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Local regulation of skin blood flow during cooling involving presynaptic P2 purinoceptors in rats

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1 This study investigated a local effect of cooling on the plantar skin blood flow (PSBF) of tetrodotoxin-treated rats by laser-Doppler flowmetry.

2 When the air temperature around the left foot was locally cooled from 25 to 10°C, the PSBF of the left foot decreased.

3 The response was inhibited by the α-adrenoceptor antagonist phentolamine, the α_1-adrenoceptor antagonist bunazosin, the α_2-adrenoceptor antagonist RS79948, and bretylium and guanethidine that inhibit noradrenaline release from sympathetic nerves. Adrenalectomy of the rats did not affect the cooling-induced response.

4 The P2 purinoceptor antagonists suramin and PPADS also significantly suppressed the cooling-induced reduction of PSBF. However, the inhibitory effect of PPADS on the cooling-induced response was abolished after the treatment with phentolamine. Intra-arterial injections of ATPγS, a stable P2 purinoceptor agonist, at 25°C caused a transient decrease in PSBF in a dose-dependent manner, which was significantly inhibited by phentolamine and guanethidine.

5 These results suggest a novel mechanism for local cooling-induced reduction of skin blood flow *in vivo*; moderate cooling of the skin induces the release of ATP, which stimulates presynaptic P2 purinoceptors on sympathetic nerve terminals and facilitates the release of noradrenaline, thereby causing contractions of skin blood vessels *via* the activation of α_1-and α_2-adrenoceptors.
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Abbreviations: HR, heart rate; MAP, mean arterial pressure; PPADS, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid); PSBF, plantar skin blood flow; TTX, tetrodotoxin

Introduction

Local cooling of the skin causes constriction of cutaneous vessels, which prevents excessive heat loss. The response is achieved by a reflex increase in the sympathetic tone and also by a direct local effect of cooling on cutaneous vessels. The latter view has been derived from a number of observations made in isolated large vessels; moderate cooling enhances postsynaptic α_2-adrenergic contractile activity in the saphenous vein from dogs (Flavahan *et al*., 1985; Vanhoutte *et al*., 1985) and humans (Harker *et al*., 1990), and in the tail artery from rats (Harker *et al*., 1991). The cold-induced constriction seems to be mediated by α_{2C}-adrenoceptors, which are localized to the *trans*-Golgi and do not function at 37°C (Chotani *et al*., 2000). Recent studies have suggested that the cold-induced constriction is mediated by redox signaling in smooth muscle cells and is initiated by the generation of reactive oxygen species in mitochondria, which stimulate RhoA-Rho kinase signaling and the subsequent mobilization of α_{2C}-adrenoceptors to the cell surface (Jeyaraj *et al*., 2001; Bailey *et al*., 2004; 2005). In contrast, much less information is available about the effect of cooling on cutaneous microcirculation *in vivo*. Most *in vivo* studies have investigated the effect of cooling on finger or forearm cutaneous blood flow of human subjects (Ekenvall *et al*., 1988; Lindblad *et al*., 1990; Freedman *et al*., 1992; Pérgola *et al*., 1993; Johnson *et al*., 2005). As the protocol of the experiments in human subjects was limited, the detailed mechanism for the cooling-induced response *in vivo* has not been elucidated.

The purpose of the present study was therefore to elucidate the local regulation of cutaneous microcirculation during cooling *in vivo*. The mechanism for local cooling induced reduction of skin blood flow was investigated in the rats treated with tetrodotoxin (TTX), which allows us to stably measure skin blood flow (Chino *et al*., 2000) and to eliminate the influence of sympathetic tone. We suggest a novel mechanism of cold-induced vasoconstriction *in vivo* involving presynaptic P2 purinoceptors on sympathetic nerve terminals, which facilitate noradrenaline release from the nerve.

Methods

Rats were treated as approved by the Institutional Animal Care and Use Committee and according to the Guidelines for

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Animal Experiments established by the Japanese Pharmacological Society. TTX-treated rats were prepared as described previously (Chino *et al*., 2000). Briefly, male Wistar rats weighing 250–350 g (SLC, Hamamatsu, Japan) were anesthetized with pentobarbital sodium (50 mg kg^{-1}, i.p.), and placed on a heating pad in the dorsal position. After TTX (50 μg kg^{-1}, i.v.) was injected through a polyethylene tube inserted in the left jugular vein, the animal was artificially ventilated at a stroke volume of 1 ml 100 g^{-1} body weight and a rate of 85 strokes min^{-1} with room air by a respirator (SN-480-7; Shinano, Tokyo, Japan).

A polyethylene tube was placed in the right carotid artery and connected to a pressure transducer (TDN-R; Gould, Oxnard, CA, U.S.A.) for the measurement of the mean arterial pressure (MAP) and heart rate (HR). A laser Doppler flow probe (NS type; Omega Wave, Tokyo, Japan) was placed at the surface of the plantar of the left foot to measure the plantar skin blood flow (PSBF) with a laser Doppler flow meter (ALF 2100; Advance, Tokyo, Japan). The right foot served as the control. The microvessels with the blood flow between 20 and 30 ml 100 g^{-1} min^{-1} were selected for the measurement. The skin temperature of the plantar was measured using a thermosensor (AW-601H, Nihon Kohden, Tokyo, Japan). The data were stored and analyzed on a Macintosh computer with an AD converter (Lab Stack; Keisoku Giken, Tokyo, Japan). A polyethylene tube inserted in the left jugular vein was used for the administration of drugs and saline. In some experiments, to investigate local effects of drugs, a catheter was retrogradely inserted into the right iliac artery for intra-arterial injection of drugs into the left iliac artery.

The cooling apparatus for the rat foot, made of plastic in a volume of 25 ml, was made in our laboratory. A rubber tube was coiled around the apparatus, and water was perfused in the tube by a roller pump (PA-12; Cole Parner Instrument, Chicago, IL, U.S.A.). The air temperature in the apparatus was continuously monitored with a thermosensor (SXB-54; Techno-Seven, Yokohama, Japan), and regulated by changing the temperature of the perfusing water. The left foot was placed in the apparatus to apply local cooling. The temperature and humidity of the laboratory were maintained at 24±2°C and 55±10%, respectively.

In some experiments, the rats were adrenalectomized 2 days before the experiments. The bilateral removal of adrenals was achieved *via* a dorsal approach through two small lateral skin incisions under the anesthesia with pentobarbital sodium (50 mg kg^{-1}, i.p.). The adrenals were pulled out through the incision by holding the periadrenal fat and severed with scissors. After each excision surgery, incisions were appropriately sutured. The adrenalectomized rats were given free access to 0.9% saline to maintain their electrolyte balance. The accomplishment of adrenalectomy was confirmed by the measurement of the serum adrenaline concentration, which was performed by a company which provides clinical testing services (SRL, Tokyo, Japan). The serum concentration of adrenaline was less than the detection limit (5 pg ml^{-1}) in all of the adrenalectomized rats ($n=7$), while it was 18.3±4.8 pg ml^{-1} ($n=8$) in the sham-operated rats.

Drugs

The following drugs were used: TTX and clonidine hydrochloride (Wako, Osaka, Japan); phentolamine mesylate (Ciba-Geigy, Hyogo, Japan); RS79948 hydrochloride ((8aR,12aS,13aS)-5,8,8a,9,10,11,12,12a,13,13a-decahydro-3-methoxy-12-(ethylsulfonyl)-6H-isoquino[2,1-g][1,6]naphthyridine hydrochloride) and rauwolscine hydrochloride (Tocris, Ballwin, MO, U.S.A.); and adenosine 5′-[γ-thio]triphosphate tetralithium salt (ATPγS), bretylium tosylate, guanethidine monosulfate, phenylephrine hydrochloride, pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt (PPADS), suramin sodium salt, and tyramine hydrochloride (Sigma, St Louis, MO, U.S.A.). Bunazosin hydrochloride was kindly donated to us by Eisai (Tokyo, Japan).

TTX and bunazosin were dissolved in distilled water. The other drugs were dissolved in physiological salt solution. TTX, phenylephrine, and clonidine were i.v. administered as a bolus injection of 0.5 ml 100 g^{-1} body weight, and the other drugs as a bolus injection of 0.1 ml 100 g^{-1} body weight. ATPγS was intra-arterially (i.a.) administered as a bolus injection of 10 μl 100 g^{-1} body weight. The appropriate vehicle controls showed no apparent effect.

Statistical analysis

All data are expressed as mean±s.e.m. The statistical significance was evaluated by Student's paired or unpaired *t*-test or one-way analysis of variance (ANOVA) followed by the Bonferroni method. A probability of $P<0.05$ was accepted as the level of statistical significance.

Results

Changes in plantar skin blood flow induced by local cooling

Figure 1 shows the changes in the HR, MAP, and PSBF induced by local cooling of the left foot. When the air temperature in the apparatus was changed from 25 to 10°C, the PSBF of the left foot decreased and reached a plateau within 10 min. In contrast, the PSBF of the right foot did not change during cooling of the left foot. When the air temperature in the apparatus was returned to 25°C, the PSBF of the left foot recovered to the basal level within 15 min. We confirmed that there was a linear relationship between the air temperature in the apparatus and the skin temperature of the plantar, and that cooling the air temperature in the apparatus from 25 to 20, 15, 10, and 5°C decreased the PSBF in a temperature-dependent manner (data not shown). As the maximum response was reached by the cooling to 10°C, following studies were performed using this condition.

Role of sympathetic nerve endings in the cooling-induced response

To elucidate the mechanism for the cooling-induced reduction of PSBF, the effects of α-adrenoceptor antagonists on the response were first examined. The α-adrenoceptor antagonist phentolamine (10 mg kg^{-1}, i.v.) and α_1-adrenoceptor antagonist bunazosin (5 mg kg^{-1}, i.v.) *per se* caused a sustained decrease in HR, a transient increase in MAP by averages of 76 and 58%, respectively, and a transient small decrease in PSBF; MAP and PSBF almost recovered within 5 min. The α_2-adrenoceptor antagonist RS79948 (1 mg kg^{-1}, i.v.) *per se*

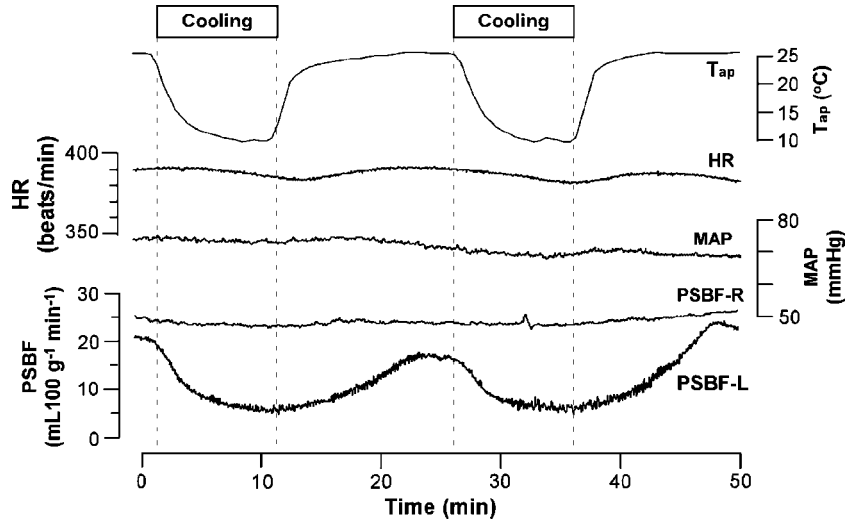

Figure 1 Typical traces of changes in the heart rate (HR), mean arterial blood pressure (MAP), and plantar skin blood flow of the left (PSBF-L) and right (PSBF-R) feet induced by local cooling of the left foot in tetrodotoxin-treated rats. T_{ap}: Air temperature in the apparatus.

Table 1 Changes in basal levels of heart rate, mean arterial pressure, and plantar skin blood flow after treatment with various drugs in tetrodotoxin-treated rats

	HR (beats min^{-1})		*MAP* (mmHg)		*PSBF* (ml min^{-1} 100 g^{-1})	
	Control	*After*	*Control*	*After*	*Control*	*After*
Vehicle	364±8	363±9	64.9±2.4	64.4±1.6	18.9±1.4	20.1±0.4
Phentolamine 10 mg kg^{-1}	373±11	283±6**	68.2±2.3	74.6±4.6	18.2±1.4	18.6±3.1
Bunazosin 5 mg kg^{-1}	372±3	322±5**	68.9±1.4	68.2±1.4	14.4±1.3	15.7±1.4
RS79948 1 mg kg^{-1}	320±16	319±15	61.0±1.4	61.9±1.5	23.0±1.2	24.3±1.1
Bretylium 10 mg kg^{-1}	368±4	447±9**	70.1±1.0	90.0±7.3*	19.0±0.7	19.8±3.3
Guanethidine 10 mg kg^{-1}	370±3	434±6**	67.8±3.0	86.0±8.3*	22.5±1.9	19.6±2.3
Suramin 30 mg kg^{-1}	353±3	350±2	60.7±1.2	60.9±1.9	22.6±1.4	26.3±2.8**
PPADS 10 mg kg^{-1}	340±3	333±3	66.9±3.6	72.8±3.1	22.8±1.3	27.6±1.1**
PPADS 30 mg kg^{-1}	340±3	336±5	66.9±3.6	72.3±2.5	22.8±1.3	33.5±2.2**

Values show the stable basal levels of HR, MAP, and PSBF before (control) and 5–10 min following (after) the administration of drugs, that is, just before the first and second application of cooling. Each drug was injected i.v.
Data represent mean±s.e.m. ($n=4$–7).
*$P<0.05$, **$P<0.01$ vs corresponding control.

caused no remarkable changes in these parameters. Table 1 shows the basal levels of HR, MAP, and PSBF just before the application of cooling. The second application of cooling after the treatment with each drug was made after these parameters reached a plateau. As shown in Figure 2, phentolamine (10 mg kg^{-1}, i.v.), bunazosin (5 mg kg^{-1}, i.v.), and RS79948 (1 mg kg^{-1}, i.v.) all significantly inhibited the reduction of PSBF induced by the cooling to 10°C. We confirmed the specificity of the antagonists (Table 2): Bunazosin (5 mg kg^{-1}, i.v.) abolished the pressor response to phenylephrine (5 μg kg^{-1}), an α_1-adrenoceptor agonist, but only partly suppressed that to clonidine (3 μg kg^{-1}), an α_2-adrenoceptor agonist (Table 2), while RS79948 (1 mg kg^{-1}, i.v.) inhibited the pressor response to clonidine (3 μg kg^{-1}), but was without any effect on that to phenylephrine (5 μg kg^{-1}). Phentolamine (10 mg kg^{-1}, i.v.) abolished both the pressor response to phenylephrine and the response to clonidine.

In the present study, the rats were treated with TTX to completely block sympathetic tone (Chino *et al*., 2000). Nevertheless, the cooling-induced reduction of PSBF was

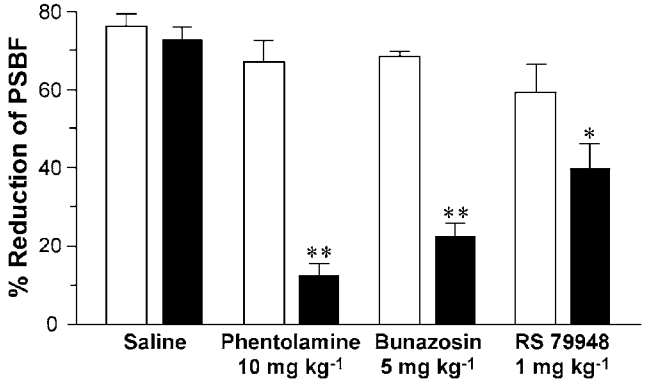

Figure 2 Effects of α-adrenoceptor antagonists on local cooling-induced reduction of PSBF in tetrodotoxin-treated rats. Reduction of PSBF induced by local cooling to 10°C is expressed as a percentage of the basal PSBF at 25°C before (open columns) and after (closed columns) treatment with phentolamine (10 mg kg^{-1}, i.v.), bunazosin (5 mg kg^{-1}, i.v.), and RS79948 (1 mg kg^{-1}, i.v.). Data represent mean±s.e.m. ($n=4$–5). *$P<0.05$, **$P<0.01$ vs before the administration of each drug.

Table 2 Effects of α-adrenoceptor antagonists on increases in mean arterial pressure induced by phenylephrine and clonidine in tetrodotoxin-treated rats

	Control	*Phentolamine* (*10* mg kg^{-1})	*Bunazosin* (*5* mg kg^{-1})	*RS79948* (*1* mg kg^{-1})
Phenylephrine 5 μg kg^{-1}	70.5±7.9	0.8±1.6**	1.9±0.7**	70.1±6.1
Clonidine 3 μg kg^{-1}	69.5±5.5	4.2±0.6**	44.0±0.9**	22.2±2.9**

Values show increases in MAP (mmHg) induced by phenylephrine or clonidine before (control) and after the treatment with each α-adrenoceptor antagonist. Each drug was injected i.v.
Data represent mean±s.e.m. ($n=4$–7).
**$P<0.01$ vs corresponding control.

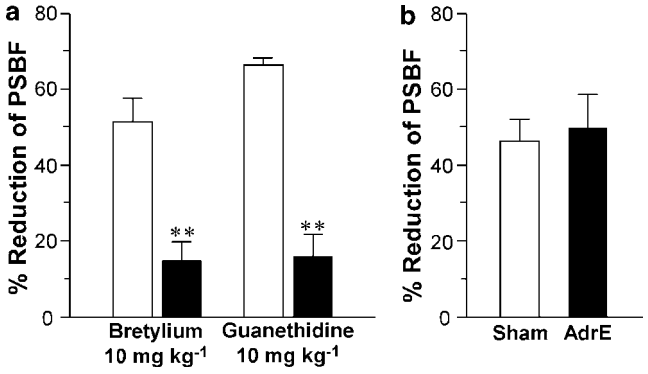

Figure 3 Contribution of noradrenaline release from sympathetic nerve terminals to reduction of PSBF induced by local cooling to 10°C in tetrodotoxin-treated rats. (a) Effects of bretylium (10 mg kg^{-1}, i.v.) and guanethidine (10 mg kg^{-1}, i.v.) on the cooling-induced response are summarized as described in Figure 2. (b) The cooling-induced responses in sham-operated (Sham; open column) and adrenalectomized (AdrE; closed column) rats are summarized. Data represent mean±s.e.m. ($n=5$–7). **$P<0.01$ vs before the administration of each drug.

inhibited by the α-adrenoceptor antagonists, suggesting the contribution of noradrenaline or adrenaline to the response. Thus, we investigated whether catecholamines participate in the response. First, we examined the effects of bretylium and guanethidine that inhibit the release of noradrenaline from sympathetic nerves. Bretylium (10 mg kg^{-1}, i.v.) and guanethidine (10 mg kg^{-1}, i.v.), inhibitors of noradrenaline release from sympathetic nerves, *per se* caused a sustained increase in HR and a transient large increase in MAP by averages of 86 and 143%, respectively, and a transient small increase in PSBF; PSBF recovered within 5 min, while MAP partially recovered and reached a plateau higher than that of the control (Table 1). Bretylium (10 mg kg^{-1}, i.v.) and guanethidine (10 mg kg^{-1}, i.v.) significantly inhibited the cooling-induced reduction of PSBF (Figure 3a). Second, we examined the influence of the removal of the adrenal gland to the response to local cooling. After the treatment with TTX, the HR was not different between the adrenalectomized (363±7 beats min^{-1}, $n=8$) and sham-operated rats (352±5 beats min^{-1}, $n=7$), but the MAP of the adrenalectomized rats (44±2 mmHg, $n=7$) was significantly lower than that of sham-operated ones (67±3 mmHg, $n=8$; $P<0.01$). There was no apparent difference in the cooling-induced reduction of PSBF between the adrenalectomized and sham-operated rats (Figure 3b).

Involvement of P2 purinoceptors in the cooling-induced responses

ATP has been shown to facilitate noradrenaline release *via* presynaptic P2X purinoceptors on sympathetic nerve terminals (Boehm, 1999; von Kügelgen *et al*., 1999; Sperlágh *et al*., 2000). Thus, we investigated the contribution of presynaptic P2X purinoceptors to the cooling-induced response. The P2 purinoceptor antagonists suramin (30 mg kg^{-1}, i.v.) and PPADS (10 mg kg^{-1}, i.v.) *per se* caused an increase in PSBF by averages of 18.6 and 35.0%, respectively; PSBF partially recovered and reached a plateau higher than that of the control (Table 1). PPADS, but not suramin, also caused a transient increase in MAP by an average of 51%; MAP recovered within 10 min. No apparent changes in HR were produced by suramin or PPADS. The reduction of PSBF induced by cooling to 10°C was significantly suppressed by suramin (30 mg kg^{-1}, i.v.) and by PPADS (10 and 30 mg kg^{-1}, i.v.) in a dose-dependent manner (Figure 4a). Our preliminary experiments showed that PPADS (100 mg kg^{-1}, i.v.) did not cause further inhibition of the response, indicating that the inhibitory effect of PPADS reaches maximum at the dose of 30 mg kg^{-1}. PPADS (10 mg kg^{-1}, i.v.) given in addition to phentolamine (10 mg kg^{-1}, i.v.) did not cause an additional decrease in the cooling-induced response (Figure 4b). Moreover, PPADS (10 mg kg^{-1}, i.v.) did not affect the dose–response relation for phenylephrine-induced decreases in PSBF (i.a.; Figure 4c). Although suramin has been shown to uncouple G proteins from their associated receptors (Chung & Kermode, 2005), we confirmed that suramin (30 mg kg^{-1}) did not affect the pressor response to phenylephrine (5 μg kg^{-1}, i.v.; data not shown).

Finally, the effect of ATPγS, a stable P2 purinoceptor agonist, on the PSBF was investigated at 25°C in TTX-treated rats. Injections (i.a.) of ATPγS into the iliac artery caused a transient decrease in PSBF in a dose-dependent manner, followed by a transient increase in it (Figure 5a). Both the decrease and increase in PSBF were largely suppressed by suramin (30 mg kg^{-1}, i.v.; Figure 5a). The reduction, but not the subsequent elevation, of PSBF induced by ATPγS was significantly suppressed by phentolamine (10 mg kg^{-1}, i.v.; Figure 5b) and guanethidine (10 mg kg^{-1}, i.v.; Figure 5c), but was not affected by the vehicle (Figure 5d).

Discussion

The present *in vivo* study confirms that cutaneous microcirculation is locally regulated by a direct local effect of cooling on the skin. Local cooling of the skin caused reduction of PSBF in animals in which the sympathetic tone was blocked by TTX. Nevertheless, the contractile response was inhibited by α_1- and α_2-adrenoceptor antagonists and inhibitors of noradrenaline release from sympathetic nerves, suggesting a contribution of noradrenaline released from sympathetic nerve terminals in the local cooling-induced response. We further provide pharmacological evidence that the release of noradrenaline in response to local cooling is under the control of presynaptic purinoceptors located on sympathetic nerve terminals.

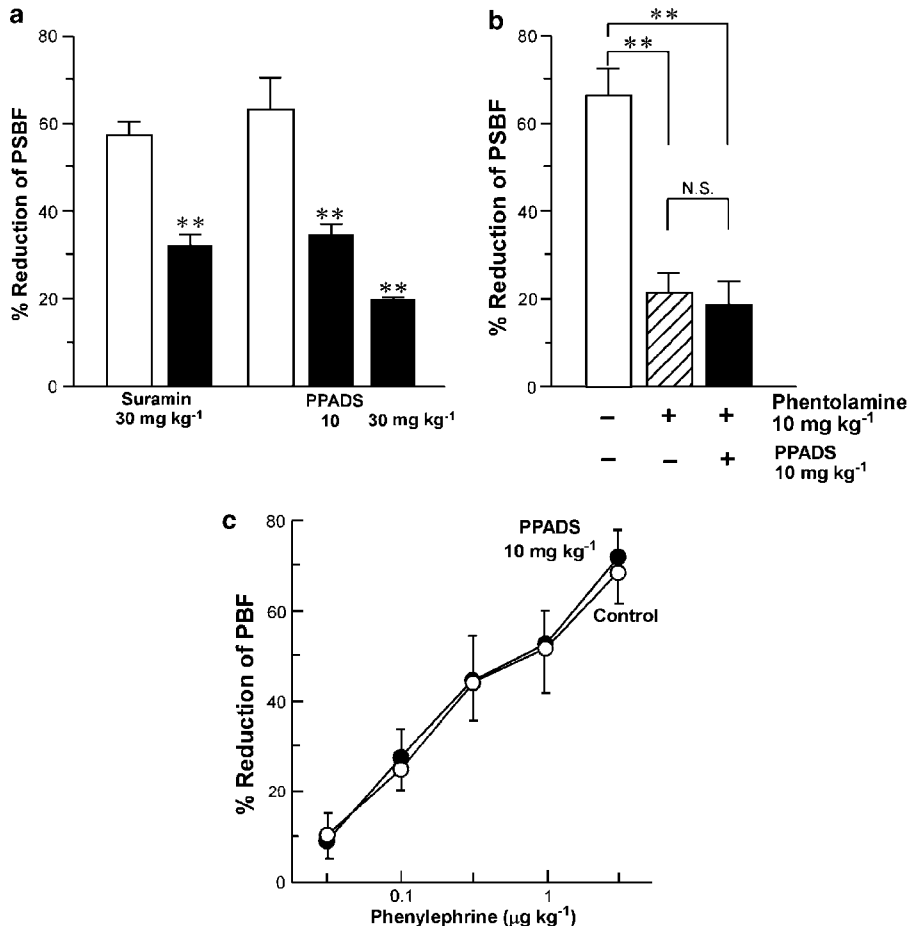

Figure 4 Effect of P2 purinoceptor antagonists on local cooling-induced reduction of PSBF in tetrodotoxin-treated rats. (a, b) Effects of suramin (30 mg kg^{-1}, i.v.) and pyridoxal-5′-phosphate-6-azophenyl-2′,4′-disulphonate (PPADS; 10 or 30 mg kg^{-1}, i.v.; a) or phentolamine (10 mg kg^{-1}, i.v.) and phentolamine plus PPADS (10 mg kg^{-1}, i.v.; b) on the cooling-induced response are summarized as described in Figure 2. (c) Dose–response relation for phenylephrine-induced reduction of PSBF. The reduction of PSBF induced by phenylephrine (i.a.) is expressed as a percentage of the basal PSBF before (open circles) and after (closed circles) the treatment with PPADS (10 mg kg^{-1}, i.v.; closed columns) at 25°C. Data represent mean ± s.e.m. ($n = 4$–5). **$P < 0.01$ vs before the administration of drugs. NS: not significant.

The cooling-induced reduction of PSBF was inhibited not only by RS79948 but also by bunazosine, implying that the response is provoked by noradrenaline acting *via* both α_1- and α_2-adrenoceptors. These results appear to be inconsistent with those of the earlier *in vitro* studies in isolated large cutaneous vessels (Flavahan *et al*., 1985; Harker & Vanhoutte, 1988; Harker *et al*., 1991) and *in situ* study in the rat cremaster muscle microvessels with intact circulation (Faber, 1988), which show that lowering the tissue bath temperature augments the responses to exogenously applied α-adrenergic agonists *via* α_2-adrenoceptors, but not *via* α_1-adrenoceptors. It is noteworthy, however, that both α_1- and α_2-adrenoceptors are distributed in cutaneous blood vessels (Vanhoutte & Janssens, 1980; Johnson *et al*., 1986), and that noradrenaline contracts cutaneous arteries, primarily by activating α_1-adrenoceptors at normal temperature (Harker & Vanhoutte, 1988; Harker *et al*., 1991). Thus, if the release of noradrenaline from sympathetic nerves increased during cooling, it could cause constriction *via* α_1-adrenoceptors in addition to α_2-adrenoceptors that are sensitized by cooling.

However, cooling *per se* rather decreases the release of noradrenaline from sympathetic nerves (Janssens & Vanhoutte, 1978; Vanhoutte & Janssens, 1980; Janssens *et al*., 1981). It is, thus, more likely that cooling causes noradrenaline release indirectly *via* the effect of other endogenous substances in cutaneous microcirculation. ATP has been shown to facilitate noradrenaline release *via* presynaptic P2X purinoceptors located on sympathetic nerve terminals (Boehm, 1999; von Kügelgen *et al*., 1999; Sperlágh *et al*., 2000), and to be released from various types of cells, for example, neurons, endothelial cells, and Merkel cells, by various stimulations, for example, by nociceptive stimulation, inflammation, ischemia, and trauma (Abbracchio & Burnstock, 1998). We, therefore, hypothesized that ATP is released during cooling from some cells around sympathetic nerve terminals or from sympathetic nerves *per se* and facilitates noradrenaline release *via* presynaptic P2X purinoceptors. This hypothesis is supported by the findings that the cooling-induced reduction of PSBF was suppressed by suramin and PPADS and the inhibitory effect of PPADS was abolished after the treatment with phentolamine. These P2 purinoceptor antagonists act on some types of P2Y purinoceptors as well. It is less likely, however, that the increased noradrenaline release is mediated by presynaptic P2Y purinoceptors, since the activation of

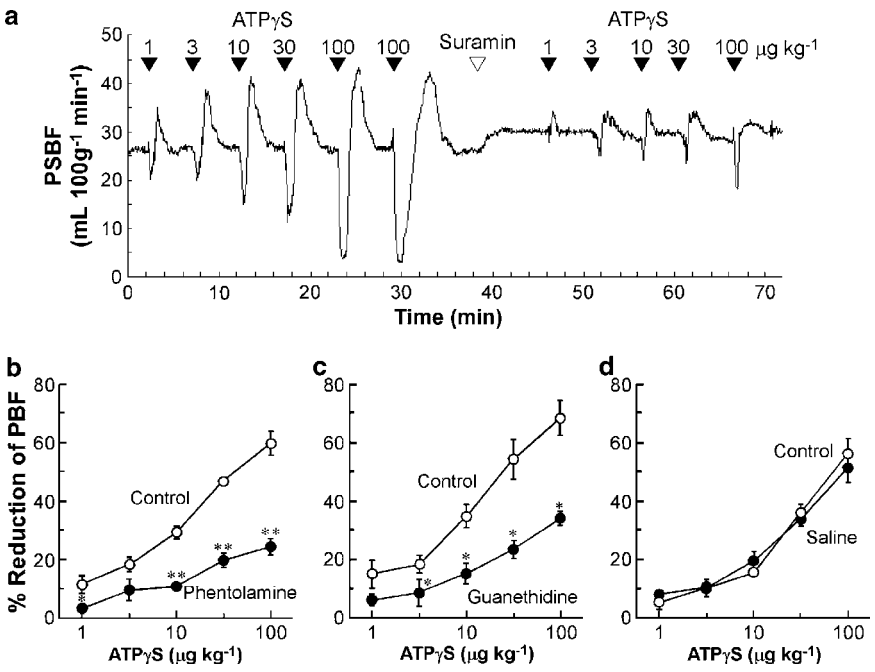

Figure 5 Effect of a P2 purinoceptor agonist on PSBF in tetrodotoxin-treated rats. (a) Typical traces of changes in PSBF induced by ATPγS (1 to 100 μg kg^{-1}, i.a.) at 25°C. Both the decrease and increase in PSBF were largely suppressed by suramin (30 mg kg^{-1}, i.v.). The traces are representative of four independent experiments. (b–d) Dose–response relation for ATPγS-induced reduction of PSBF. The initial reduction of PSBF induced by ATPγS (i.a.) is expressed as a percentage of the basal PSBF at 25°C before (open circles) and after (closed circles) the treatment with phentolamine (10 mg kg^{-1}, i.v.; b), guanethidine (10 mg kg^{-1}, i.v.; c), or saline (d). Data represent mean ± s.e.m. ($n = 4$–6). *$P < 0.05$, **$P < 0.01$ vs control.

presynaptic P2Y purinoceptors rather reduces the release of neurotransmitters (Boehm, 1999).

An alternative interpretation of the results with suramin and PPADS might be conceivable; noradrenaline elicits the release of ATP *via* α-adrenoceptors and the released ATP subsequently produces vasoconstriction *via* postsynaptic P2 purinoceptors. The activation of postsynaptic α-adrenoceptors may elicit the release of ATP from endothelial and smooth muscle cells in sympathetically innervated tissues (Shinozuka *et al*., 1994; Vizi & Sperlágh, 1999). However, the present study clearly showed that the decrease in PSBF induced by an i.a. injection of phenylephrine was not affected by PPADS, while that induced by an i.a. injection of ATPγS was inhibited by phentolamine and guanethidine. These results suggest that ATP causes constriction of cutaneous vessels by releasing noradrenaline from sympathetic nerve terminals in the skin. Altogether, we propose a novel role of ATP acting *via* presynaptic P2 purinoceptors, most likely P2X purinoceptors, in the local regulation of cutaneous microcirculation during cooling.

The constrictor response to ATPγS was not completely inhibited by phentolamine or guanethidine, implying that ATPγS also causes constriction of cutaneous microvessels by another mechanism, most probably *via* postsynaptic P2X purinoceptors (Burnstock & Warland, 1987; Evans & Surprenant, 1992). In the isolated saphenous vein from dogs, cooling increases the contractile responses evoked by ATP and α,β-methylene ATP (Flavahan & Vanhoutte, 1986). In the isolated rabbit central ear artery, endothelin-1 enhances the constriction induced by sympathetic nerve stimulation during cooling by increasing the response *via* postsynaptic P2 purinoceptors (Garcia-Villalón *et al*., 1997). These findings imply that postsynaptic purinoceptors contribute to the enhanced constrictor response during cooling. It is thus possible that ATP released by cooling stimulation also causes constriction *via* postsynaptic P2 purinoceptors. However, the inhibitory effect of PPADS on the cooling-induced reduction of PSBF was abolished by phentolamine treatment in the present study, indicating that postsynaptic purinoceptors play a minor role in the cooling-induced constriction of rat plantar cutaneous microvessles.

The present results suggest that ATP acting *via* presynaptic P2 purinoceptors causes reduction of PSBF by releasing noradrenaline from sympathetic nerves. As P2X purinoceptors form nonselective cation channels (Khakh *et al*., 2001), the activation of P2X purinoceptors would lead to Ca^{2+} influx through the receptor itself and membrane depolarization, the latter activating voltage-dependent Ca^{2+} channels and increasing Ca^{2+} influx, as demonstrated in rat cultured sympathetic neurons (Boehm, 1999; von Kügelgen *et al*., 1999). In Ca^{2+}-free conditions, the ATP-induced release of noradrenaline is abolished, indicating the requirement of Ca^{2+} influx (Boehm, 1999; von Kügelgen *et al*., 1999; Sperlágh *et al*., 2000). However, Papp *et al*. (2004) recently showed that the ATP-induced noradrenaline outflow from rat hippocampal slices was not affected by the external Ca^{2+} removal, but was abolished by the external Na^{+} removal and the noradrenaline transporter inhibitor desipramine. These authors proposed that the ATP-induced, Ca^{2+}-independent release of noradrenaline is mediated by the Na^{+}-dependent reversal of noradrenaline transporters. However, it remains to be elucidated whether such Ca^{2+}-independent mechanism functions in the peripheral sympathetic nervous system.

The treatment of rats with TTX, a voltage-dependent Na^+ channel blocker, enabled us to analyze the local regulation of skin blood flow *in vivo*. We have previously shown that the bolus injection of TTX ($50\,\mu g\,kg^{-1}$, i.v.) abolishes the pressor response induced by electrical stimulation of the spinal cord in rats (Chino *et al*., 2000), which indicates that the sympathetic nerve conduction is totally blocked by the TTX treatment. In the present study, the abolition of sympathetic tone by the TTX treatment was confirmed by the failure of α-adrenoceptor antagonists to lower blood pressure in the TTX-treated rats. Since TTX only blocks the nerve conduction by blocking voltage-dependent Na^+ channels on the axon, the toxin does not inhibit all mechanisms of release of noradrenaline from sympathetic nerves. In fact, we confirmed that the injection of tyramine ($100\,\mu g\,kg^{-1}$, i.a.), which releases noradrenaline from sympathetic nerves, into the iliac artery caused a reduction of PSBF in TTX-treated rats (data not shown). The present study also provided evidence that ATP elicits the release of noradrenaline from sympathetic nerves in the TTX-treated rats. This is in accord with earlier observations showing that ATP-induced release of noradrenaline from sympathetic nerves are not inhibited by TTX (Boehm, 1999; von Kügelgen *et al*., 1999; Sperlágh *et al*., 2000). In human skin, the local cutaneous vasoconstriction associated with direct cooling of the skin requires an intact sympathetic vasoconstrictor system, because brethylium abolishes the response (Pérgola *et al*., 1993; Johnson *et al*., 2005). In accordance with these findings, the cooling-induced reduction of PSBF was inhibited by bretylium and guanethidine in the TTX-treated rats. These findings indicate that intact sympathetic nerve terminals are required for the local cooling-induced constriction of cutaneous microvessels, independently of sympathetic tone.

It is of interest that guanethidine inhibited the vasoconstrictor responses induced by cooling and ATPγS, which seem to be mediated by noradrenaline release, in the rats treated with TTX. These results are incompatible with a general view that an inhibition of noradrenaline release by guanethidine is confined to the action potential-dependent exocytotic release mechanism. In the isolated guinea-pig right atrium, ATP-evoked release of noradrenaline has been shown to be insensitive to ω-conotoxin-GVIA and Cd^{2+}, but abolished by Ca^{2+} removal, suggesting that ATP promotes the release of noradrenaline *via* the direct influx of Ca^{2+} through P2X purinoceptors, rather than through N-type voltage-operated Ca^{2+} channels (Sperlágh *et al*., 2000). Thus, guanethidine could also inhibit the Ca^{2+}-induced release of noradrenaline *via* the mechanism other than action potential- and voltage-operated Ca^{2+} channel-dependent one.

Very recently, Johnson *et al*. (2005) showed that the initial vasoconstriction induced by local cooling was eliminated not only by bretylium but also by a local anaesthetic cream consisting of lidocaine and prilocaine in the human forearm skin. From these results, the authors proposed a novel neuronal mechanism for local cooling-induced vasoconstriction, although further evidence would be required to prove this; local cooling stimulates cold-sensitive sensory afferents, which act on sympathetic vasoconstrictor nerves locally to stimulate noradrenaline release. It is well known that P2X purinoceptors are localized in peripheral sensory nerve terminals (Ralevic & Burnstock, 1998) and the nociceptive sensory neurons express TTX-resistant Na^+ channels in addition to TTX-sensitive ones (McCleskey & Gold, 1999). Thus, the presynaptic purinoceptors located on sensory nerve terminals in addition to those on sympathetic ones may also be involved in the cooling-induced response.

In the present study, we observed several unexpected effects of receptor antagonists *per se* in the TTX-treated rats. Firstly, phentolamine and bunazosin, but not RS79948, caused a transient increase in MAP. Phentolamine and prazosin, but not yohimbine, have been shown to increase the spontaneous outflow of noradrenaline from sympathetic nerves (Ellis *et al*., 1990). Therefore, the blockade by phentolamine and bunazosin of presynaptic α_1-adrenoceptors may increase spontaneous release of vasoconstrictor cotransmitters such as ATP and neuropeptide Y (Lundberg, 1996) from sympathetic nerves, causing a transient pressor response. Secondly, phentolamine and bunazosin also caused a sustained decrease in HR. This may be due to the inhibition of postsynaptic α_1-adrenoceptors which mediate a positive chronotropic response in rats (Rand *et al*., 1986). Thirdly, bretylium and guanethidine caused increases in MAP and HR. These responses may be explained by the release of noradrenaline by these drugs (Rand *et al*., 1986). Finally, suramin and PPADS caused a transient increase in PSBF, while PPADS, but not suramin, caused a transient increase in MAP. Suramin and PPADS are P2 purinoceptor antagonists with wide subtype specificity (Ralevic & Burnstock, 1998). ATP facilitates noradrenaline release from sympathetic nerves *via* presynaptic P2X purinoceptors (Boehm, 1999; von Kügelgen *et al*., 1999; Sperlágh *et al*., 2000) and causes vasoconstriction *via* postsynaptic P2X purinoceptors (Burnstock & Warland, 1987; Evans & Surprenant, 1992). These vasoconstrictor responses to ATP endogenously produced in cutaneous microcirculation may be inhibited by the P2 purinoceptor antagonists, resulting in the elevation of PSBF. In contrast, ATP inhibits noradrenaline release *via* presynaptic P2Y purinoceptors (Boehm, 1999) and causes endothelium-dependent vasodilatation *via* P2Y purinoceptors on endothelial cells (Kennedy *et al*., 1985; Keef *et al*., 1992). These vasodilator responses to ATP endogenously produced in the systemic circulation may be inhibited by PPADS, resulting in the elevation of MAP. The selectivity at P2X purinoceptor subtypes is comparable between suramin and PPADS, while that at P2Y purinoceptor subtypes is different (Ralevic & Burnstock, 1998). The different selectivity at P2Y purinoceptor subtypes may be responsible for the different effects on MAP. It is necessary to note that these unexpected effects were observed in the TTX-treated rats. Under physiological conditions, these effects may be masked by the sympathetic or other nerves.

In summary, our results suggest that (1) intact sympathetic nerve terminals are required for the constriction of cutaneous microvessels in response to local cooling; (2) the vasoconstriction induced by local cooling is mediated by noradrenaline released from sympathetic nerve terminals and acting *via* both α_1- and α_2-adrenoceptors; (3) cooling-induced facilitation of noradrenaline release is mediated by ATP acting *via* presynaptic P2 purinoceptors, most likely P2X purinoceptors, on sympathetic nerve terminals.

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