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RESEARCH PAPER

Urethane, but not pentobarbitone, attenuates presynaptic receptor function in rats: a contribution to the choice of anaesthetic

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Background and purpose: We examined whether cannabinoid CB_1 and histamine H_3 receptors resemble α_2-adrenoceptors in that their presynaptically mediated cardiovascular effects are less marked in urethane- than in pentobarbitone-anaesthetized pithed rats.
Experimental approach: Effects of the cannabinoid agonist CP-55,940 and the H_3 receptor agonist imetit on electrically induced tachycardic and vasopressor responses, respectively, was compared in pithed rats anaesthetized with urethane or pentobarbitone. The affinity of urethane for the three receptors was measured by radioligand binding studies in rat brain cortex membranes and its potency assessed in superfused mouse tissues preincubated with ^{3}H-noradrenaline.
Key results: The neurogenic tachycardic response was less markedly inhibited by CP-55,940 in urethane- than in pentobarbitone-anaesthetized pithed rats. Imetit inhibited the neurogenic vasopressor response after pentobarbitone but not after urethane. The catecholamine-induced tachycardic and vasopressor response did not differ between rats anaesthetized with either compound. Urethane 10 mM (plasma concentration reached under anaesthesia) did not affect binding to CB_1 or H_3 receptors and α_2 adrenoceptors, nor did it alter the inhibitory effect of agonists at the three receptors on electrically evoked ^{3}H-noradrenaline release.
Conclusions and implications: Urethane, but not pentobarbitone, abolished the H_3 receptor-mediated vascular response in pithed rats and attenuated the CB_1 receptor-mediated cardiac response much more than pentobarbitone. The weaker effects of CB_1, H_3 and α_2 receptor agonists cannot be explained by antagonism by urethane at the three receptors *in vitro*. Pentobarbitone, but not urethane, is suitable as an anaesthetic for investigations of inhibitory presynaptic receptor function in pithed and anaesthetized rats.
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Keywords: urethane anaesthesia; pentobarbitone anaesthesia; pithed rat; ^{3}H-rimonabant binding; ^{3}H-N$^\alpha$-methylhistamine binding; ^{3}H-rauwolscine binding; ^{3}H-noradrenaline release; CB_1 receptor; H_3 receptor; α_2-adrenoceptor

Abbreviations: AM-281, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide; CNS, central nervous system; CP-55,940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethyl-heptyl)phenyl]trans-4-(3-hydroxypropyl)-cyclohexanol; DBP, diastolic blood pressure; ES, electrical stimulation; HR, heart rate; PSS, physiological salt solution; S_n, n^{th} electrical or chemical stimulation; t_1, basal tritium efflux after 55–60 min of superfusion; t_2, basal tritium efflux after 55–90 min of superfusion; WIN 55,212-2, R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]-pyrrolo[1,2,3-de]1,4-benzoxazinyl](1-naphthalenyl)methanone mesylate

Introduction

Pithed animals offer the opportunity to study the influence of pre- (Armstrong and Boura, 1973) and postsynaptic receptors (Shipley and Tilden, 1947) on cardiovascular parameters under conditions that resemble the *in vivo* situation in many respects. One major advantage of such preparations is the fact that basal blood pressure and heart rate (HR) are very stable since they are no longer under the control of reflex loops involving the central nervous system (CNS). Prior to pithing, the animals have to be anaesthetized. The choice of the anaesthetic is of crucial importance on the effect of test drugs on cardiovascular parameters studied later in the pithed animal. For instance, in pithed rats the cardiovascular responses to α_2-adrenoceptor agonists acting at pre- and postsynaptic α_2-adrenoceptors were

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found to be weaker in urethane than in pentobarbitone anaesthesia (Armstrong *et al.*, 1982). Recently, we found that the cannabinoid receptor agonist R-(+)-methanandamide by stimulating presynaptic cannabinoid CB_1 receptors (receptor nomenclature follows Alexander *et al.*, 2008) inhibited the neurogenic tachycardia in pithed rats anaesthetized with pentobarbitone but not in animals treated with urethane (Baranowska *et al.*, 2008); the inhibition of the neurogenic tachycardia observed in pentobarbitone-anaesthetized pithed rats is the end organ response to the receptor-mediated reduction of noradrenaline release. On the other hand, the β_1-adrenoceptor-mediated tachycardia induced by isoprenaline did not differ between rats anaesthetized with either urethane or pentobarbitone.

The first aim of the present study was to extend the above observations: (i) to another agonist at presynaptic CB_1 receptors; (ii) to an additional inhibitory presynaptic receptor, that is, the histamine H_3 receptor; and (iii) to another relevant end organ system, namely vascular tissue. These extensions are expected to provide a rational basis for the choice of anaesthetic for the determination of inhibitory presynaptic receptors in pithed rats. In detail, we examined whether the inhibitory cardiac response to another cannabinoid receptor agonist of a different chemical class, CP-55,940, also differs between urethane- and pentobarbitone-anaesthetized rats. With respect to the additional receptor system, we studied whether the inhibitory effect of imetit, a potent and selective agonist at histamine H_3 receptors, on the neurogenic vasopressor response differs between pithed rats treated with either anaesthetic. As the study by Armstrong *et al.* (1982) and our own data suggested that cardiovascular effects of α_2-adrenoceptor agonists and CB_1 and H_3 receptor agonists were blunted in urethane-anaesthetized pithed rats, the fourth aim of our study was to examine *in vitro* whether this phenomenon can be explained by an antagonistic property of urethane at these receptors. For this purpose, the affinity and potency of urethane at the three receptors were determined by radioligand binding and superfusion studies respectively. Irrespective of the mechanism of action, the fifth and ultimate aim of this study was to provide evidence for the practically important general recommendation towards the choice of pentobarbitone or urethane as an anaesthetic for investigations of presynaptic inhibitory receptor function in pithed animals. Our results show that the attenuated effect of agonists at three presynaptic receptors after anaesthesia with urethane cannot be explained by an *in vitro* affinity or potency of this anaesthetic at the respective receptors. If experiments on presynaptic receptors in pithed and anaesthetized rats are planned, pentobarbitone may be used and urethane should be avoided to anaesthetize the animals prior to pithing.

Methods

Experiments were approved by the Local Animal Ethics Committee in Białystok (Poland). They have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Studies on the pithed rat

Male Wistar rats weighing 180–300 g were anaesthetized with either urethane 14 mmol·kg^{-1} or pentobarbitone 300 μmol·kg^{-1} i.p. and injected with atropine 2 μmol·kg^{-1} i.p. The depth of anaesthesia during the short time period before pithing was assessed by monitoring the reflex responses to nociceptive stimuli. After cannulation of the trachea, the rats were pithed through the orbit with a stainless steel rod inserted into the spinal canal and then artificially ventilated (10 mL·kg^{-1}, 60 strokes·min^{-1}) using the Ugo Basile rodent respiratory system (Hugo Sachs, March-Hugstetten, Germany). Both vagal nerves were cut in the neck. HR was derived from the electrocardiogram recorded via subcutaneous electrodes. Diastolic blood pressure (DBP) was measured from the right carotid artery via the DTX pressure transducer (Spectramed, Bromma, Sweden). Body temperature was kept constant at 37 ± 1°C using an electric heating pad (Bio-Sys-Tech, Białystok, Poland) and monitored by a rectal probe transducer. The transducers were connected to a Trendscope 8031 monitor (S&W Vickers, Białystok, Poland). The left femoral vein was cannulated for i.v. administration of drugs in a volume of 0.5 mL·kg^{-1}. Animals were allowed to stabilize for 20–30 min after the end of surgical preparation.

Experiments were initiated by i.v. injection of pancuronium (0.8 μmol·kg^{-1}) to avoid muscle twitches associated with the electrical stimulation. It was also administered to those rats that did not undergo electrical stimulation to ascertain identical experimental conditions in all animals. Five minutes later, the first electrical or chemical stimulus (S_1; to increase HR or DBP) was administered and another 5 min later, CP-55,940 or imetit was administered i.v. After 5, 10, 20 and 30 min, four additional stimuli (S_2–S_5) were administered. Electrical stimulation of the preganglionic sympathetic efferent nerves was generated between the pithing rod and an indifferent electrode placed ventrally; for chemical stimulation, single i.v. injections of isoprenaline or phenylephrine were carried out (for details, see Table 1). The doses of isoprenaline and phenylephrine were chosen so that the increases in HR and DBP were similar to those induced by electrical stimulation. For the evaluation of the results, the ratios S_2/S_1 S_3/S_1 S_4/S_1 and S_5/S_1 were determined. These ratios were

Table 1 Details of studies on the pithed rat

	Electrically induced tachycardia	*Electrically induced vasopressor response*
Reference	Baranowska *et al.* (2008)	Malinowska and Schlicker (1991)
Pithing rod	Enamelled except for a 1 cm section 7 cm from the tip with the uncovered segment situated at vertebra C7–T1	Distal part not enamelled
Electrical stimulation	10 s trains of pulses at 1 Hz, 1 ms and 50 V	7 s trains of pulses at 1 Hz, 1 ms and 50 V
Chemical stimulation	Isoprenaline 0.1–0.2 nmol·kg^{-1}	Phenylephrine 10 nmol·kg^{-1}

Table 2 Details of binding studies[1]

	^{3}H-rimonabant	*^{3}H-rauwolscine*	*^{3}H-N$^\alpha$-methylhistamine*
Labelled receptor	CB_1	α_2	H_3
Corresponding saturation study from our laboratory	Kathmann *et al.* (1999)	Schlicker *et al.* (1994)	Kathmann *et al.* (1993)
Dissociation constant (K_D, nM)	1.55 ± 0.10	1.2 ± 0.2	0.70 ± 0.03
Density (B_{max}, fmol·mg protein^{-1})	510 ± 30	42.7 ± 0.09	97.7 ± 5.7
Specific activity of radioligand (Ci·mmol^{-1})	44	73	70
Concentration of radioligand in assay (nM)	0.5	1	0.2
Amount of protein in assay (μg)	70–90	180–260	180–260
Incubation conditions	25°C, 60 min	23°C, 30 min	30°C, 40 min
Determination of specific binding	CP-55,940 3 μM	Noradrenaline 100 μM	R-α-Methylhistamine 2 μM
Amount of non-specific binding (%)	46 ± 1	17 ± 1	22 ± 1

[1]Conditions and values in the present and our previous studies are identical with the exception of the specific activities of the radioligands and the amounts of non-specific binding, which differ to some extent.

expressed as percentages of the corresponding ratios obtained from the vehicle-treated animals.

Binding studies

Cerebral cortices from male Wistar rats were homogenized (Potter-Elvehjem, 10 up-and-down strokes during 1 min) in 25 volumes of ice-cold Tris-HCl buffer (Tris 50 mM, pH 7.5; EDTA 5 mM) containing sucrose 10.27% and centrifuged at 1000× *g* for 10 min (4°C). The supernatant was centrifuged at 35 000× *g* for 10 min and the pellet was resuspended in (sucrose-free) Tris-HCl buffer and frozen at –80°C. Protein content was determined using the Bradford method.

For binding experiments, membranes were incubated with Tris-HCl buffer in a final volume of 0.5 mL. Radioligand concentration, protein content, temperature and duration of the binding experiment differed for the three radioligands and are given in Table 2. The incubation was terminated by filtration through polyethyleneimine (0.3%)-pretreated Whatman GF/C filters. The drugs used to determine specific binding and the amounts of non-specific binding are given in Table 2.

Superfusion studies

Cerebral cortex slices (0.3 mm thick, diameter 3 mm) and vas deferens pieces from male C57BL/6J mice were incubated (37°C, 60 min) with physiological salt solution (PSS; Ca^{2+} 1.3 mM) containing ^{3}H-noradrenaline 0.025 μM. Subsequently, the preparations were transferred to superfusion chambers and superfused (1 mL·min^{-1}) with PSS (37°C). Ca^{2+} concentration was 1.3 and 3.25 mM for experiments on superfused cortex slices and vas deferens pieces respectively. The superfusate was collected in 5 min samples; experiments lasted for 110 min. The drugs under study were present in the medium either throughout superfusion or from 62 min of superfusion onward, as indicated under *Results*. Desipramine 1 μM was present throughout superfusion in all experiments to block the neuronal noradrenaline transporter. The α_2-adrenoceptor antagonist rauwolscine 0.1 or 1 μM was present throughout superfusion in part of the experiments (as indicated below). Tritium overflow was evoked by two 2 min periods of electrical field stimulation after 40 and 90 min of superfusion (S_1 and S_2). Stimulation parameters were 0.3 Hz, 50 mA, 2 ms for cortex slices and 3 Hz, 200 mA, 2 ms for vas deferens pieces. The PSS was of the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3 or 3.25 (see above), KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, ascorbic acid 0.06, disodium EDTA 0.03, glucose 10; the solution was aerated with 95% O_2 and 5% CO_2 (pH 7.4).

Tritium efflux was calculated as the fraction of the tritium content in the tissues at the beginning of the respective collection period (fractional rate of tritium efflux). To quantify effects of drugs on basal efflux, the ratio of the fractional rates in the 5 min period prior to S_2 (t_2) and in the 5 min period 15–20 min after the onset of S_1 (t_1) was determined (for drugs added to the PSS from 62 min of superfusion onward) or the t_1 values obtained in the absence or presence of a given drug were directly compared with each other (for drugs present in the PSS throughout superfusion). Stimulation-evoked tritium overflow was calculated by subtraction of basal from total efflux during stimulation and the subsequent 13 min and expressed as per cent of the tritium present in the tissue at the onset of stimulation (basal efflux was assumed to decline linearly from the 5 min period before to that 15–20 min after onset of stimulation). To quantify drug-induced effects on the stimulated tritium overflow, the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1) (for drugs added to the PSS from 62 min of superfusion) or the S_1 values obtained in the absence or presence of a given drug were directly compared with each other (for drugs present throughout superfusion).

Statistics

Data were analysed using the GraphPadPrism software (Prism; GraphPad Software, San Diego, CA, USA). Results are given as means ± SEM of *n* experiments (pithed rats, superfusion) and of *n* experiments in triplicate (binding). For comparison of mean values, the *t*-test for unpaired data was used; when two or more treatment groups were compared with the same control, the Bonferroni correction was used. The *F*-test was applied in order to evaluate whether the inhibition of radioligand binding by drugs is better fitted by a one- or a two-site model.

Materials

(R)-(-)-[ring-2,5,6-^{3}H]-noradrenaline (spec. act. 53 Ci·mmol^{-1}), ^{3}H-N$^\alpha$-methylhistamine, ^{3}H-rauwolscine (PerkinElmer, Zaventem, Belgium); ^{3}H-SR141716A (^{3}H-rimonabant) (Amersham, Braunschweig, Germany); AM-281 (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide) (Tocris, Bristol, England); atropine sulphate, desipramine hydrochloride, (-)-isoprenaline bitartrate, pancuronium bromide, urethane, WIN 55,212-2 (R(+)-[2,3-dihydro-5-methyl-3-[(morpholinyl) methyl]-pyrrolo[1,2,3-de]1,4-benzoxazinyl](1-naphthalenyl) methanone mesylate) (Sigma, München, Germany); CP-55,940 ((-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl] trans-4-(3-hydroxypropyl)-cyclo-hexanol) (Biotrend, Köln, Germany); imetit dihydrobromide (Professor C.R. Ganellin, University College, London, England); R-α-methylhistamine dihydrogenmaleate (Professor W. Schunack, Institut für Pharmazie, Freie Universität Berlin); L(-)-noradrenaline (Sanofi-Aventis, Frankfurt, Germany); pentobarbitone sodium (Biowet, Puławy, Poland or Sigma); rauwolscine hydrochloride (Roth, Karlsruhe, Germany); rimonabant hydrochloride (SR 141716A; Sanofi-Aventis, Montpellier, France). Drugs needed for the experiments on pithed rats were dissolved in saline; CP-55,940 was dissolved in a 19% w/v solution of cyclodextrin in saline. Stock solutions of the drugs needed for *in vitro* experiments were prepared with water or dimethylsulphoxide (rimonabant, CP-55,940, WIN 55,212-2) and diluted with Tris-HCl buffer (binding experiments) or PSS (superfusion experiments) to the concentration required. Cyclodextrin solution caused a short-lasting decrease in HR and DBP followed by an increase in DBP by about 10 mmHg, which turned to basal values within 1–2 min. The other solvents by themselves did not affect the parameters under study.

Results

Studies on the pithed rat

Basal HR and DBP did not differ between pithed rats anaesthetized with urethane or pentobarbitone (Table 3). Electrical stimulation or isoprenaline 0.1–0.2 nmol·kg^{-1} increased HR by about 80 beats·min^{-1}; for both types of stimulation, there was no difference between pithed rats anaesthetized with urethane and those anesthetized with pentobarbitone (Table 3). DBP was increased by electrical stimulation or phenylephrine 10 nmol·kg^{-1} by about 30 mmHg; again, there was no difference between urethane- and pentobarbitone-anaesthetized pithed animals (Table 3).

In control groups, the degree of tachycardia and vasopressor responses did not markedly change upon repeated electrical stimulation or repeated addition of isoprenaline or phenylephrine (S_2–S_5); in other words, the ratios S_n/S_1 were close to unity (data not shown). The cannabinoid receptor agonist CP-55,940 at 1 μmol·kg^{-1} did not influence the tachycardia induced by isoprenaline (Malinowska *et al.*, 2001) but inhibited that elicited by electrical stimulation (Figure 1A). The extent of inhibition was about 20% and 40% in urethane- and pentobarbitone-anaesthetized pithed rats respectively, and remained stable over a time period of at least 30 min (Figure 1A). The histamine H_3 receptor agonist imetit 1 μmol·kg^{-1} did not affect the increase in DBP induced by noradrenaline (Godlewski *et al.*, 1997). The drug decreased the electrically induced vasopressor response in pentobarbitone-anaesthetized pithed rats by 20%; this effect remained stable over a time period of at least 30 min (Figure 1B); by contrast, the same dose of imetit failed to influence the electrically induced vasopressor response in urethane-anaesthetized pithed animals (Figure 1B).

Binding studies

Saturation binding studies with the three radioligands ^{3}H-rimonabant, ^{3}H-rauwolscine and ^{3}H-N$^\alpha$-methylhistamine have been carried out on rat brain cortex membranes in previous studies from our laboratory (see Table 2 for references). Scatchard analysis revealed straight lines with Hill coefficients (n_H) not different from unity for each of the three ligands. The affinity constants (K_D) and maximum numbers of binding sites (B_{max}) are given in Table 2.

In *competition* experiments, the three radioligands were used at the concentrations indicated in Table 2. The cannabinoid CB_1 receptor antagonist AM-281, unlabelled rauwolscine and the histamine H_3 receptor agonist imetit potently and monophasically inhibited the binding of ^{3}H-rimonabant, ^{3}H-rauwolscine and ^{3}H-N$^\alpha$-methylhistamine respectively (Figure 2A–C); the negative logarithms of the inhibition constants (pK_i) were 7.5 ± 0.1, 8.5 ± 0.2 and 9.5 ± 0.1 respectively. Urethane, studied at concentrations up to 10 mM, did not affect the binding of each of the three radioligands (Figure 2A–C). Pentobarbitone, studied up to 1 mM, did not affect the binding of ^{3}H-rauwolscine and

Table 3 Influence of urethane and pentobarbitone on the basal, electrically (ES) and chemically [by isoprenaline (ISO) or phenylephrine (PHE)] induced increase in heart rate and diastolic blood pressure in pithed and vagotomized rats

	Heart rate (beats·min^{-1})				*Diastolic blood pressure (mmHg)*			
	Basal	*ES-induced increase*[1]	*Basal*[2]	*ISO-induced increase*[1,2]	*Basal*	*ES-induced increase*[1]	*Basal*	*PHE-induced increase*[1]
Urethane	341.4 ± 7.3	75.4 ± 2.9	324.4 ± 6.3	78.4 ± 4.2	46.0 ± 2.1	34.4 ± 3.2	44.3 ± 1.5	25.2 ± 1.9
Pentobarbitone	331.6 ± 5.8	75.4 ± 4.2	339.9 ± 7.7	85.8 ± 5.6	48.8 ± 3.9	31.2 ± 2.8	48.7 ± 2.0	27.9 ± 2.0

Means ± SEM of 6–17 experiments.
[1]Induced by the first electrical or chemical stimulation (S_1).
[2]From Baranowska *et al.* (2008).

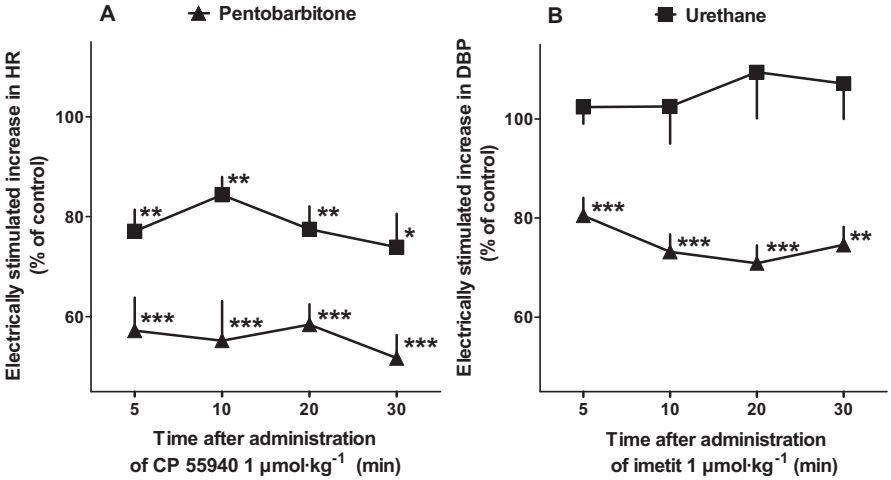

Figure 1 Effect of CP-55,940 on the electrically induced tachycardia and of imetit on the electrically induced vasopressor response in urethane- and pentobarbitone-anaesthetized pithed and vagotomized rats. Five periods of electrical stimulation were administered, one 5 min before (S_1) and another four 5, 10, 20 and 30 min after (S_2–S_5) i.v. injection of the drug under study. To quantify the effects of drugs, the ratios of S_2, S_3, S_4 and S_5 over S_1 were determined (S_n/S_1). S_n/S_1 values are expressed as percentages of the corresponding ratios in controls (injection of vehicle; not shown). Means ± SEM of 4–10 experiments. *P < 0.05, **P < 0.01, ***P < 0.001, compared with the corresponding control.

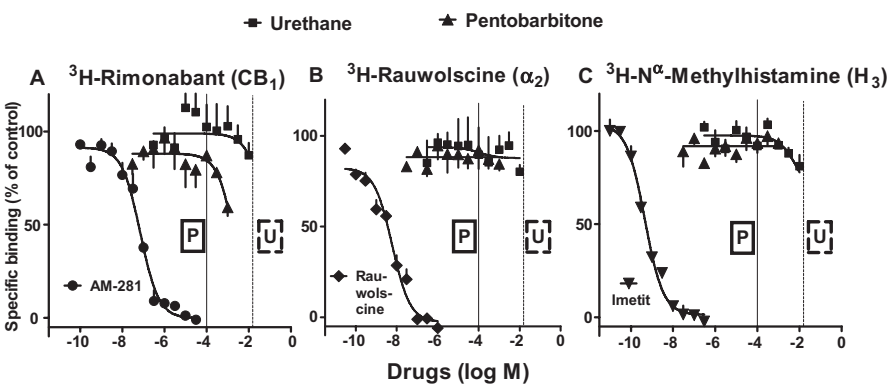

Figure 2 Inhibition of specific ^{3}H-rimonabant (A), ^{3}H-rauwolscine (B) and ^{3}H-N$^\alpha$-methylhistamine binding (C) to rat brain cortex membranes by urethane, pentobarbitone and selective ligands at the three labelled receptors. Details of the experimental protocols are given in Table 2. Means ± SEM of three to four experiments in triplicate (for some data points, SEM is smaller than symbols). The solid and interrupted line marked by P and U, respectively, represents the plasma concentration obtained under pentobarbitone administration in the study by Hatanaka *et al.* (1988) and under urethane administration in the studies by Boyland and Rhoden (1949) and O'Flaherty and Sichak (1983) (for further details, see *Discussion*).

^{3}H-N$^\alpha$-methylhistamine (Figure 2B and C); the same held true for ^{3}H-rimonabant binding, although 1 mM pentobarbitone inhibited the binding of the latter radioligand (Figure 2A).

Superfusion studies

Basal tritium efflux, expressed as t_2/t_1, was 0.80 ± 0.02 in superfused mouse brain cortex slices preincubated with ^{3}H-noradrenaline (nine controls); the fractional rate of tritium efflux during t_1 was 0.0036 ± 0.0002 min^{-1}. Similar values were obtained in vas deferens pieces and in the presence of the drugs under study (not shown). Only pentobarbitone 1 mM increased basal tritium efflux (t_2/t_1) in cerebral cortex slices by 20%. The *electrically evoked tritium overflow*, expressed as S_2/S_1, was 1.05 ± 0.03 and 0.86 ± 0.08 in control experiments on cortex slices (n = 9) and vas deferens pieces (n = 8) respectively; the amount of tritium evoked by S_1 was 3.65 ± 0.29% and 3.23 ± 0.33% of tissue tritium respectively.

The effects of the test drugs on the electrically evoked tritium overflow were studied in three experimental series. In the first one, the effect of urethane and pentobarbitone (present in the medium from 62 min of superfusion onward) on the evoked overflow (S_2/S_1) in mouse brain cortex slices was studied. The evoked overflow was not affected by urethane 0.1–10 mM and by the lower concentrations of pentobarbitone, but inhibited by 10% and 46% by 0.32 and 1 mM of this anaesthetic respectively (n = 4–6; results not shown). In mouse vas deferens pieces, urethane 10 mM (present together with rauwolscine 1 µM throughout superfusion) did not affect the electrically evoked tritium overflow (S_1; n = 7; not shown).

In the second series, the interaction of urethane 10 mM (present in the medium throughout superfusion) with ago-

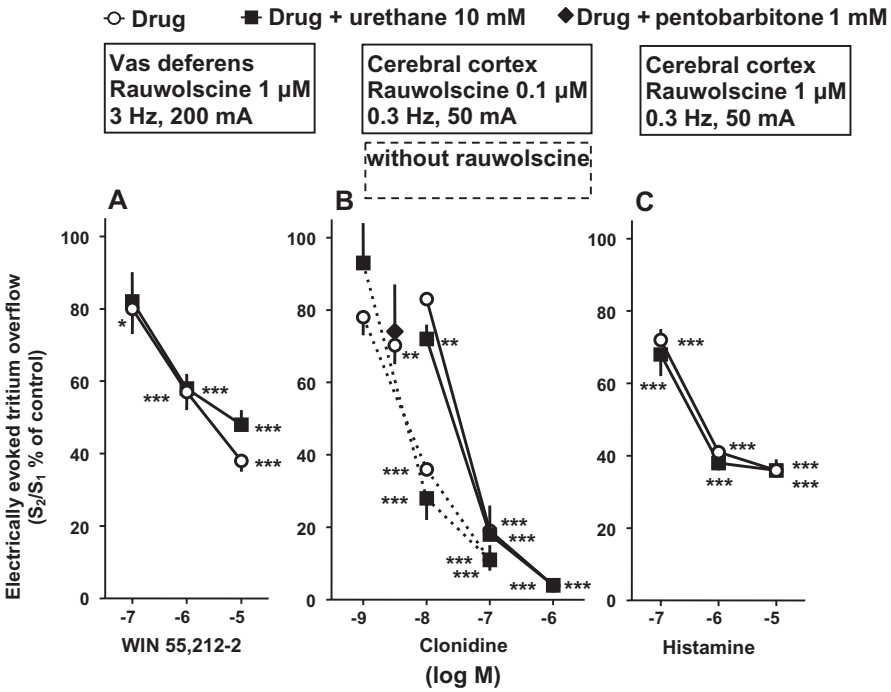

Figure 3 Effect of three agonists on the electrically evoked tritium overflow from superfused mouse tissues preincubated with ^{3}H-noradrenaline, and interaction with urethane or pentobarbitone. The effect of the cannabinoid receptor agonist WIN 55,212-2 was studied in pieces of vas deferens (A) whereas the effect of the α_2-adrenoceptor agonist clonidine (B) and of histamine (C) was studied in cerebral cortex slices. The superfusion medium contained the respective agonist from 62 min of superfusion onward and urethane or pentobarbitone (and, when relevant, rauwolscine) throughout superfusion. Tritium overflow was evoked after 40 and 90 min of superfusion (S_1, S_2), and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined (S_2/S_1). S_2/S_1 values are expressed as percentages of the corresponding ratios in controls (not shown). Means ± SEM of 4–10 experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with the corresponding control.

nists at three presynaptic receptors (present in the medium from 62 min of superfusion onward) was studied; rauwolscine, when relevant, was present throughout superfusion (as indicated in the three panels of Figure 3). Cannabinoid CB_1 receptors were studied in vas deferens pieces (agonist WIN 55,212-2); α_2-adrenoceptors (agonist clonidine) and histamine H_3 receptors (agonist histamine) were studied in cerebral cortex slices. Figure 3A shows that the effect of WIN 55,212-2 on the evoked overflow (S_2/S_1) was not affected by urethane 10 mM; likewise, the anaesthetic did not influence the concentration–response curves of clonidine (studied both in the absence and presence of rauwolscine) and of histamine (Figure 3B and C). Rauwolscine 0.1 and 1 µM by itself increased the evoked overflow (S_1) by 250% and 310% respectively (n = 9–10; results not shown).

In the third series, the interaction of pentobarbitone (present in the medium throughout superfusion) with clonidine (present in the medium from 62 min of superfusion onward) was studied in mouse brain cortex slices. The effect of clonidine on the evoked overflow (S_2/S_1) was not affected by pentobarbitone 0.32 mM (results not shown) and 1 mM (Figure 3B).

Discussion

As briefly outlined in the *Introduction*, the present investigation was based on two independent observations in pithed rats, namely that, depending on the choice of anaesthetic, entirely different results could be obtained. In other words, the choice of anaesthetic plays a surprisingly important role for the outcome of the investigation. In the pithed rat anaesthetized with urethane, the α_2-adrenoceptor agonist-induced decrease in neurogenic tachycardia was clearly less pronounced than in pithed rats anaesthetized with pentobarbitone (Armstrong *et al.*, 1982). Similarly and even more drastically, in pithed rats R-(+)-methanandamide, a cannabinoid receptor agonist, did not decrease the neurogenic tachycardia at all when rats were anaesthetized with urethane, but R-(+)-methanandamide reduced the neurogenic tachycardia when pentobarbitone was used (Baranowska *et al.*, 2008). In both cases, the function of the relevant inhibitory presynaptic receptor (α_2 and CB_1 respectively) on the sympathetic nerve terminals, that is, inhibition of stimulation-evoked noradrenaline release, was determined indirectly as inhibition of neurogenic tachycardic responses at the level of the innervated end organ. On the basis of the qualitatively and quantitatively different effects of the anaesthetics, it was tempting to formulate the working hypothesis that pentobarbitone is better suited as an anaesthetic than urethane when inhibitory presynaptic receptors on sympathetic nerve endings are identified and characterized.

In order to provide evidence in favour of this hypothesis, the aim of the present study was to extend the results with the anaesthetics in three directions. First, the effect of a second cannabinoid receptor agonist from a different chemical class was examined. It was found that the cannabinoid receptor

agonist CP-55,940 inhibited the neurogenic tachycardic response to a more marked extent in pentobarbitone- than in urethane-anaesthetized pithed rats, showing that the effects of agonists from different chemical classes acting via CB_1 receptors crucially depend on the anaesthetic selected. Second, another inhibitory presynaptic receptor on the sympathetic nerve terminals, the histamine H_3 receptor, was included in the study. Our experiments revealed that the inhibitory effect of the H_3 receptor agonist imetit is abolished when urethane instead of pentobarbitone is used to anaesthetize the rats. Third, the study with imetit provides a further extension of the experiments discussed so far in that neurogenic changes of the function of the sympathetically innervated vasculature were evaluated. The vasculature represents another end organ system that, by the modulation of the neurogenic pressor response, offers the possibility to indirectly determine the influence of anaesthetics on the function of inhibitory presynaptic receptors.

In the context of all previous and present experiments discussed so far, the increases in HR and DBP under the experimental conditions of our study are related to the activation of postsynaptic β_1- and α_1-adrenoceptors as they were almost completely (by about 85% and 95%) diminished by propranolol and prazosin respectively (10 $\mu mol \cdot kg^{-1}$ each; data not shown). The effects of agonists at the latter two receptors do not differ in urethane- and pentobarbitone-anaesthetized pithed rats, suggesting: (i) that it is possible to monitor the effects of CP-55,940 and imetit via the end organ response; and (ii) that the differential influence of the two anaesthetics is not a general phenomenon but specific to certain receptors, such as several of the inhibitory presynaptic receptors on sympathetic nerve endings.

In view of the considerable differences between both anaesthetics, the question of the underlying mechanism arises. As a matter of fact, the two compounds differ qualitatively with regard to their influence on the sympathetic outflow from the CNS to the periphery; urethane has a stimulatory and pentobarbitone has an inhibitory influence (Maggi and Meli, 1986a; Shimokawa *et al.*, 1998). However, after mechanical destruction of the spinal cord in pithed rats, the influence of the CNS can be excluded. Therefore, as the fourth aim of our study, we examined the possibility that the urethane-induced attenuation of the cardiovascular effects of CB_1, α_2 and H_3 receptor agonists might be explained by a so far unknown antagonistic effect of urethane at the respective receptors. For this purpose, various *in vitro* experiments were carried out. Thus, we determined specific ^{3}H-rimonabant, ^{3}H-rauwolscine and ^{3}H-N$^\alpha$-methylhistamine binding to rat brain cortex membranes. Saturation binding studies for each of the three radioligands have been carried out in our laboratory in the past (Table 2). We found the binding of each radioligand to be potently inhibited by selective ligands at their respective receptors and the results closely conform to previously published data (AM-281 – Gifford *et al.*, 1997; rauwolscine – Schlicker *et al.*, 1994; imetit – Kathmann *et al.*, 1993). However, even high concentrations of the two anaesthetics had virtually no effect on binding; the only exception was pentobarbitone, which inhibited ^{3}H-rimonabant binding by about 50% at 1 mM. This concentration is, however, much higher than the plasma concentration of 100 μM obtained in rats 1 h after i.v. administration of pentobarbitone 50 $mg \cdot kg^{-1}$ (Hatanaka *et al.*, 1988; solid line in Figure 2). The standard dose range for i.p. administration of pentobarbitone to rats is 40–60 $mg \cdot kg^{-1}$ (Wixson and Smiler, 1997); in our study 68 $mg \cdot kg^{-1}$ were used. The highest concentration of urethane examined in our binding studies, that is, 10 mM, is close to the plasma concentration of 15–16 mM obtained in rats 1 h after subcutaneous administration of 1 $g \cdot kg^{-1}$ (Boyland and Rhoden, 1949) or in mice obtained 1 h after i.p. administration of 1.2 $g \cdot kg^{-1}$ (O'Flaherty and Sichak, 1983) (interrupted line in Figure 2). The standard dose range for i.p. administration of urethane to rats is 1–1.5 $g \cdot kg^{-1}$ (Wixson and Smiler, 1997); in our study 1.25 $g \cdot kg^{-1}$ were used.

In order to confirm the lack of any direct interaction of urethane and pentobarbitone with the receptors studied in our radioligand binding experiments, functional *in vitro* studies on CB_1, α_2 and H_3 receptors were carried out as well. Mouse tissues were used for this purpose as, at least in our hands, the extent of inhibition of noradrenaline release via presynaptic receptors is generally more marked than in rat tissues; this has been published for the H_3 receptor in the cerebral cortex (Schlicker *et al.*, 1992a). The functional properties of the three types of inhibitory presynaptic receptors, however, do not differ between the two rodent species (CB_1 receptors: rat – Ishac *et al.*, 1996; mouse – Trendelenburg *et al.*, 2000; alpha$_2$-adrenoceptors: rat – Trendelenburg *et al.*, 1993; mouse – Limberger *et al.*, 1995; H_3 receptors: rat – Schlicker *et al.*, 1989; mouse – Schlicker *et al.*, 1992b). In one experimental series, the effect of the two anaesthetics on basal and electrically evoked tritium overflow from mouse brain cortex slices preincubated with ^{3}H-noradrenaline was studied; under the present conditions, the electrically evoked tritium overflow reflects quasi-physiological noradrenaline release (Schlicker *et al.*, 1992c). Urethane failed to affect either parameter. Pentobarbitone inhibited the electrically evoked tritium overflow in a very high (toxic) concentration range in which this anaesthetic simultaneously increased basal tritium efflux. The latter phenomenon *per se* might explain the inhibitory effect of pentobarbitone on the evoked tritium overflow as calculation of the evoked release is affected by a simultaneous increase in the basal values.

The possibility that urethane attenuates the effect of agonists at the three receptors *in vitro* was studied in three protocols previously described by our laboratory. Functional CB_1 receptors (agonist WIN 55,212-2) were examined in pieces of vas deferens (Schlicker *et al.*, 2003) whereas functional H_3 (agonist imetit) and α_2 receptors (agonist clonidine) were studied in cerebral cortex slices preincubated with ^{3}H-noradrenaline (Schlicker *et al.*, 1992b,c) In the two former models, rauwolscine was added as an auxiliary drug as it increases the extent of the CB_1 (Schlicker and Göthert, 1998) and H_3 receptor-mediated effect (Schlicker *et al.*, 1992c). Urethane at 10 mM did not affect the concentration–response curves of the respective agonists at the three receptors.

The interaction of the two anaesthetics with clonidine has been studied in more detail in two additional series of experiments. In the first one, the interaction of urethane with clonidine has been determined in the presence of rauwolscine, which, as expected, caused a rightward shift of the concentration–response curve of clonidine and increased the

electrically evoked tritium overflow (Weitzell *et al*., 1979). The competitive α_2-adrenoceptor antagonist rauwolscine has been chosen as urethane might allosterically interact with α_2-adrenoceptors; combination of a competitive antagonist with a negative allosteric modulator will result in a supra-additive antagonism (Christopoulos and Kenakin, 2002). However, the effect of clonidine was not affected by urethane in the presence of rauwolscine. In the second series of experiments, the possibility has been taken into consideration that the differential effects of an α_2-adrenoceptor agonist in the presence of different anaesthetics are related to the fact that the response to the agonist may be increased by pentobarbitone rather than decreased by urethane. This possibility, however, does not appear to hold true as the effect of clonidine was not affected by pentobarbitone.

Taken together, the radioligand and functional experiments *in vitro* exclude the possibility that urethane exhibits antagonist properties at the three inhibitory presynaptic receptors under consideration or that the anaesthetics might interact with these receptors in another unknown way. The possibility has to be considered that the effect of urethane is not related to the compound itself but rather to a degradation product (urethane is indeed metabolized, although slowly; Maggi and Meli (1986b). One might also argue that urethane, due to its potentiating effect on nicotinic receptors (Hara and Harris, 2002), causes sympathetic neurones to fire at high rates, at which the action of presynaptic agonists is weak. This possibility is, however, not plausible as the extent of the neurogenic vasopressor response and tachycardia as well as the α_1- and β_1-adrenoceptor-mediated postsynaptic effects were not affected by urethane.

Irrespective of the mechanism(s) involved, the fifth and final aim of the present study could be reached, namely to contribute to the development of a rational basis for the choice of anaesthetic for *in vivo* investigation of these inhibitory presynaptic receptors. Thus, urethane anaesthesia would lead to the wrong conclusion that the sympathetic nerve terminals of the rat cardiovascular system are not endowed with inhibitory receptors or that they are of minor importance. By contrast, from data obtained from pithed rats anaesthetized with pentobarbitone the correct conclusion would be drawn. Although this interpretation is based on data obtained with α_2, CB_1 and H_3 receptors only, it may be hypothesized that this interpretation also holds true for other inhibitory presynaptic receptors. Therefore, pentobarbitone is a better anaesthetic than urethane for investigations of the function of inhibitory presynaptic receptors on sympathetic nerve terminals of pithed and anaesthetized rats.

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Conflict of interest

The authors state no conflict of interest.

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