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Vasorelaxant properties of norbormide, a selective vasoconstrictor agent for the rat microvasculature

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1 The effects of norbormide on the contractility of endothelium-deprived rat, guinea-pig, mouse, and human artery rings, and of freshly isolated smooth muscle cells of rat caudal artery were investigated. In addition, the effect of norbormide on intracellular calcium levels of A7r5 cells was evaluated.

2 In resting rat mesenteric, renal, and caudal arteries, norbormide (0.5–50 μM) induced a concentration-dependent contractile effect. In rat caudal artery, the contraction was very slowly reversible on washing, completely abolished in the absence of extracellular calcium, and antagonized by high concentrations (10–800 μM) of verapamil. The norbormide effect persisted upon removal of either extracellular Na^+ or K^+. The contractile effect of norbormide was observed also in single, freshly isolated smooth muscle cells from rat caudal artery.

3 In resting rat and guinea-pig aortae, guinea-pig mesenteric artery, mouse caudal artery, and human subcutaneous resistance arteries, norbormide did not induce contraction. When these vessels were contracted by 80 mM KCl, norbormide (10–100 μM) caused relaxation. Norbormide inhibited the response to Ca^{2+} of rat aorta incubated in 80 mM KCl/Ca^{2+}-free medium. Norbormide (up to 100 μM) was ineffective in phenylephrine-contracted guinea-pig and rat aorta.

4 In A7r5 cells, a cell line from rat aorta, norbormide prevented high K^+- but not 5-hydroxytryptamine-induced intracellular calcium transients.

5 These findings indicate that *in vitro*, norbormide induces a myogenic contraction, selective for the rat small vessels, by promoting calcium entry in smooth muscle cells, presumably through calcium channels. In rat aorta and arteries from other mammals, norbormide behaves like a calcium channel entry blocker.

Keywords: Norbormide; vascular smooth muscle rings; vascular smooth muscle cells; Ca^{2+} influx; intracellular Ca^{2+} levels; calcium channel entry blocker

Introduction

Norbormide, 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidene) 5-norbornene-2,3-dicarboximide, is a rat toxicant described for the first time by Roszkowski (Roszkowski *et al.*, 1964; Poos *et al.*, 1966). Its toxicological profile is very peculiar, for norbormide is the most selective toxicant so far known in mammals (Roszkowski, 1965), its toxicity being confined to a single genus, *viz. Rattus*. Data from *in vivo* experiments indicate that the toxicity of norbormide in rat is accounted for by tissue ischaemia due to a generalized vasoconstriction (Roszkowski, 1965). Furthermore, the vasospastic effect of norbormide occurs only in rat vessels of relatively small calibre (i.e. mesenteric, ear, and coronary arteries) since the drug does not contract rat aortic segments (Roszkowski, 1965). Thus, norbormide seems to be a vasoactive agent, very selective for the microvessels of the rat.

Neither the mechanism(s) underlying the vasoconstrictor effect of norbormide nor the basis of its high selectivity for the rat microvasculature have been ever investigated. Furthermore, reported *in vivo* experiments give no indication as to whether norbormide exerts other effects different from vasoconstriction on vessels of other species.

This *in vitro* study has been undertaken to investigate the mechanism(s) of the vasospastic effect of norbormide on rat microvasculature and its species selectivity. In addition, we have investigated whether norbormide could affect the contractile function of vessels different from rat microvasculature. For this purpose, norbormide has been tested on rings of both conductance and resistance vessels isolated from rat (aorta, mesenteric, renal, and caudal arteries), guinea-pig (aorta and mesenteric artery), mouse (caudal artery), and man (subcutaneous arteries), on freshly isolated smooth muscle cells of rat caudal artery, and on intracellular calcium levels of A7r5 cells.

Our results confirm the selectivity of the vasospastic effect of norbormide for the rat microvasculature and demonstrate that the drug acts as a vasodilator on all the other arteries tested.

Methods

Arterial rings

Male Sprague-Dawley rats (250–300 g), guinea-pigs (300–350 g) and mice (40–50 g) were killed by decapitation, exsanguinated, and the thoracic aorta (rat and guinea-pig), the ventral caudal artery (rat), the renal (rat), and the first branch of the mesenteric artery (rat and guinea-pig) were removed. Human subcutaneous resistance arteries were obtained from biopsies of anterior abdominal wall fat taken during routine abdominal surgery on patients who were receiving no medication. All the vessels were cleaned of connective tissue and cut into rings of 2 mm length. The endothelium was removed by gently rubbing the lumen of the rings with a round wooden stick (aorta) or a very thin rough-surfaced tungsten wire (caudal, mesenteric, renal, and subcutaneous arteries). The rings were vertically suspended between steel hooks (aortic rings) or tungsten wires (caudal, mesenteric, renal, and subcutaneous artery rings) in an organ bath filled with 18 ml of physiological salt solution (PSS). The PSS was of the following composition (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, main-

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tained at 37°C and bubbled with 95% O_2/5% CO_2. Tension was recorded on a pen recorder *via* an isometric force displacement transducer. Rings were stretched passively to impose a resting tension (1.5 g for the aortic rings and 0.5 – 1 g for the caudal, mesenteric, renal, and subcutaneous artery rings) and were allowed to equilibrate for 60 min. After equilibration, each ring was repeatedly stimulated with 10 μM phenylephrine until a reproducible response was obtained. To verify the absence of the endothelium, rings contracted with 10 μM phenylephrine were exposed to 1 – 2 μM carbamylcholine. The absence of the endothelium was revealed by the lack of carbamylcholine-induced relaxation. All experiments were performed in the presence of 10 μM indomethacin, to avoid any possible release of cyclo-oxygenase products.

Some experiments were performed on arterial rings bathed in modified physiological salt solutions:

Na^+-free solution NaCl was omitted, sucrose was added to maintain osmolarity, and $KHCO_3$ was substituted for $NaHCO_3$ in the PSS.

K^+-free solution KCl was omitted and NaH_2PO_4 was substituted for KH_2PO_4 in the PSS.

Ca^{2+}-free solution $CaCl_2$ was omitted and 1 mM ethylene glycol *bis* (β-aminoethyl ether) N,N′-tetraacetic acid (EGTA) was added to the PSS.

High-K^+ solution KCl concentration was increased to 80 mM in the PSS.

A7r5 cells

Cell culture The A7r5 cell line was obtained from Aviano Oncology Centre (Aviano, Italy). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co. St. Louis, MO, U.S.A.) completed with 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5 μg ml^{-1} fungizone, and 10% foetal bovine serum (FBS) (Sigma Chemical Co. St. Louis, MO, U.S.A.) at 37°C under an atmosphere of 95% air/5% CO_2. Before use, cells were allowed to reach confluence, after which they were maintained in 0.5% FBS for 7 – 12 days. Cells were then trypsinized and replated in 8 × 30 – mm glass coverslips at a density of 150,000 cells ml^{-1}. The coverslips were incubated overnight with DMEM plus 10% FBS to allow a better attachment of the cells; afterwards FBS concentration was again reduced to 0.5% until the end of the experiment.

Measurement of intracellular Ca^{2+} levels in A7r5 cells $[Ca^{2+}]_i$ was measured by fluorometry in a double-wavelength mode in a Perkin Elmer LS-50B spectrofluorometer equipped with a magnetic stirrer and a temperature regulator. Cells were loaded with fura-2 acetoxymethyl ester (fura-2/AM) (Molecular Probes Inc., Eugene, OR, U.S.A.) as previously described (Goldman *et al.*, 1990). Briefly, A7r5 cells grown as monolayers on glass coverslips were incubated with 2 ml of complete DMEM containing 10 μM fura-2/AM and 2.5 μl of 25% w/w pluronic F-127 for 120 min at room temperature. Coverslips were then transferred to a customized holder, inserted in a quartz cuvette, and placed in the spectrofluorometer where they were superfused at a rate of 5 ml min^{-1} for 30 – 40 min with modified Krebs solution (MKS) of the following composition (mM): NaCl 140, KCl 5.9, NaH_2PO_4 1.2, $MgCl_2$ 1.4, $CaCl_2$ 1.8, [4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid] (HEPES) 5 and glucose 11.5, at pH 7.4 and 37°C.

$[Ca^{2+}]_i$ was calculated by the formula of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = \frac{R - R_{min}}{R_{max} - R} \times \frac{S_f}{S_b} \times K_d$$

where K_d is the dissociation constant of fura-2 and was assumed to be 225 nM, R is the 340/380 nm ratio of fura-2 fluorescence measured in the cells, R_{max} is the 340/380 nm ratio in the presence of a saturating calcium concentration, R_{min} is the 340/380 nm ratio of fura-2 fluorescence in a Ca^{2+}-free solution containing K_2H_2-EGTA, and S_f/S_b is the ratio of Ca^{2+}-free to Ca^{2+}-bound fura-2 at 380 nm. Calibration was performed by using fura-2 free acid in 10% glycerol, that simulates the cell cytosol. R_{min} was determined in a solution containing (in mM): KCl 115, NaCl 10, $MgSO_4$ 2, K_2H_2-EGTA 10, and K_2-3-(N-morpholino) propanesulphonic acid (MOPS) 10 at pH 7.2. For the determination of R_{max}, 2 mM $CaCl_2$ was added to the medium and CaK_2-EGTA was substituted for K_2H_2-EGTA. R_{max}, R_{min}, and S_f/S_b values were found to be 17.36, 1, and 7.81, respectively.

In some experiments, A7r5 cells were depolarized by high-K^+ solution (60 mM KCl), made by substituting KCl for equimolar NaCl in the MKS.

Rat caudal artery cells

Isolation procedure Single smooth muscle cells from rat caudal artery were obtained from adult male Sprague-Dawley rats (250 – 300 g) as described by Furspan (1992). After excision of the ventral caudal artery, proximal segments (about 6 mm long) were carefully cleaned and incubated in a digestion medium containing 0.3 units ml^{-1} collagenase B (Boehringer Mannheim GmbH, Biochemica, Germany), 0.3 mg ml^{-1} trypsin inhibitor, 0.4 mg ml^{-1} papain, 0.3 mg ml^{-1} dithiothreitol, and 7.5 mg ml^{-1} bovine serum albumin (fatty acid free) for 90 min at 37°C. The segments were then transferred to a 35 mm culture dish and gently shaked to obtain isolated smooth muscle cells.

Evaluation of cell contraction Culture dishes, superfused with MKS (see above) at a rate of 5 ml min^{-1} at room temperature, were mounted on the stage of an inverted microscope (Nikon TMD) connected to a video imaging system able to display images of single cells.

Drugs

Norbormide was a kind gift of India S.p.A. (Padova, Italia). Phenylephrine hydrochloride, yohimbine hydrochloride, carbamylcholine chloride, verapamil hydrochloride, 5-hydroxytryptamine hydrochloride, and indomethacin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Indomethacin was dissolved in ethanol and norbormide in dimethylformamide. At the concentrations reached in the medium, ethanol and dimethylformamide had no effect either on resting or stimulated tissue tone. All the other drugs were dissolved in twice-distilled water.

Data and statistics

Contractile responses of arterial rings were measured as mg of developed tension. The concentrations of norbormide causing the half-maximal contraction of rat caudal, mesenteric, and renal artery rings or the half relaxation of 80 mM KCl-contracted rat and guinea-pig aortic rings (EC_{50} values) were calculated for each experiment. Data are expressed as the mean ± s.e. mean.

Results

Vasoconstrictor effect of norbormide

Rat caudal, mesenteric, and renal artery rings responded similarly to norbormide (0.5 – 50 μM) with concentration-dependent contractions (EC_{50}s 2.49 ± 0.23 μM, $n = 8$; 0.41 ± 0.12 μM, $n = 3$; 0.90 ± 0.33 μM, $n = 3$, respectively) (Figure 1), in agreement with Roszkowski's (1965) observations *in vivo*.

The effect of each drug concentration reached equilibrium within 15–20 min and was slowly reversible on drug washout, the maximally contracted rings taking about 90 min to relax.

To investigate further the contractile effect of norbormide, the rat caudal artery was chosen as an experimental model in the following experiments. In the absence of external calcium, norbormide was unable to induce contraction, while a transient contraction was elicited by phenylephrine (Figure 2). Pretreatment (30 min) of the artery rings with the selective α_2-adrenoceptor antagonist, yohimbine (10 μM) did not affect the concentration-response curve to norbormide (data not shown). The rat caudal artery rings maximally contracted by norbormide were relaxed by verapamil (10–800 μM) (Figure 3).

Norbormide contracted rat caudal artery rings incubated either in Na^+- or K^+-free medium (Figure 4).

The contractile effect of norbormide, evaluated as shortening of the cell, was also observed in vascular smooth muscle cells freshly isolated from rat caudal artery (Figure 5b) and was not reversed by washout (Figure 5c).

Vasorelaxant effect of norbormide

Norbormide did not affect the resting tone of rings from rat and guinea-pig aorta, guinea-pig mesenteric artery, mouse caudal artery and human subcutaneous arteries. However, when these vessels were contracted by 80 mM KCl, norbormide (10–100 μM) induced a concentration-dependent relaxation. Figure 6 shows the concentration-response curves to norbormide of 80 mM KCl-contracted rat and guinea-pig aortic rings. Norbormide concentrations inducing half relaxation (EC_{50}) were 27.80 ± 2.88 μM and 45.21 ± 3.19 μM for rat and guinea-pig aortic rings, respectively. The same results were obtained when verapamil (0.1–10 μM) was used instead of norbormide (not shown). Furthermore, norbormide antagonized the contractile responses to calcium of rat aortic rings depolarized with 80 mM KCl (Figure 7). In rat and guinea-pig aortic rings contracted with 10 μM phenylephrine, 100 μM norbormide was ineffective.

Effect of norbormide on intracellular calcium transients in stimulated A7r5 cells

Exposure of resting A7r5 cells to 60 mM KCl or 1 μM 5-hydroxytryptamine raised $[Ca^{2+}]_i$ from 107 ± 6 nM (n = 18) to 293 ± 23 nM (n = 8) or 269 ± 6 nM (n = 6), respectively. Pretreatment of the cells with 50 μM norbormide completely

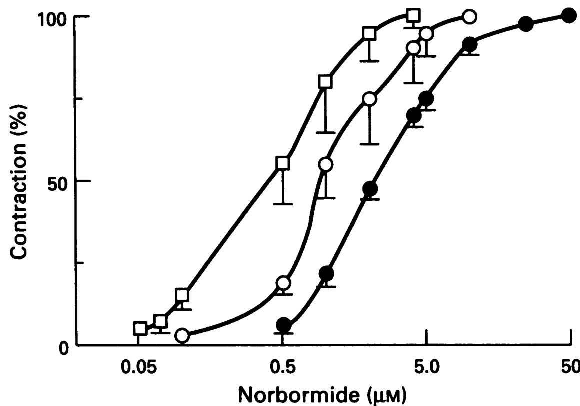

Figure 1 Cumulative concentration-response curves to norbormide of resting rat caudal (●), mesenteric (□), and renal (○) artery rings. Each point is the mean ± s.e. mean of eight, three, and three separate experiments, respectively. Responses are normalized for the maximal response to norbormide (2081 ± 336 mg, 783 ± 167 mg, and 1600 ± 253 mg, for rat caudal, mesenteric, and renal artery rings, respectively).

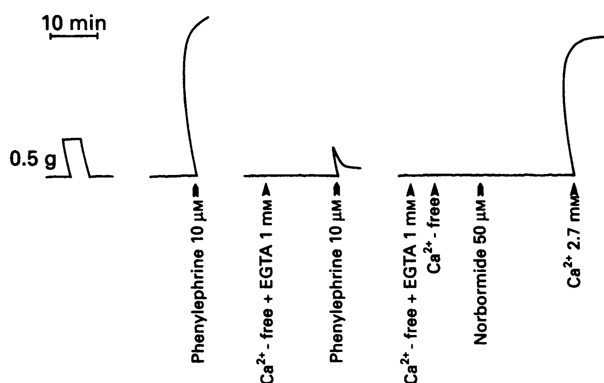

Figure 2 Recording from an experiment on one rat caudal artery ring. The ring was first stimulated with 10 μM phenylephrine in the PSS and then in Ca^{2+}-free/1 mM EGTA medium. After a 60 min washout in the PSS, the ring was restimulated with phenylephrine (not shown), re-exposed to Ca^{2+}-free solutions (Ca^{2+}-free/1 mM EGTA medium for 5 min and Ca^{2+}-free medium for 10 min), and treated with 50 μM norbormide for 20 min. Then $CaCl_2$ (2.7 mM, final concentration) was added.

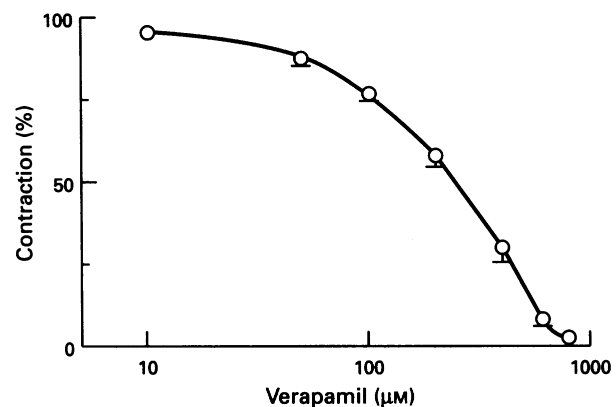

Figure 3 Concentration-response curve to verapamil of rat caudal artery rings contracted by 50 μM norbormide. Each point is the mean ± s.e. mean of six separate experiments. The tension developed by the rings in response to 50 μM norbormide was 1519 ± 387 mg.

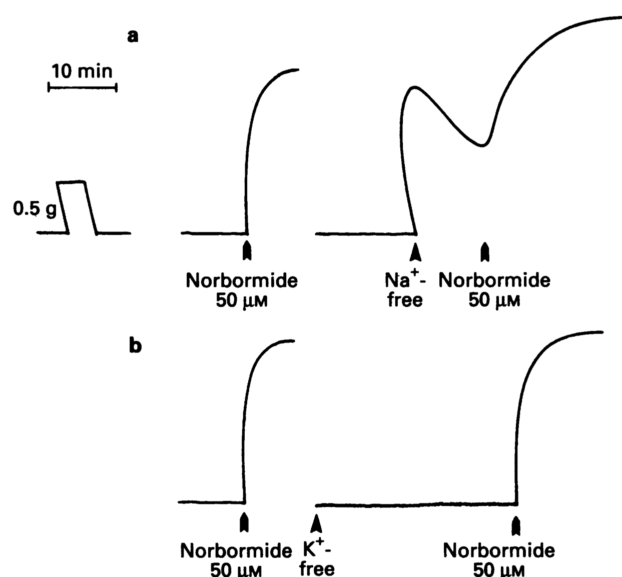

Figure 4 Recordings of the contractile responses induced by 50 μM norbormide in two separate rat caudal artery rings incubated in Na^+-free (a) or K^+-free solution (b). Note that the Na^+-free solution elicits a contractile response, while the K^+-free solution does not.

prevented the increase in $[Ca^{2+}]_i$ induced by 60 mM KCl (Figure 8), but did not affect the response to 5-hydroxytryptamine (not shown). The same results were obtained when 1 μM verapamil was used instead of norbormide.

Discussion

Vasoconstrictor effect of norbormide

This study shows that norbormide selectively contracts rat caudal, mesenteric, and renal artery rings *in vitro*, in agreement with the vasoconstriction of rat small vessels induced by the drug *in vivo* (Roszkowski, 1965). This effect is neither mediated by a release of noradrenaline from nerve endings, since norbormide also induces contraction of single cells isolated from rat caudal artery, nor by a release of products of cyclo-oxygenase activity from the vascular myocytes, as the contraction also occurs in the presence of indomethacin. Therefore, the contractile effect of norbormide is probably due to an action on the vascular smooth muscle cells.

Rat caudal artery rings bathed in calcium-free medium do not respond to norbormide, while they respond to phenylephrine with a transient contraction. Thus norbormide, unlike agonists acting by mobilizing intracellular Ca^{2+}, elicits a contractile response entirely dependent on external calcium. This observation is not consistent with a calcium release from

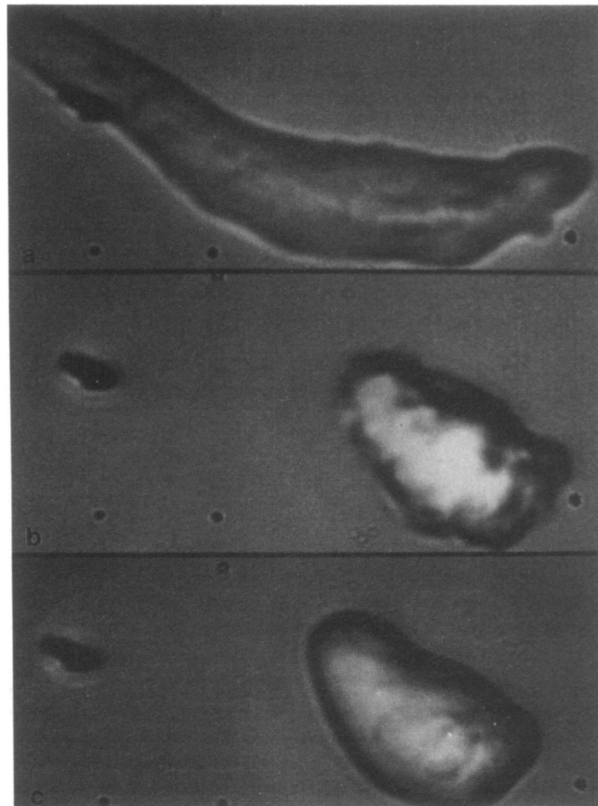

Figure 5 Video images of a rat caudal artery cell in resting state (a), after 1 min exposure to 50 μM norbormide (b), and after 15 min washout (c).

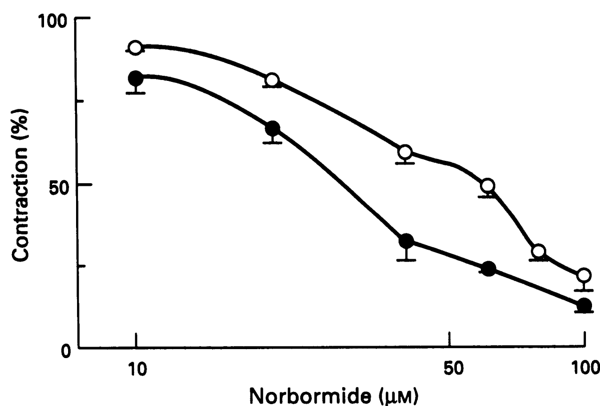

Figure 6 Concentration-response curves to norbormide of rat (●) and guinea-pig (○) aortic rings contracted by depolarization (80 mM KCl). Each point is the mean ± s.e.mean of at least seven separate experiments. The tensions developed by the aortic rings from rat and guinea-pig in response to 80 mM KCl were 865 ± 103 mg (n = 7) and 1037 ± 84 mg (n = 10), respectively.

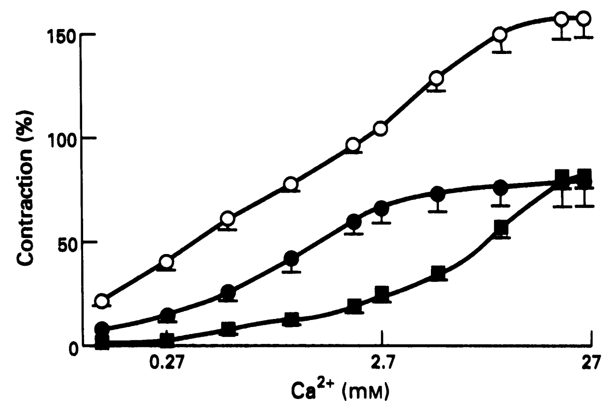

Figure 7 Concentration-response curves to calcium of rat aortic rings depolarized by 80 mM KCl in the absence (○) and presence of 25 μM (●) or 50 μM (■) norbormide. Each point is the mean ± s.e. mean of at least six separate experiments. In each ring, two cumulative concentration-response curves to $CaCl_2$ were obtained first in the absence and then in the presence of norbormide. Data are normalized, so that contraction of untreated ring in 2.7 mM $CaCl_2$ (final concentration in the PSS) is taken as the 100% contraction. The actual value of developed tension of the untreated rings in 2.7 mM $CaCl_2$ was 1063 ± 119 mg (n = 11).

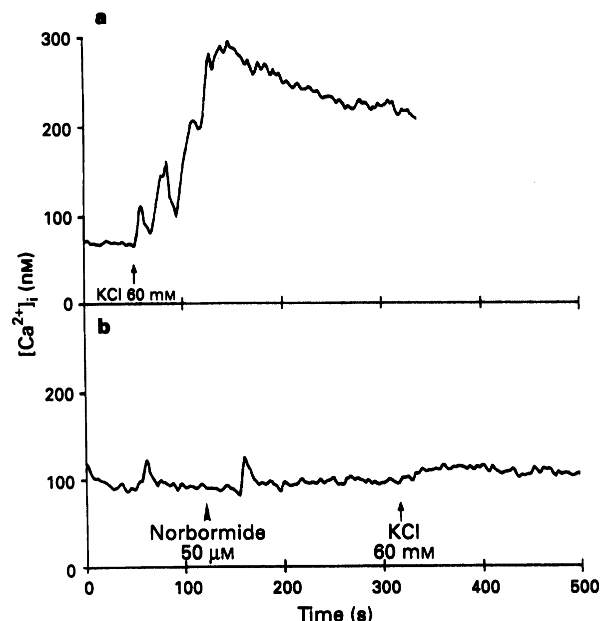

Figure 8 Recordings of $[Ca^{2+}]_i$ increase induced by 60 mM KCl (a) and prevention of this increase by 50 μM norbormide (b) in A7r5 cells. $[Ca^{2+}]_i$ was determined in fura-2-loaded A7r5 monolayers as described under Methods. The traces are representative of at least five similar experiments.

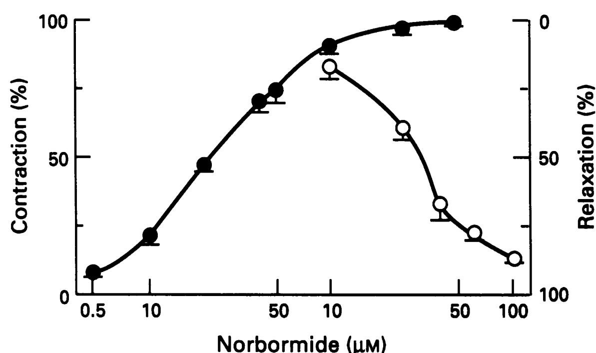

Figure 9 Vasoconstriction of rat caudal artery rings (●) and vasorelaxation of depolarization-contracted rat aortic rings (○) induced by norbormide.

the sarcoplasmic reticulum induced by the drug, either through stimulation of receptors coupled to IP_3 (van Breemen & Saida, 1989; Khalil *et al.*, 1990), or by inducing IP_3 production *via* activation of some step beyond the agonist receptors, or by a caffeine-like action. Therefore, it appears that norbormide activates contraction by promoting calcium influx into vascular smooth muscle cells.

It is well established that calcium enters vascular smooth muscle cells through calcium channels and, under some circumstances, through a Na^+/Ca^{2+} exchange mechanism (Ashida & Blaustein, 1987; Carafoli *et al.*, 1990). It has been reported that calcium channel activation is primarily involved in the contraction of vascular smooth muscle induced by postjunctional α_2-adrenoceptor stimulation (Langer & Shepperson, 1982; Jim & Matthews, 1985). An interaction of norbormide with these receptors can be ruled out, since the contractile effect of the drug is not inhibited by the selective α_2-adrenoceptor antagonist, yohimbine. We suggest that norbormide may operate as a calcium channel opener, although a functional approach provides only indirect evidence on the mechanism of action of a drug. Rat caudal artery rings contracted by norbormide are relaxed by verapamil at concentrations higher than those antagonizing the contraction consequent to the opening of calcium channels by high potassium. As high concentrations of calcium entry blockers may have intracellular effects (Cauvin *et al.*, 1983), it could be that verapamil reverses the norbormide effect through an intracellular mechanism. On the other hand, the contracted caudal artery rings take a long time to relax when norbormide is removed from the medium, indicating a long lasting action and suggesting that the drug dissociates very slowly from its binding site(s). This could explain why high concentrations of verapamil, acting as a calcium entry blocker, are needed to counteract the norbormide action, whatever the mechanism of calcium channel activation by norbormide and whichever the site(s) of norbormide binding. Furthermore, the proposal that norbormide-induced contraction is due to the activation of calcium channels could be consistent with the large doses of vasodilators such as organic nitrates or isoprenaline needed to alter the threshold of norbormide vasoconstriction *in vivo* (Roszkowski, 1965), as these vasodilators are poorly effective on the contraction dependent on calcium channel activation by high potassium (Schultz *et al.*, 1979; Lincoln, 1983; Jones *et al.*, 1984).

An alternative explanation has been considered, taking into account that the increase in intracellular sodium concentration induces myogenic contractions in several isolated vascular smooth muscle preparations as a consequence of calcium entry through a Na^+/Ca^{2+} exchange (Karaki *et al.*, 1978; Ozaki *et al.*, 1978; Bova *et al.*, 1988; Nabel *et al.*, 1988; Woolfson *et al.*, 1990). Intracellular sodium can be increased by inhibiting the Na^+/K^+ ATPase activity of vascular smooth muscle cell plasmalemma. However, the contractile response of rat caudal artery to norbormide cannot result from this mechanism, since rat caudal artery rings do not contract when the Na^+/K^+ ATPase activity is inhibited by means of extracellular potassium removal and norbormide is still effective in K^+-free medium. Intracellular sodium concentration can also be increased by promoting sodium entry (Shinjoh *et al.*, 1991), but it is unlikely that norbormide contracts rat caudal artery rings by this mechanism, as the effect of the drug is still evident in the absence of external sodium.

Vasorelaxant effect of norbormide

This study shows that norbormide behaves like the calcium entry blocker verapamil in vessels other than the small vessels of the rat (e.g. caudal artery), since it relaxes rat aorta and both large and small vessels from other species (aorta and mesenteric artery from guinea-pig, caudal artery from mouse, and subcutaneous arteries from humans) contracted by depolarization. Norbormide, although less potent than verapamil, is as effective as verapamil in relaxing these vessels. In guinea-pig and rat aorta, norbormide, at concentrations effective against the contraction induced by depolarization, is ineffective on the contraction induced by phenylephrine, a feature that has been observed also for verapamil (Bova *et al.*, 1994) and diltiazem (Cauvin *et al.*, 1984). Likewise, in A7r5 cells both norbormide and verapamil inhibit intracellular calcium transients induced by depolarization, while they do not affect the increase in $[Ca^{2+}]_i$ elicited by 5-hydroxytryptamine. The similarity between the effects of verapamil and norbormide suggests that the two drugs share a common mechanism of action. However, the patterns of action of norbormide and verapamil do not overlap completely. A simple competition mechanism between calcium and verapamil has been reported (Asano & Hidaka, 1984), while norbormide inhibits the maximal response to calcium, suggesting a non-surmountable antagonism.

Altogether these data indicate that norbormide can elicit opposite effects in isolated vascular smooth muscle from different species and, within the same species, in different vascular areas (Figure 9). The finding that norbormide *in vitro* contracts small calibre vessels from rat but not from guinea-pig, mouse, or man is consistent with the selectivity of its toxic action *in vivo* and indicates the myogenic nature of this action. The *in vitro* vasorelaxant effect of norbormide could account for the hypotension observed in human subjects treated with norbormide (see Pelfrene, 1991). Present data suggest that the vasorelaxant and vasoconstrictor properties of norbormide are mediated by an action on calcium movements across the sarcolemma, most likely through calcium channels, without affecting the intracellular calcium stores. In this context, it is noteworthy that the vasorelaxant effect of norbormide occurs in a range of concentrations very close to those inducing vasoconstriction (Figure 9).

In conclusion, the *in vitro* results confirm *in vivo* observations (Roszkowski, 1965) and show that norbormide has a selective, myogenic, vasoconstrictor effect on rat microvasculature by promoting calcium entry. On the other hand, in rat aorta, guinea-pig vessels, mouse caudal artery as well as human subcutaneous arteries, norbormide behaves as a calcium entry blocker. The selectivity of norbormide vasoconstriction indicates that the mechanisms regulating calcium handling exhibit some anomalous features in rat microvasculature like those that occur in rat heart (Noble & Powell, 1985). Furthermore, one can wonder whether some relationship exists between the peculiar behaviour of rat microvasculature and the fact that mammalian models of primary hypertension have been developed only in the rat.

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