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Relaxant and Ca^{2+} channel blocking properties of norbormide on rat non-vascular smooth muscles

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Abstract

We have investigated the effects of the rat-specific vasoconstrictor agent norbormide on the mechanical and electrophysiological properties of rat non-vascular smooth muscles. Norbormide (50 μM) did not affect the resting tone of urinary bladder, tracheal, and duodenal rings. In all tissues, KCl-induced concentration–response curves were shifted downward by norbormide (5 and 50 μM). In urinary bladder and tracheal rings, norbormide inhibited contractile responses to carbachol only at the higher concentration (50 μM). In single gastric fundus myocytes, 50 μM norbormide inhibited L-type Ca^{2+} current ($I_{Ca(L)}$) by about 60%, neither affecting both activation and inactivation rates of the current nor the current–voltage curve along the voltage axis. Our results indicate that rat non-vascular smooth muscles are relaxed by norbormide with a mechanism likely involving a reduction of Ca^{2+} entry through L-type Ca^{2+} channels.

Keywords: Norbormide; (Rat); Trachea; Urinary bladder; Duodenum; Ca^{2+} channel L-type

1. Introduction

Norbormide, 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene)-5 norbormene-2,3-dicarboximide (Fig. 1), is a selective rat toxicant described for the first time by Roszkowski et al. (1964). In vivo studies have shown that norbormide causes death in the rat species by inducing a severe and irreversible vasoconstriction of the peripheral arteries which has never been observed in non-rat species (Roszkowski, 1965). In vitro studies have revealed that norbormide, at concentrations almost overlapping those inducing the contractile effect in rat peripheral arteries, exhibits vasorelaxant properties in small and large arteries of several non-rat species (Bova et al., 1996). Surprisingly, a relaxant action of norbormide has been demonstrated also in rat aorta, indicating that the drug possesses opposite effects in the rat arterial bed (Bova et al., 1996).

On the basis of these observations, we have wondered which effect norbormide could induce in rat non-vascular smooth muscles. In this study, we have therefore investigated the actions of norbormide on rat respiratory, urinary, and gastrointestinal smooth muscles. Our results demonstrate that none of these muscles is contracted by norbormide. Furthermore, our data show that all smooth muscles tested are relaxed by the drug with a pattern of action similar to that described for well known Ca^{2+} entry blocker agents.

2. Material and methods

The experiments have been carried out in accordance with the European Community guidelines for the use of experimental animals and have been approved by the Italian Ethics Committee.

2.1. Recording of mechanical force in tracheal, urinary bladder, and duodenal rings

Contractions of rat tracheal, urinary bladder, and duodenal smooth muscle rings were recorded as previously described for aortic and rat caudal rings (Bova et al., 1996). Briefly, male Wistar–Kyoto rats (250–300 g) were

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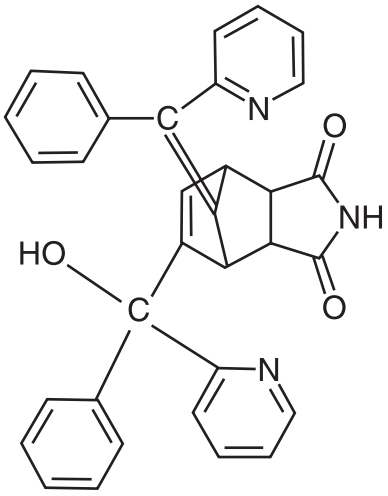

Fig. 1. Structure of norbormide.

killed by cervical dislocation and trachea, urinary bladder, and duodenum were removed, cleaned of the connective tissue, and cut into rings of 2-mm length. The rings were vertically suspended between 100 μm tungsten wires in organ baths filled with 15 ml of physiological salt solution (PSS) (see below for composition) and tension was recorded on a pen recorder (Ugo Basile, Italy) via an isometric force displacement transducer (Ugo Basile). Rings were stretched passively to impose resting tensions (2.0 g, trachea; 1.5 g, urinary bladder; 1.0 g, duodenum) found to be optimal by testing their response to carbachol at different imposed tensions. Rings were allowed to equilibrate for 60 min and then repeatedly stimulated with carbachol (10 μM) until reproducible contractile responses were obtained. In the case of duodenal rings, to minimize their spontaneous oscillatory contractions, they were left at 4 °C for 24 h before use.

2.2. Contraction experiments

The effects of norbormide (5 and 50 μM) on either KCl- or agonist-induced contractions have been studied using the following general experimental protocol: (1) induction of a concentration–response curve for KCl or carbachol; (2) washout for 2 h; (3) pre-treatment of the preparations with norbormide for 30 min; (4) induction of a second concentration–response curve for KCl or carbachol.

The higher norbormide concentration used in this study (50 μM) was the same that induced maximal relaxation in rat aorta pre-contracted by KCl (Bova et al., 1996); the lower concentration of the drug was arbitrarily chosen.

The concentration–response curves were constructed cumulatively for either KCl or carbachol. In the case of duodenum, norbormide was tested only on KCl-induced contraction due to the fact that, in this muscle, carbachol could induce only phasic, oscillatory contractile responses. Furthermore, since norbormide is hardly washed-out, each concentration of the drug was tested in separate rings.

2.3. Patch-clamp experiments

2.3.1. Cell isolation procedure

Smooth muscle cells were freshly isolated by collagenase treatment of gastric fundus strips obtained from male rats (250–300 g), anaesthetised with ketamine (Gellini, Italy), decapitated, and drained of blood as previously described (Fusi et al., 2001). Tissue specimens were digested for 40–45 min at 37 °C in 2 ml of nitrate-rich digestion solution (see below for composition) containing 2 mg collagenase (type I), 2.5 mg bovine serum albumin, and 3 mg soybean trypsin inhibitor, bubbled with 95% O_2 and 5% CO_2.

Thereafter, cells were mechanically dispersed in a Kraft-brühe (KB) solution (Isenberg and Klockner, 1982), modified with a plastic pipette, and used for experiments within 10 h after isolation. During this time, they were stored at 4 °C in KB solution containing bovine serum albumin.

An aliquot of the cell suspension (40–150 μl) was transferred to a small recording chamber (250 μl) mounted on the stage of an inverted phase-contrast microscope (TE300, Nikon, Japan) and monitored with a video camera (JVC TK-1280E). Immediately after harvest, smooth muscle cells were relaxed and elongated in shape, 3–10 μm wide and 150–300 μm long. They responded to mechanical and pharmacological stimulation (e.g. 1 μM acetylcholine) by contracting and changing the texture of the cell surface from a smooth contour to one overwhelmed by bulbous and mound-like evaginations, as previously described (Fay and Delise, 1973).

After adhesion of the cells to the glass bottom of the chamber (20 min), they were continuously superfused with external solution (see below) by means of a peristaltic pump (LKB 2132) at a flow rate of 500 μl min^{-1}. Electrophysiological responses were tested at room temperature (22–24 °C) only in cells that were phase dense.

2.3.2. Whole-cell patch-clamp recording

Conventional (Hamill et al., 1981) whole-cell patch-clamp method was employed to voltage-clamp smooth muscle cells. Recording electrodes were pulled from borosilicate glass capillaries (WPI, Berlin, Germany) and fire-polished to create a pipette resistance of 2–5 MΩ when filled with internal solution. A low-noise, high-performance Axopatch 200B (Axon Instruments, USA) patch-clamp amplifier driven by an IBM computer in conjunction with an A/D, D/A board (DigiData 1200 A/B series interface, Axon Instruments) was used to generate and apply voltage pulses to the clamped cells and record the corresponding membrane currents. Current signals, after compensation for whole-cell capacitance, series resistance, and liquid junction potential, were low-pass filtered at 1 kHz and digitised at 3 kHz prior to being stored on the computer hard disk. Long-lasting inward $I_{Ca(L)}$ was always recorded in 2.5 mM Ca^{2+}-containing external solution.

$I_{Ca(L)}$ was measured over a range of test potentials (250 ms) from −50 to 70 mV from a holding potential (V_h) of

− 50 mV. Data were collected once the current amplitude had been stabilized (usually 5–10 min after the whole-cell configuration had been obtained). Under these conditions, $I_{Ca(L)}$ did not run down during the following 30–40 min.

K^+ currents were blocked with 30 mM tetraethylammonium in the external solution and Cs^+ in the internal solution. Current values were corrected for leakage using the P/4 protocol.

The current–voltage relationships were calculated on the basis of the peak values (leakage corrected) from the original currents.

2.4. Solutions

Standard PSS contained (mM): NaCl 125, KCl 5, $CaCl_2$ 2.7, $MgSO_4$ 1, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11, at pH 7.35, maintained at 37 °C and bubbled with 95% O_2 and 5% CO_2. High potassium solutions were made by adding KCl from a concentrated solution (2.4 M) to the organ bath. Nitrate-rich digestion solution, to isolate myocytes, contained the following compounds (in mM): 55 NaCl, 65 $NaNO_3$, 5 KCl, 5 Na-pyruvate, 10 glucose, 10 taurine, 10 HEPES, and 1.2 $MgCl_2$; pH was adjusted to 7.4 with NaOH. The modified KB solution used to foster tissue disaggregation contained 1 mg bovine serum albumin and (in mM) 105 NaCl, 7 KH_2PO_4, 5 KCl, 5 glucose, 10 taurine, 10 HEPES, 1.6 $MgCl_2$, 2.5 Na-pyruvate, 1.7 creatine, 2 oxalacetate, 1.5 Na_2ATP, and 0.1 EGTA; pH was adjusted to 7.25 with NaOH. To isolate $I_{Ca(L)}$, the pipette was filled with a high Cs^+ solution (internal solution) of the following composition (in mM): 105 CsCl, 10 HEPES, 11 EGTA, 2 $MgCl_2$, 1 $CaCl_2$, 5 Na-pyruvate, 5 succinic acid, 5 oxalacetic acid, 3 Na_2ATP, and 5 phosphocreatine; pH was adjusted to 7.4 with CsOH. The free Ca^{2+} concentration in this solution was estimated to be less than 4 nM. The bath solution (external solution) contained (in mM): 135 NaCl, 5.6 KCl, 5 Na-pyruvate, 20 glucose, 30 tetraethylammonium, 10 HEPES, 2.5 Ca^{2+}, and 1.2 $MgCl_2$; pH was adjusted to 7.4 with NaOH. Osmolarity of the external solution was adjusted to 315 mosM, and that of the internal one to 290 mosM with the use of an osmometer (Osmostat OM 6020, Menarini Diagnostics, Italy). After control measurements, each cell was exposed to drugs by perfusing the experimental chamber with a drug-containing external solution.

2.5. Drugs

Norbormide was a kind gift of I.N.D.I.A. (Padova, Italy). Carbachol chloride, collagenase (type I), tetraethylammonium, bovine serum albumin, trypsin inhibitor, and nifedipine were from Sigma (St. Louis, MO, USA). Norbormide was dissolved in dimethylformamide and nifedipine in ethanol to obtain stock solutions of 50 and 1 mM, respectively. The maximum concentration of dimethylformamide was 0.1% and had no effect on the contractile responses of the smooth muscle rings to KCl or carbachol as well as on $I_{Ca(L)}$. All the other drugs were dissolved in twice distilled water.

2.6. Statistical analysis

Data are reported as mean ± S.E.M. Statistical analyses and significance, as measured by the Student's *t*-test for paired samples or analysis of variance (ANOVA) followed by Bonferroni post-test, as appropriate, were performed using GraphPad InStat version 3.05 (GraphPad Software, USA). In all comparisons, $P < 0.05$ was considered significant.

3. Results

3.1. Effect of norbormide on resting tension of rat tracheal, duodenal, and urinary bladder smooth muscle rings

Resting tracheal, duodenal, and urinary bladder muscular rings exposed to a norbormide concentration that maximally contracts rat peripheral arteries (Bova et al., 1996) did not show any change in baseline tension (Fig. 2). On the other hand, these preparations were strongly contracted by 10 μM carbachol, an agonist of muscarinic acetylcholine receptors coupled to the phospholipase C pathway.

3.2. Effect of norbormide on KCL- and carbachol-induced contractions in rat tracheal rings

In the rat trachea, both KCl (30, 60, and 90 mM) and carbachol (0.1–100 μM) induced a concentration-dependent

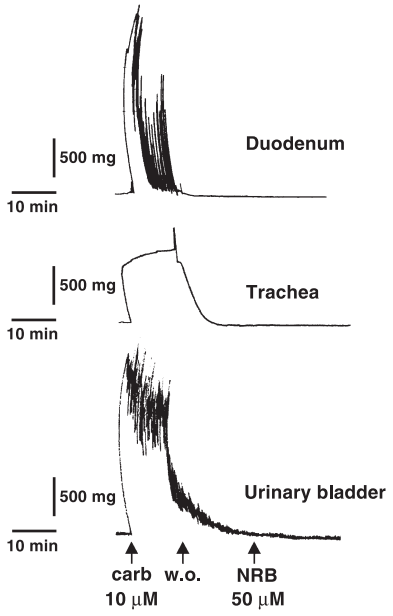

Fig. 2. Original records showing the lack of contractile effect of 50 μM norbormide (NRB) on resting rat duodenal, tracheal, and urinary bladder smooth muscle rings. Carbachol (carb) was used to test the capacity of the preparations to contract in response to stimulation with a phospholipase C-coupled receptor agonist.

contractile effect whose maximum values were 600 ± 50 mg for KCl ($n = 17$) and 1150 ± 80 mg for carbachol ($n = 17$). Thirty minutes of pre-treatment with either 5 or 50 μM norbormide significantly shifted downward the concentration–response curve to KCl with a maximum inhibitory effect of 25% at the lower concentration and 56% at the higher concentration (Fig. 3A). Carbachol concentration–response curve was not significantly affected by 5 μM norbormide, while it was shifted downward by 50 μM norbormide with a 10% decrease of the maximal contractile response (Fig. 3B). The EC_{50s} in the absence and the presence of 50 μM norbormide were 0.8 ± 0.2 and 1.2 ± 0.3 μM, respectively.

3.3. Effect of norbormide on KCL- and carbachol-induced contractions in rat urinary bladder rings

In the rat urinary bladder, both KCl (30, 60, and 90 mM) and carbachol (0.05–50 μM) induced a concentration-dependent contractile effect whose maximum values were 1693 ± 177 mg for KCl ($n = 17$) and 2150 ± 158 mg for carbachol ($n = 17$). Thirty minutes of pre-treatment with either 5 or 50 μM norbormide significantly shifted downward the concentration–response curve to KCl with a maximum inhibitory effect of 32% at the lower concentration and 68% at the higher concentration (Fig. 4A). Carbachol concentration–response curve was not significantly affected by 5 μM norbormide, while it was shifted downward by 50 μM norbormide with a 45% decrease of the maximal contractile response (Fig. 4B). The EC_{50s} in the absence and the presence of 50 μM norbormide were 1.1 ± 0.12 and 1.0 ± 0.2 μM, respectively.

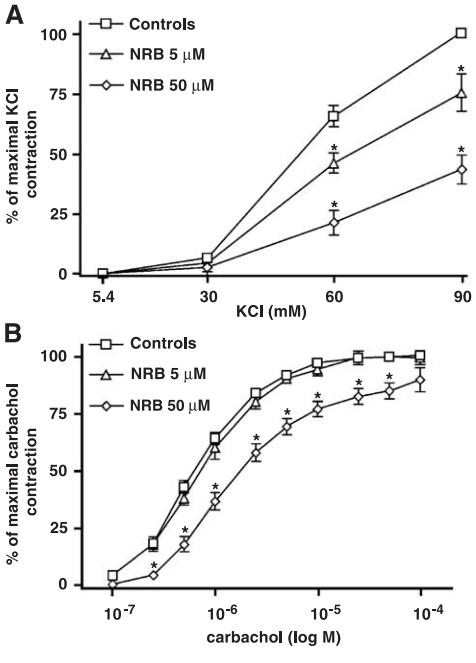

Fig. 3. Effect of norbormide (NRB) on KCl (panel A) and carbachol (panel B) concentration–response curves in rat tracheal smooth muscle rings. Data are expressed as percent of the maximum contractile response to KCl (90 mM) or carbachol (100 μM). Each point represents mean ± S.E.M. of 17 rings. *$P < 0.05$, Bonferroni post-test.

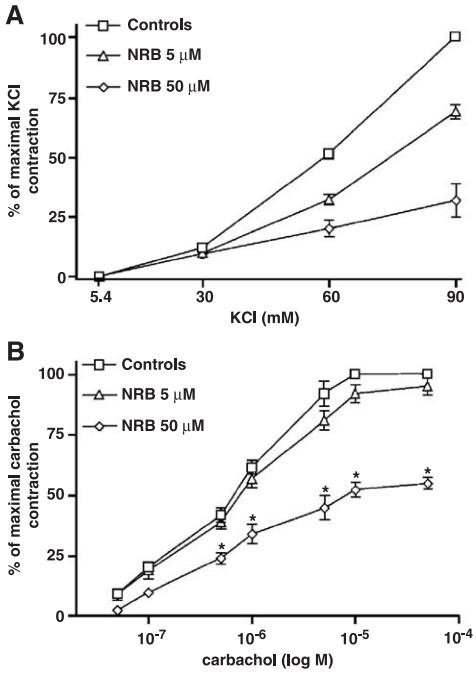

Fig. 4. Effect of norbormide (NRB) on KCl (panel A) and carbachol (panel B) concentration–response curves in rat urinary bladder smooth muscle rings. Data are expressed as percent of the maximum contractile responses elicited by KCl (90 mM) or carbachol (10 μM). Each point represents mean ± S.E.M. of 17 rings. *$P < 0.05$, Bonferroni post-test.

3.4. Effect of norbormide on KCL-induced contractions in rat duodenal rings

In rat duodenum, KCl (30, 60, and 90 mM) induced a concentration-dependent contractile effect whose maximum value was 943 ± 108 mg ($n = 17$). Thirty minutes of pre-treatment with either 5 or 50 μM norbormide significantly shifted downward the concentration–response curve to KCl with a maximum inhibitory effect of 44% at the lower concentration and 74% at the higher concentration (Fig. 5).

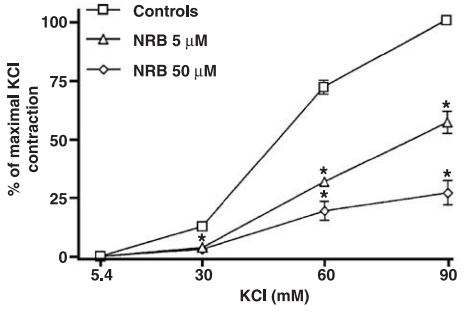

Fig. 5. Effect of norbormide (NRB) on KCl concentration–response curve in rat duodenal smooth muscle rings. Data are expressed as percent of the maximum contractile response elicited by KCl (90 mM). Each point represents mean ± S.E.M. of 17 rings. *$P < 0.05$, Bonferroni post-test.

3.5. Effect of norbormide on $I_{Ca(L)}$

Fig. 6A shows a typical recording of $I_{Ca(L)}$ under control conditions, in the presence of 50 μM norbormide and following washout of the drug. Fig. 6B shows the time course of the effects of norbormide on the peak currents. After $I_{Ca(L)}$ had reached steady values, 50 μM norbormide was added to the bath solution. This produced a gradual decrease of the current (over 5 min) and was only partially reversible upon washout.

The effect of norbormide on the kinetic of $I_{Ca(L)}$ activation and inactivation was analysed. Activation and inactivation rates were measured under control conditions as well as at the plateau of norbormide inhibition. The current evoked at 10 mV starting from $V_h = -50$ mV activated and then inactivated with time courses that could be fitted by mono-exponential equations, with a τ of activation of 1.95 ± 0.15 ms and a τ of inactivation of 72.75 ± 6.82 ms ($n = 4$) (Fig. 6C). The addition of 50 μM norbormide to the perfusion medium had no effect on both rates (τ of activation of 2.40 ± 0.19 ms and τ of inactivation of 87.81 ± 14.04 ms; $P > 0.05$, Student's t-test for paired samples).

Norbormide significantly inhibited the peak inward current at all potentials tested (Fig. 6D); this inhibition, however, was not accompanied by a significant shift of the current–voltage curve along the voltage axis. Norbormide (50 μM) inhibited $I_{Ca(L)}$ evoked by depolarisation step to 10 mV ($V_h = -50$ mV) by 60.9%.

4. Discussion

Norbormide is the most selective toxicant known so far due to its property to induce death exclusively in rats by determining a peripheral vasoconstriction (Roszkowski, 1965; Bova et al., 1996) that has been ascribed to the activation of a phospholipase C-coupled receptor expressed in the myocytes of the rat peripheral arteries (Bova et al., 2001). The data presented in this paper suggest that such a putative receptor may not be expressed in tracheal, urinary bladder, and duodenal smooth muscle cells, as indicated by the absence of any contractile effect induced by the drug even at concentrations producing the maximal contractile response in the peripheral arteries. This finding provides evidence that norbormide contractile action is not only species-specific but also tissue-specific, thus indicating the existence of species- and tissue-selective mechanisms that can be modulated by pharmacological agents.

In rat aorta and in non-rat arteries, norbormide, at concentrations that contract rat peripheral vessels, induces a relaxing effect which has been suggested to be mediated

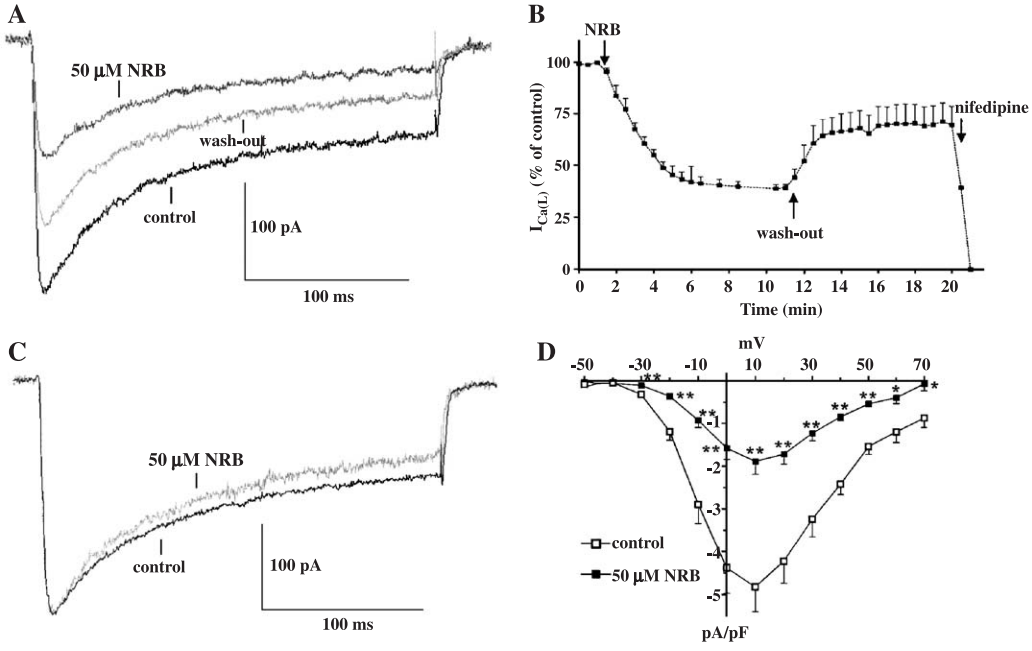

Fig. 6. Inhibition by norbormide of $I_{Ca(L)}$ in rat gastric fundus myocytes. Panel A: original recordings of conventional whole-cell $I_{Ca(L)}$ elicited with 250-ms clamp pulses to 10 mV from a V_h of −50 mV measured in the absence (control) or presence of 50 μM norbormide (NRB) as well as after washout of the drug. Panel B: time course of $I_{Ca(L)}$ inhibition induced by norbormide. Norbormide (50 μM) was applied, at the time indicated by the arrow, and peak currents were recorded during a typical depolarisation from −50 to 10 mV, applied every 30 s, and subsequently normalised according to the current recorded just prior to norbormide application. Drug washout is also shown. Nifedipine was added at the end of the experiment to show that I_{Ca} flows fully through L-type Ca^{2+} channels. Data points are mean ± S.E.M. of 3–4 cells ($n = 3$). Panel C: average traces measured from four cells ($n = 3$) in the absence (control, black) or presence of 50 μM norbormide (grey). The drug trace is magnified (magnification is 2.38) so that the peak amplitudes of the traces before and after drug are matched. Vertical bar refers only to control trace. Panel D: current–voltage relationships constructed prior to the addition of norbormide (control, □) and in the presence of 50 μM norbormide (■). Data points are mean ± S.E.M. of four cells ($n = 3$). *$P < 0.05$, **$P < 0.01$, Student's t-test for paired samples.

by a block of L-type Ca^{2+} channels (Bova et al., 1996). An inhibition of L-type Ca^{2+} channels has been recently shown also in rat caudal artery myocytes where the drug can simultaneously activate contraction (Fusi et al., 2002), thus indicating that the two actions are mediated by different binding sites and that in norbormide-contracted arteries, the vasoconstrictor mechanism overwhelms the inhibition of L-type Ca^{2+} current.

The results presented in the present paper show that, also in rat non-vascular smooth muscles, norbormide elicits relaxant and Ca^{2+} channel blocker effects. In rat tracheal, duodenal, and urinary bladder rings, contractile responses to KCl and carbachol were inhibited by norbormide in the same range of concentrations used to relax rat aorta and non-rat arteries (Bova et al., 1996). The inhibitory effect was more pronounced on KCl-induced contraction compared to contraction induced by agonists. This behaviour has been described for several L-type Ca^{2+} channel blockers in vascular and non-vascular smooth muscles (Karaki et al., 1997; Chang et al., 1993; Baba et al., 1985; Cheng and Townley, 1983; Lowe and Noronha-Blob, 1991) and has been ascribed to the different Ca^{2+} sources driving KCl- and agonist-induced contractions (Karaki et al., 1997; Sato et al., 2000). Indeed, the contractile response of smooth muscles to agonists depends both on Ca^{2+} release from the sarcoplasmic reticulum and on extracellular Ca^{2+} influx through either Ca^{2+} entry blocker-sensitive and -insensitive Ca^{2+} channels (Karaki et al., 1997), whereas KCl-induced contraction is exclusively dependent upon extracellular Ca^{2+} entry through L-type Ca^{2+} channels that are mostly sensitive to Ca^{2+} entry blockers (Karaki et al., 1997; Bolton, 1979).

The sensitivity to Ca^{2+} entry blockers of the agonist-induced contractions is different in different smooth muscles (Karaki et al., 1997). In this regard, it has been reported that in rat and guinea-pig tracheal muscle, both verapamil and nifedipine were poorly effective in inhibiting contractions induced by agonists (Ahmed et al., 1985; Baba et al., 1985). Our results show that this was also the case for norbormide that, even at the maximal concentration used in this study, induced only a slight inhibitory effect on carbachol-induced contraction in tracheal rings, further supporting a similarity in the mechanism of action between norbormide and L-type Ca^{2+} channel blockers.

The hypothesis of an antagonism exerted by norbormide on L-type Ca^{2+} channels was confirmed by the electrophysiological data presented here, since norbormide did inhibit $I_{Ca(L)}$ in gastric smooth muscle cells. The features of norbormide Ca^{2+} channel antagonism described here, such as the extent of inhibition, time for maximal effect development, depression of current–voltage relationship as well as the lack of effect on $I_{Ca(L)}$ activation and inactivation rates, looked similar to those observed previously in guinea-pig cardiac and rat tail artery myocytes (Bova et al., 1997; Fusi et al., 2002). Rat caudal artery myocytes, however, displayed a full reversibility of the inhibition upon washout that was only partial in cardiac (Bova et al., 1997) and in gastric fundus myocytes, as shown in the present experiments.

Drugs possessing Ca^{2+} channel blocking activity can either act directly at the channel protein or exert their effect via diffusible intracellular components. The electrophysiological data obtained with the conventional whole-cell patch-clamp method support the former mechanism, since the cell cytoplasm underwent extensive dialysis which should have washed away any cytoplasmic messenger already after the first few minutes of current recordings in the whole-cell configuration (Penner, 1995).

In conclusion, this study shows that norbormide, at concentrations that contract rat peripheral arteries, fails to activate contraction in rat extravascular smooth muscles but rather inhibits their contractile response to both KCl and carbachol, possibly by reducing Ca^{2+} entry by L-type Ca^{2+} current; furthermore, it strongly supports the idea that the vascular myocytes of the rat peripheral arteries represent the only muscular cells contracted by the drug.

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