

Materials

Fura-2-AM was purchased from Molecular Probes (Eugene, OR, U.S.A.). Dimethylsulphoxide was obtained from Prolabo (Paris, France), Pluronic F-127 from BASF (Wyandotte, MI, U.S.A.), sodium pentobarbitone from Sanofi S.A. (Paris, France) and Tris from Merck (Darmstadt, Germany). All other chemicals were from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Statistical analysis

Data are given as mean ± s.e.mean; n = the number of experiments. Means were compared using ANOVA plus Bonferroni tests. The level of significance was $P<0.05$.

Results

Baseline perfusion pressure was stable throughout; values in time controls ($n=12$) were between 13 ± 1 and 15 ± 1 mmHg before equilibration and up to the final challenge with KCl and NA (80 min).

Loading with fura-2 produced a 3 to 4 fold increase in fluorescence (Table 1). There was a slight but a significant ($P<0.05$) linear fall in baseline fluorescence (global averages: 83.3 c.p.s. s^{-1} at 340 nm and 62.5 c.p.s. s^{-1} at 380 nm; $n=12$), but this fall did not affect baseline $[Ca^{2+}]_i$ (69 ± 4 at the beginning and 74 ± 9 nM at the end of the experiment). Treatment with PTX had no effect on fura-2 loading and calibration (Table 1) or baseline perfusion pressure.

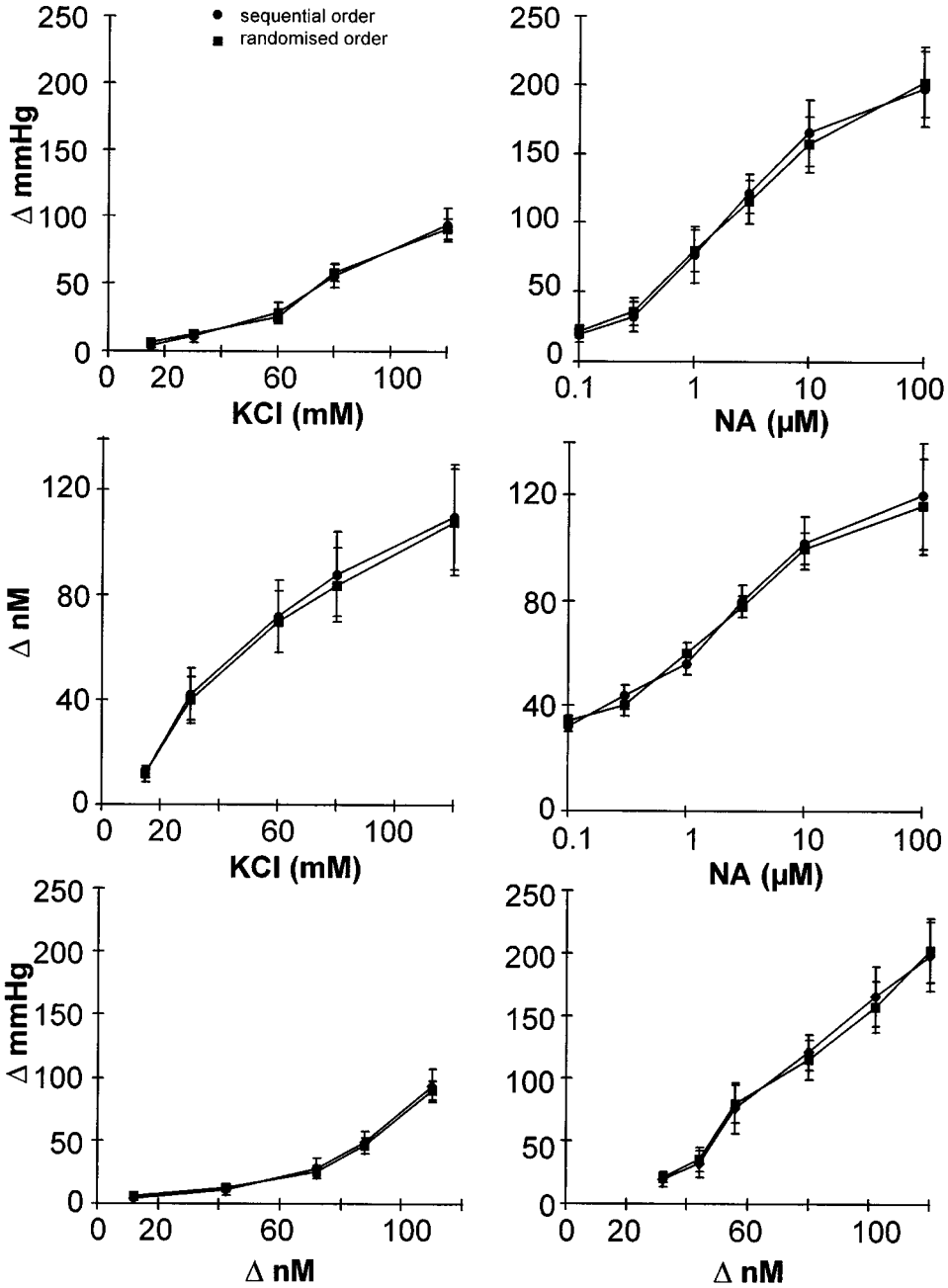


Figure 2 Dose-response curves for increases in perfusion pressure (Δ mmHg, upper), changes in $[Ca^{2+}]_i$ (Δ nM, middle) and $[Ca^{2+}]_i$ sensitivities of vasoconstriction (lower) following KCl (left) and NA (right) administered in a sequential or a randomized order ($n=6-9$ per experiment).

KCl produced a biphasic increase in perfusion pressure and monophasic $[Ca^{2+}]_i$ change; NA produced a monophasic increase in perfusion pressure but biphasic $[Ca^{2+}]_i$ change (Figure 1). For all further calculations, maximal peak values were taken.

In time controls, responses were for KCl: 41 ± 4 mmHg, 99 ± 7 nM $[Ca^{2+}]_i$, and for NA: 113 ± 8 mmHg, 85 ± 2 nM $[Ca^{2+}]_i$. Responses did not vary significantly with time and the order of challenge had no significant effect (results not shown).

Randomized or sequential administration of concentrations gave similar results (Figure 2). When arteries were perfused with the dialysis solution used to eliminate DTT, vasoconstrictor responses to KCl (80 mM, 45 ± 5 mmHg, $n=9$) and NA (3 μM, 121 ± 5 mmHg, $n=9$) were not significantly altered.

PTX had no effect on baseline perfusion pressure (global mean, 14 ± 1 mmHg, $n=34$; see above for control values) or on fluorescence (Table 1). PTX did not modify baseline $[Ca^{2+}]_i$: 67 ± 16 nM in the absence, 56 ± 18 nM in the presence of PTX (30 ng ml^{-1}), 67 ± 11 nM at 100, 58 ± 11 nM at 300, and 69 ± 18 nM at 1000 ng ml^{-1}. PTX had no effect on baseline $[Ca^{2+}]_i$ sensitivity of tension (results not shown).

At low doses ($\leqslant 30$ ng ml^{-1}) PTX had no effect on vasoconstriction induced by either KCl or NA. Higher concentrations ($\geqslant 100$ ng ml^{-1}) of PTX reduced ($P<0.05$) the vasoconstrictor response to NA but had no effect on that to KCl (Figures 3 and 4). PTX did modify neither the pattern of the vasoconstrictor response to NA (Figure 1) nor $[Ca^{2+}]_i$ changes. However it lowered $[Ca^{2+}]_i$ sensitivity of tension elicited by NA.

Western blots of proteins extracted from these arteries revealed the presence of $G_{i\alpha}$-and $G_{o\alpha}$-proteins in membranes (Figure 5 upper and lower, respectively). Given the selectivity of the antibodies and the molecular markers used, Figure 5 (upper) shows a single band the molecular weight of which was approximately 41–42 kDa, corresponding to $G_{i\alpha}$ subunits. A weak single band at 40 kDa corresponding to $G_{o\alpha}$ subunits was seen (Figure 5, lower). In comparison to G_i-proteins, only traces of G_o-proteins were detected.

Perfusion with PTX (1000 ng ml^{-1}) induced ADP-ribosylation of $G_{i\alpha}$ (and/or $G_{o\alpha}$) in rat tail artery (Figure 6). One band, at about 42 kDa, was labelled in the autoradiographs.

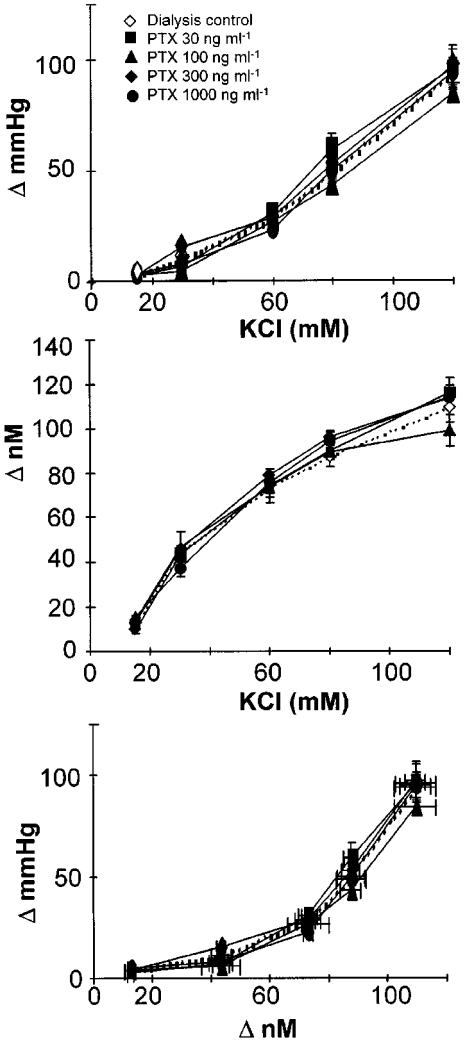


Figure 3 Increases in perfusion pressure (Δ mmHg, upper), changes in $[Ca^{2+}]_i$ (Δ nM, middle) and $[Ca^{2+}]_i$ sensitivities of vasoconstriction (lower) produced by KCl in rat tail artery segments in the absence (dialysis control, $n=9$) or presence of PTX (30 ng ml^{-1}, $n=6$; 100 ng ml^{-1}, $n=7$; 300 ng ml^{-1}, $n=7$; 1000 ng ml^{-1}, $n=6$).

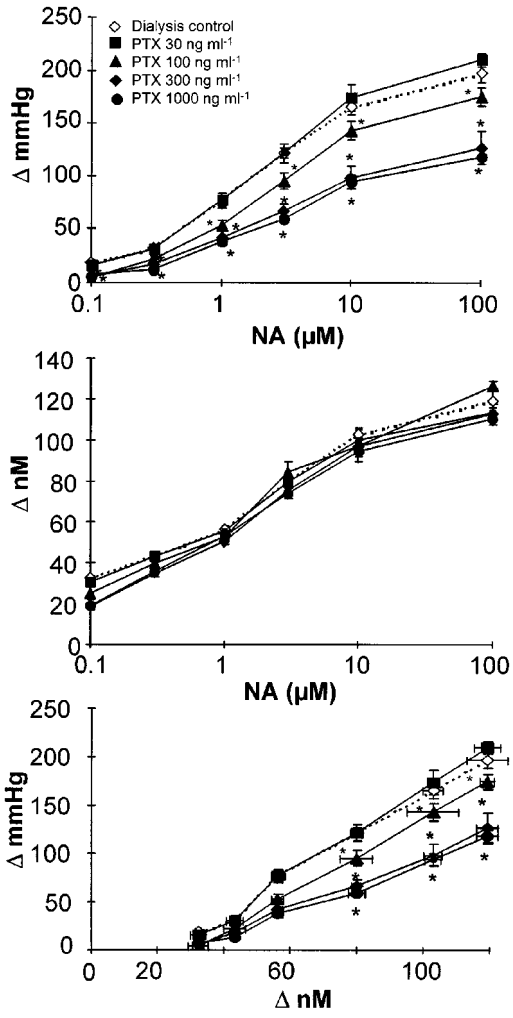
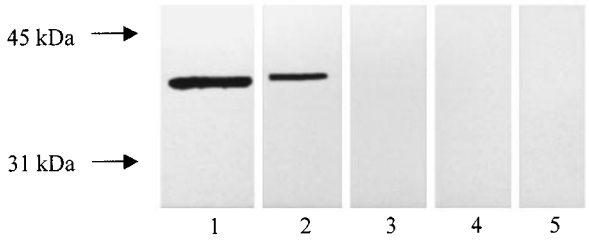


Figure 4 Increases in perfusion pressure (Δ mmHg, upper), changes in $[Ca^{2+}]_i$ (Δ nM, middle) and $[Ca^{2+}]_i$ sensitivities of vasoconstriction (lower) produced by NA in rat tail artery segments in the absence (dialysis control, $n=9$) or presence of PTX (30 ng ml^{-1}, $n=6$; 100 ng ml^{-1}, $n=7$; 300 ng ml^{-1}, $n=7$, 1000 ng ml^{-1}, $n=6$). *$P<0.05$ *versus* dialysis control.

Lane	1	2	3	4	5
Purified G	+	-	-	+	-
Membrane preparations	-	+	-	-	+
Antibodies $G_{i\alpha 1\text{-}2} + G_{i\alpha 3}$	+	+	+	-	-
Pixels	4138	2067	0	0	0



Lane	1	2	3	4	5
Purified G	+	-	-	+	-
Membrane preparations	-	+	-	-	+
Antibodies $G_{o\alpha}$	+	+	+	-	-
Pixels	3419	709	0	0	0

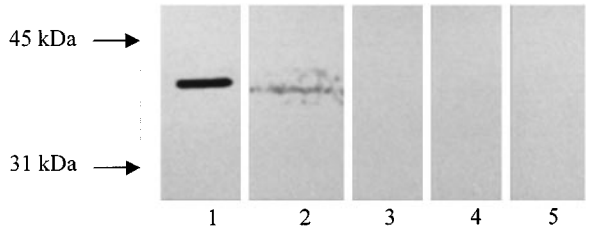


Figure 5 Membrane preparations (10 μg) of rat tail artery labelled with $G_{i\alpha}$- (upper) and $G_{o\alpha}$ (lower)-antibodies. Control was bovine brain purified G-proteins subunits. Lanes: 1, bovine brain purified G-proteins subunits with polyclonal rabbit antibodies directed against $G_{i\alpha 1\text{-}2} + G_{i\alpha 3}$ (upper) or monoclonal mouse antibody directed against $G_{o\alpha}$ (lower), 2, membrane preparation of the rat tail artery with polyclonal rabbit antibodies directed against $G_{i\alpha 1\text{-}2} + G_{i\alpha 3}$ (upper) or monoclonal mouse antibody directed against $G_{o\alpha}$ (lower), 3, polyclonal rabbit antibodies directed against $G_{i\alpha 1\text{-}2} + G_{i\alpha 3}$ (upper) or monoclonal mouse antibody directed against $G_{o\alpha}$ (lower), 4, bovine brain purified G-proteins subunits, 5, membrane preparation of the rat tail artery. The arrows indicate the positions of the molecular markers ovalbumin (45 kDa) and carbonic anhydrase (31 kDa).

Lane	1	2	3
PTX (1000 ng ml^{-1})	+	-	+
Tail artery	+	+	+
$[\alpha^{32}P]NAD^+$	+	+	-
Pixels	25053	3150	3101

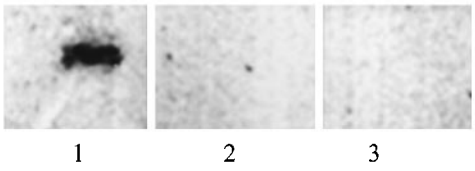


Figure 6 Autoradiographs showing ADP-ribosylation of G_i-proteins induced by perfusion (2 h) with PTX (1000 ng ml^{-1}) in rat tail artery. Lanes: 1, rat tail artery perfused with PTX and ADP-ribosylation mixture containing $[\alpha^{32}P]NAD^+$ (30 Ci $mmol^{-1}$), 2, rat tail artery perfused with ADP-ribosylation mixture containing $[\alpha^{32}P]NAD^+$, 3, rat tail artery perfused with PTX.

Discussion

Our results show that in the *in vitro* de-endothelialized tail artery, PTX attenuates the vasoconstriction induced by the agonist, NA, but has no effect on baseline vasomotion or on KCl-induced vasoconstriction. PTX did not modify baseline $[Ca^{2+}]_i$ or changes in $[Ca^{2+}]_i$ associated with vasoconstriction.

The absence of effect of PTX on $[Ca^{2+}]_i$ cannot be explained by interference of PTX with the fura-2 technique. PTX had no effect on the parameters generally taken as criteria for satisfactory use of this technique, namely, R'_{max}, R'_{min}, β' and the loading ratio F_{360}/AF_{360}. This absence of effect of PTX on $[Ca^{2+}]_i$ is not surprising given the lack of effect of PTX on either calcium channels or inositol phosphate metabolism (Cheung *et al*., 1990; LaBelle & Murray, 1990; Takuwa *et al*., 1990; Ohya & Sperelakis, 1991). The possibility exists that the PTX B-oligomer could modify $[Ca^{2+}]_i$ by triggering phospholipase C (Wong & Rosoff, 1996), but this does not appear to be the case in our preparation. Another hypothesis is that of a toxic effect of PTX at a concentration of 1000 ng ml^{-1}, but some authors (Day *et al*., 1995; Mader *et al*., 1996) have used even higher doses with no toxic effects.

Although PTX (100 ng ml^{-1}) lowered the myogenic tone of rat cerebral arteries *in vitro* (Osol *et al*., 1993), it did not lower baseline perfusion resistance in the rat tail artery even at a 10 fold higher dose (present results). This suggests that PTX-sensitive G-proteins are not involved in the maintenance of baseline tone of the rat tail artery.

PTX did not lower vasoconstriction induced by perfusion of KCl. This observation suggests that PTX-sensitive G-proteins play no role in the low $[Ca^{2+}]_i$ sensitivity of myosin light chain phosphorylation and of the contractile elements involved in KCl-induced contraction.

PTX lowered vasoconstriction induced by the agonist, NA, confirming results previously published by several laboratories (Abebe *et al*., 1995) but not by all (Gong *et al*., 1996).

Our results show that PTX reduces vasoconstriction by lowering the $[Ca^{2+}]_i$ sensitivity of tension without changing $[Ca^{2+}]_i$ mobilization. As described in the Introduction, agonist-induced increases in smooth muscle tension operate at a much higher level of $[Ca^{2+}]_i$ sensitivity compared to KCl-induced contraction. High signal amplification (=contraction/$[Ca^{2+}]_i$ mobilization) involves G_i-protein-mediated modulation of MLC_{20}-phosphatase and -kinase (see Introduction). We suggest that this system is involved in α_1-adrenoceptor-mediated vasoconstriction of the rat tail artery *in vitro*. Several lines of evidence argue in favour of this working hypothesis. It has previously been showed that α-adrenoceptors and especially α_1-adrenoceptors, can couple to multiple transduction pathways (Orowio *et al*., 1992; Garcia-Sainz, 1993) including G_i-protein-mediated pathways (Fujiwara *et al*., 1989; Aburto *et al*., 1993; D'Angelo & Osol, 1994). PTX-sensitive G_i-proteins have been isolated from the rat tail artery smooth muscle cell (Weber & MacLeod, 1996; Petitcolin *et al*., 1998). The present results show that in the de-endothelialized rat tail artery, PTX ADP-ribosylates G_i-proteins.

Differences in the association between $[Ca^{2+}]_i$ and smooth muscle contraction (when comparing NA with KCl or the effects of PTX) can be explained in terms of $[Ca^{2+}]_i$-sensitivity of contraction (which is our working hypothesis) or in terms of artefacts in the $[Ca^{2+}]_i$ measurements (Karaki *et al*., 1997). Although the parameters generally taken as criteria for a satisfactory use of fura-2 were not modified by PTX (see above) this does not exclude the fact that there may be differences between the fura-2 signal and the $[Ca^{2+}]_i$ associated with the contractile apparatus of the cell. Van Breemen (1977) suggested that functional $[Ca^{2+}]_i$ domains can be separated into a cytoplasmic contractile domain ($[Ca^{2+}]_c$) and a subplasmalemmal domain ($[Ca^{2+}]_{pm}$).

Other authors have suggested that the latter domain does not contain contractile elements (Abe *et al*., 1995; 1996). As (1) sarcoplasmic reticulum function regulates both $[Ca^{2+}]_{pm}$ and $[Ca^{2+}]_c$ and (2) agonists such as histamine inhibit whereas high KCl enhances sarcoplasmic filling with Ca^{2+} (Rembold & Chen, 1998) a case could be made for the possibility that calcium microdomains in the cytosol might be partly responsible for the differences observed between the NA and KCl $[Ca^{2+}]_i$ signals. Although contraction is closely associated with the fura-2 signal which is primarily a measure of $[Ca^{2+}]_c$ (Rembold & Chen, 1998), determination of the aequorin/fura-2 ratio (as an indicator of focal increases in $[Ca^{2+}]_i$ (Van Riper *et al*., 1996)), could be used to evaluate potentially different effects of NA and KCl on calcium microdomains.

In conclusion, we have shown that PTX lowers $[Ca^{2+}]_i$ sensitivity of vasoconstriction in the presence of the agonist, NA, and demonstrated the existence of a PTX-sensitive G_i-protein system in rat tail artery smooth muscle cells. We suggest that the amplification of $[Ca^{2+}]_i$ sensitivity of vasoconstriction involves a PTX-sensitive G_i-protein linked to the α_1-adrenoceptor.

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