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Mechanisms involved in the vasorelaxant effect of (−)-stepholidine in rat mesenteric small arteries

Shi Lei [a,1], Luis M. Orensanz [b], Michael J. Mulvany [a], Ulf Simonsen [a,*]

[a] *Department of Pharmacology, University of Aarhus, DK-8000 Aarhus C, Denmark*
[b] *Departamento de Investigación, Hospital Ramon y Cajal, 28034 Madrid, Spain*

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Abstract

The purpose of the present investigation was to clarify whether the hypotensive action of the protoberberine alkaloid, and dopamine receptor antagonist, (−)-stepholidine, can be ascribed to an effect on peripheral small arteries. For this purpose isolated mesenteric small arteries were suspended in microvascular myographs for isometric tension recording. Relaxations mediated by dopamine D_1 receptors were antagonized by (−)-stepholidine. (−)-Stepholidine inhibited in a concentration-dependent manner the contractile responses evoked by noradrenaline (10^{-6} M), but not the contractile responses evoked by depolarizing solution (KCl, 60 mM) or 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619, 10^{-7} M). Mechanical endothelial cell removal, blockade of K^+ channels, muscarinic receptors or adrenoceptors did not influence the inhibitory effect of (−)-stepholidine on the contractile response evoked with noradrenaline in the segments.(−)-Stepholidine caused rightward shifts of the concentration–response curves for noradrenaline and phenylephrine. The pA_2 values for (−)-stepholidine were 6.05 and 5.94 against noradrenaline and phenylephrine, respectively. Electrical field stimulation induced prazosin-sensitive frequency-dependent contractions in mesenteric small arteries. These contractions were significantly inhibited by 10^{-6} and 10^{-5} M (−)-stepholidine. In membranes from the rat cerebral cortex labelled with [^{3}H]prazosin, (−)-stepholidine (10^{-7}–10^{-4} M) completely inhibited the specific binding of the ligand with a pK_i of 5.6. The present investigation suggests the inhibitory effect of (−)-stepholidine on the α_1-adrenoceptor-mediated contractions induced by exogenously added and nerve-released noradrenaline in peripheral small arteries might contribute to a hypotensive effect of the drug.

Keywords: (−)-Stepholidine; (Rat); Mesenteric small artery; Adrenoceptor; Dopamine receptor; Electrical field stimulation

1. Introduction

(−)-Stepholidine is a protoberberine alkaloid which has been isolated from the Chinese herb *Stephania intermedia* and from opium (Cava et al., 1968; Brochmann-Hansen and Richter, 1975). (−)-Stepholidine consists of an isoquinoline ring with methoxy groups at C_3 and C_9 positions and hydroxyl groups at C_2 and C_{10} positions (Fig. 1; Sun and Jin, 1992). Tetrahydroprotoberberines and (−)-stepholidine share a naloxone resistant analgesic action which is mediated through inhibition of central postsynaptic and presynaptic dopamine receptors (Jin, 1987; Sun and Jin, 1992). (−)-Stepholidine has been suggested to be useful in the treatment of extrapyramidal diseases and vascular headache, but it has also a pronounced hypotensive effect (Jin, 1987). Central dopaminergic pathways in striatum, the chemotrigger zone, and from hypothalamus to the pituitary gland are of importance in control of movements, emesis and prolactin release, but it is unlikely that antagonism of dopamine receptors localized in these areas produces the blood pressure fall observed to (−)-stepholidine (Singewald and Philippu, 1994; Luchsinger et al., 1995). In peripheral sympathetic nerves, dopamine is a precursor for noradrenaline (Soares da Silva, 1988), and dopamine has a vasodilator effect either through activation of postjunctional dopamine D_1 receptors (Brodde et al., 1981; Illes and Nörenberg, 1989; Hughes and Sever, 1989; Münch et al., 1991) or prejunctional dopamine D_2 receptors inhibiting the release of noradrenaline (Hahn and MacDonald, 1984; Lokhandwala and DeFeao, 1988). However, the result of activation of peripheral dopamine receptors would be blood pressure fall as found with

* Corresponding author. Tel. +45-89-42-17-13; Fax: +45-86-12-88-04
[1] Present address: Department of Pharmacology, Xuzhou Institute of Medical Sciences, Hubei Road 2, Jiangsu, Xuzhou, China.

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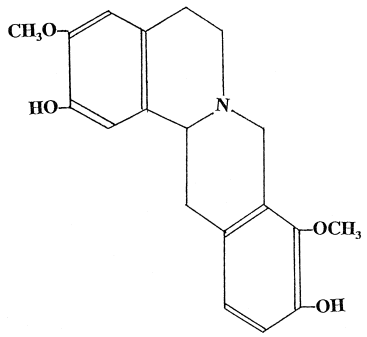

Fig. 1. Structure of (−)-stepholidine.

dopamine agonists such as fenoldopam (Clark et al., 1991). Therefore, it is also unlikely that peripheral dopamine receptors are involved in the hypotensive action of (−)-stepholidine.

Benzylisoquinolones such as papaverine and laudanosine, and aporphine alkaloids such as glaucine and boldine, which are structurally related to protoberberines, have been demonstrated to inhibit cyclic nucleotide phosphodiesterases (Ivorra et al., 1992; Chuliá et al., 1997), displace [^{3}H]prazosin binding to α_1-adrenoceptors (Schott et al., 1988; Ivorra et al., 1992, 1993; Chuliá et al., 1994; Madrero et al., 1996), and [^{3}H](+)-diltiazem binding to the benzothiazepine recognition site in Ca^{2+} channels (Ivorra et al., 1992). (−)-Stepholidine was reported to inhibit the contractions induced by phenylephrine in the rabbit aorta, and suggested to be a Ca^{2+} channel blocker, since it induced rightward shifts of concentration–response curves in aorta for $CaCl_2$ in high K^+ (Yang et al., 1993). Thus, a variety of vasodilator mechanisms could contribute to the hypotensive effect of (−)-stepholidine.

The effect of (−)-stepholidine on the small arteries, which are of importance in the control of the peripheral resistance and, hence, blood pressure control (Christensen and Mulvany, 1993) remains unclear. Therefore, we have examined the effect of (−)-stepholidine in rat mesenteric small arteries in an attempt to clarify the underlying mechanisms such as the involvement of the endothelium, K^+ channels, Ca^{2+} entry through voltage-dependent Ca^{2+} channels, adrenoceptors, dopamine, 5-hydroxytryptamine (5-HT) and muscarinic receptors.

2. Materials and methods

Aorta and the mesenteric bed of 12–16 week old male Wistar rats killed with CO_2 were used. Throughout the subsequent dissection, the mesenteric vascular bed was bathed in cold physiological salt solution (PSS, 4°C) of the following composition (mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $CaCl_2$ 2.5, ethylenediaminetetraacetic acid (EDTA) 0.026 and glucose 5.5. The solution was gassed with 5% CO_2 in O_2 to maintain pH at 7.4.

Segments (ca. 2 mm long) of third order branches of the rat superior mesenteric artery or aorta were mounted as ring preparations on two 40 μm wires on an isometric double myograph (JP Trading, Aarhus, Denmark) by fixing one of the wires to a force transducer and the second wire to a length displacement device (Mulvany and Halpern, 1977). The vessels were allowed to equilibrate in PSS, 37°C, pH 7.4 for about 30 min. The relation between resting wall tension and the internal circumference was determined, and the internal circumference, L_{100}, corresponding to a transmural pressure of 100 mm Hg for a relaxed vessel in situ was calculated (Mulvany and Halpern, 1977). The vessels were set to the internal circumference L_1, given by $L_1 = 0.09L_{100}$. Previous studies have shown that the force development is close to maximal at this internal circumference (Mulvany and Warshaw, 1979). The effective internal lumen diameter was determined as $l_1 = L_1/\pi$.

2.1. Experimental protocols

2.1.1. Evaluation of the effects of (−)-stepholidine on dopamine, muscarinic and β-adrenoceptors

With the aim of clarifying whether dopamine receptors play a role in the vascular effects of (−)-stepholidine, the dopamine receptors were characterized in rat mesenteric arteries by first evaluating the effect of dopamine added at resting tension and on vessels contracted with 9, 11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619, 10^{-7} M). Dopamine D_1 and D_2 receptor agonists, *R*(+)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride (SKF-38393) and LY 171555 hydrochloride (quinpirole), respectively, were applied. Moreover, the receptors mediating dopamine-induced relaxations were characterized by using a dopamine D_1 receptor antagonist, *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzapine hydrochloride (SCH-23390, 10^{-9}–10^{-7} M), and a dopamine D_2 receptor antagonist, (−)-sulpiride (10^{-7}–10^{-5} M). Finally the effect of (−)-stepholidine (10^{-7}–10^{-6} M) on the dopamine concentration–response curves was evaluated. A first control curve for dopamine (10^{-8}–3×10^{-5} M) was constructed in the absence of antagonist. After washing, the vessel was incubated with the antagonist for 30 min and a new concentration–response curve for dopamine was constructed. All responses induced by dopamine were obtained in the presence of prazosin (10^{-6} M), yohimbine (10^{-6} M), propranolol (10^{-6} M), cocaine (10^{-6} M), and corticosterone (10^{-6} M) to avoid activation of α- and β-adrenoceptors and tissue uptake of dopamine.

Experiments were performed to clarify whether (−)-stepholidine antagonizes the relaxations of mesenteric small arteries to acetylcholine and isoprenaline. The vessels were incubated for 30 min with 10^{-6} M phentolamine to

block the contractile α-adrenoceptors, contracted with (10^{-8}–10^{-7} M) U46619 giving a stable contraction corresponding to 50–60% of the response to 125 mM KPSS, and concentration–response curves for isoprenaline were constructed. A first, cumulative control concentration–response curve for isoprenaline in the absence of antagonist was obtained. After washing, the vessel was incubated with the antagonist for 30 min and a new concentration–response curve for isoprenaline was constructed in the presence of antagonist. The isoprenaline concentration–response curves were repeated in the presence of a maximum of two different concentrations of antagonist for each preparation.

2.1.2. Evaluation of vasorelaxant effect of (−)-stepholidine

To determine whether (−)-stepholidine induced vasorelaxation, the vessels were contracted with either noradrenaline, the thromboxane analogue, U46619, or 60 mM K^+, and increasing concentrations of (−)-stepholidine were added in steps of half log units. To assess whether the relaxations induced by (−)-stepholidine of contracted mesenteric small arteries were dependent on the presence of the endothelium, the vessels were contracted by noradrenaline and a first cumulative concentration–response curve was obtained for (−)-stepholidine (10^{-7}–10^{-4} M). The endothelial cells were removed by introducing into the lumen a human scalp hair and rubbing back and forth several times. The effectiveness of this procedure was assessed by absence of relaxation to acetylcholine in noradrenaline contracted arteries. Then a second concentration–relaxation curve for (−)-stepholidine was obtained.

To evaluate an involvement of K^+ channel opening in the relaxations induced by (−)-stepholidine, a first concentration–relaxation curve to (−)-stepholidine was constructed in noradrenaline activated segments, and a second concentration–relaxation curve in the presence of either a raised K^+ concentration (20 mM), the non-selective K^+ channel blocker, tetraethylammonium (TEA, 10^{-3} or 10^{-2} M), or a blocker of ATP-sensitive K^+ channels, glibenclamide (10^{-6} M).

2.1.3. Evaluation of effect of (−)-stepholidine on 5-HT and adrenoceptors

The effect of (−)-stepholidine on concentration–response curves for 5-hydroxytryptamine (5-HT, 10^{-8}–10^{-5} M) was also evaluated. The vessels were incubated with phentolamine (10^{-6} M) and cocaine (10^{-6} M) to inhibit α-adrenoceptors and tissue uptake, respectively, and a concentration–response curve for 5-HT was constructed and served as control. After washing, the vessel was incubated within (−)-stepholidine (10^{-6}–10^{-5} M) for 15 min and a new concentration–response curve for 5-HT was constructed.

Cumulative concentration–response curves for different adrenoceptor agonists were constructed in the absence and presence of (−)-stepholidine. These experiments were performed in endothelium denuded vessels in the presence of cocaine (10^{-6} M), corticosterone (10^{-6} M) and propranolol (10^{-6} M) to inhibit uptake and β-adrenoceptors, respectively. A first concentration–response curve which served as control was obtained for the adrenoceptor agonist (noradrenaline, phenylephrine) in the absence of antagonist, and the following curves in the presence of increasing concentrations of (−)-stepholidine (10^{-6}–3×10^{-5} M). Parallel time control curves for noradrenaline and phenylephrine in the absence of antagonist were also obtained. The effect of (−)-stepholidine ($10^{-6} \times 3$–10^{-5} M) on the contractions induced by phenylephrine in rat aorta was also evaluated with a similar protocol as the one described for the mesenteric small arteries.

2.1.4. Evaluation of the effect of (−)-stepholidine on responses to electrical field stimulation

For electrical field stimulation (EFS), rings of mesenteric small arteries were mounted as described above with two electrodes made of platinum plate, approximately 2 mm apart from each other. The endothelial cells were removed, the vessels were incubated with propranolol (10^{-6} M) and cocaine (10^{-6} M) for 20 min, and a first frequency–response curve was constructed by stimulating with 20 s trains with 0.25 ms square pulses applied at frequencies of 2–16 Hz with a Cibertec CS20 stimulator (Letica, Barcelona, Spain) with constant current output adjusted to 60 mA. Then the arteries were stimulated with noradrenaline (10^{-5}) for 2 min and relaxed in PSS for 15 min to replenish amine stores. After a first control frequency–response curve, the arteries were incubated with increasing concentrations of (−)-stepholidine (10^{-7}–10^{-5} M) for 20 min before a second frequency–response curve was obtained. The neurogenic component of the response to EFS was determined following 30 min incubation of the arteries with tetrodotoxin (10^{-6} M). Frequency–response curves in the absence of treatment were run in parallel.

2.1.5. Evaluation of the effect of (−)-stepholidine on Ca^{2+} channels

The involvement of Ca^{2+} channel inhibition was tested in vessels incubated with the α-adrenoceptor blocker, phentolamine (10^{-6} M), activated by 125 mM KPSS, and stepwise increasing the external concentration of $CaCl_2$ (10^{-5}–10^{-2} M). A first concentration–response curve was performed and served as control for the following curves which were constructed in the presence of (−)-stepholidine (10^{-5}, 3×10^{-5} or 10^{-4} M). Preparations incubated with nifedipine (10^{-7} M) were included as positive controls.

2.2. [^{3}H]prazosin binding studies

Binding assays were performed as previously described (Ambrosio et al., 1984). Male rats weighing 180–200 g

were decapitated and their cerebral cortices were dissected over ice. The tissue was homogenised in 20 vol of ice-cold buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, pH = 7.7 at 25°C and centrifuged at 46,000 g for 10 min, then washed twice by resuspension in buffer and centrifugation. Finally, the pellets were resuspended in 50 vol of buffer. The assays for 0.2 nM [^{3}H]prazosin were performed in a total volume of 2 ml with a protein concentration determined with the method of Bradford (1976) of 150 μg/ml incubation medium. The assay tubes were incubated in the absence or presence of (−)-stepholidine for 30 min at 25°C, since preliminary experiments showed that prolonged incubation with (−)-stepholidine up to 120 min did not cause further displacement. The binding reaction was terminated by rapid filtration under vacuum through GF/B filters presoaked for 3 h in 0.05% polyethylenimine, followed by two 5 ml washes with buffer. Specific binding was defined as binding in the presence of 10^{-5} M phentolamine and represented 90% of total [^{3}H]prazosin binding. Assays were conducted in triplicate.

2.3. Drugs

The following drugs were use: (−)-stepholidine (a gift from Dr. Jin Guo Zhang, Shanghai Institute of Materia Medica, Chinese Academy of Sciences); noradrenaline hydrochloride, phenylephrine hydrochloride, dopamine hydrochloride, prazosin hydrochloride, propranolol, 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619), isoprenaline, cocaine, corticosterone, glibenclamide, 5-hydroxytryptamine HCl (5-HT), yohimbine, acetylcholine, tetrodotoxin, TEA, and phentolamine were purchased from Sigma, St. Louis, MO, USA; *R*(+)-1-phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrochloride (SKF-38393), (−)-sulpiride, LY 171555 hydrochloride (quinpirole), *R*(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzapine hydrochloride (SCH-23390), and WB4101 hydrochloride (2-2,6-dimethoxyphenoxyethyl aminomethyl-1,4-benzodioxane) were from Research Biochemicals International, Natrick, MA, USA; [furanyl-5-^{3}H]prazosin (24 Ci/mmol) was purchased from Amersham.

(−)-Stepholidine was dissolved in a small amount of 10% phosphoric acid and water, then pH was adjusted to 5.0 by adding 1 N sodium hydroxide and finally, water was added to obtain a stock solution of 5×10^{-2} M. The pH of the myograph solution was checked and was found only to be affected at concentrations of (−)-stepholidine above 3×10^{-4} M. Further dilutions of (−)-stepholidine were made in distilled water. For the binding experiments, (−)-stepholidine was dissolved in ethanol, which represented 1% in all assay samples. This ethanol concentration had no effect on the specific binding of [^{3}H]prazosin. Noradrenaline and phenylephrine were prepared in 0.25 N HCl and further diluted in twice distilled water. Prazosin was dissolved in warm water (50°C) at pH 4–5 with constant agitation. Glibenclamide was prepared as a 10^{-2} M stock solution in dimethylsulphoxide. The other drugs were dissolved in distilled water. None of the solvent, in the concentration applied, had any effect on the preparations.

2.4. Analysis of data

The mechanical responses of the vessels were measured as force and expressed as active wall tension, ΔT, which is

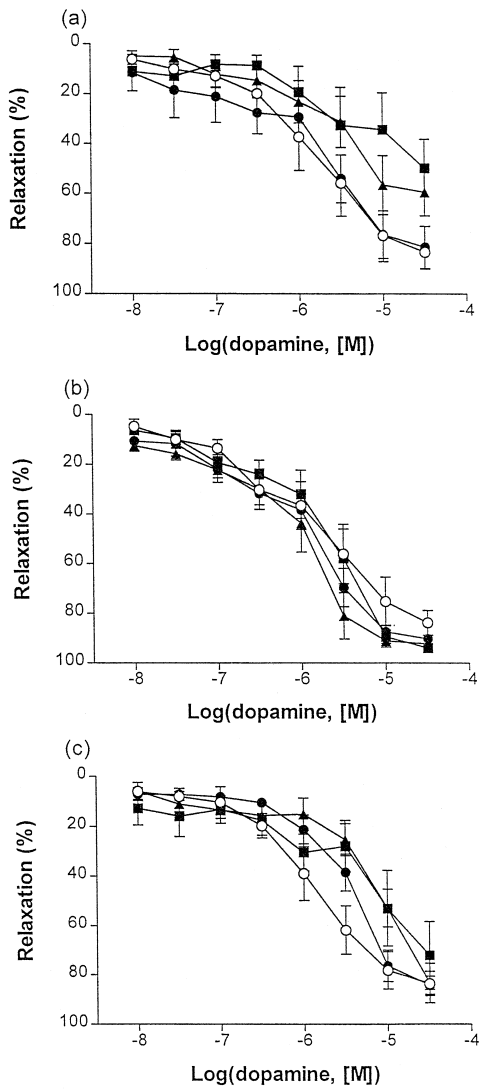

Fig. 2. Relaxation–concentration curves obtained on rat mesentric small artery to dopamine following precontraction with 10^{-7} M U46619 in the presence of prazosin (10^{-6} M), yohimbine (10^{-6} M), corticosterone (10^{-6} M), cocaine (10^{-6} M) and propranolol (10^{-6} M). A curve for dopamine was constructed in control conditions (open circles) and in the presence of (a) 10^{-9} M (closed circles), 10^{-8} M (closed triangles) and 10^{-7} M (closed squares) SCH-23390; (b) 10^{-7} M (closed circles), 10^{-6} M (closed triangles) and 10^{-5} M (closed squares) (−)-sulpiride; and (c) 10^{-7} M (closed circles), 3×10^{-7} M (closed triangles) and 10^{-6} M (closed squares) (−)-stepholidine. The pD_2 values for dopamine in the absence were 5.81±0.19 (n = 7) and in the presence of 10^{-7} M (−)-stepholidine 5.55±0.14 (n = 6), 3×10^{-7} M 5.00±0.16 (n = 4) and 10^{-6} M 4.87±0.26 (n = 5). The results are expressed as percentage of the contraction induced by U46619. Each point represents the mean± S.E.M. of six to seven experiments.

the increase in measured force, ΔF, divided by twice the segment length (Mulvany and Halpern, 1977). Using a computer programme (GraphPad, Institute for Scientific Information, San Diego, CA, USA), the concentration–response curves were fitted to the classical Hill equation: $R/R_{max} = A(M)^n/(A(M)^n + EC_{50}(M)^n)$, where R/R_{max} is the relative response to the effective concentration of drug, $A(M)$, and $EC_{50}(M)$ is the concentration of agonist required to give half maximal vessel response (R_{max}) when $A(M)$ and $EC_{50}(M)$ are given in molar concentration. n is a curve-fitting parameter or Hill coefficient.

To assess the effect of (−)-stepholidine, at least three different concentrations of antagonist were examined. A maximum of two concentrations were studied per mesenteric arterial ring and the results compared to those in the first concentration-response curve for the agonist (noradrenaline, phenylephrine or dopamine) performed in the absence of the antagonist. Concentration ratios (CR) were calculated at the EC_{50} level, and the pA_2 values of the competitive antagonists, defined as the negative logarithm of the molar antagonist concentration, in the presence of which twice the original agonist concentration is needed for the original effect, was determined by plotting log (CR-1) against log antagonist concentration (Arunlakshana and Schild, 1959). The slope and pA_2 value were determined by least square fitting of a regression line to the points.

IC_{50} values in the binding experiments were determined from a Hill plot, and K_i values calculated by the method of Cheng and Prusoff (1973). The [^{3}H]prazosin dissociation constant, K_D at the α_1-adrenoceptors was 0.2 nM.

The results are expressed as means ± S.E.M., and the response curves presented on a semi-logarithmic scale. Differences between means were analyzed using either one way analysis of variance, Student's two tailed *t*-test or paired *t*-test as appropriate. Probability levels under 5% were considered significant. N.S. indicates no significant difference.

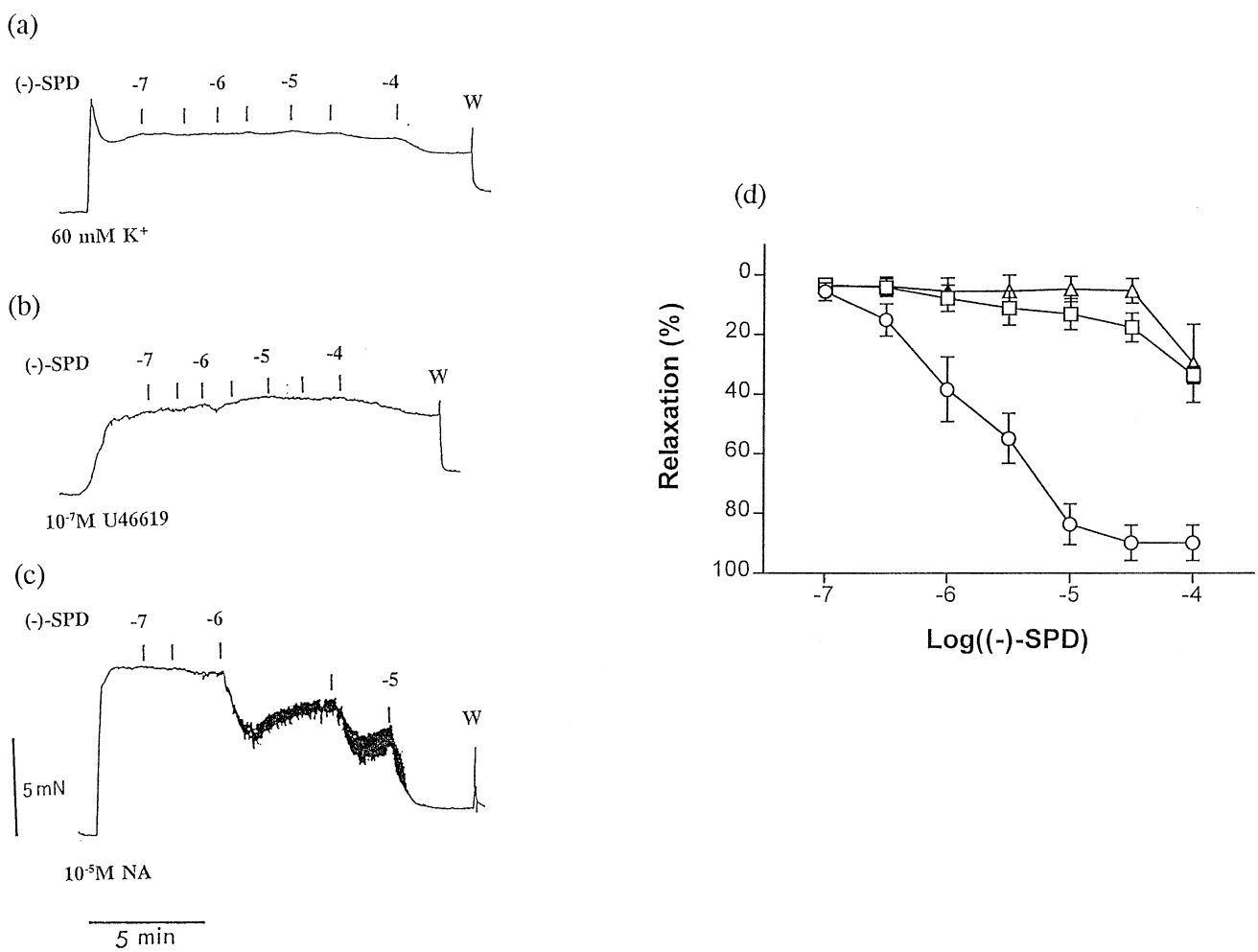

Fig. 3. Original trace recordings showing the effect of (−)-stepholidine ((−)-SPD) in rat mesenteric small arteries contracted to either (a) 60 mM potassium-rich solution, (b) 10^{-7} M U46619 or (c) 10^{-5} M noradrenaline and exposed to increasing concentrations of (−)-stepholidine. W = wash. (d) Average results of the effects of (−)-stepholidine in mesenteric small arteries contracted to either noradrenaline (10^{-5} M, open circles), U44619 (10^{-6} M, closed triangles) or K^+-rich solution (60 mM, open squares). The results are means ± S.E.M. of five to six experiments.

3. Results

Mesenteric vessels with an internal diameter of 246 ± 4 μm were mounted, and 125 mM KPSS induced contractions of 3.2 ± 0.1 Nm^{-1} ($n = 93$) in these arteries.

3.1. Effect of (−)-stepholidine on dopamine receptors in mesenteric small arteries

Dopamine (10^{-9}–10^{-4} M) did not have any contractile effect in mesenteric small arteries ($n = 6$). However, in vessels incubated with prazosin (10^{-6} M), yohimbine (10^{-6} M), corticosterone (10^{-6} M), cocaine (10^{-6} M), and propranolol (10^{-6} M), and contracted by U46619 (10^{-7} M), dopamine (10^{-9}–10^{-5} M) induced concentration-dependent relaxations. The partial dopamine D_1 receptor agonist, SKF-38393, induced relaxations with pD_2 and maximum relaxation of 7.87 ± 0.29 and 75 ± 11% ($n = 6$), respectively, while the dopamine D_2 receptor agonist, quinpirole (10^{-9}–3×10^{-5} M) had no relaxant effect ($n = 6$). The dopamine D_1 receptor antagonist, SCH-23390 (10^{-8} and 10^{-7} M), caused rightward shifts of the concentration–response curves for dopamine, while the dopamine D_2 receptor antagonist, (−)-sulpiride, did not change the concentration–response curves for dopamine (Fig. 2a,b). The pA_2 values for SCH-23390 against dopamine were 8.67 ± 0.51 ($n = 6$).

(−)-Stepholidine (10^{-7}–10^{-6} M) caused rightward shifts of the concentration–response curves for dopamine (Fig. 2c). The pA_2 values for (−)-stepholidine against dopamine were 6.85 ± 0.16.

3.2. Vasorelaxant effect of (−)-stepholidine in mesenteric small arteries

Noradrenaline, U46619 and 60 mM potassium-rich solutions induced stable contractions of 3.0 ± 0.6 Nm^{-1}, 1.4 ± 0.3 Nm^{-1}, and 1.8 ± 0.2 Nm^{-1}, respectively ($n = 6$). (−)-Stepholidine caused relaxations in vessels contracted by noradrenaline with pD_2 values and maximal relaxations of 5.59 ± 0.14 and 89.8 ± 5.9% ($n = 6$), respectively. However, in the vessels contracted by U46619 and 60 mM K^+-rich solution, the relaxant effect of (−)-stepholidine was small (Fig. 3). Thus, the relaxations induced by 10^{-4} M (−)-stepholidine in U44619 and 60 mM K^+-contracted preparations were 29.9 ± 13.0% ($n = 5$) and 33.9 ± 2.8% ($n = 5$).

In noradrenaline contracted arteries, (−)-stepholidine induced relaxations which were unchanged after mechanical removal of the endothelium (Fig. 4). Acetylcholine (10^{-5} M) relaxed endothelium intact segments with 53.7 ± 10.4% ($n = 6$), but it did not cause significant relaxation (1.8 ± 1.0%, $n = 6$) in endothelium denuded arteries (Fig. 4).

The muscarinic receptor antagonist, atropine (10^{-7} M), and the β-adrenoceptor antagonist, propranolol (10^{-6} M),

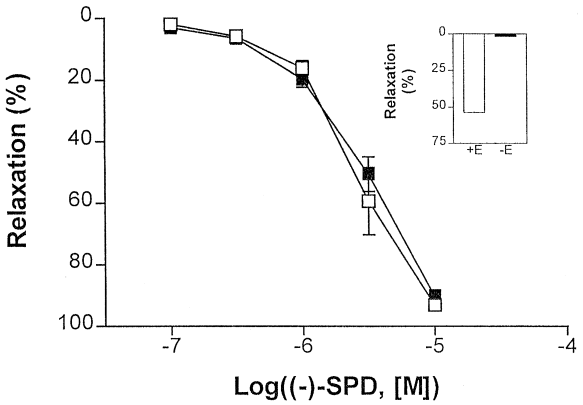

Fig. 4. The average relaxations induced (−)-stepholidine in endothelium-intact (+E, open squares) and endothelium-denuded (−E, closed squares) rat mesenteric small arteries contracted by 10^{-6} M noradrenaline. The pD_2 values and maximal relaxations induced by (−)-stepholidine were 5.51 ± 0.11 and 93.1 ± 2.3%, and 5.41 ± 0.11 and 91.9 ± 2.3% ($n = 6$) in endothelium-intact and -denuded arteries, respectively. Inset shows the relaxations induced by acetylcholine (10^{-5} M) before and after endothelial cell removal. Relaxations are expressed as percentage of the response induced by noradrenaline. The results are means ± S.E.M. of six experiments.

caused significant rightward shifts of the concentration–response curves for acetylcholine and isoprenaline, respectively, but did not inhibit the relaxations induced by (−)-stepholidine (10^{-7}–10^{-5} M) in noradrenaline contracted preparations (six experiments with each treatment). Conversely, in arteries contracted with U46619 (10^{-7} M), acetylcholine (10^{-9}–10^{-5} M) induced relaxations with similar potency and maximal relaxations in the absence and presence of (−)-stepholidine (10^{-6}–10^{-5} M). The pD_2 values and maximal responses were 7.52 ± 0.16 and 80.7 ± 6.5% ($n = 6$) in the absence, and 7.43 ± 0.39 and 85.3 ± 4.3% ($n = 6$) in the presence of 10^{-5} M (−)-stepholidine. In arteries treated with phentolamine (10^{-6} M) and contracted with U46619 (10^{-8}–10^{-7} M), the β-adrenoceptor agonist, isoprenaline (10^{-9}–10^{-5} M) induced concentration-dependent relaxations. The pD_2 values and maximal relaxations were 7.44 ± 0.32 and 86.5 ± 6.2%, respectively, in the absence, and 7.59 ± 0.14 and 93.1 ± 1.9% ($n = 6$), respectively, in the presence of 10^{-5} M (−)-stepholidine.

(−)-Stepholidine (10^{-7}–10^{-4} M) relaxed vessels contracted with either noradrenaline or noradrenaline and 20 mM K^+ with the same potency and maximum relaxation, but the relaxations induced with the K^+ channel opener, pinacidil (10^{-7}–10^{-4} M), were significantly reduced in noradrenaline and 20 mM K^+ contracted preparations compared to preparations only contracted with noradrenaline (Table 1). Glibenclamide (10^{-6} M), a blocker of ATP-sensitive K^+ channels, did also inhibit pinacidil-induced relaxations, but did not change the (−)-stepholidine elicited relaxations (Table 1). TEA, a blocker of Ca^{2+}-activated K^+ channels, caused a small shift in the concen-

Table 1
The effect of 20 mM K^+, 10^{-6} glibenclamide and tetraethylammonium (TEA) on the relaxations induced by (−)-stepholidine and the K^+ channel opener, pinacidil, in rat mesenteric small arteries contracted with noradrenaline

	(−)-Stepholidine		Pinacidil	
	pD_2 ($-\log(EC_{50})$)	Maximum relaxation (%)	pD_2 ($-\log(EC_{50})$)	Maximum relaxation (%)
Control	5.40 ± 0.15 (16)	9.57 ± 0.8 (16)	5.12 ± 0.14 (6)	96.3 ± 0.7 (6)
20 mM K^+	5.03 ± 0.12 (6)	92.8 ± 3.0 (6)	4.24 ± 0.10 (6)[a]	87.6 ± 2.1 (6)[a]
Glibenclamide	5.57 ± 0.15 (6)	97.5 ± 0.6 (6)	4.52 ± 0.13 (6)[a]	92.8 ± 2.5 (6)
TEA 10^{-3} M	5.22 ± 0.23 (9)	90.6 ± 3.0 (9)	–	–
TEA 10^{-2} M	4.86 ± 0.23 (7)	90.5 ± 2.5 (7)	–	–

Values are means ± S.E.M.
n = number of vessels.
EC_{50} is the concentration of agonist required to produce half-maximal relaxation and maximum relaxation is the response obtained at the highest concentration of agonist applied.
[a] $P < 0.05$ compared to the first control curve response (repeated measure ANOVA, followed by paired t-test).

tration–relaxation curves to (−)-stepholidine only at the highest concentration of TEA (10^{-2} M) (Table 1).

3.3. Effect of (−)-stepholidine on contractions induced by 5-HT, noradrenaline, and EFS

5-HT did not induce stable contractions, and therefore, the effect of (−)-stepholidine was evaluated on concentration–response curves for 5-HT. In the presence of 10^{-6} M cocaine and 10^{-6} M phentolamine, 5-HT induced reproducible contractions in mesenteric small arteries, pD_2 maximum response being 5.82 ± 0.05 and 3.2 ± 0.1 Nm^{-1} ($n = 6$). These contractions were unchanged in the presence of (−)-stepholidine (10^{-6}–10^{-5} M). Thus, in the presence of 10^{-5} M (−)-stepholidine, 5-HT induced con-

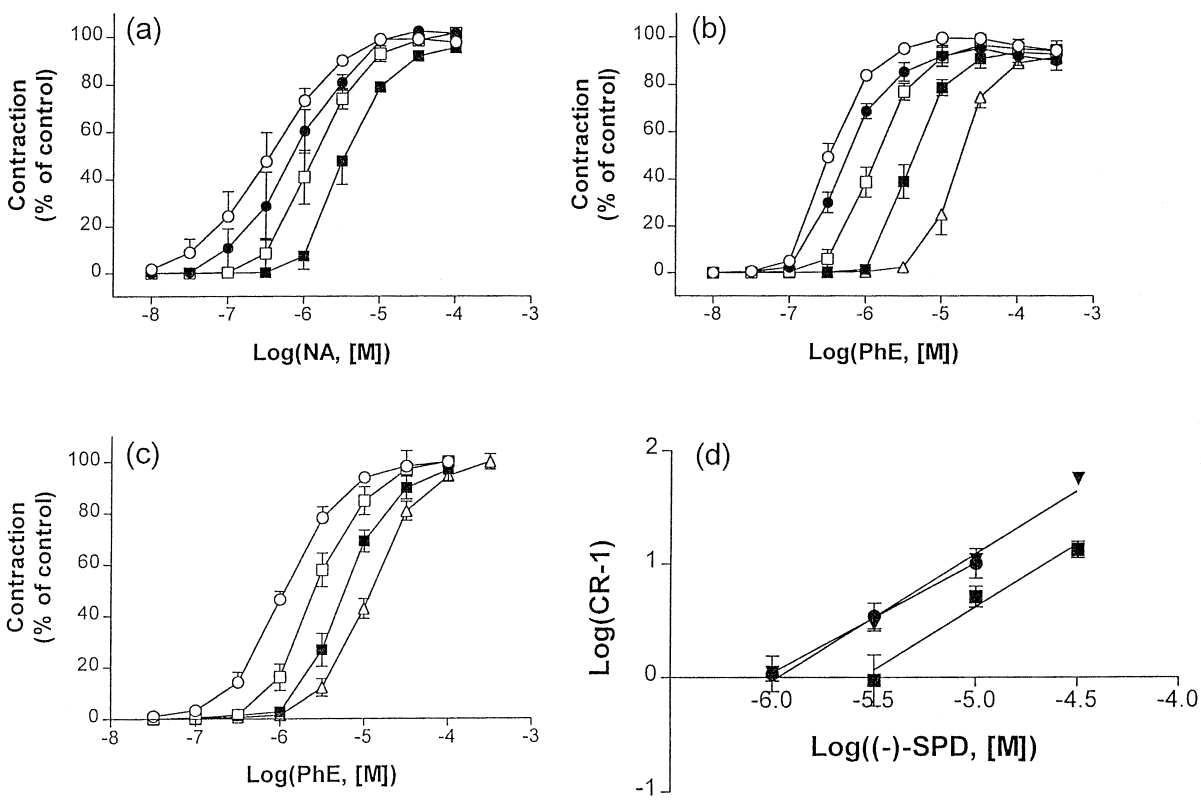

Fig. 5. Cumulative concentration–response curves for (a) noradrenaline (NA); (b) phenylephrine (PhE) in endothelium-denuded rat mesenteric small arteries; (c) phenylephrine in rat aorta segments, obtained in the absence (open circles) and presence of 10^{-6} M (closed circles), 3×10^{-6} M (open squares), 10^{-5} M (closed squares) and 3×10^{-5} M (open triangles) (−)-stepholidine; and (d) the Schild regressions for the effect of (−)-stepholidine on concentration–response curves for noradrenaline (closed circles) and phenylephrine (closed squares) in mesenteric small arteries, and for phenylephrine in aorta (closed squares). The experiments were performed in the presence of 10^{-6} M cocaine, 10^{-6} M propranolol and in aorta also in the presence of 10^{-7} M yohimbine. The results are means ± S.E.M. of 6–10 experiments.

tractions with pD_2 values of 5.75 ± 0.06 and maximum of 3.1 ± 0.1 Nm^{-1} ($n = 6$). However, the 5-HT induced contractions were significantly inhibited by the 5-hydroxytryptamine 5-HT_2 receptor antagonist, ketanserin (10^{-8} M) (data not shown).

Noradrenaline and phenylephrine produced reproducible concentration–response curves in rat mesenteric small arteries. (−)-Stepholidine (10^{-6}–10^{-5} M) competitively antagonized the contractions to noradrenaline and phenylephrine in rat mesenteric small arteries (Fig. 5a,b). The Schild plots for (−)-stepholidine against noradrenaline and phenylephrine gave slopes (0.97 ± 0.15 and 1.12 ± 0.10 against noradrenaline and phenylephrine, respectively) not different from unity and pA_2 values of 6.05 ± 0.10 ($n = 6$, noradrenaline) and 5.94 ± 0.16 ($n = 10$, phenylephrine) (Fig. 5d).

In a separate series of experiments, it was clarified whether (−)-stepholidine has the same affinity to the α_1-adrenoceptor subtype in rat aorta. (−)-Stepholidine also caused rightward displacements of the phenylephrine concentration–response curves in aorta segments (Fig. 5c). The Schild plot of (−)-stepholidine against phenylephrine gave a straight line with a slope (1.13 ± 0.17) not different from unity and a pA_2 value of 5.56 ± 0.11 ($n = 14$, Fig. 5d).

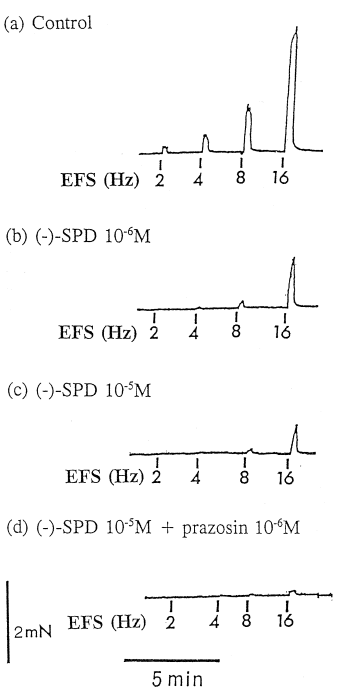

Fig. 6. The effect of (−)-stepholidine in rat mesenteric small arteries stimulated by increasing frequencies (2–16 Hz) of electrical field stimulation (EFS) with 0.25 ms pulse duration in 20 s trains and current adjusted to 60 mA. Isometric tension recordings showing the response in (a) control conditions, (b) in the presence of 10^{-6} M (−)-stepholidine ((−)-SPD), (c) 10^{-5} M (−)-stepholidine and (d) 10^{-6} M prazosin. The experiments were performed in the presence of 10^{-6} M cocaine, 10^{-6} M corticosterone and 10^{-6} M propranolol. W = wash.

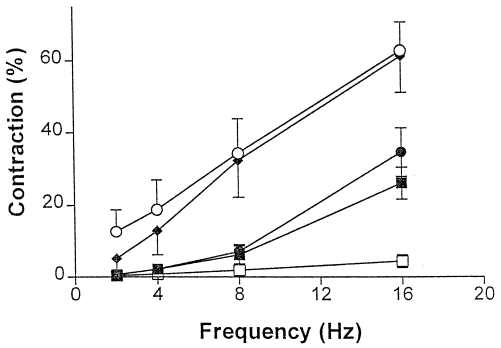

Fig. 7. The average of the responses to electrical field stimulation (EFS) in the absence (open circles) and the presence of 10^{-7} M (closed diamonds), 10^{-6} M (closed circles) and 10^{-5} M (closed squares) (−)-stepholidine ((−)-SPD), and 10^{-6} M prazosin (open squares). The experiments were performed in the presence of 10^{-6} M cocaine, 10^{-6} M corticosterone and 10^{-6} M propranolol. The contractions to EFS are expressed as percentage of the response induced by 10^{-5} M noradrenaline. Results are means ± S.E.M. of six experiments.

In endothelium-denuded mesenteric small arteries and in the presence of propranolol (10^{-6} M), corticosterone (10^{-6} M) and cocaine (10^{-6} M), EFS (0.25 ms, 20 s, 60 mA) induced tetrodotoxin-sensitive frequency-dependent (2–16 Hz) reproducible contractions in mesenteric small arteries. The contractions induced by 16 Hz in control conditions corresponded to $69 \pm 16\%$ ($n = 7$) of the response to 125 mM K^+. These contractions were unchanged in the presence of 10^{-7} M (−)-stepholidine, but significantly inhibited by 10^{-6} M (−)-stepholidine, and totally abolished in the presence of the α_1-adrenoceptor antagonist prazosin (Figs. 6 and 7).

3.4. Binding studies

The specific binding of 0.2 nM [^{3}H]-prazosin to the α_1-adrenoceptors of the cerebral cortex membranes was completely inhibited by WB4101 yielding an IC_{50} value of 0.8 nM ($pK_i = 9.39$, three experiments, results not shown). (−)-Stepholidine (10^{-7}–10^{-4} M) did also completely inhibit the specific binding of [^{3}H]prazosin with a half-maximal effect of $4.7 \pm 0.2 \times 10^{-6}$ M ($n = 4$) (Fig. 8). The calculated pK_i for (−)-stepholidine was 5.63 ± 0.23 ($n = 4$).

3.5. Effect of (−)-stepholidine on $CaCl_2$-induced contractions

(−)-Stepholidine (3×10^{-5}–10^{-4} M) induced small relaxations in vessels contracted with 125 mM K^+ even in the presence of phentolamine to block contractile α-adrenoceptors. Therefore, the effect of (−)-stepholidine was evaluated on the contractions induced by increasing concentrations of $CaCl_2$ (10^{-5}–10^{-2} M) in high K^+ solution (125 mM). In control conditions, the pD_2 values and maximal contractions were 3.05 ± 0.08 and 3.7 ± 0.4

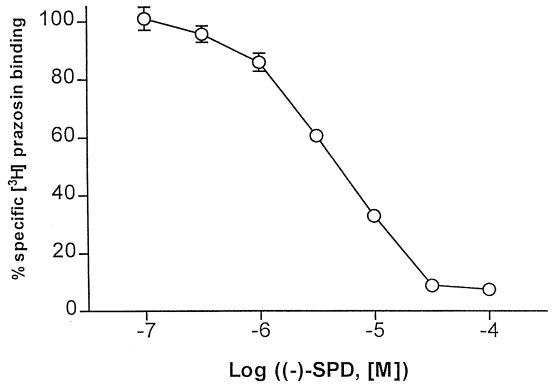

Fig. 8. Displacement of specific [^{3}H]prazosin (0.2 nM) binding from rat cerebral cortex membranes by (−)-stepholidine (open circles). Values represent the means ± S.E.M. of four individual experiments performed in triplicate.

Nm^{-1} ($n = 6$). (−)-Stepholidine (10^{-5}–10^{-4} M) had no influence on the contractile concentration–response curves for $CaCl_2$. The pD_2 and maximum were 2.90 ± 0.08 and 4.2 ± 0.4 Nm^{-1} ($n = 6$), respectively, in the presence of 10^{-4} M (−)-stepholidine. Nifedipine (10^{-7} M), a blocker of L-type voltage-dependent Ca^{2+} channels, abolished the contractions of mesenteric small arteries induced by $CaCl_2$ solution in high K^+ solution ($n = 6$).

4. Discussion

The present investigation suggests that the hypotensive action of the dopamine antagonist (−)-stepholidine might, in part, be explained by an antagonistic effect of α-adrenoceptors in peripheral arteries such as in mesenteric small arteries, where it inhibits the contractions to both exogenous and endogenous noradrenaline. Several other possibilities such as endothelium-dependent relaxation, K^+ channel opening and blockade of Ca^{2+} entry have been excluded.

4.1. Effect of (−)-stepholidine on dopamine receptors in mesenteric small arteries

Dopamine receptors were originally classified into two main groups, and the five cloned dopamine receptors fall into these classes, such that the dopamine D_1-like receptors include D_1 and D_5, and dopamine D_2-like include D_2, D_3 and D_4 receptors (Seeman and Van Tol, 1994). α_1-Adrenoceptors were shown to mediate vasoconstriction, and both postjunctional dopamine D_2 receptors were suggested to mediate the vasorelaxant responses to dopamine in rabbit mesenteric small arteries (Münch et al., 1991), although dopamine induced repolarization of these arteries was only found to be mediated by dopamine D_1 receptors (Illes and Nörenberg, 1989). Moreover, in cultures of rat mesenteric artery smooth muscle the dopamine receptor coupled to cyclic AMP formation was suggested to belong to the dopamine D_1 receptor family (Hall et al., 1993). The present agonist study suggests that dopamine does not induce contraction, and that dopamine D_1 receptors mediate dopamine-induced relaxations, since dopamine and the partial dopamine D_1 receptor agonist, SKF-38393, induced relaxations, while the dopamine D_2 receptor agonist, quinpirole, had no relaxant effect. SCH-23390, which is a potent and selective dopamine D_1 receptor antagonist (Hyttel, 1983), competitively antagonized the dopamine-induced relaxations in rat mesenteric arteries, giving pA_2 values (8.67) in the range for postjunctional dopamine D_1 receptors (Hyttel, 1983; Hall et al., 1993). The present finding that dopamine induced potent relaxations in the presence of the dopamine D_2 receptor selective antagonist, (−)-sulpiride, suggests dopamine D_2 receptors are not involved in these relaxations. Thus, the present results suggest that dopamine D_1 receptors mediate the relaxations induced by dopamine in rat mesenteric small arteries.

In radioligand experiments, (−)-stepholidine was found to have high affinity for both dopamine D_1 and D_2 receptors with preferential affinity to dopamine D_1 receptors (Jin, 1987). The aporphines, which are structurally related to the protoberberines, such as apomorphine are dopamine agonists which preferentially activate D_1 receptors (Seeman and Van Tol, 1994). (−)-Stepholidine induces contralateral rotation in 6-hydroxydopamine lesioned rats and was suggested also to be a dopamine D_1 receptor agonist (Jin et al., 1992; Sun and Jin, 1992), but (−)-stepholidine antagonizes dopamine induced increases in cyclic AMP formation, which is a dopamine D_1 receptor mediated effect (Jin, 1987). Our finding in isolated rat mesenteric arteries that (−)-stepholidine, in contrast to dopamine and SKF-38393, did not induce relaxations of U46619 contracted preparations suggests that (−)-stepholidine is not a dopamine D_1 receptor agonist. (−)-Stepholidine competitively antagonized the dopamine-induced relaxations, although with less potency than the dopamine D_1 receptor selective antagonist, SCH-23390, but these results suggest that (−)-stepholidine behave as a dopamine D_1 receptor antagonist in rat mesenteric small arteries.

The present study suggests that (−)-stepholidine is selectively inhibiting the dopamine-induced relaxations, since (−)-stepholidine did not change the relaxations induced by the muscarinic receptor agonist, acetylcholine, or the adrenoceptor agonist, isoprenaline. However, the effect of (−)-stepholidine on the dopamine receptors cannot explain a hypotensive action of the drug.

4.2. Vasorelaxant effect of (−)-stepholidine in mesenteric small arteries

(−)-Stepholidine concentration-dependently relaxed noradrenaline contracted mesenteric small arteries, while it did not relax vessels contracted by the thromboxane ana-

logue, U46619. The endothelium-dependent relaxations in rat mesenteric small arteries appear to be mediated through the release of both endothelium-derived relaxing factor (EDRF) and endothelium-derived hyperpolarizing factor (EDHF) dependent on the agonist (i.e., noradrenaline or U46619) applied to contract the vessel segment (Plane and Garland, 1996). Moreover, rutaecarpine, an quinazoline alkaloid, was recently shown to induce endothelium-dependent relaxations in rat mesenteric arteries (Chiou et al., 1997). However, since mechanical endothelial cell removal, which abolished the relaxations to acetylcholine, did not change the relaxations to (−)-stepholidine, this suggests that release of endothelium-derived relaxing factors does not play a role in these relaxations.

The benzylisoquinolines such as papaverine and aporphines such as glaucine, which are structurally related to the protoberberines, inhibit phosphodiesterases isolated from vascular smooth muscle (Ivorra et al., 1992; Chuliá et al., 1994). However, the lack of effect of (−)-stepholidine on relaxations induced by acetylcholine or isoprenaline, suggest that (−)-stepholidine does not have a main inhibitory effect on the phosphodiesterases.

The isoquinazolinocarboline alkaloid, dehydroevodiamine, was recently shown to induce relaxation through opening of K^+ channels (Chiou et al., 1996). A possible role for K^+ channel involvement in (−)-stepholidine-induced relaxation comes from our finding that (−)-stepholidine did not relax small arteries contracted by raising extracellular K^+ in contrast to noradrenaline contracted vessels. In contrast to the K^+ channel opener, pinacidil, the (−)-stepholidine-induced relaxations remained unchanged when raising extracellular K^+ concentration to 20 mM. Moreover, since the non-selective K^+ channel blocker, TEA (Benham et al., 1985), only had a small effect at the highest concentration, while the blocker of ATP-sensitive K^+ channels, glibenclamide (Quast and Cook, 1989) had no effect, on the relaxations to (−)-stepholidine, these results suggest that glibenclamide and TEA-sensitive K^+ channels are not involved in the vasorelaxant effect of (−)-stepholidine in rat mesenteric small arteries.

4.3. Effect of (−)-stepholidine on contractions induced by noradrenaline and EFS

The finding of a selective inhibitory effect of (−)-stepholidine in noradrenaline- and phenylephrine-contracted compared to U44619, 5-HT, and K^+-activated preparations could be ascribed to an action on α-adrenceptors. The antagonism of (−)-stepholidine against the α-adrenoceptor agonists appeared competitive, since it caused parallel rightward shifts in the concentration–response curves without changing the maximal responses. The slopes of the Arunlakshana–Schild regression lines for (−)-stepholidine did not significantly differ from unity, which suggests that the contractions to noradrenaline and phenylephrine are mediated by a homogenous population of α_1-adrenoceptors now identified as α_{1A}-adrenoceptors (Chen et al., 1996; Ipsen et al., 1997). Noradrenaline can cause relaxation of rat mesenteric small arteries through activation of dopamine D_1 receptors and only α-adrenoceptor antagonists, which are also dopamine antagonists, produce a Schild plot with unit slope, when the arteries are activated with noradrenaline (Van der Graaf et al., 1995). In rat mesenteric small arteries, the contractions to noradrenaline are competitively antagonized by the selective α_1-adrenoceptor antagonist, prazosin (Nielsen et al., 1991), while postjunctional α_2-adrenoceptors are not directly coupled to a contractile mechanism (Heesen and De Mey, 1990; Nielsen et al., 1991). In human subcutaneous small arteries where postjunctional α_2-adrenoceptors are present (Nielsen et al., 1991), (−)-stepholidine antagonized with estimated pK_B values of 5.76 the contractions to the α_2-adrenoceptor agonist, 2-amino-6-ethyl-4,5,6,7-tetrahydro-6*H*-oxozalo-(5,4-d)-azepin dihydrochloride (BHT 933) (Lei et al., unpublished observations). In rat mesenteric small arteries, (−)-stepholidine was less potent (6.0) compared to prazosin (9.2; Nielsen et al., 1991), but the present results suggest that it is an α-adrenoceptor antagonist.

Aporphines such as glaucine, and boldine, which are structurally related to the protoberberines, exhibited selectivity for α_{1A}-adrenoceptors compared to other α_1-adrenoceptor subtypes in [^{3}H]prazosin competition binding in rat cerebral cortex (Madrero et al., 1996). In contrast to rat mesenteric small arteries, where the α_{1A}-adrenoceptors mediate contractions to noradrenaline (Chen et al., 1996; Ipsen et al., 1997), the α_{1D}-adrenoceptors appear to predominate in rat aorta (Han et al., 1990; Testa et al., 1995; Deng et al., 1996; Ipsen et al., 1997). In the present study, the pA_2 values obtained for (−)-stepholidine in rat aorta rings were only slightly different from those of (−)-stepholidine against phenylephrine in rat mesenteric small arteries, and this suggests that (−)-stepholidine is a general α_1-adrenoceptor blocker. This is further supported by the binding experiments in the present study showing that (−)-stepholidine displayed a monophasic competition curve for [^{3}H]prazosin binding to rat cortical membranes.

EFS of mesenteric small arteries performed at resting tension induces release of mainly noradrenaline, which mediates the increases in force, while the other co-released neurotransmitters such as ATP and neuropeptide Y modulate the response to noradrenaline (Angus et al., 1988; Sjöblom-Widfeldt, 1990; Donoso et al., 1997). Prazosin almost abolished the neurogenic contractions in the mesenteric small arteries in the present study supporting that α_1-adrenoceptors mediate these responses. (−)-Stepholidine also inhibited the responses to EFS in rat mesenteric arteries suggesting that it antagonizes contractions both to exogenously added and endogenously released noradrenaline. This latter effect might contribute to the hypotensive effect of the drug.

4.4. Involvement of potential-operated Ca^{2+} channels in the effect of (−)-stepholidine

(−)-Stepholidine in high concentrations (10^{-5}–10^{-4} M) was reported to induce rightward shifts in the concentration–response curves to $CaCl_2$ in aorta (Yang et al., 1993) suggesting that it could cause relaxation through inhibition of Ca^{2+} entry. The highest concentrations of (−)-stepholidine applied did induce relaxations of 60 mM K^+-induced contractions in mesenteric arteries suggesting an additional relaxation mechanisms of the alkaloid. The antagonist of L-type voltage operated Ca^{2+} channels, nifedipine, blocked the contractions of rat mesenteric arteries evoked by increasing the external Ca^{2+} concentration in a high K^+ solution, but (−)-stepholidine had no effect suggesting that this mechanism is not implicated in the relaxations to (−)-stepholidine. The discrepancy between the earlier mentioned studies suggesting that (−)-stepholidine is a Ca^{2+} antagonist (Yang et al., 1993) and the present study can probably be ascribed to the presence of the α-adrenoceptor blocker, phentolamine, in the present study. This excludes the contribution from nerve-released noradrenaline in the contractile concentration–response curves obtained to $CaCl_2$ in high K^+. Therefore, these results suggest that the vasorelaxant effects of (−)-stepholidine are not due to blocking of voltage-dependent Ca^{2+} entry.

In summary, the present investigation suggests that (−)-stepholidine is a competitive antagonist of vasoconstrictory α_1-adrenoceptors and also inhibits vasodilating dopamine receptors in mesenteric small arteries. The inhibitory effect of (−)-stepholidine on the contractions to exogenously added and nerve-released noradrenaline in peripheral small arteries might contribute to the hypotensive effect of the drug.

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