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Pertussis toxin-induced histamine sensitisation: an aspecific phenomenon independent from the nitric oxide system?

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Abstract

Mechanisms were studied initially to develop an in vitro safety test for detecting pertussis toxin toxicity in acellular pertussis vaccines based on the histamine sensitisation test. Maximal contractions and sensitivities to different agonists and adrenoceptor-induced contractions in Ca^{2+}-free medium of isolated rat small mesenteric resistance arteries were significantly reduced by in vivo [30 μg/kg, intravenously (i.v.), day 5] or in vitro (10 μg/ml, 2 h) pertussis toxin pretreatment. Pertussis toxin-induced decrease in sensitivity of small mesenteric resistance arteries to noradrenaline was endothelium-dependent. N^{ω}-nitro-L-arginine methyl ester (L-NAME) (100 μM, 20 min) did not reestablish the sensitivity to noradrenaline. In vivo L-NAME treatment (0, 1, 10 or 30 mg/kg) of pertussis toxin-pretreated (15 μg/kg) rats did not reduce pertussis toxin-induced enhancement of the histamine-induced decrease in blood pressure and histamine (10, 30, 100 or 300 mg/kg) induced mortality. Finally, in vivo pertussis toxin pretreatment sensitises rats for sodium nitroprusside (50 μg/kg/min). We conclude that pertussis toxin-induced histamine sensitisation is caused by an interference of pertussis toxin with the contractile mechanisms of vascular smooth muscle of resistance arteries which indicates only an indirect role for histamine in the histamine sensitisation test.

Keywords: Histamine sensitisation; Pertussis toxin; Small mesenteric resistance artery; Nitric oxide synthase

1. Introduction

Pertussis toxin, one of the major toxic components of *Bordetella pertussis* whole-cell vaccines, is very important for the development of immunity to whooping cough (Cherry, 1996; Edwards and Karzon, 1990; Pittman, 1979). Acellular pertussis vaccines contain detoxified antigens, i.e. detoxified pertussis toxin and pertussis toxoid (Anderson et al., 1994; Robinson and Funnell, 1992; Wardlaw, 1992). Regulatory authorities require safety testing of acellular pertussis vaccines in order to confirm absence of residual pertussis toxin toxicity. Currently, the histamine sensitisation test is the only test considered by the regulatory authorities to be suitable for this purpose (EP, 2003; WHO, 1998). The test is based on the principle that mice vaccinated with biologically active pertussis toxin are sensitised for histamine, resulting in a decrease of the lethal dose of histamine (Parfentjev and Goodline, 1948). The test gives rise to major animal welfare concern. Therefore, we started with the development of an in vitro safety test for detecting biologically active pertussis toxin in acellular pertussis vaccines based on mechanisms involved in the histamine sensitisation test. Ultimately, we want to reproduce a mechanistic effect of in vivo pertussis toxin with in vitro pertussis toxin treatment.

Mechanisms were studied initially to clarify which receptors, signal transduction systems and physiological (cardiovascular and pulmonary) systems are involved in pertussis toxin-induced histamine sensitisation. Previous studies had shown that vaccination of rats with biologi-

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cally active pertussis toxin decreases diastolic blood pressure, enhances histamine-induced decrease in mean arterial blood pressure, decreases the lethal dose of histamine and that this histamine sensitisation mainly involved histamine H_1 receptors (Vleeming et al., 2000a). In addition, we found that in vivo pertussis toxin pretreatment of male Wistar rats reduced maximal noradrenaline- or KCl-induced contraction of isolated small mesenteric resistance arteries, decreased sensitivity to noradrenaline of isolated rat small mesenteric resistance arteries and did not affect histamine- or acetylcholine-induced relaxations (Van Meijeren et al., 2004). It is known that vascular contraction induces endothelial nitric oxide release (Dora et al., 2000; Fleming et al., 1999; Kim and Greenburg, 2001) including noradrenaline-induced vasoconstriction (Bruck et al., 2001; Calderone et al., 2002). In addition, one of the signal transduction pathways that can be activated upon histamine H_1-receptor stimulation is the nitric oxide synthase-cyclic GMP system (Hill, 1990; Hill et al., 1997), which is thought to play an important role in the regulation of vascular tone and blood pressure (Corbin and Francis, 1999; Das and Kumar, 1995; Hussain et al., 1999; Leurs et al., 1995; Lin et al., 2000). We studied whether nitric oxide synthase could be involved in pertussis toxin-induced histamine sensitisation and/or in pertussis toxin-induced decreased noradrenaline-induced contraction of small mesenteric resistance arteries. Secondly, as mentioned, in vivo pertussis toxin pretreatment significantly decreased contractile properties of rat isolated small mesenteric resistance arteries. We hypothesise that vasoconstriction-regulating mechanisms of vascular smooth muscle cells could be involved in pertussis toxin-induced histamine sensitisation. We determined receptor, Ca^{2+}, endothelium, nitric oxide synthase and vessel diameter dependency of in vivo pertussis toxin pretreatment effects on contractile properties of isolated arteries and if these effects could be reproduced with in vitro pertussis toxin treatment. The intention was to generate mechanism-based information that can be used for the development of an in vitro pertussis toxin toxicity test for acellular pertussis vaccine safety testing.

2. Materials and methods

2.1. Animals

Male SPF Wistar rats, weighing 250–350 g each, were used. They were obtained from the SPF breeding colony of Harlan, The Netherlands.

The animals were SPF-housed under constant conditions, a relative humidity of 30–60% and a temperature of 22–24 °C. Food and water were available ad libitum. The experiments were reviewed and approved by the Animal Ethics Committee of the National Institute for Public Health and the Environment.

2.2. In vivo PT-pretreatment

Rats were injected i.v. with either saline or pertussis toxin 30 μg/kg (batch PTPU 1049). This dose is based on the fact that 15 μg/kg proved to sensitise rats for histamine. At 5 days after pretreatment with pertussis toxin or saline, the rats were killed, 10 min after intraperitoneal (i.p.) injection of heparin, by decapitation and exsanguination. Small mesenteric resistance arteries or thoracic aortas were removed as described in the following sections.

2.3. Experimental protocol for small mesenteric resistance arteries

Small mesenteric resistance arteries were prepared, mounted in a Mulvany–Halpern myograph and equilibrated as described previously (Van Meijeren et al., 2004). Cumulative concentration–response curves were made in small mesenteric resistance arteries of control or pertussis toxin-pretreated animals for noradrenaline, phenylephrine, BHT 933 (2-amino-6-ethyl-4,5,7,8-tetrahydro-6H-oxazolo-(5,4-a)-azepine dihydrochloride), serotonin or KCl. Also, they were incubated 3 min in Ca^{2+}-free Krebs–Ringer containing 0.3 mM EGTA and contracted with 10 μM noradrenaline or 10 μM phenylephrine. After the transient contractile response, agonists were added for the second time to the organ baths in order to determine the depletion of the respective agonist-sensitive intracellular Ca^{2+} stores. In another series of experiments, contractile concentration–response curves for noradrenaline or KCl were made in small mesenteric resistance arteries without endothelium of control or pertussis toxin-pretreated animals. Endothelium was removed by gently rubbing the intraluminal surface with crest horsehair. Sufficient removal of endothelium was checked by adding a single dose of 0.1 μM acetylcholine to small mesenteric resistance arteries precontracted with 60 mM KCl. They were excluded from the experiment when they relaxed more than 10% of precontraction. Small mesenteric resistance arteries without endothelium that lost more than 25% of their functionality were also excluded from the experiment. Functionality was determined by comparing the maximal KCl-induced contraction of one small mesenteric resistance artery segment without endothelium to the maximal KCl-induced contraction of the small mesenteric resistance artery with endothelium from the same animal. Furthermore, small mesenteric resistance arteries from control or pertussis toxin-pretreated rats without endothelium were incubated in Ca^{2+}-free Krebs–Ringer and contracted with 10 μM noradrenaline as described above. Finally, small mesenteric resistance arteries were incubated in vitro for 2 h with 10 μg/ml pertussis toxin (Aventis Pasteur) or saline. Cumulative concentration–response curves for noradrenaline, phenylephrine, serotonin or KCl were made. Subsequently, contractile responses for noradrenaline of control or in vitro pertussis toxin-pretreated small mesenteric resistance arteries in Ca^{2+}-free Krebs–

Ringer were performed as described above. Also, the involvement of nitric oxide-synthase in pertussis toxin-induced decreased sensitivity to noradrenaline of small mesenteric resistance arteries was investigated as follows. A cumulative concentration–response curve for noradrenaline was performed. After exhaustive washing and precontraction with 60 mM KCl, maximal acetylcholine-induced relaxation of all control and in vitro pertussis toxin-pretreated small mesenteric resistance arteries was determined. Following this, small mesenteric resistance arteries were incubated 20 min in Krebs–Ringer with 100 μM of the nitric oxide synthase inhibitor L-NAME. Sufficient inhibition of nitric oxide synthase was checked by administering a single dose of 0.1 μM acetylcholine to small mesenteric resistance arteries precontracted with 60 mM KCl. After washing with 100 μM L-NAME Krebs–Ringer solution, a cumulative concentration–response curve for noradrenaline in control or pertussis toxin-pretreated small mesenteric resistance arteries was performed in the continuous presence of L-NAME.

2.4. Experimental protocol for aortic rings

The thoracic aorta was removed, immersed in cold, gassed (95% O_2 and 5% CO_2) Krebs–Ringer solution (pH 7.4) of the following composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 11.0, prepared free of fat and remaining connective tissue and cut into rings of approximately 2 mm long. Endothelium of some of the aortic rings was removed by gently rubbing the intraluminal surface. The rings were mounted between two stainless-steel wire hooks, the lower fixed to the organ bath and the upper to an isometric force transducer (Harvard Bioscience). The 10-ml organ bath was filled with oxygenated (95% O_2 and 5% CO_2) Krebs–Ringer and maintained at 37 °C. The rings were set at an initial resting tension of 1.5 g. Removal of endothelium was confirmed by the absence of acetylcholine- or histamine-induced relaxation of KCl-precontracted aortic rings. Aortic rings were stabilised under a resting tension of 1.5 g for 45 min with changes of Krebs–Ringer solution every 15 min. After stabilisation, aortic rings were challenged threefold with 80 mM KCl with threefold washout periods of 10 min. Cumulative concentration–response curves for noradrenaline, phenylephrine, BHT 933, serotonin or KCl were performed in aortic rings with or without endothelium from control or pertussis toxin-pretreated rats. Furthermore, after precontraction with 30 or 40 mM KCl, cumulative concentration–response curves for histamine or acetylcholine were constructed. Stability of precontraction was evaluated by adding saline. Finally, aortic rings with or without endothelium of control or pertussis toxin-pretreated rats were incubated for 15 min in Ca^{2+}-free Krebs–Ringer containing 1.0 mM EGTA. Ca^{2+}-free Krebs–Ringer was refreshed after 7 min. Following incubation, 10 μM noradrenaline or 10 μM phenylephrine was administered to the organ bath. After the transient contractile response, agonists were added for the second time to the organ baths in order to determine the depletion of the respective agonist-sensitive intracellular Ca^{2+} stores.

2.5. Study design for pretreatment of rats with pertussis toxin and treatment with L-NAME or challenge with sodium nitroprusside

Male Wistar rats were injected i.v. with either saline or pertussis toxin (15 μg/kg; RIVM standard PTPU 1049 or batch from Aventis Pasteur for studies with sodium nitroprusside). At 5 days after pretreatment, animals were anaesthetised with urethane (1.5 g/kg of body weight, i.p.). Systolic blood pressure and diastolic blood pressure were continuously recorded from a cannulated carotid artery via a pressure transducer connected to a HSE System (Hugo Sachs Electronic, Germany). Furthermore, the left jugular vein was cannulated for administration of saline, L-NAME, sodium nitroprusside or histamine by the use of infusion pumps. After 30 min of stabilisation, animals were i.v. treated with saline or L-NAME (1, 10 or 30 mg/kg). Subsequently, 20 min after treatment with saline or L-NAME, the effect of histamine (0–300 mg/kg, i.v.) or sodium nitroprusside (50 μg/kg/min) on mean arterial blood pressure and mortality was evaluated. Mean arterial blood pressure was calculated as diastolic blood pressure + 1/3(systolic blood pressure − diastolic blood pressure). Rectal temperature was monitored throughout the experiment, and the body temperature of the animals was kept between 37 and 38 °C by placing the animals on heated pads and by radiant.

2.6. Pertussis toxin and drugs

Pertussis toxin (RIVM standard PTPU 1049) was isolated and purified from *B. pertussis Tohama* and obtained from the RIVM (Bilthoven, The Netherlands) and dissolved in saline before injection. Pertussis toxin (batch FA120066) of Aventis Pasteur (France) was used for the experiments with in vitro pertussis toxin incubation and for the experiments with sodium nitroprusside. Both batches proved to exhibit sufficient histamine sensitising potencies in histamine sensitisation tests in mice (data not shown). Acetylcholine, BHT 933, EGTA, histamine, 6-hydroxydopamine, L-NAME, phenylephrine and serotonin [5-hydroxytryptamine (5-HT)] were obtained from Sigma-Aldrich, The Netherlands. Noradrenaline was obtained from Centrafarm, The Netherlands. KCl came from Merck, Germany.

2.7. Data and statistical analysis

Data are presented as means ± S.E.M. All EC_{50} values, the effective concentration causing 50% of the maximal response (E_{max}) and E_{max} values were obtained from the calculation of EC_{50} and E_{max} values for each individual

representative curve and then averaged ± S.E.M. Results were tested with one-way analysis of variance (ANOVA) (95%). Differences were considered to be significant when $P < 0.05$.

3. Results

3.1. Effect of in vivo pertussis toxin pretreatment on contractile responses of small mesenteric resistance arteries or aortic rings with or without endothelium in normal or Ca^{2+}-free Krebs–Ringer

In small mesenteric resistance arteries of control or pertussis toxin-pretreated rats (30 μg/kg; PT PU1049), noradrenaline, phenylephrine, serotonin or KCl produced concentration-dependent contractions (Fig. 1). BHT 933 did not elicit contraction in small mesenteric resistance arteries from control or pertussis toxin-pretreated rats (data not shown). In vivo pertussis toxin pretreatment significantly decreased E_{max} values of noradrenaline-, 5-HT- or KCl-induced contractions and increased EC_{50} values of noradrenaline-, phenylephrine- or 5-HT-induced contractions of small mesenteric resistance arteries (Fig. 1, Table 1).

In absence of extracellular Ca^{2+}, 10 μM noradrenaline or 10 μM phenylephrine induced transient contractile responses of small mesenteric resistance arteries from control or pertussis toxin-pretreated rats (Fig. 2). Maximal contraction to 10 μM noradrenaline in Ca^{2+}-free Krebs–Ringer solution of in vivo pertussis toxin-pretreated small mesenteric resistance arteries was significantly ($P < 0.05$) reduced as compared to control (5.2 ± 0.8 mN, $n = 6$; 10.5 ± 0.7 mN, $n = 6$, respectively). Maximal contraction to 10 μM phenylephrine in Ca^{2+}-free Krebs–Ringer solution of in vivo pertussis toxin-pretreated small mesenteric resistance arteries was also significantly ($P < 0.05$) reduced as compared to control (2.8 ± 0.4 mN, $n = 6$; 7.6 ± 1.6 mN, $n = 5$, respectively). A second application of 10 μM noradrenaline or 10 μM phenylephrine in Ca^{2+}-free Krebs–Ringer solution did not induce contractions in small mesenteric resistance arteries from control or pertussis toxin-pretreated rats.

In small mesenteric resistance arteries without endothelium, in vivo pertussis toxin pretreatment significantly decreased E_{max} values of noradrenaline- or KCl-induced contraction, but did not affect sensitivity to noradrenaline of small mesenteric resistance arteries without endothelium (Fig. 3, Table 2). Furthermore, maximal contraction to 10 μM noradrenaline in Ca^{2+}-free Krebs–Ringer solution of small mesenteric resistance arteries from in vivo pertussis toxin-pretreated or control rats with (6.2 ± 0.7 mN, $n = 9$; 8.8 ± 0.5 mN, $n = 14$, respectively) or without endothelium (4.3 ± 0.3 mN, $n = 7$; 6.2 ± 0.6 mN, $n = 7$, respectively) was significantly ($P < 0.05$) reduced. A second application of 10 μM noradrenaline in Ca^{2+}-free Krebs–Ringer solution did not induce contractions in small mesenteric resistance arteries with or without endothelium from control or pertussis toxin-pretreated rats.

In vivo pertussis toxin pretreatment did not affect noradrenaline-, phenylephrine-, serotonin- or KCl-induced cumulative contractions of aortic rings with or without endothelium and did not affect histamine- or acetylcholine-induced relaxations of KCl precontracted aortic rings (data not shown). BHT 933 did not elicit contraction of aortic rings with or without endothelium from control or pertussis toxin-pretreated rats. Finally, in vivo pertussis toxin pretreatment did not affect noradrenaline- or phenylephrine-induced transient contractions of aortic rings with or

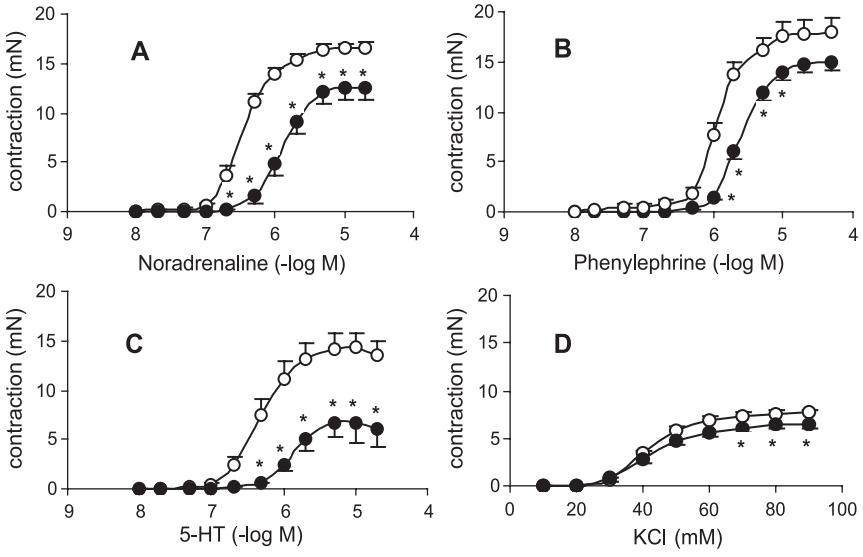

Fig. 1. Concentration–response curves for (A) noradrenaline, (B) phenylephrine, (C) serotonin (5-HT) and (D) KCl of isolated small mesenteric resistance arteries from pertussis toxin (30 μg/kg; black circles) or saline (open circles) pretreated male Wistar rats. Values are expressed as means ± S.E.M for 9–15 small mesenteric resistance arteries. *$P < 0.05$, significantly different from control.

Table 1
EC_{50} and E_{max} values of contraction-inducing agonists for small mesenteric resistance arteries from control or pertussis toxin (30 μg/kg, i.v.) pretreated male Wistar rats

Agonist	Treatment	n	EC_{50} (M)	E_{max} (mN)
Noradrenaline	control	15	$3.92 \times 10^{-7} \pm 0.5 \times 10^{-7}$	16.72 ± 0.6
	pertussis toxin	10	$14.5 \times 10^{-7} \pm 2.0 \times 10^{-7}$*	12.88 ± 1.2*
Phenylephrine	control	11	$1.30 \times 10^{-6} \pm 0.2 \times 10^{-6}$	18.14 ± 1.5
	pertussis toxin	11	$2.88 \times 10^{-6} \pm 0.2 \times 10^{-6}$*	15.12 ± 0.7 (n.s.)
Serotonin	control	9	$6.81 \times 10^{-7} \pm 1.7 \times 10^{-7}$	14.48 ± 1.5
	pertussis toxin	9	$15.0 \times 10^{-7} \pm 1.0 \times 10^{-7}$*	7.35 ± 1.8*
KCl	control	15	$42.1 \times 10^{-3} \pm 1.0 \times 10^{-3}$	7.76 ± 0.3
	pertussis toxin	10	$43.1 \times 10^{-3} \pm 2.3 \times 10^{-3}$ (n.s.)	6.68 ± 0.4*

Data are presented as means ± S.E.M. n indicates the number of small mesenteric resistance arteries used.
*$P < 0.05$, significantly different from control; n.s.: not significantly different from control.

without endothelium in Ca^{2+}-free Krebs–Ringer (data not shown).

3.2. Effect of in vitro pertussis toxin pretreatment on contractile properties of small mesenteric resistance arteries in normal or Ca^{2+}-free Krebs–Ringer in absence or presence of L-NAME

In vitro pertussis toxin pretreatment (10 μg/ml, 2 h; Aventis Pasteur) significantly decreased E_{max} values and increased EC_{50} values of noradrenaline-, phenylephrine- or serotonin-induced contractions of isolated rats small mesenteric resistance arteries as compared to control (Fig. 4, Table 3). KCl-induced contraction was not affected by in vitro pertussis toxin pretreatment (Fig. 4, Table 3). In absence of extracellular Ca^{2+}, in vitro pertussis toxin pretreatment significantly reduced the transient contractile response of small mesenteric resistance arteries to 10 μM noradrenaline as compared to control (3.6 ± 0.5 mN, $n = 5$; 9.8 ± 0.5 mN, $n = 7$, respectively; Fig. 5). A second application of 10 μM noradrenaline in Ca^{2+}-free Krebs–Ringer solution did not induce contractions in control or in vitro pertussis toxin-pretreated small mesenteric resistance arteries.

Incubation with 100 μM L-NAME for 20 min significantly increased sensitivity to noradrenaline of small mesenteric resistance arteries from control rats, but did not affect noradrenaline-induced maximal contraction (Fig. 6, Table 4). L-NAME treatment significantly reduced noradrenaline-induced maximal contraction and increased EC_{50} values for noradrenaline of rat small mesenteric resistance arteries pretreated in vitro with pertussis toxin,

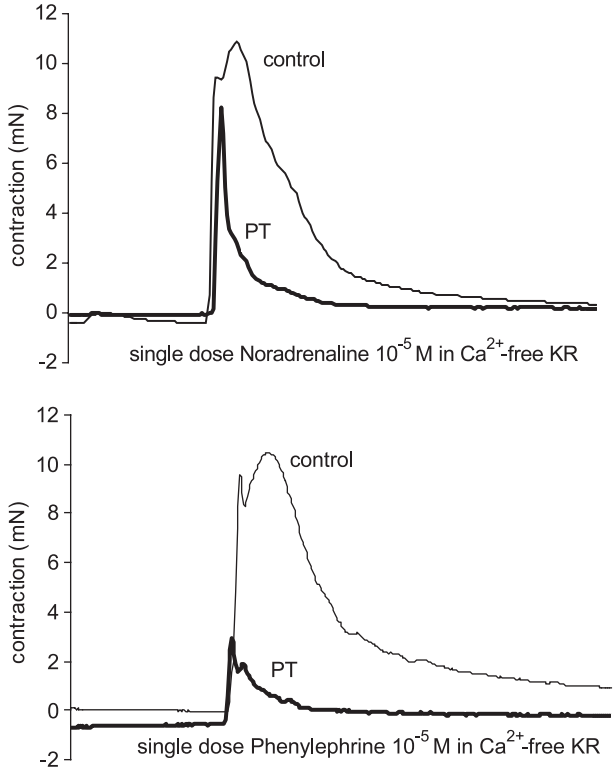

Fig. 2. Example of contractile response of small mesenteric resistance arteries from control or pertussis toxin-pretreated rats to noradrenaline (upper graph) or phenylephrine (lower graph) in Ca^{2+}-free Krebs–Ringer.

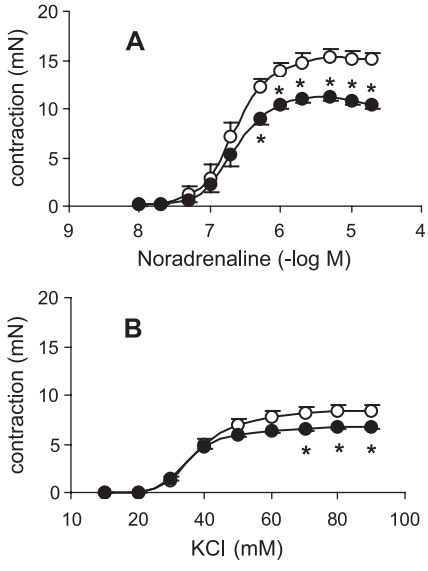

Fig. 3. Concentration–response curves for (A) noradrenaline and (B) KCl of isolated small mesenteric resistance arteries without endothelium from pertussis toxin (30 μg/kg; black circles) or saline (open circles) pretreated male Wistar rats. Values are expressed as means ± S.E.M for seven small mesenteric resistance arteries. *$P < 0.05$, significantly different from control.

Table 2
EC_{50} and E_{max} values of contraction-inducing agonists for small mesenteric resistance arteries without endothelium from control or pertussis toxin-pretreated (30 μg/kg, i.v.) male Wistar rats

Agonist	Treatment	n	EC_{50} (M)	E_{max} (mN)
Noradrenaline	control	7	$2.44 \times 10^{-7} \pm 0.4 \times 10^{-7}$	15.33 ± 0.7
	pertussis toxin	7	$2.63 \times 10^{-7} \pm 0.5 \times 10^{-7}$ (n.s.)	11.40 ± 0.4*
KCl	control	7	$38.7 \times 10^{-3} \pm 1.3 \times 10^{-3}$	8.49 ± 0.6
	pertussis toxin	7	$35.9 \times 10^{-3} \pm 0.5 \times 10^{-3}$ (n.s.)	6.71 ± 0.2*

Data are presented as means ± S.E.M. n indicates the number of small mesenteric resistance arteries used.
*$P < 0.05$, significantly different from control; n.s.: not significantly different from control.

as compared to control pertussis toxin-pretreated small mesenteric resistance arteries (Fig. 6, Table 4). Furthermore, E_{max} and EC_{50} values of in vitro pertussis toxin-pretreated and L-NAME-treated small mesenteric resistance arteries were significantly decreased and increased, respectively, as compared to control L-NAME-treated small mesenteric resistance arteries (Fig. 6, Table 4).

3.3. Effect of in vivo pertussis toxin pretreatment and L-NAME treatment of rats on blood pressure

Fig. 7 shows diastolic blood pressure and systolic blood pressure measured 5 days after pretreatment with saline or pertussis toxin (15 μg/kg; PT PU 1049) before (Fig. 7; $t = 0$) as well as 10 and 20 min after i.v. administration of saline or L-NAME (1, 10 or 30 mg/kg) (Fig. 7; $t = 10$ and $t = 20$). Compared with saline, pertussis toxin significantly decreased diastolic blood pressure from 68 ± 3 mm Hg (mean of four saline groups) to 32 ± 1 mm Hg (mean of four pertussis toxin-pretreated groups; Fig. 7A, $t = 0$) and did not change systolic blood pressure (Fig. 7B, $t = 0$). All doses of L-NAME significantly increased diastolic blood pressure in saline-pretreated animals as compared to saline-pretreated animals and treated with saline on $t = 0$ (saline-control) (Fig. 7A, $t = 10$, $t = 20$). In pertussis toxin-pretreated animals, L-NAME also caused a significant increase in diastolic blood pressure as compared to pertussis toxin-pretreated animals and treated with saline on $t = 0$ (pertussis toxin-control) (Fig. 7A, $t = 10$, $t = 20$). In saline-pretreated animals, 10 or 30 mg/kg but not 1 mg/kg L-NAME caused a significant increase in systolic blood pressure as compared to saline-control animals (Fig. 7B, $t = 10$, $t = 20$). In pertussis toxin-pretreated animals, 10 min after treatment with 1, 10 or 30 mg/kg L-NAME, systolic blood pressure was significantly increased as compared to pertussis toxin-control animals (Fig. 7B, $t = 10$). Systolic blood pressure was still significantly elevated 20 min after treatment with 10 or 30 mg/kg but not with 1 mg/kg L-NAME in pertussis toxin-pretreated animals as compared to pertussis toxin-control animals (Fig. 7B, $t = 20$).

3.4. Effect of in vivo pertussis toxin pretreatment and L-NAME treatment of rats on histamine-induced changes in blood pressure and mortality

Fig. 8 shows the effect of histamine on mean arterial blood pressure, in the absence or presence of saline or L-NAME 5 days after pretreatment of animals with saline or pertussis toxin. Before administration of histamine, mean arterial blood pressure of pertussis toxin-pretreated animals and treated with saline (pertussis toxin-control) was signif-

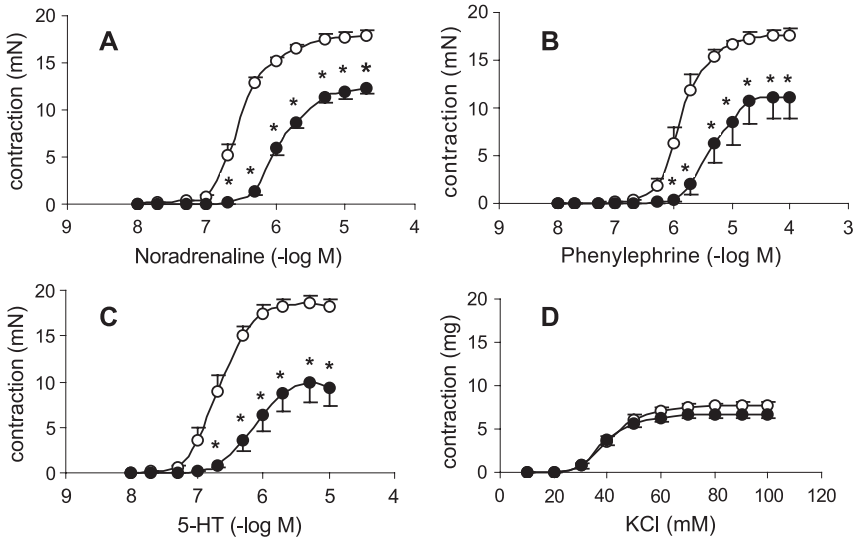

Fig. 4. Concentration–response curves for (A) noradrenaline, (B) phenylephrine, (C) serotonin (5-HT) and (D) KCl of isolated small mesenteric resistance arteries from male Wistar rats treated in vitro with saline (open circles) or pertussis toxin (10 μg/ml for 2 h; black circles). Each point represents the mean for five to eight small mesenteric resistance arteries ± S.E.M. *$P < 0.05$, significantly different from saline.

Table 3
EC_{50} and E_{max} values of contraction-inducing agonists for Wistar rat small mesenteric resistance arteries treated in vitro with saline or 10 μg/ml pertussis toxin for 2 h

Agonist	Treatment	*n*	EC_{50} (M)	E_{max} (mN)
Noradrenaline	control	7	$3.42 \times 10^{-7} \pm 0.4 \times 10^{-7}$	17.96 ± 0.6
	pertussis toxin	5	$11.1 \times 10^{-7} \pm 1.4 \times 10^{-7}$*	12.39 ± 0.6*
Phenylephrine	control	8	$2.31 \times 10^{-6} \pm 0.8 \times 10^{-6}$	17.72 ± 0.6
	pertussis toxin	8	$7.59 \times 10^{-6} \pm 2.0 \times 10^{-6}$*	11.41 ± 2.3*
Serotonin	control	8	$2.57 \times 10^{-7} \pm 0.4 \times 10^{-7}$	18.63 ± 0.9
	pertussis toxin	7	$8.94 \times 10^{-7} \pm 1.3 \times 10^{-7}$*	9.89 ± 2.2*
KCl	control	7	$41.3 \times 10^{-3} \pm 1.7 \times 10^{-3}$	7.76 ± 0.3
	pertussis toxin	5	$39.4 \times 10^{-3} \pm 1.4 \times 10^{-3}$ (n.s.)	6.77 ± 0.4 (n.s.)

Data are presented as means ± S.E.M. *n* indicates the number of small mesenteric resistance arteries used.
*$P < 0.05$, significantly different from control; n.s.: not significantly different from control.

icantly lower compared to saline-pretreated animals and treated with saline (saline-control) (Fig. 8A). Mean arterial blood pressure of pertussis toxin-pretreated animals and treated with L-NAME (1, 10 or 30 mg/kg, i.v.) was significant higher compared to pertussis toxin-control animals (Fig. 8A).

Histamine caused in saline or pertussis toxin-pretreated animals, treated with saline or L-NAME, a dose-dependent decrease in mean arterial blood pressure (Fig. 8). In pertussis toxin-control animals, histamine caused a significantly more progressive decrease in mean arterial blood pressure as compared to saline-control animals (Fig. 8B), i.e. histamine sensitisation. L-NAME treatment of pertussis toxin-pretreated animals did not reduce the enhanced histamine-induced decrease in mean arterial blood pressure as compared to pertussis toxin-control animals (Fig. 8B).

Table 5 shows histamine-induced mortality expressed as survival time and the effect of L-NAME on this parameter in saline or pertussis toxin-pretreated animals. Total observation time measured 50 min. Histamine evoked no mortality in saline-pretreated animals. In pertussis toxin-pretreated animals, histamine caused a significant decrease in survival time as compared to saline-pretreated animals, i.e. histamine sensitisation. L-NAME treatment did not significantly affect histamine-induced mortality in pertussis toxin-pretreated animals as compared to pertussis toxin-control animals (Table 5).

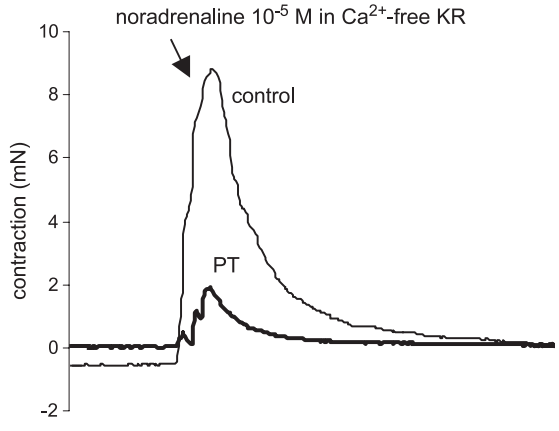

Fig. 5. Example of contractile response to noradrenaline in Ca^{2+}-free Krebs–Ringer of small mesenteric resistance arteries treated in vitro with saline or 10 μg/ml pertussis toxin.

3.5. Effect of in vivo pertussis toxin pretreatment of rats and challenge with sodium nitroprusside on blood pressure and mortality

Fig. 9 shows the effect of sodium nitroprusside on mean arterial blood pressure 5 days after pretreatment of animals with saline or pertussis toxin (15 μg/kg; Aventis Pasteur). Before administration of sodium nitroprusside or histamine, mean arterial blood pressure value of pertussis toxin-pretreated animals was significant lower compared to saline pretreated animals (Fig. 9A; histamine data not shown). Sodium nitroprusside or histamine caused in saline or pertussis toxin-pretreated animals a dose-dependent decrease in mean arterial blood pressure (Fig. 9). Total sodium nitroprusside infusion time measured 80 min. In pertussis toxin-pretreated animals, sodium nitroprusside or histamine caused a significantly more progressive decrease in mean

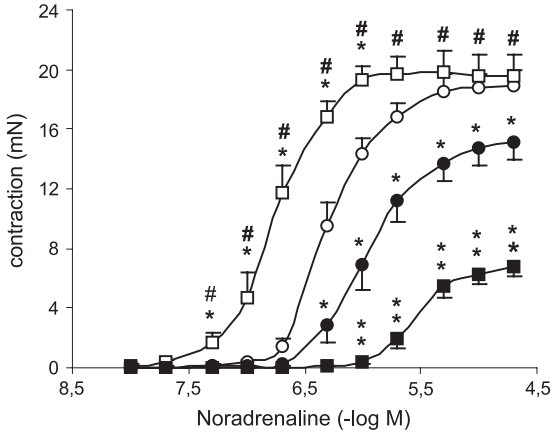

Fig. 6. Concentration–response curve for noradrenaline of isolated small mesenteric resistance arteries treated in vitro with saline (open circles and open squares) or 10 μg/ml pertussis toxin (black circles and black squares) for 2 h and incubated with 100 μM L-NAME (open and black squares) for 20 min. Each point represents the mean for eight small mesenteric resistance arteries ± S.E.M. *$P < 0.05$, significantly different from saline. **$P < 0.05$, significantly different from pertussis toxin. #$P < 0.05$, significantly different from pertussis toxin + L-NAME.

Table 4
EC_{50} and E_{max} values of noradrenaline-induced contraction of Wistar rat small mesenteric resistance arteries treated in vitro with saline or 10 μg/ml pertussis toxin for 2 h, incubated or not with 100 μM L-NAME for 20 min

Agonist	Treatment	n	EC_{50} (M)	E_{max} (mN)
Noradrenaline	control	8	$5.34 \times 10^{-7} \pm 0.7 \times 10^{-7}$	19.03 ± 1.1
	pertussis toxin	8	$12.0 \times 10^{-7} \pm 1.7 \times 10^{-7}$*	15.22 ± 1.2*
Noradrenaline	control + L-NAME	8	$2.56 \times 10^{-7} \pm 1.0 \times 10^{-7}$*	20.46 ± 1.1
	pertussis toxin + L-NAME	8	$34.1 \times 10^{-7} \pm 3.2 \times 10^{-7}$**,***	6.89 ± 0.7**,***

Data are presented as means ± S.E.M. n indicates the number small mesenteric resistance arteries used.
* $P < 0.05$, significantly different from control.
** $P < 0.05$, significantly different from control + L-NAME.
*** $P < 0.05$, significantly different from pertussis toxin.

arterial blood pressure as compared to saline-pretreated animals (Fig. 9B).

Sodium nitroprusside (50 μg/kg/min) or histamine (10, 30, 100 or 300 mg/kg) evoked no mortality in saline-pretreated animals ($n = 6$ and $n = 5$, respectively). In pertussis toxin-pretreated animals, sodium nitroprusside or histamine caused a significant increase in mortality as compared to saline-pretreated animals (4 out of $n = 5$, 5 out of $n = 5$, respectively).

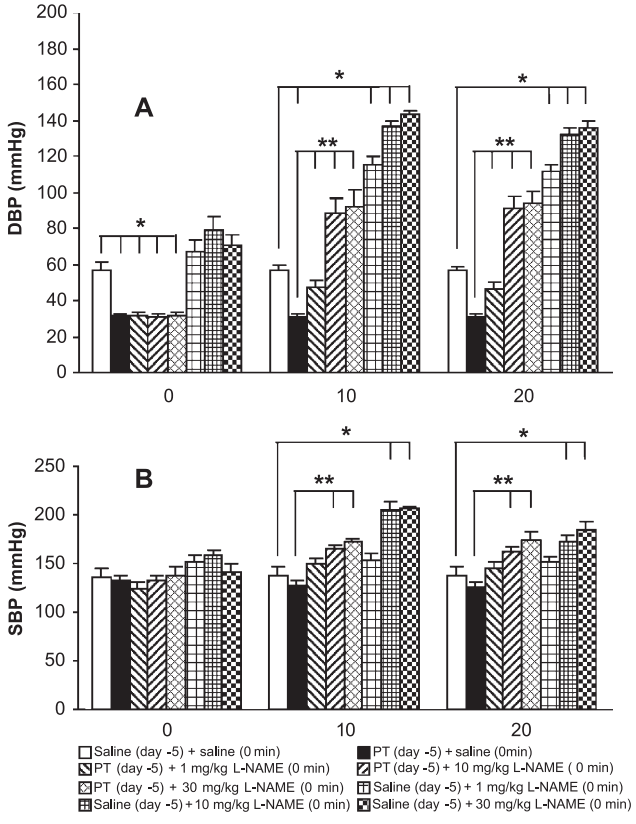

Fig. 7. Baseline ($t = 0$ min) diastolic blood pressure (DBP; A) and systolic blood pressure (SBP; B) 5 days after administration of saline or pertussis toxin (15 μg/kg, i.v.) and the effect of L-NAME (1, 10 or 30 mg/kg, i.v.) 10 and 20 min after administration to pertussis toxin- or saline-pretreated Wistar rats. Values are expressed as means ± S.E.M for 13 (pertussis toxin-pretreated and saline-treated) or 6 animals (all other groups). *$P < 0.05$, significantly different from saline + saline group. **$P < 0.05$, significantly different from pertussis toxin + saline group.

4. Discussion

We want to develop an in vitro safety test for acellular pertussis vaccine quality control based on the histamine sensitisation test in mice. Therefore, we searched for a mechanistic effect of pertussis toxin in vivo, which can be reproduced with in vitro pertussis toxin treatment. We observed that both in vivo and in vitro pertussis toxin pretreatment significantly reduced maximal agonist-induced contraction and sensitivities to contraction-inducing agonists of isolated rat small mesenteric resistance arteries. Data

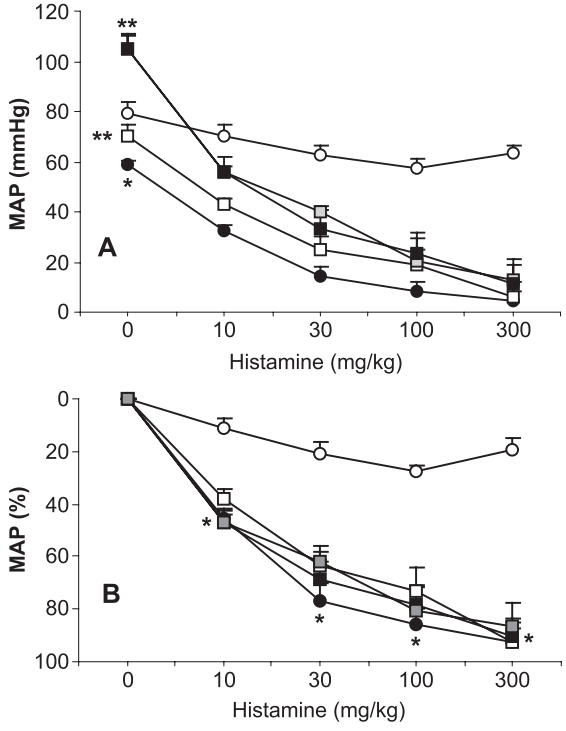

Fig. 8. Effect of histamine (0–300 mg/kg, i.v.) on mean arterial blood pressure (MAP) 5 days after administration of saline (open circles) or pertussis toxin (closed circles, white, grey and black squares) and 20 min after administration of saline (open and closed circles) or L-NAME (1 mg/kg (open squares), 10 mg/kg (grey squares) or 30 mg/kg (black squares). B shows decrease in mean arterial blood pressure expressed as percentage of the mean arterial blood pressure at 0 mg/kg histamine. Values are expressed as means ± S.E.M.; pertussis toxin + saline: $n = 13$; all other groups: $n = 6$. *$P < 0.05$, significantly different from saline + saline group. **$P < 0.05$, significantly different from pertussis toxin + saline group.

Table 5
Histamine-induced mortality of control or pertussis toxin-pretreated (15 μg/kg, i.v.) male Wistar rats and treated i.v. with saline or L-NAME 20 min before histamine challenge

	n	Survival time (min)
Control + saline	6	>50
Pertussis toxin + saline	13	35 ± 3*
Pertussis toxin + 1 mg/kg L-NAME	6	41 ± 3 (n.s.)
Pertussis toxin + 10 mg/kg L-NAME	6	42 ± 3 (n.s.)
Pertussis toxin + 30 mg/kg L-NAME	6	42 ± 4 (n.s.)
Control + 1 mg/kg L-NAME	6	>50
Control + 10 mg/kg L-NAME	6	>50
Control + 30 mg/kg L-NAME	6	>50

Mortality is expressed as survival time during infusion with histamine ($t=0$: 0 mg/kg; $t=10$: 10 mg/kg; $t=20$: 30 mg/kg; $t=30$: 100 mg/kg; $t=40$: 300 mg/kg, i.v.).
Data are presented as means ± S.E.M. *n* indicates the number of animals.
* $P<0.05$, significantly different from control + saline group; n.s.: not significantly different from pertussis toxin + saline group.

support the conclusion that in vitro pertussis toxin pretreatment is able to affect contractile properties of small diameter arteries (Boonen and De Mey, 1990; Petitcolin et al., 2001a; Spitzbarth-Régrigny et al., 2000). The majority of the biological effects of pertussis toxin are assumed to be the result of pertussis toxin-catalysed ADP-ribosylation of guanine-nucleotide-binding proteins (G-proteins), which uncouples them from the corresponding receptors (Gierschik, 1992). A pertussis toxin-sensitive G-protein signal transduction pathway coupled to adrenoceptors has been shown in smooth muscle cells from small diameter vessels (Petitcolin et al., 2001a). We observed similar effects of both in vivo and in vitro pertussis toxin pretreatment on contractile properties of isolated rat small mesenteric resistance arteries. Therefore, we suppose that pertussis toxin uncouples G-proteins in vitro from the investigated receptors in vascular smooth muscle cells of rat small resistance arteries in the same way as in vivo pertussis toxin pretreatment does. So far, we seem to have succeeded in our objective to define a mechanistic effect of in vivo pertussis toxin that can be reproduced with in vitro pertussis toxin treatment.

More mechanistic studies were performed to generate tools for our in vitro safety test. Endothelium, Ca^{2+}, vessel diameter and nitric oxide synthase dependency of pertussis toxin effects on agonist-induced contraction of small resistance arteries was investigated. In vivo pertussis toxin pretreatment did not affect contraction and relaxation properties of a large diameter artery, the rat aorta. Several authors showed that in vitro pertussis toxin pretreatment reduces contractile properties of isolated rat aortic rings (Abebe et al., 1995; Gurdal et al., 1997), which is in contrast with the results of others (Chiou et al., 1997; Lyles et al., 1998; Petitcolin et al., 2001b). A pertussis toxin-sensitive G-protein signal transduction pathway coupled to adrenoceptors has also been shown in rat aortic smooth muscle cells (Abebe et al., 1995; Petitcolin et al., 2001b). However, the conflicting observations in rat aorta regarding the effect of pertussis toxin on agonist-induced contraction suggest that the pertussis toxin-sensitive G-protein signal transduction pathway does not play important role in rat aorta. Furthermore, in vivo pertussis toxin pretreatment did not affect histamine- or acetylcholine-induced relaxation of aortic rings. Also, we did not find effect of in vivo pertussis toxin pretreatment on histamine- or acetylcholine-induced relaxation of isolated rat small mesenteric resistance arteries (Van Meijeren et al., 2004). These results support our hypothesis that vasoconstriction-regulating mechanisms are predominantly involved in pertussis toxin-induced histamine sensitisation.

We also investigated whether the reduced noradrenaline-induced contraction of small mesenteric resistance arteries by pertussis toxin pretreatment involved α_1- or α_2-adrenoceptors. In our study, the α_2-adrenoceptor agonist BHT 933 did not induce contractions in both blood vessels neither from control nor from pertussis toxin-pretreated rats. In small diameter vessels, α_2-adrenoceptor stimulation by clonidine induced vasoconstriction, which could be reduced by in vivo pertussis toxin pretreatment (Heesen and De Mey, 1990; Li and Triggle, 1993). However, an involvement of α_1-adrenoceptors in clonidine-induced contraction cannot be excluded since BHT 933 is more selective for α_2-adrenoceptors as compared to clonidine (Timmermans and Van Zwieten, 1980). The decreased noradrenaline-induced

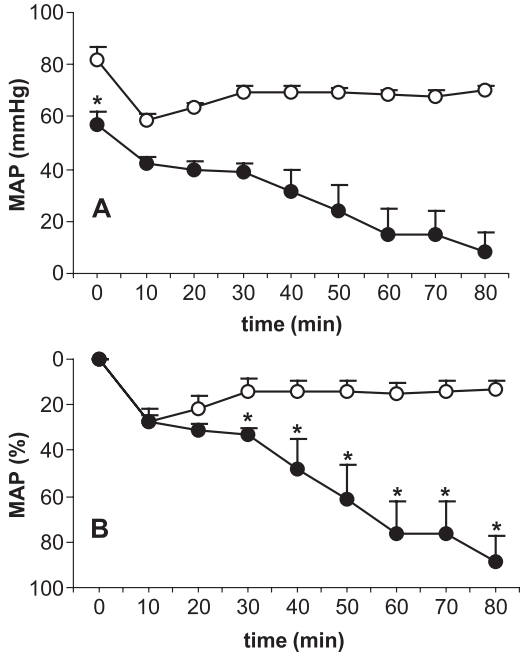

Fig. 9. Effect of sodium nitroprusside (50 μg/kg/min, i.v.) on mean arterial blood pressure (MAP) 5 days after administration of saline (open circles; $n=6$) or pertussis toxin (15 μg/kg; closed circles; $n=5$). B shows decrease in mean arterial blood pressure expressed as percentage of the mean arterial blood pressure at $t=0$. Values are expressed as means ± S.E.M. *$P<0.05$, significantly different from saline-pretreated animals.

contraction of small mesenteric resistance arteries by in vivo pertussis toxin pretreatment seems so far to involve α_1- and not α_2-adrenoceptors. Moreover, we observed that also serotonin- or KCl-induced contractions of small mesenteric resistance arteries were reduced by in vivo pertussis toxin pretreatment, indicating the disturbance of a more general vasoconstricting mechanism in pertussis toxin-induced histamine sensitisation.

Furthermore, in vivo or in vitro pertussis toxin pretreatment significantly reduced adrenoceptor-induced maximal contractions of small mesenteric resistance arteries in the absence of extracellular Ca^{2+}. Adrenoceptor-induced transient contractions in the absence of extracellular Ca^{2+} are due to receptor-mediated activation of the enzyme phospholipase C through G-proteins (Orallo, 1996). Phospholipase C catalyses from the membrane lipid phosphatidylinositol-bisphosphate the formation of the second messenger inositol 1,4,5-triphosphate, which diffuses from the cell membrane into the cytosol and releases Ca^{2+} from intracellular Ca^{2+} stores leading to an increase in cytosolic Ca^{2+} concentration (Orallo, 1996). The rise in cytosolic Ca^{2+} concentration together with the increase in sensitivity to Ca^{2+} of the contractile elements elicits vasoconstriction (Karaki et al., 1997; Orallo, 1996). Our observations of the pertussis toxin-induced reduced adrenoceptor-induced contractions of isolated rat small mesenteric resistance arteries in absence of extracellular Ca^{2+} could be the consequence of reduced cytosolic Ca^{2+} levels and/or reduced sensitivities to Ca^{2+}. However, it was shown that noradrenaline-induced vasoconstriction of rat tail artery segments without endothelium was decreased by in vitro pertussis toxin pretreatment with no modification of intracellular Ca^{2+} concentration (Spitzbarth-Régrigny et al., 2000). This suggests that the decreased adrenoceptor-stimulated vasoconstriction of pertussis toxin-treated small mesenteric resistance arteries in absence of extracellular Ca^{2+} is due to a reduction of sensitivity of small mesenteric resistance artery vascular smooth muscle cells to intracellular Ca^{2+} rather than to an effect on Ca^{2+} mobilisation. This is supported by the finding that adrenoceptor-induced contraction of vascular smooth muscle involves two G-protein coupled signal transduction pathways, one involved in Ca^{2+} mobilisation and insensitive to pertussis toxin and, a second one, sensitive to pertussis toxin and involved in enhancing sensitivity of contractile elements to intracellular Ca^{2+} (Gurdal et al., 1997; Petitcolin et al., 2001a). Also serotonin mediates vasoconstriction through G-protein coupled receptors, which activate phospholipase C what ultimately leads to intracellular Ca^{2+} mobilisation and smooth muscle contraction (Martin, 1994). However, serotonin contractile responses in Ca^{2+}-free medium could not be obtained because of the tachyphylactic nature of the contractile response to serotonin (Boonen and De Mey, 1990). Taken together, we hypothesise that pertussis toxin decreases agonist-induced maximal contractions and sensitivities for contraction-inducing agonists of small mesenteric resistance arteries by reducing sensitivities of small mesenteric resistance artery contractile elements to intracellular Ca^{2+}. Another possibility is that pertussis toxin reduces the functionality of the contractile apparatus by damaging the vascular wall and/or vascular smooth muscle cell structures.

Finally, endothelium dependency of the decreased contractile properties of small mesenteric resistance arteries by in vivo pertussis toxin pretreatment was determined. The reduced sensitivities to contraction-inducing agonists, but not the decreased agonist-induced maximal contractions in normal or Ca^{2+}-free Krebs–Ringer, of small mesenteric resistance arteries from pertussis toxin-pretreated rats proved to be endothelium-dependent. These results are supported by other research in which in vitro pertussis toxin pretreatment of rat de-endothelialised tail artery decreased maximal noradrenaline-induced contractions, but did not affect the sensitivity to noradrenaline (Spitzbarth-Régrigny et al., 2000). Vascular endothelium contains the enzyme nitric oxide synthase, which catalyses the formation of nitric oxide from L-arginine. Nitric oxide activates soluble guanylate cyclase located in the adjacent vascular smooth muscle cells, which catalyses the formation of the intracellular second messenger cyclic GMP. Elevation of cyclic GMP induces relaxation of vascular smooth muscle, resulting in vasodilatation and a decrease of blood pressure. This nitric oxide synthase pathway is thought to play a major role in the regulation of vascular tone and blood pressure (Corbin and Francis, 1999; Das and Kumar, 1995; Hussain et al., 1999; Leurs et al., 1995; Lin et al., 2000) and is one of the signal transduction pathways that can be activated upon histamine H_1-receptor stimulation (Hill, 1990; Hill et al., 1997). Furthermore, it is known that vascular contraction induces endothelial nitric oxide release (Dora et al., 2000; Fleming et al., 1999; Kim and Greenburg, 2001) including noradrenaline-induced vasoconstriction (Bruck et al., 2001; Calderone et al., 2002). We studied whether nitric oxide synthase could be involved in pertussis toxin-induced decreased sensitivity of small mesenteric resistance arteries to noradrenaline and in pertussis toxin-induced histamine sensitisation. Inhibition of nitric oxide formation did not reestablish sensitivity to noradrenaline of small mesenteric resistance arteries pretreated with pertussis toxin. More than that, inhibition of nitric oxide synthase in small mesenteric resistance arteries pretreated in vitro with pertussis toxin further decreased sensitivity to noradrenaline. Normally, inhibition of nitric oxide synthase increases sensitivity of vascular smooth muscle to contraction-inducing agonist (Calderone et al., 2002). We do not have a proper explanation for the L-NAME-induced decreased sensitivity of pertussis toxin-pretreated small mesenteric resistance arteries to noradrenaline. However, this effect could not be attributed to a nonreversible toxic effect of L-NAME on pertussis toxin-pretreated small mesenteric resistance arteries because viability of the preparations at the end of the experiment turned out to be unaffected. Viability of small mesenteric resistance arteries was checked at the end of each experi-

ment by adding a single dose KCl and comparing the contractile response with the KCl-induced contraction of the small mesenteric resistance arteries at the beginning of the experimental protocol. So far, we consider the L-NAME-induced enhanced decreased sensitivity to noradrenaline of isolated rat small mesenteric resistance arteries as a nonspecific effect of L-NAME probably caused by the high concentration mainly used in vitro to inhibit nitric oxide synthase (Hussain et al., 1999; Mendizabal et al., 2001).

Also, in vivo, we did not find any indication of an involvement of nitric oxide synthase in pertussis toxin-induced histamine sensitisation. This is not caused by unsatisfactorily pretreatment with pertussis toxin. Pertussis toxin pretreatment proved to be sufficient as was shown by the decrease in diastolic blood pressure, an important outcome of pertussis toxin pretreatment of rats, and the well sensitisation for histamine, as is shown by the enhanced histamine-induced decrease of mean arterial blood pressure and a reduction of the lethal dose of histamine corresponding with preceding research (De Wildt et al., 1986; Vleeming et al., 2000a,b). Finally, L-NAME treatment was also sufficient as is indicated by the elevations in diastolic and systolic blood pressure, a well-known effect of L-NAME (Handy et al., 1996; Janssen et al., 2000; Szabo et al., 1993). However, no protection against pertussis toxin-induced histamine sensitisation is achieved by inhibiting nitric oxide synthesis, even despite the L-NAME-induced increases in blood pressure, which could exert a possible protective effect of L-NAME on pertussis toxin-induced histamine sensitisation.

Because (a) pertussis toxin decreases contractile properties of vascular smooth muscle of resistance arteries, (b) pertussis toxin does not affect relaxation properties of arteries and (c) no involvement of nitric oxide synthase in pertussis toxin-induced histamine sensitisation could be found, we hypothesised if pertussis toxin would be able to sensitise rats for other vasodilatation-inducing compounds than histamine. This would mean that histamine is only indirectly involved in the histamine sensitisation test and that the pertussis toxin-induced enhanced histamine-induced decrease in blood pressure and the increased histamine-induced mortality is a consequence of the inability of the vascular system to respond to the histamine-induced vasodilatation. Indeed, we observed that pertussis toxin was able to induce sensitisation of rats for sodium nitroprusside resulting in an enhanced sodium nitroprusside-induced decrease in blood pressure and an increase in sodium nitroprusside-induced mortality.

In conclusion, we suggest that pertussis toxin induces histamine sensitisation probably by interfering with the contractile mechanisms of vascular smooth muscle with only an indirect role for histamine. The contractile mechanisms of the vascular smooth muscle cells of resistance arteries are considered to be the basis for a possible in vitro alternative to the histamine sensitisation test.

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