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ORIGINAL ARTICLE

Prostaglandins of the E series inhibit monoamine release via EP_3 receptors: proof with the competitive EP_3 receptor antagonist L-826,266

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Abstract Prostaglandin E_2 (PGE_2) and its analogue sulprostone inhibit noradrenaline and serotonin release in rodent tissues. We examined whether the receptor involved is blocked by the EP_3 antagonist L-826,266, whether such receptors also occur on central cholinergic neurones and retinal dopaminergic cells, whether PGE_2 is produced by the degradation of the endocannabinoid virodhamine and whether EP_3 receptor activation stimulates ^{35}S-GTPγS binding. Transmitter release was studied as electrically evoked tritium overflow in superfused tissues preincubated with ^{3}H-noradrenaline (which in the guinea pig retina labels dopaminergic cells), ^{3}H-serotonin or ^{3}H-choline. ^{35}S-GTPγS binding, a measure of G protein activation, was studied in mouse and guinea pig hippocampal membranes. L-826,266 antagonised the effect of sulprostone on noradrenaline release in the rat cortex, yielding a Schild plot-based pA_2 value of 7.56. Apparent pA_2 values in mouse cortex and rat vas deferens (noradrenaline release) and rat cortex (serotonin release) were 7.55, 7.87 and 7.67, respectively. PGE_2 did not affect acetylcholine release in rat brain and dopamine release in guinea pig retina. In seven mice tissues, noradrenaline release was inhibited by sulprostone but not affected by virodhamine. ^{35}S-GTPγS binding was not altered by sulprostone but stimulated by the cannabinoid agonist WIN 55,212-2. Prostaglandins of the E series inhibit monoamine release via EP_3 receptors at which L-826,266 is a competitive antagonist. EP_3 receptors that inhibit transmitter release are not present on central cholinergic neurones and retinal dopaminergic cells. Virodhamine is not converted to PGE_2. An EP_3 receptor model based on ^{35}S-GTPγS binding could not be identified.

Keywords L-826,266 · Virodhamine · Presynaptic EP_3 receptor · Noradrenaline release · Serotonin release · ^{35}S-GTPγS binding

Introduction

Prostaglandins of the E series have been shown to inhibit the release of noradrenaline (Starke 1977 (review); Reimann et al. 1981; Exner and Schlicker 1995) and serotonin (Schlicker et al. 1987) in the brain and to inhibit noradrenaline and acetylcholine release in autonomically innervated tissues (for review, see Güllner 1983; Fuder and Muscholl 1995; Boehm and Kubista 2002). Prostaglandins of the E series act via four receptor subtypes, termed EP_1 to EP_4 (for review, see Alexander et al. 2008), and there is evidence that the presynaptic receptor involved in the above effects is an EP_3 receptor. The latter conclusion is based on data with subtype-selective agonists and on the lack of effect of antagonists with a preference for other EP_x receptor subtypes including SC 19220 (EP_1) and AH 6809 (EP_1 and EP_2; Schlicker et al.

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1987; Coleman et al. 1990 (review); Exner and Schlicker 1995). EP_3 receptor-selective antagonists became available in recent years only (for review, see Jones et al. 2009). One of them, L-826,266 (Juteau et al. 2001), was used in the present study to finally prove the receptor subtype for the presynaptic prostaglandin receptors involved in the inhibition of noradrenaline release in the rat and mouse brain cortex, of serotonin release in the rat brain cortex and of noradrenaline release in the rat vas deferens.

The question whether dopamine release is also subject to inhibition by prostaglandins of the E series has led to contradictory results. Bergström et al. (1973) found that dopamine release from rat striatal slices is inhibited by prostaglandin E_2 (PGE_2), whereas Reimann et al. (1981) did not find such an effect. In an in vivo study on cats, an effect of PGE_2 on hypothalamic dopamine release could also not be detected (von Voigtlander 1976). With respect to acetylcholine release in the brain, only few data are available from the literature. Thus, Harsing et al. (1979) found an inhibitory effect of PGE_1 in the cat brain in vivo. The second aim of the present study was to examine whether PGE_2 also inhibits acetylcholine release in rat brain slices and dopamine release in guinea pig retina discs. The latter preparation was chosen since it had proven to be particularly suited for the identification of presynaptic receptors (Weber and Schlicker 2001).

Endocannabinoids are, like prostanoids, formed from arachidonic acid (Di Marzo et al. 2005). Although endocannabinoids act via cannabinoid CB_1 and CB_2 receptors, endothelial cannabinoid receptors and $TRPV_1$ receptors (Pertwee 2005), some of their effects are related to conversion products, including prostanoids (e.g. Grainger and Boachie-Ansah 2001; van Dross 2009). This is also true for the endocannabinoid virodhamine (Porter et al. 2002), which in the human pulmonary artery may be converted to a prostacyclin-like compound, which, in turn, contributes to the direct vasodilatory effect of virodhamine (Kozłowska et al. 2008). Therefore, the third aim of our study was to examine whether virodhamine inhibits noradrenaline release due to the formation of a prostaglandin of the E series in the pulmonary artery from the mouse; other noradrenergically innervated tissues from the mouse were studied as well. Tissues from the mouse were used for this purpose to allow for experiments with knockout animals in the case of positive results.

Our experiments with L-826,266 revealed that the presynaptic receptors for the prostaglandins of the E series are indeed EP_3 receptors. Since EP_3 receptors are G protein-coupled (for review, see Hatae et al. 2002; Alexander et al. 2008), the fourth aim of our study was to examine in mouse and guinea pig hippocampal membranes whether the $EP_{1/3}$ receptor agonist sulprostone is capable of stimulating the binding of ^{35}S-GTPγS, which represents a method of determination of G protein activation (for review, see Wieland and Jakobs 1994). In these experiments, the cannabinoid receptor agonist WIN 55,212-2, for which a marked stimulation of ^{35}S-GTPγS binding has been shown frequently (see Breivogel et al. 2004 for references), served as a positive control.

Materials and methods

Superfusion studies

Slices from cerebral cortex (0.3 mm thick, diameter 3 mm), hippocampus and striatum (0.3 mm thick, diameter 2 mm) and peripheral tissues (dimensions approximately 1×1×1 mm) were prepared from male Wistar rats and from C57BL/6J mice of either sex (Table 1), and retinal discs (diameter 3 mm) were prepared from male Dunkin–Hartley guinea pigs. Preparations were incubated (37°C) for 60 min with physiological salt solution (PSS; Ca^{2+} 1.3 mM) containing ^{3}H-noradrenaline 0.025 μM, ^{3}H-serotonin 0.025 μM or ^{3}H-choline 0.1 μM (Table 1). Subsequently, the preparations were transferred to superfusion chambers and superfused with PSS (37°C; for auxiliary drugs, flow rate and Ca^{2+} concentration, see Table 1). The superfusate was collected in 5-min samples; experiments lasted for 110 min (in the case of guinea pig retinal discs for 120 min). Tritium overflow was evoked by two 2-min periods of electrical field stimulation (pulses of 2 ms were administered consistently; for stimulation frequency and current strength, see Table 1); the two stimulation periods (S_1 and S_2) started after 40 and 90 min (retinal discs 60 and 100 min) of superfusion. The drugs under study were present in the medium either throughout superfusion or from 62 min (retinal discs 82 min) of superfusion onwards, as indicated under "Results" section. Due to its known long equilibration time, the exposure of preparations to L-826,266 had to be extended under some experimental conditions (Jones et al. 2008). The PSS was composed as follows (millimolars): NaCl 118, KCl 4.8, $CaCl_2$ 1.3 or 3.25 (as indicated in Table 1), KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, ascorbic acid 0.06, disodium ethylenediaminetetraacetic acid (EDTA) 0.03 and 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 preparations 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 or 82 min of superfusion onwards), or the t_1 values obtained in the absence or presence of a given drug were directly

Table 1 Experimental details of superfusion studies, absolute values of basal and electrically evoked tritium overflow and effect of L-826,266

Results in	Tissue	^{3}H-Ligand	Auxiliary drug(s) (μM)	Flow rate (ml min^{-1})	Ca^{2+} concentration (mM)	Frequency (Hz); current strength (mA)	Basal tritium efflux (t_1; min^{-1})[a]		Electrically evoked tritium overflow (S_1; % of tissue tritium)[c]	
							No L-826,266	L-826,266[b] 0.32μM	No L-826,266	L-826,266[b] 0.32μM
Fig. 1b	Rat cortex	^{3}H-NA	Desipramine 1, rauwolscine 1, naproxene 10	1	1.3	0.3; 50	0.0043±0.0002	0.0045±0.0003	10.29±0.64	11.05±0.58
Fig. 2a	Mouse cortex						0.0032±0.0002	0.0037±0.0002	13.07±0.50	15.05±0.55*
Fig. 2b	Rat vas deferens			0.5	3.25	3; 200	0.0016±0.0001	0.0026±0.0004	4.44±0.70	4.32±0.86
Fig. 2c	Rat cortex	^{3}H-5-HT	Femoxetine 1, naproxene 10	1	1.3	3; 200	0.0065±0.0004	0.0080±0.0011	4.38±0.45	5.23±0.66
Fig. 3	Guinea pig retina	^{3}H-NA	–	1	1.3	3; 50	0.0060±0.0006	–	3.40±0.45	–
Fig. 4a	Rat cortex	^{3}H-Choline	Hemicholinium 10, AF-DX 384 1	0.5	3.25	3; 200	0.0023±0.0002	–	1.76±0.45	–
Fig. 4b	Rat hippocampus						0.0022±0.0003	–	1.55±0.27	–
Fig. 4c	Rat striatum						0.0020±0.0002	–	3.40±0.51	–
Fig. 5a	Mouse pulmonary artery	^{3}H-NA	Desipramine 1, rauwolscine 1	0.5	3.25	3; 200	0.0032±0.0003	–	7.08±0.61	–
Fig. 5b	Mouse aorta						0.0030±0.0004	–	2.88±0.55	–
Fig. 5c	Mouse atrium						0.0020±0.0001	–	3.94±0.53	–
Fig. 5d	Mouse kidney						0.0028±0.0002	–	6.94±0.56	–
Fig. 5e	Mouse spleen						0.0026±0.0001	–	2.38±0.25	–
Fig. 5f	Mouse cortex			0.5	1.3	0.3; 50	0.0024±0.0002	–	10.78±3.39	–
Fig. 5g	Mouse hippocampus						0.0024±0.0002	–	6.82±1.91	–

Mean ± SEM of three to 17 experiments (refers to the number of animals)

^{3} H-NA ^{3} H-noradrenaline, *^{3} H-5-HT* ^{3} H-serotonin

*$P<0.05$, compared to the corresponding value without L-826,266

[a] Basal tritium efflux was determined in the 5-min collection period from 55 to 60 min of superfusion (retina 75 to 80 min) and is given as a fraction of tissue tritium (t_1)

[b] Rat cortex slices were exposed to L-826,266 from the beginning of superfusion onwards, whereas mouse cortex slices and rat vas deferens pieces were exposed to this compound during superfusion and, in addition, during the preceding 60 min

[c] The first 2-min period of electrical stimulation (S_1) was administered 40 min (retina 60 min) after the beginning of superfusion

compared to 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 percent of the tritium present in the preparation 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 or 82 min of superfusion), or the S_1 values obtained in the absence or presence of a given drug were directly compared to each other (for drugs present throughout superfusion). The apparent pA_2 value for L-826,266 was calculated according to the formula $pA_2 = \log([A']/[A] - 1) - \log[B]$, where $[A']$ and $[A]$ are the EC_{50} values for prostaglandin E_2 or sulprostone obtained in the presence and absence of L-826,266 and $[B]$ is the concentration of L-826,266 (Furchgott 1972). In the experiments shown in Fig. 1b, various concentrations of L-826,266 were examined, and the data obtained were analysed according to the Schild regression (see, e.g. Kenakin 1993).

Binding studies

Hippocampi from C57BL/6J mice and Dunkin–Hartley guinea pigs were homogenised (Potter-Elvehjem) in 25 volumes of ice-cold Tris–EDTA–sucrose buffer (Tris 50 mM, EDTA 5 mM, pH 7.5, sucrose 10.27%) and centrifuged at 1,500×g for 10 min (4°C). The supernatant was centrifuged at 25,000×g for 25 min (4°C), and the pellet was washed twice with Tris–EDTA buffer. Finally, the pellet was resuspended in buffer and frozen at −80°C. The buffer was composed as follows (millimolars): Tris 50, pH 7.4; ethylene glycol tetraacetic acid (EGTA) 1; $MgCl_2$ 3 and NaCl 100.

Binding experiments were performed in Tris–EGTA buffer (Tris 50 mM, pH 7.4; EGTA 1 mM; $MgCl_2$ 3 mM; NaCl 100 mM; GDP 30 µM; 0.5 mg/ml bovine serum albumin) in a final volume of 0.5 ml containing 5–10 µg protein. ^{35}S-GTPγS was used at a concentration of 0.05 nM. The incubation (30°C) was terminated after 60 min by filtration through Whatman GF/B filters. Non-radioactive GTPγS (10 µM) was used to determine non-specific binding (30% of total binding).

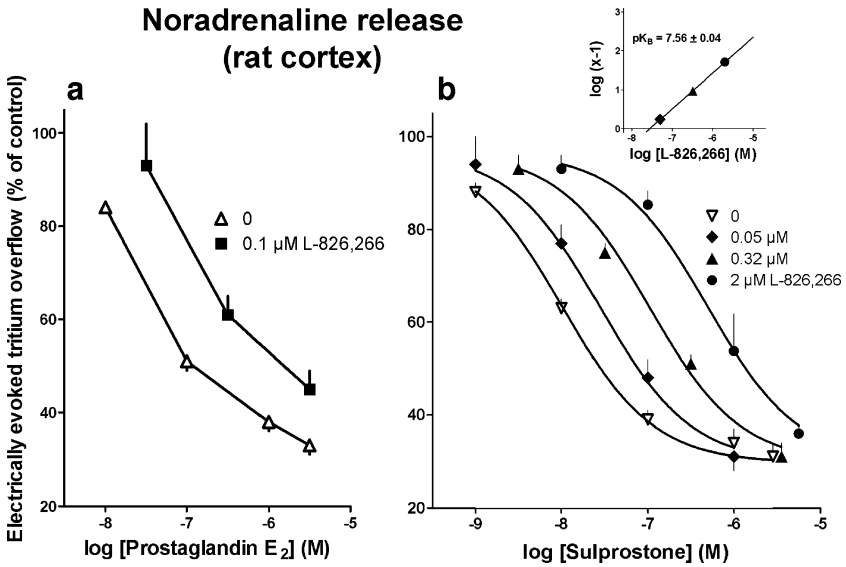

Fig. 1 Effect of **a** prostaglandin E_2 and **b** sulprostone on the electrically evoked tritium overflow from superfused rat brain cortex slices preincubated with ^{3}H-noradrenaline and interaction with L-826,266. Tritium overflow was evoked twice, after 40 and 90 min of superfusion, and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. Tritium overflow was expressed as percent of the S_2/S_1 value in controls (not shown). The superfusion medium contained prostaglandin E_2 or sulprostone from 62 min of superfusion onwards and L-826,266 (plus auxiliary drugs, see Table 1) throughout superfusion (110 min), i.e. the exposure time of the tissue to L-826,266 from the onset of superfusion until S_2 was 1.5 h. For the experiments with the highest concentration of L-826,266 (2 µM), the exposure time was increased to 6.5 h (i.e. the slices were exposed to L-826,266 during superfusion (1.5 h), the preincubation period (1 h; during which slices were preloaded with ^{3}H-noradrenaline) and an additional incubation period (4 h) preceding the preincubation period). *Inset* Schild plot for the three concentrations of L-826,266. "x" (concentration ratio) means the ratio of the EC_{50} of sulprostone in the presence over its EC_{50} in the absence of L-826,266. The slope for the regression line was 0.92 (95% confidence limits 0.79 and 1.05), and the correlation coefficient r was 0.999. Mean ± SEM of three to seven experiments

Statistics

Results are given as mean ± standard error of the mean (SEM) of *n* experiments (superfusion) and of *n* experiments in triplicate (binding experiments). For comparison of mean values, the *t* test for unpaired data was used; the Bonferroni correction was used when two or more values were compared to the same control. The GraphPad Prism[R] software package (GraphPad Software, La Jolla, CA, USA) was used to analyse the binding data and the results shown in Fig. 1b.

Drugs used

[Methyl-^{3}H]-choline chloride (specific activity 70.3 Ci/mmol), (*R*)-(−)-[ring-2,5,6-^{3}H]-noradrenaline (specific activity 53 Ci/mmol), ^{3}H-serotonin (5-[1,2-^{3}H(*N*)]-hydroxytryptamine creatinine sulphate, specific activity 28.1 Ci/mmol), ^{35}S-GTPγS ([^{35}S]-guanosine 5′-(γ-thio)triphosphate, specific activity 1,250 Ci/mmol; PerkinElmer, Zaventem, Belgium); AF-DX 384 (5,11-dihydro-11-{[(2-{2-[(dipropylamino)methyl]-1-piperidinyl}ethyl)amino]carbonyl}-6H-pyrido(2,3-β)(1,4)benzodiazepine-6-one; Boehringer-Ingelheim, Biberach an der Riss, Germany); virodhamine (Biotrend, Köln, Germany); desipramine hydrochloride (Novartis, Wehr, Germany); femoxetine hydrochloride (Ferrosan, Copenhagen, Denmark); hemicholinium-3, oxotremorine sesquifumarate, prostaglandin E_2, 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); L-826,266 (5-bromo-*N*-[3-(5-chloro-2-naphthalen-2-ylmethyl-phenyl)-acryloyl]-2-methoxy-benzenesulphonamide; Merck Frosst, Pointe-Claire-Dorval, Québec, Canada); rauwolscine hydrochloride (Roth, Karlsruhe, Germany) and sulprostone (Bayer Schering Pharma, Berlin, Germany) were the drugs used. Stock solutions of the drugs were prepared with ethanol (prostaglandin E_2, sulprostone, virodhamine), dimethyl sulphoxide (L-826,266, WIN 55,212-2) or water and diluted with PSS (superfusion experiments) or reaction buffer (binding experiments) to the concentration required. The organic solvents did not affect basal and evoked tritium outflow by themselves.

Results

The experiments of the present study comprise superfusion experiments (Figs. 1, 2, 3, 4 and 5) and binding experiments with ^{35}S-GTPγS (Fig. 6). Basal tritium efflux in the superfusion experiments (for absolute values in controls, see Table 1) was not affected by the drugs under study (Table 1 or not shown). Electrical stimulation in the superfusion studies was administered before and after addition of test drugs, i.e. from 40 to 42 min and from 90 to 92 min of superfusion (S_1 and S_2; respective time periods for retinal discs 60–62 and 100–102 min). Control S_1

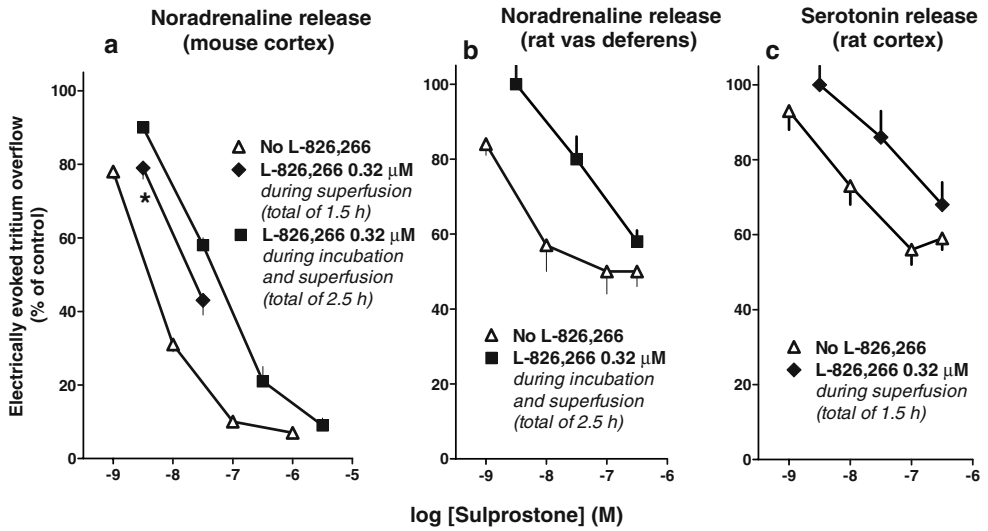

Fig. 2 Effect of sulprostone on the electrically evoked tritium overflow from superfused **a** mouse brain cortex slices and **b** rat vas deferens tissue pieces preincubated with ^{3}H-noradrenaline and **c** rat brain cortex slices preincubated with ^{3}H-serotonin and interaction with L-826,266. Tritium overflow was evoked twice, after 40 and 90 min of superfusion, and the ratio of the overflow evoked by S_2 over that evoked by S_1 was determined. Tritium overflow was expressed as percent of the S_2/S_1 value in controls (not shown). The superfusion medium contained sulprostone from 62 min of superfusion onwards and L-826,266 0.32 µM (plus auxiliary drugs, see Table 1) throughout superfusion, i.e. the exposure time of the tissue against L-826,266 from the onset of superfusion until S_2 was 1.5 h. For the experiments on mouse brain cortex slices and rat vas deferens pieces, the exposure time was increased to 2.5 h. Mean ± SEM of three to ten experiments. *$P<0.05$, compared to the corresponding value with a longer exposure time to L-826,266

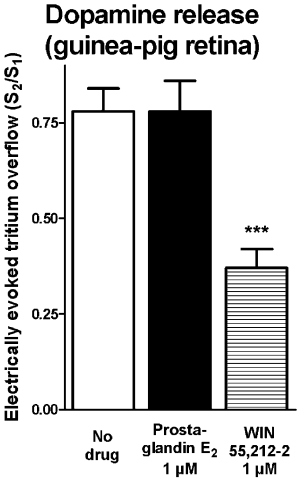

Fig. 3 Effect of prostaglandin E_2 and WIN 55,212-2 on the electrically evoked tritium overflow from superfused guinea pig retinal discs preincubated with ^{3}H-noradrenaline. Tritium overflow was evoked twice, after 60 and 100 min of superfusion, and the ratio of the tritium overflow evoked by S_2 over that evoked by S_1 is shown (S_2/S_1). The superfusion medium contained prostaglandin E_2 or WIN 55,212-2 from 82 min of superfusion onwards. Mean ± SEM of 16–17 experiments. ***$P<$ 0.001, compared to control

values are given in Table 1; some examples for control S_2/S_1 ratios are depicted in Figs. 3, 4 and 5.

Interaction of PGE_2 and sulprostone with L-826,266 in rodent tissues

In rat and/or mouse brain cortex slices and vas deferens pieces preincubated with ^{3}H-noradrenaline or ^{3}H-serotonin, PGE_2 or the $EP_{1/3}$ receptor agonist sulprostone (added to the medium after S_1) inhibited the electrically evoked tritium overflow concentration-dependently (Figs. 1 and 2). The potencies and maximum effects are given in Table 2. PGE_2, which was studied in rat brain cortex slices preincubated with ^{3}H-noradrenaline only, was less potent than sulprostone.

The EP_3 receptor antagonist L-826,266 (present in the medium throughout superfusion) shifted to the right the concentration–response curve of PGE_2 and/or sulprostone in each of the four experimental models (Figs. 1 and 2a–c). An exposure time of 90 min (1.5 h) was sufficient for rat brain cortex slices when L-826,266 concentrations up to 0.32 µM were employed (Figs. 1 and 2c). For mouse brain cortex slices and rat vas deferens pieces exposed to L-826,266 0.32 µM, the contact time had to be extended from 1.5 to 2.5 h. Figure 2a clearly shows for mouse cortex slices that the usual exposure time of 1.5 h would lead to an underestimation of the potency of L-826,266. For rat brain cortex slices exposed to the highest concentration of L-826,266, 2 µM (Fig. 1b), the contact time had even to be extended to 6.5 h. In the latter model, three concentrations of the antagonist covering a range from 0.05 to 2 µM were studied against sulprostone and the log values of the rightward shifts were correlated with the log values of the respective concentrations (Schild plot); linear regression yielded a straight line with a slope not different from unity (Fig. 1b, inset). In the experiments with PGE_2 (Fig. 1a) and in the three models shown in Fig. 2, one concentration of L-826,266 was studied only. The Schild plot-based pA_2 (pK_B) value and the apparent pA_2 values are given in Table 2; the values are similar and range from 7.55 to 7.87. L-826,266 (0.05–2 µM) by itself usually did not affect the electrically evoked tritium overflow (S_1; Table 1 or not shown); only in mouse cortex slices preincubated with ^{3}H-noradrenaline, L-826,266 (0.32 µM) increased S_1 by 15% (Table 1).

Inhibitory effect of PGE_2 on dopamine and acetylcholine release?

To investigate whether dopamine and central acetylcholine release are also inhibited by prostaglandins of the E series, tritium overflow was studied in superfused guinea pig

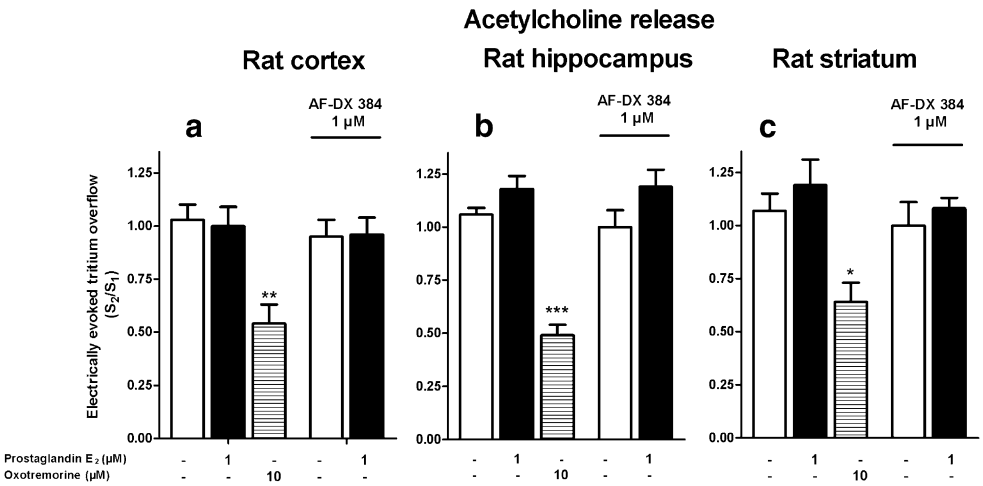

Fig. 4 Effect of prostaglandin E_2 and oxotremorine on the electrically evoked tritium overflow from superfused rat **a** cortical, **b** hippocampal and **c** striatal slices preincubated with ^{3}H-choline. Tritium overflow was evoked twice, after 40 and 90 min of superfusion, and the ratio of the tritium overflow evoked by S_2 over that evoked by S_1 is shown (S_2/S_1). The superfusion medium contained prostaglandin E_2 or oxotremorine from 62 min of superfusion onwards and hemicholinium 10 µM and AF-DX 383 1 µM (when indicated) throughout superfusion. Mean ± SEM of three to eight experiments. *$P<0.05$; **$P<$ 0.01; ***$P<0.001$, compared to control

retinal discs preincubated with ^{3}H-noradrenaline (which labels dopaminergic cells in this tissue) and in slices from three brain regions of the rat preincubated with ^{3}H-choline, respectively. PGE_2 1 μM, added to the medium after S_1, failed to affect the electrically evoked tritium overflow in guinea pig retinal discs preincubated with ^{3}H-noradrenaline, whereas the cannabinoid receptor agonist WIN 55,212-2 inhibited it (Fig. 3). The electrically evoked tritium overflow from rat cortical, hippocampal and striatal slices preincubated with ^{3}H-choline was not altered by PGE_2 1 μM, whereas the muscarine receptor agonist oxotremorine caused an inhibition (Fig. 4). The effect of PGE_2 was also studied in slices superfused in the presence of the muscarine receptor antagonist AF-DX 384, but again PGE_2 1 μM failed to inhibit the evoked overflow (Fig. 4).

Effects of virodhamine on noradrenaline release

The possibility that virodhamine affects noradrenaline release via a conversion product (prostanoid) was examined

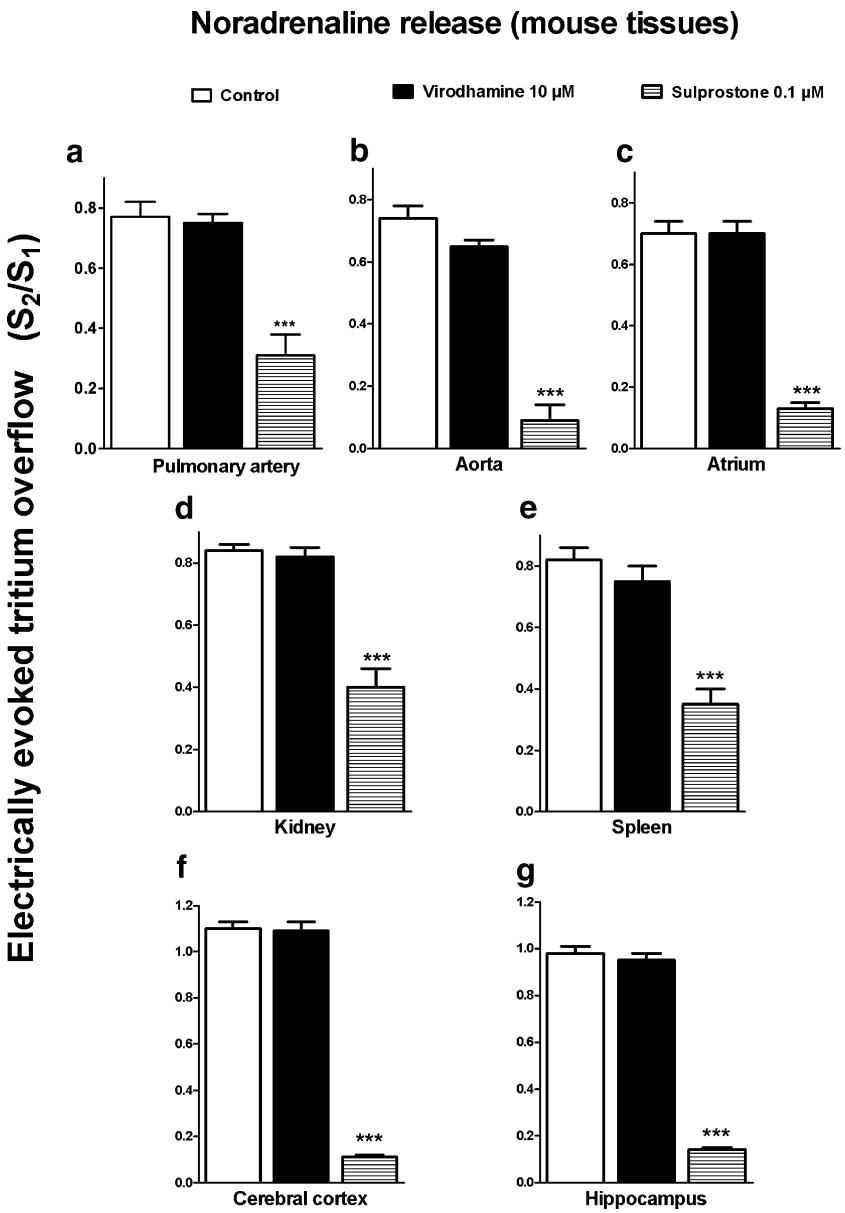

Fig. 5 Effect of virodhamine and sulprostone on the electrically evoked tritium overflow from seven superfused mouse tissues preincubated with ^{3}H-noradrenaline. Tritium overflow was evoked twice, after 40 and 90 min of superfusion, and the ratio of the tritium overflow evoked by S_2 over that evoked by S_1 is shown (S_2/S_1). The superfusion medium contained the drug under study from 62 min of superfusion onwards (for auxiliary drugs, which were present throughout superfusion, see Table 1). Mean ± SEM of four to 11 experiments. ***$P<0.001$, compared to the corresponding control

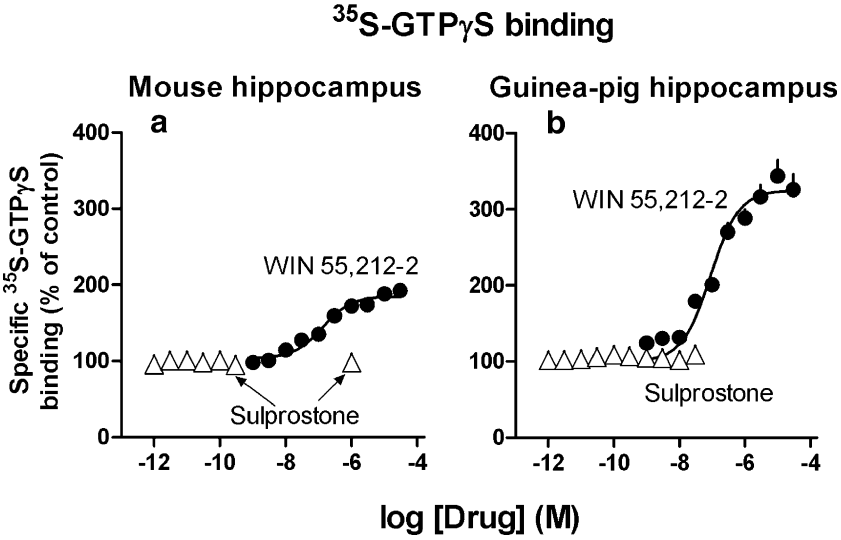

Fig. 6 Effect of sulprostone and WIN 55,212-2 on specific ^{35}S-GTPγS (0.05 nM) binding to **a** mouse and **b** guinea pig hippocampal membranes. Mean ± SEM of two to five experiments in triplicate

in tissue pieces from the mouse pulmonary artery (addition to the medium after S_1). The electrically evoked tritium overflow in this tissue was not affected by virodhamine 10 μM but inhibited by sulprostone 0.1 μM (Fig. 5a). The effects of virodhamine and sulprostone were also studied in another four sympathetically innervated mouse tissues (aorta, atrium, kidney and spleen) as well as in the mouse cerebral cortex and hippocampus. Consistently, virodhamine 10 μM had no effect, whereas sulprostone 0.1 μM caused a marked inhibition (Fig. 5b–g).

Does sulprostone affect ^{35}S-GTPγS binding?

Sulprostone did not affect ^{35}S-GTPγS binding in mouse (Fig. 6a) and guinea pig hippocampal membranes (Fig. 6b). WIN 55,212, used as a positive control, did stimulate this parameter. Its maximum stimulatory effect was much more pronounced in guinea pig (+225%) than in mouse hippocampal membranes (+85%); the pEC_{50} values were virtually identical—7.05 and 6.88, respectively (Fig. 6a, b).

Discussion

Proof that EP_3 receptors mediate the effect of PGE_2 and sulprostone on monoamine release

The major finding of the present study is that the EP_3 receptor antagonist L-826,266 antagonises the inhibitory effect of prostaglandins of the E series on noradrenaline and serotonin release. Under the experimental conditions of our study, the electrically evoked tritium overflow from tissues preincubated with ^{3}H-noradrenaline or ^{3}H-serotonin represents quasi-physiological release of the respective monoamine (Schlicker et al. 1987; Exner and Schlicker 1995; unpublished results). During superfusion, desipramine and femoxetine, inhibitors of the neuronal noradrenaline and serotonin transporter, respectively, were present in the medium to avoid an interference of the test drugs with these mechanisms. Naproxene, a cyclooxygenase inhibitor, was added to the superfusion fluid to block the formation of endogenous prostaglandins of the E series since the latter like exogenously added PGE_2 or sulprostone are expected

Table 2 Maximum inhibitory effects and potencies of prostaglandin E_2 and sulprostone and antagonistic potency of L-826,266 in superfused rat and mouse tissues preincubated with ^{3}H-noradrenaline (^{3}H-NA) or ^{3}H-serotonin (^{3}H-5-HT)

Results in	Tissue	^{3}H-Ligand	EP_3 receptor agonists			L-826,266
			Agonist	Approximate maximum effect (%)	pEC_{50}	pA_2
Fig. 1	Rat cortex	^{3}H-NA	Prostaglandin E_2	70	7.43	7.72[a]
			Sulprostone	70	8.06	7.56[b]
Fig. 2a	Mouse cortex	^{3}H-NA	Sulprostone	90	8.51	7.55[a]
Fig. 2b	Rat vas deferens	^{3}H-NA	Sulprostone	50	8.68	7.87[a]
Fig. 2c	Rat cortex	^{3}H-5-HT	Sulprostone	40	8.35	7.67[a]

[a] Apparent pA_2 values are based on one concentration of L-826,266

[b] The pA_2 value is based on three concentrations of L-826,266 and derived from the Schild plot shown in Fig. 1b (inset)

to interact with the antagonist L-826,266; this would lead to an underestimation of the potency of the antagonist.

Tissues preincubated with ^{3}H-noradrenaline were, in addition, superfused in the presence of the α_2-adrenoceptor antagonist rauwolscine to increase the extent of the PGE_2- or sulprostone-related effect. The fact that the effect mediated via presynaptic heteroreceptors on noradrenaline release is increased (or obtained at all) when the autoreceptor is blocked simultaneously has been shown for many heteroreceptors (for review, see Schlicker and Göthert 1998), including the EP_3 receptor (Exner and Schlicker 1995). One may argue that the real implication of a presynaptic effect that needs the presence of an auxiliary drug may be low; however, with respect to the EP_3 receptor-mediated inhibition of noradrenaline release in mouse cortex slices, the maximum inhibitory effect does not markedly differ in the presence and absence of rauwolscine (90% vs. 80%, respectively; Exner and Schlicker 1995).

L-826,266 antagonised the effect of sulprostone on noradrenaline release in the rat brain cortex with a pA_2 value of 7.56; similar values were obtained when (a) PGE_2 was used instead of sulprostone, (b) mouse instead of rat cortex slices were examined, (c) noradrenaline release from the vas deferens instead of the cortex was considered and (d) serotonin instead of noradrenaline was measured. Our experiments on rat brain cortex slices preincubated with ^{3}H-noradrenaline show that the nature of antagonism of L-826,266 towards EP_3 receptors is competitive as suggested by a slope of 1 in the Schild plot. In this respect, our data are compatible with those reported by Clark et al. (2008) for the human EP_3 receptor studied in a cell line. Like in previous in vitro studies (Jones et al. 2008), a long equilibration time of L-826,266 was necessary in some of our experiments. This property may be related to the highly lipophilic character of the compound (Jones et al. 2008), and care has to be taken in future in vitro studies to make sure that the exposure time of the tissues towards the antagonist will be long enough.

The pA_2 values reported herein are similar to those reported for EP_3 receptors in the guinea pig aorta and vas deferens (Jones et al. 2008). Species differences may be the reason why the pA_2 values in the study by Jones et al. (2008) and in the present work are lower than the pK_i value of 9.10 described by Juteau et al. (2001) in the human embryonic kidney 293 cell line stably expressing the human EP_3 receptor and the pK_b value of 8.35 in the study by Clark et al. (2008) in human erythroleukemia cells endogenously expressing the human EP_3 receptor. In other in vitro studies on rat (Oliveira et al. 2009), mouse (Weller et al. 2007) and guinea pig preparations (Schlemper et al. 2005), L-826,266 at a concentration of 1 μM markedly antagonised EP_3 receptor-mediated effects. The effectiveness of L-826,266 against the EP_3 receptor has also been shown in rats (Oliveira et al. 2008) and mice in vivo (Oliva et al. 2006; Kassuya et al. 2007).

Search for EP_3 receptors at dopaminergic cells and cholinergic neurones

The question whether presynaptic receptors for prostaglandins of the E series are also present on retinal dopaminergic cells and central cholinergic neurones was again studied on superfused tissues. For the experiments on guinea pig retinal discs, ^{3}H-noradrenaline was used since this tracer is transported into retinal dopaminergic cells and exocytotically released upon electrical stimulation like ^{3}H-dopamine and since the scatter of variation of results is lower (Schlicker et al. 1996). PGE_2 failed to affect dopamine release, whereas the cannabinoid receptor agonist WIN 55,212-2, as expected (Schlicker et al. 1996), inhibited the release of this monoamine.

The effect of PGE_2 on acetylcholine release was studied in slices from three brain regions (of the rat) pre-labelled with ^{3}H-choline, which is transported into the cholinergic nerve terminals via the high-affinity choline uptake (HACU), converted to acetylcholine and released upon electrical stimulation. Hemicholinium was used to avoid interference of test drugs with the HACU. PGE_2 did not affect acetylcholine release, whereas the muscarine receptor agonist oxotremorine, as expected (Starke et al. 1989), inhibited it. The possibility has to be considered that, like in the case of noradrenergic neurones (Exner and Schlicker 1995; Schlicker and Göthert 1998), an inhibitory effect of PGE_2 on acetylcholine release will be increased or occur only if the presynaptic autoreceptors are blocked simultaneously. Our study shows, however, that even when the muscarinic autoreceptors on the cholinergic neurones were blocked by AF-DX 384, PGE_2 still did not affect acetylcholine release.

Are prostaglandins of the E series formed from virodhamine?

Functional evidence that the endocannabinoid virodhamine is converted to prostacyclin or a related product in the human pulmonary artery (Kozłowska et al. 2008) prompted us to examine whether virodhamine may be also converted to prostaglandins of the E series. This question is of practical relevance since prostacyclin and analogues are used for the treatment of pulmonary hypertension (Naeije and Huez 2007). Virodhamine might be useful for the same purpose (a) due to its conversion to prostacyclin (or a similar product), (b) its agonism at the endothelial vasodilatory cannabinoid receptor (Kozłowska et al. 2008) and (c) (possibly) endogenously formed prostaglandins of the E series that inhibit noradrenaline release in vessels of the pulmonary circulation.

We preferred the pulmonary artery from the mouse over that from humans since knockout mice are available for enzymes involved in the degradation of virodhamine to arachidonic acid (fatty acid amide hydrolase; Cravatt et al. 2001) and its conversion to prostaglandins of the E series (cyclooxygenases COX-1 and COX-2; Langenbach et al. 1995; Morham et al. 1995). In addition to the pulmonary artery, another four sympathetically innervated tissues and two brain areas with noradrenergic neurones have been studied. In each experimental model, sulprostone had a marked inhibitory effect on noradrenaline release, whereas virodhamine did not affect it.

Does sulprostone increase ^{35}S-GTPγS binding?

In the final part of our study, we tried to identify an additional functional EP_3 receptor model in mouse hippocampal tissue. Such a model would be interesting per se but might also be helpful in order to further characterise the compound L-826,266. Although our study revealed that L-826,266 is a competitive EP_3 receptor antagonist, the question whether this drug is a neutral antagonist or an inverse agonist cannot easily be answered by superfusion experiments. (Some) EP_3 receptors are $G_{i/o}$ protein-coupled (Hatae et al. 2002; Alexander et al. 2008), and for this reason, we used the ^{35}S-GTPγS binding assay, which measures G protein activation and is particularly suited for the identification of functional $G_{i/o}$ protein-coupled receptors (Wieland and Jakobs 1994), including the CB_1 receptor (for review, see Breivogel et al. 2004). However, sulprostone failed to increase ^{35}S-GTPγS binding in mouse hippocampal membranes and was also without effect when studied in guinea pig hippocampal membranes in which the cannabinoid receptor agonist WIN 55,212-2, used here as a positive control, has an even more pronounced effect on ^{35}S-GTPγS binding compared to the mouse.

Conclusions

First, the present study proves, on the basis of a selective antagonist, that the presynaptic prostaglandin E receptors involved in the inhibition of noradrenaline and serotonin release in rodent tissues belong to the EP_3 receptor subtype. The study also shows that L-826,266, the drug used for this purpose (Juteau et al. 2001), acts as a competitive antagonist. Second, presynaptic receptors for prostaglandins of the E series could not be detected on retinal dopaminergic cells of the guinea pig and on cholinergic neurones of three brain regions of the rat. Third, virodhamine is not converted in a functionally relevant manner to a prostaglandin of the E series in seven noradrenergically innervated tissues of the mouse. Fourth, an additional EP_3 receptor model, based on ^{35}S-GTPγS binding, could not be identified in the mouse and guinea pig hippocampus.

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