Junctional and nonjunctional effects of heptanol and glycyrrhetinic acid derivates in rat mesenteric small arteries

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1 Heptanol, 18α-glycyrrhetinic acid (18αGA) and 18β-glycyrrhetinic acid (18βGA) are known blockers of gap junctions, and are often used in vascular studies. However, actions unrelated to gap junction block have been repeatedly suggested in the literature for these compounds. We report here the findings from a comprehensive study of these compounds in the arterial wall.

2 Rat isolated mesenteric small arteries were studied with respect to isometric tension (myography), $[Ca^{2+}]_i$ (Ca^{2+}-sensitive dyes), membrane potential and – as a measure of intercellular coupling – input resistance (sharp intracellular glass electrodes). Also, membrane currents (patch-clamp) were measured in isolated smooth muscle cells (SMCs). Confocal imaging was used for visualisation of $[Ca^{2+}]_i$ events in single SMCs in the arterial wall.

3 Heptanol (150 μM) activated potassium currents, hyperpolarised the membrane, inhibited the Ca^{2+} current, and reduced $[Ca^{2+}]_i$ and tension, but had little effect on input resistance. Only at concentrations above 200 μM did heptanol elevate input resistance, desynchronise SMCs and abolish vasomotion.

4 18βGA (30 μM) not only increased input resistance and desynchronised SMCs but also had nonjunctional effects on membrane currents. 18αGA (100 μM) had no significant effects on tension, $[Ca^{2+}]_i$, total membrane current and synchronisation in vascular smooth muscle.

5 We conclude that in mesenteric small arteries, heptanol and 18βGA have important nonjunctional effects at concentrations where they have little or no effect on intercellular communication. Thus, the effects of heptanol and 18βGA on vascular function cannot be interpreted as being caused only by effects on gap junctions. 18αGA apparently does not block communication between SMCs in these arteries, although an effect on myoendothelial gap junctions cannot be excluded.
British Journal of Pharmacology (2004) **142**, 961–972. doi:10.1038/sj.bjp.0705870

Keywords: Heptanol; membrane potential; $[Ca^{2+}]_i$; ion current; vasomotion; glycyrrhetinic acid; gap junctions; smooth muscle

Abbreviations: 18αGA, 18α-glycyrrhetinic acid; 18βGA, 18β-glycyrrhetinic acid; NA, noradrenaline

Introduction

In vascular smooth muscle (VSM), gap junctions with varying densities have been demonstrated by immunohistochemistry (Yeh *et al.*, 1997; Ko *et al.*, 1999; Berman *et al.*, 2002; Hill *et al.*, 2002) and electron microscopy (Hill *et al.*, 2002). The presence of gap junctions or connexins in the media of arteries has been further substantiated through evidence of mRNA for connexins (Haefliger *et al.*, 1997a; Rummery *et al.*, 2002; Sandow *et al.*, 2003) and connexin proteins (Haefliger *et al.*, 1997b; Ko *et al.*, 1999; Sandow *et al.*, 2003). Functionally, it is well known that the VSM cells are electrically coupled, and it has been inferred that gap junctions mediate this intercellular communication (Bény & Pacicca, 1994; Xia & Duling, 1995; Welsh & Segal, 1998).

To obtain functional evidence for the role of gap junctions, a number of drugs with effects on gap junctions have been used, including the long-chain alcohol heptanol and derivatives of glycyrrhetinic acid. Heptanol and 18β-glycyrrhetinic acid (18βGA) are commonly used to block electrical cell-to-cell communication in a number of cells including smooth muscle cells. The effects of heptanol and 18βGA on vascular tone have often been interpreted in terms of the role of gap junctions in maintaining vascular tone (Tsai *et al.*, 1995; Watts & Webb, 1996; Christ *et al.*, 1999; Christ & Brink, 2000; Jiang *et al.*, 2001; Leite & Webb, 2001; Coleman *et al.*, 2002; Lagaud *et al.*, 2002). However, there are concerns that heptanol and 18βGA have effects unrelated to their supposed effects on gap junctions in concentrations close to those needed to block gap junctions (Javid *et al.*, 1996; Chaytor *et al.*, 1997; 1998; Yamamoto *et al.*, 1998; Murai *et al.*, 1999; Santicioli & Maggi, 2000; Coleman *et al.*, 2001; Tare *et al.*, 2002). The α-form of glycyrrhetinic acid (18αGA) has been suggested to be a more specific and less toxic inhibitor of gap junctions than other glycyrrhetinic acid compounds (Davidson *et al.*, 1986; Davidson & Baumgarten, 1988), although its specificity has also been questioned (Chaytor *et al.*, 2000; Schuster *et al.*, 2001; Tare *et al.*, 2002). Surprisingly, despite the suggested nonjunctional effects of heptanol and glycyrrhetinic acid derivates in vascular preparations, these compounds are still extensively used in vascular studies to block gap junctions without providing direct evidence of specificity.

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In the present study we tested the specificity of heptanol and glycyrrhetinic acid derivates by investigating their concentration-dependent effects on smooth muscle cell input resistance (as an indicator of gap junctional conductance), force, intracellular free calcium ($[Ca^{2+}]_i$), and membrane potential in rat mesenteric small arteries. In addition, we measured membrane currents in isolated smooth muscle cells from these arteries. The results demonstrate that heptanol and $18\beta GA$ block intercellular communication in mesenteric small arteries, but that at lower concentrations several nonjunctional effects appear. These findings strongly indicate that caution is necessary when ascribing an inhibitory effect on vascular tone to a purely junctional effect of these compounds. Moreover, these results have general implications when interpreting works published with these compounds.

Methods

Preparation

All experiments were conducted in accordance with the Danish national guidelines for animal research. Male Wistar rats, 12–18 weeks old, were killed with CO_2. The mesentery was removed from the rats and placed into ice-cold physiological salt solution (PSS). Third-generation branches of small mesenteric arteries were dissected out and cleaned of fat and connective tissue. The isolated arterial segments were used either for mounting into a myograph or for cell isolation for patch-clamp studies.

Isometric force, $[Ca^{2+}]_i$ and membrane potential measurements

Simultaneous measurements of isometric force and $[Ca^{2+}]_i$ or isometric force and membrane potential were made at $37^\circ C$ in isolated rat mesenteric resistance arteries. An approx. 2-mm long arterial segment was mounted as a ring preparation in the myograph (Danish Myo Technology, Aarhus, Denmark) for isometric force development. The internal circumference of the mounted artery was normalised on the basis of the passive tension–length curve to a value that gives maximal force development (Mulvany & Halpern, 1977). $[Ca^{2+}]_i$ in the outer layers of the smooth muscle cells was measured with Fura-2 after loading with $2-5\,\mu M$ of the acetoxymethyl ester form of the dye (we have previously demonstrated that applying Fura-2/AM to the myograph bath causes loading of only the outer smooth muscle cells (Peng *et al.*, 1998)). Membrane potential was measured using KCl-filled microelectrodes with resistance in the range of $40-100\,M\Omega$. In these experiments cell input resistance was semiquantitatively evaluated by injecting 1 nA current pulses (25 ms) and measuring the subsequent potential change; electrode resistance was routinely compensated by balancing the Wheatstone bridge of the amplifier (Intro-710, WPI) before impalements. All arteries were stimulated with $10\,\mu M$ noradrenaline (NA), which is a supramaximal NA concentration in these arteries (Mulvany *et al.*, 1980). NA in concentrations of $0.7-2\,\mu M$ was used to elicit a submaximal response.

Confocal measurements

Arteries, mounted as described above for isometric force development, were loaded with $3\,\mu M$ Calcium Green-1 acetoxymethyl ester. Calcium Green-1/AM was dissolved in DMSO with 0.1% $(w\,v^{-1})$ cremophor and 0.02% $(w\,v^{-1})$ Pluronic F-127 and the arteries were loaded (final DMSO concentration 0.5%) for 1.5 h at $37^\circ C$. The myograph was placed on the stage of an inverted confocal laser scanning microscope (ODYSSEY XL, Noran). Confocal optical sections were acquired with a water immersion objective ($\times 60$, NA 1.2, Nikon). A 77×58-μm image (640×480 8-bit pixels) was obtained every 266 ms by using a 100-ns time scan mode and 8-frame averaging (in some experiments 16-frame averaging with 533 ms time interval was used). The emission signals at 530 nm (after excitation at 488 nm) were stored on a computer together with simultaneous force measurements. For image analysis, the programs Intervision (Noran) and Image Space (Molecular Dynamics) were used. The $[Ca^{2+}]_i$ changes within cells were estimated as changes in the mean intensity of Calcium Green-1 fluorescence within regions of interests (ROIs) in which all pixel values were averaged (Peng *et al.*, 2001).

Patch-clamp recordings

To assess membrane currents, smooth muscle cells were isolated from rat mesenteric small arteries as described previously (Matchkov *et al.*, 2004). A few branches of mesenteric small arteries were placed into a microtube containing an enzyme solution and stored overnight at $4^\circ C$. The enzyme solution contained (in mM): NaCl 110, KCl 5, $MgCl_2$ 2, KH_2PO_4 0.5, NaH_2PO_4 0.5, $NaHCO_3$ 10, $CaCl_2$ 0.16, EDTA 0.49, Na-HEPES 10, glucose 10, taurine 10 at pH 7.0, as well as $1.5\,mg\,ml^{-1}$ papain, $1.6\,mg\,ml^{-1}$ albumin, and $0.4\,mg\,ml^{-1}$ DL-dithiothreitol. On the following day, the microtube with the vessels was incubated for 5–10 min at $37^\circ C$. The vessels were removed from the enzyme solution and stored in standard bath solution at $4^\circ C$. Single cells were released by trituration with a polyethylene pipette into the bath solution.

To assess calcium currents, smooth muscle cells were isolated from rat mesenteric resistance arteries by a method modified from Petkov *et al.* (2001). A few branches of mesenteric resistance arteries were placed into a microtube containing the patch clamp bath solution (for compositions see below) for 30 min at $37^\circ C$ gently bubbled with 95% O_2/5% CO_2. The solution was then replaced with 1 ml of calcium-free collagenase/elastase enzyme solution for 30–50 min at $37^\circ C$ gently bubbled with 95% O_2/5% CO_2. The latter solution contained (in mM): NaCl 110, KCl 5.6, $MgCl_2$ 1.2, Na-HEPES 10, glucose 20, taurine 20, Na-pyruvate 5 at pH 7.4, as well as $1\,mg\,ml^{-1}$ collagenase type 1 (Worthington Biochemical Corporation, Lakewood, NJ, U.S.A.), $4.85\,\mu g\,ml^{-1}$ elastase type I (Sigma Chemical, Denmark), $1\,mg\,ml^{-1}$ soybean trypsin inhibitor and $1\,mg\,ml^{-1}$ albumin. Single cells were released by trituration with a polyethylene pipette into the standard bath solution.

All patch-clamp recordings were made at room temperature ($22-24^\circ C$). Whole-cell recordings were made with patch pipettes having resistances in the range of $2-5\,M\Omega$. For these experiments, only cells with low access resistance ($5-10\,M\Omega$) were used.

Recordings were made with an Axopatch 200B amplifier (Axon Instruments, U.S.A.) in whole-cell configuration. Data were sampled at a rate of 2 kHz. Data acquisition and analysis were carried out with the software package Clampex 7 for Windows (Axon Instruments). Series resistance and capacitative current were routinely compensated. To compare ionic currents from different recordings, the current amplitudes (recorded in pA) were normalised to the cell capacitance (recorded in pF) and expressed as current density ($A\,F^{-1}$).

Currents were recorded at a holding potential of $-60\,mV$, and current–voltage characteristics were obtained from ramp protocols. The voltage ramps were performed from -60 to $+20\,mV$ with duration of 140 ms.

Solutions

The PSS used contained (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, EDTA 0.026, glucose 5.5, gassed with 5% CO_2/95% O_2 and pH 7.4. K-PSS was PSS where NaCl was substituted with an equimolar amount of KCl and $1\,\mu M$ phentolamine was added.

In the patch clamp experiments the standard bath solution contained (in mM): NaCl 135, KCl 6, Na-HEPES 10, $MgCl_2$ 1, $CaCl_2$ 0.1 at pH 7.4. The standard pipette solution contained (in mM): NaCl 10, KCl 122, K-HEPES 10, $MgCl_2$ 1, $CaCl_2$ 0.01, BAPTA 0.1 at pH 7.4. In potassium-free solution the bath solution contained (in mM): HEPES 10, CsCl 140, $CaCl_2$ 0.1 and pH was 7.4, whereas the pipette solution contained (in mM): HEPES 10, BAPTA 10, CsCl 140, $CaCl_2$ 0.01 and pH was 7.4.

Calcium currents were recorded using the following solutions (Petkov *et al.*, 2001). Bath solution contained (in mM): NaCl 110, KCl 5.6, $CaCl_2$ 5, $MgCl_2$ 1.2, Na-HEPES 10, glucose 20, taurine 20, Na-pyruvate 5 at pH 7.4. The pipette solution contained (in mM): CsCl 102, HEPES 10, EGTA 11, $CaCl_2$ 1, Na-pyruvate 5, succinic acid 5, oxalacetic acid 5, phosphocreatine 5, MgATP 3 at pH 7.4.

All stock solutions were at least 1000-fold more concentrated than final bath concentration. Stock solutions of 18α-GA (100 mM), 18β-GA (10–100 mM) and glibenclamide (10 mM) were dissolved in DMSO (final DMSO concentration ⩽0.1%). Stock solutions of heptanol (10–100 mM) and nifedipine (10 mM) were dissolved in ethanol (final ethanol concentration ⩽0.5%). Stock solutions of charybdotoxin (0.1 mM) and iberiotoxin (0.1 mM) were made up in 150 mM NaCl solutions.

Charybdotoxin and iberiotoxin were used to block large-conductance, calcium-activated potassium channels (K_{Ca}) in smooth muscle cells. These drugs were always used at a concentration of 100 nM, which is known to produce substantial block of K_{Ca} in smooth muscle (Nelson & Quayle, 1995). Glibenclamide at a concentration of $10\,\mu M$ was used to block ATP-sensitive potassium channels (K_{ATP}) (Nelson & Quayle, 1995).

Drugs and chemicals

Glibenclamide, heptanol, nifedipine, NA, 18α-GA, 18β-GA and papain were obtained from Sigma (Sigma-Aldrich, Denmark), charybdotoxin from Alamone Labs (Alamone Labs Ltd, Israel), iberiotoxin from Latoxan (Latoxan, France) and phentolamine from Ciba-Geigy (Basel, Switzerland).

Statistics

All data are mean ± s.e.m. In the patch clamp experiments only one experimental recording was taken from each cell, thus *n* is the number of cells; in other experiments *n* is the number of arteries. Differences between means were tested with a paired two tailed Student's *t*-test; $P < 0.05$ was considered significant.

Results

Effects on membrane potential and input resistance

Arteries stimulated with a submaximal concentration of NA responded by oscillating in force and membrane potential, as described previously (Peng *et al.*, 2001). Heptanol relaxed the arteries and abolished oscillation in both force and membrane potential (Figure 1). The effect of heptanol was reversible: depolarisation and contraction to a second NA stimulation after a 20-min washout period was not changed significantly (Figure 1b; $n = 4$). The relaxation induced by $150\,\mu M$ heptanol in the presence of a submaximal NA concentration was associated with repolarisation (change in membrane potential (ΔU_m) was $-23 \pm 3.2\,mV$, $n = 6$ cells/6 arteries). There was no measurable increase of cell input resistance in this situation (Figure 1a and 2b). In nonstimulated arteries $150\,\mu M$ heptanol caused hyperpolarisation ($\Delta U_m = -6.5 \pm 1.6\,mV$, $n = 6$ cells/4 arteries) (Figure 1c and 2a) but had no effect on input resistance (Figure 1c and 2b). With $500\,\mu M$ heptanol arteries depolarised (Figure 1 and 2a) by $16.6 \pm 4.0\,mV$ ($n = 5$ cells/5 arteries) and by $33.7 \pm 9.6\,mV$ ($n = 3$ cells/3 arteries) for NA activated and nonactivated arteries, respectively. In both NA-activated and nonactivated arteries input resistance increased substantially (Figure 1 and 2b) at concentrations higher than $200\,\mu M$.

18βGA had a biphasic effect on the membrane potential. When arteries were activated with NA (membrane potential $-37 \pm 1.7\,mV$, $n = 21$ cells/12 arteries) $30\,\mu M$ 18βGA evoked a transient repolarisation by $-13 \pm 6.3\,mV$ ($n = 4$ cells/3 arteries) followed by depolarisation to membrane potential $-25 \pm 2.3\,mV$ ($n = 11$ cells/7 arteries) (Figure 3a). In nonactivated arteries membrane potential was $-57 \pm 1.4\,mV$ ($n = 23$ cells/12 arteries) and $30\,\mu M$ 18βGA evoked hyperpolarisation ($\Delta U_m = -7 \pm 2.1\,mV$, $n = 4$ cells/3 arteries) followed by depolarisation to membrane potential $-27 \pm 2.7\,mV$ ($n = 9$ cells/7 arteries) (Figure 3c). 18βGA depolarised and increased input resistance in a concentration-dependent manner (Figure 2c and d). The input resistance was increased significantly by concentrations of 18βGA higher than $3\,\mu M$. At $6\,\mu M$ 18βGA there was no significant change in membrane potential ($-37 \pm 1.7\,mV$ ($n = 21$ cells/12 arteries) and $-32 \pm 1.5\,mV$ ($n = 3$ cells/3 arteries) in control NA conditions and in the presence of $6\,\mu M$ 18βGA, respectively), but a significant increase in input resistance (Figure 2d). The effects of 18βGA on the membrane potential and force were reversible (Figure 3b; $n = 7$).

Effects on membrane currents

Whole-cell membrane currents were used to determine the ionic currents that are involved in the membrane potential changes seen with heptanol and glycyrrhetinic acid. At $150\,\mu M$
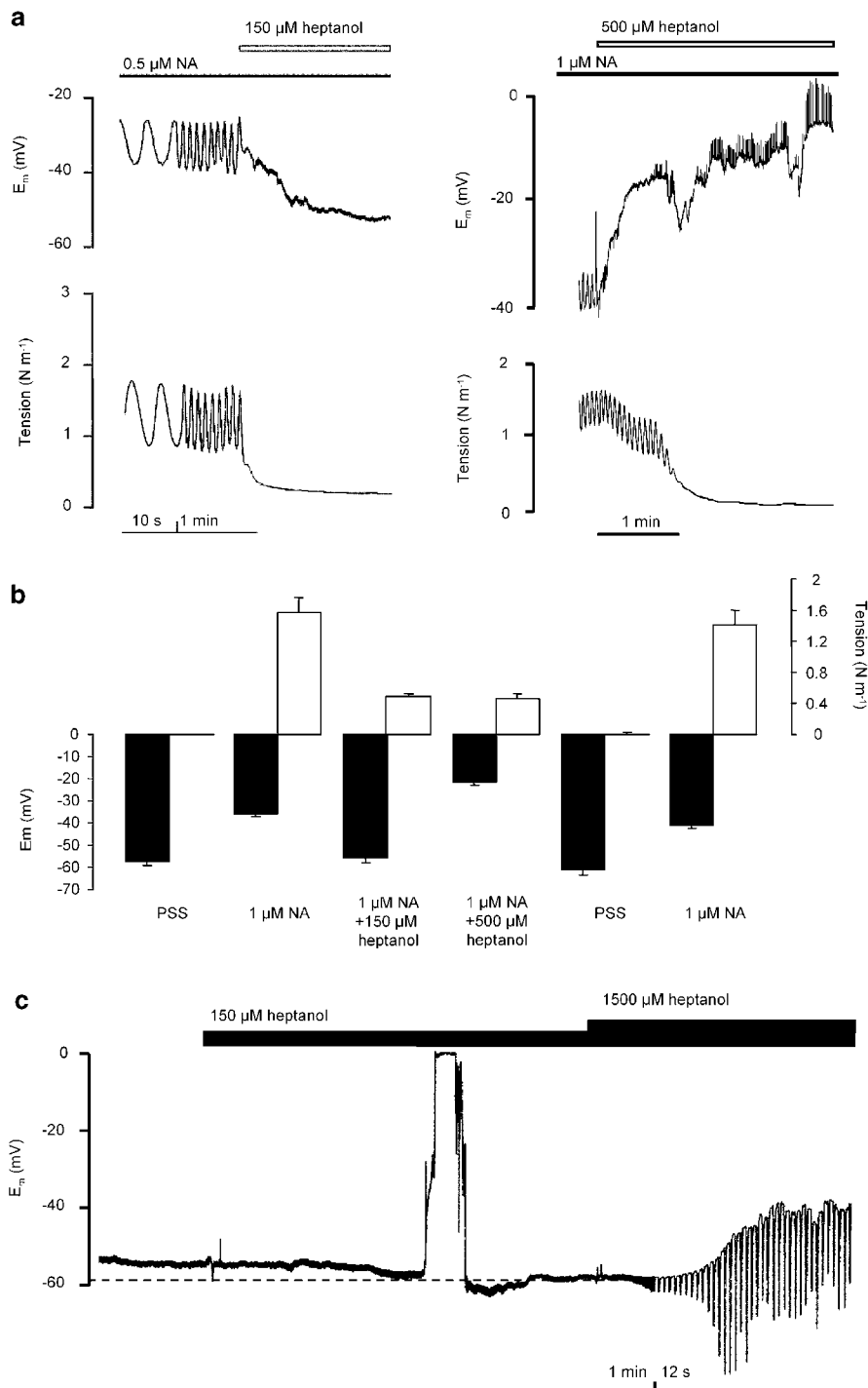
Figure 1 Effect of heptanol on membrane potential, cell input resistance and tension. (a) Original recordings of simultaneous measurements of membrane potential and isometric tension in isolated mesenteric small arteries activated with noradrenaline (NA). (b) Bars illustrate the reversibility of heptanol responses on membrane potential and tension: arteries ($n = 4$) were stimulated with NA, superfused with 150 and 500 μM heptanol, rinsed for 20 min and stimulated with NA again. No significant difference between first and second NA stimulation was found. (c) Original recordings of the effects of heptanol on membrane potential and cell input resistance (rapid deflections) in isolated mesenteric small arteries under resting conditions. Heptanol was present as indicated. Note the change in time scale.

heptanol activated an outward current that reversed below −60 mV and was inhibited by 100 nM iberiotoxin (Figure 4a). The current densities at a membrane potential of +20 mV were 2.5±0.4, 4.7±0.8 and 2.0±0.3 A F^{-1} under control conditions, after superfusion with 150 μM heptanol and following application of 100 nM iberiotoxin, respectively ($n = 5$, cell capacitance was 22.1±1.7 pF). This indicates that heptanol at this concentration activates large-conductance,

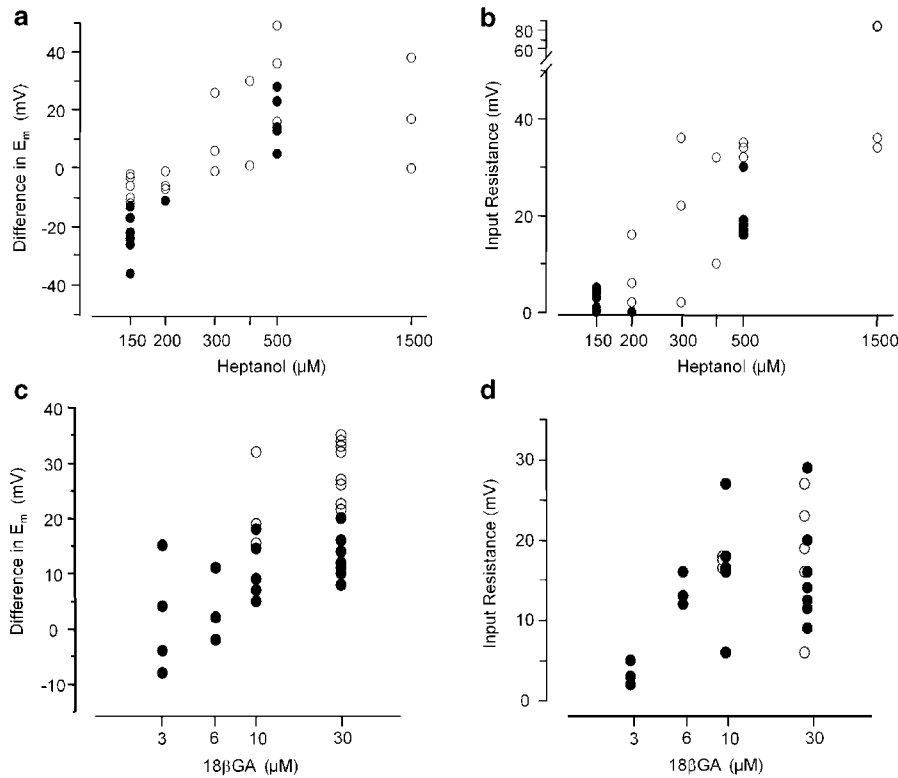**Figure 2** Concentration–response relations for effects of heptanol (a, b) and 18β-glycyrrhetinic acid (c, d) on membrane potential (a, c) (differences in membrane potential after and before addition of chemicals) and on cell input resistance (b, d) (the increase in amplitude of voltage responses to 1 nA current injection). Experiments were performed under resting conditions (open circles) and during activation with noradrenaline (filled circles).

Ca^{2+}-activated potassium channels. At $500\,\mu M$ heptanol, another membrane current was evoked which reversed at $-34\pm7.6\,mV$ ($n=5$) and, therefore, could depolarise the membrane at physiological membrane potentials: inward current densities at $-60\,mV$ were $-0.3\pm0.1\,A\,F^{-1}$ in control and $-1.7\pm0.4\,A\,F^{-1}$ after $500\,\mu M$ heptanol application (cell capacitance $17.5\pm2.2\,pF$; $n=5$) (Figure 4b).

At concentrations up to $100\,\mu M$ 18α-GA did not modify membrane conductance at membrane potentials between -60 and $+20\,mV$ (Figure 4c; $n=4$). However, at more positive membrane voltages $18\alpha GA$ had a tendency to activate outward currents: current densities were 3.7 ± 0.5, 11.9 ± 3.9 and $16.8\pm5.2\,A\,F^{-1}$ at $+60\,mV$ membrane potential under control conditions and in the presence of $30\,\mu M$ and $100\,\mu M$ $18\alpha GA$, respectively (NS; $n=4$; cell capacitance $26.8\pm2.6\,pF$).

In contrast to the α-isomer, $18\beta GA$ increased membrane conductance at negative potentials. The net current activated by $18\beta GA$ reversed at about $-26\,mV$ (Figure 4d). Under physiological conditions this current would lead to membrane depolarisation, which is consistent with the membrane potential measurements. The inward current densities at $-60\,mV$ were $-0.4\pm0.1\,A\,F^{-1}$ in control and $-4.0\pm1.1\,A\,F^{-1}$ after $30\,\mu M$ $18\beta GA$ application (cell capacitance $18.3\pm3.2\,pF$; $n=3$).

Activation of potassium channels by heptanol was also observed in membrane potential measurements on artery segments. NA depolarised arteries from -62.0 ± 3.6 to $-35.4\pm1.6\,mV$; addition of $150\,\mu M$ heptanol repolarised the membrane to $-55.4\pm4.4\,mV$, a membrane potential change of

$-20.0\pm4.3\,mV$ ($n=6$ cells/6 arteries). The repolarisation by heptanol was antagonised by $100\,nM$ of either iberiotoxin or charybdotoxin: the change in potential in response to heptanol was $-12.0\pm1.7\,mV$ ($n=7$ cells/6 arteries). The remaining repolarisation may partly be due to activation of K_{ATP} channels, since the repolarisation was only $-9.3\pm3.7\,mV$ ($n=4$ cells/4 arteries) in the presence of $10\,\mu M$ glibenclamide together with either $100\,nM$ iberiotoxin or $100\,nM$ charybdotoxin.

On the basis of these results we expected the opening of K_{Ca} channels to contribute to the relaxation induced by $150\,\mu M$ heptanol: blocking these channels would, therefore, reduce the heptanol relaxant effect. However, as seen in Figure 5, this was not the case. Force was similarly affected by heptanol in the absence and presence of $100\,nM$ iberiotoxin. This observation prompted us to investigate additional effects of heptanol in these arteries.

Effects on force and $[Ca^{2+}]_i$

Arteries stimulated with a submaximal concentration of NA responded with an increase in force and $[Ca^{2+}]_i$ with superimposed oscillations on both (Figure 6a–c). Both force and $[Ca^{2+}]_i$ elevations were inhibited by $150\,\mu M$ heptanol (Figure 6a) and by $30\,\mu M$ $18\beta GA$ (Figure 6c) but were not affected by $18\alpha GA$ at concentrations up to $100\,\mu M$ (Figure 6b). Heptanol reduced both tension and $[Ca^{2+}]_i$ in a concentration-dependent manner, and was less potent in arteries activated with high NA concentrations (compare $10\,\mu M$ in Figure 7a

with $2 \mu M$ in Figure 7b). In potassium-activated arteries heptanol also reduced both tension and $[Ca^{2+}]_i$ (Figure 7c) although this inhibition was less than in NA-activated arteries. $18\beta GA$ also concentration-dependently reduced both tension

and $[Ca^{2+}]_i$ in arteries activated with NA or potassium (Figure 7e and f). In contrast, 30–$100 \mu M$ $18\alpha GA$ had no effect on either tension or $[Ca^{2+}]_i$ (Figure 7d).

In the presence of $150 \mu M$ heptanol tension could be recovered by increasing the NA concentration: oscillations also reappeared in four out five experiments but with reduced amplitude (Figure 6d). With $500 \mu M$ heptanol present oscillations did not reappear (not shown), nor was it possible to restore oscillations in the presence of $30 \mu M$ $18\beta GA$ (Figure 6e). $18\alpha GA$ at concentrations 30–$100 \mu M$ had no effect on oscillation amplitude or frequency ($n = 9$).

Effects on calcium currents

Since iberiotoxin did not reverse the effect of $150 \mu M$ heptanol on tension in NA-activated arteries, and because $150 \mu M$ heptanol reduced $[Ca^{2+}]_i$ (and tension) in potassium-activated arteries, it seemed possible that heptanol could inhibit Ca^{2+} entry in these arteries by a mechanism distinct from its effect on membrane potential. We therefore tested the effect of heptanol on whole-cell voltage-sensitive Ca^{2+} currents (Figure 8a). These experiments showed that heptanol concentration-dependently inhibited the nifedipine-sensitive Ca^{2+} current, and that this effect was significant at $150 \mu M$ ($P < 0.05$, $n = 5$). $18\beta GA$ also inhibited the whole-cell voltage-sensitive Ca^{2+} current in a concentration-dependent manner ($P < 0.05$, $n = 5$, Figure 8b) with a significant effect at $10 \mu M$.

Effects on calcium transient synchronisation – confocal experiments

Heptanol and $18\beta GA$ disrupted the synchronisation of calcium signals between smooth muscle cells (Figure 9). Synchronised $[Ca^{2+}]_i$ oscillations in smooth muscle cells were observed in arteries activated with a submaximal NA concentration ($n = 10$). Heptanol at $150 \mu M$ partly desynchronised the $[Ca^{2+}]_i$ oscillations while $500 \mu M$ heptanol led to complete desynchronisation (Figure 9a, $n = 4$). In the presence of $10 \mu M$ $18\beta GA$ $[Ca^{2+}]_i$ transients were partly desynchronised and $30 \mu M$ $18\beta GA$ completely abolished synchronisation (Figure 9b, $n = 6$).

Discussion

We evaluated the specificity of heptanol, $18\alpha GA$ and $18\beta GA$ in blocking gap junctions in mesenteric small artery smooth muscle. The specificity for gap junctions of heptanol (Javid
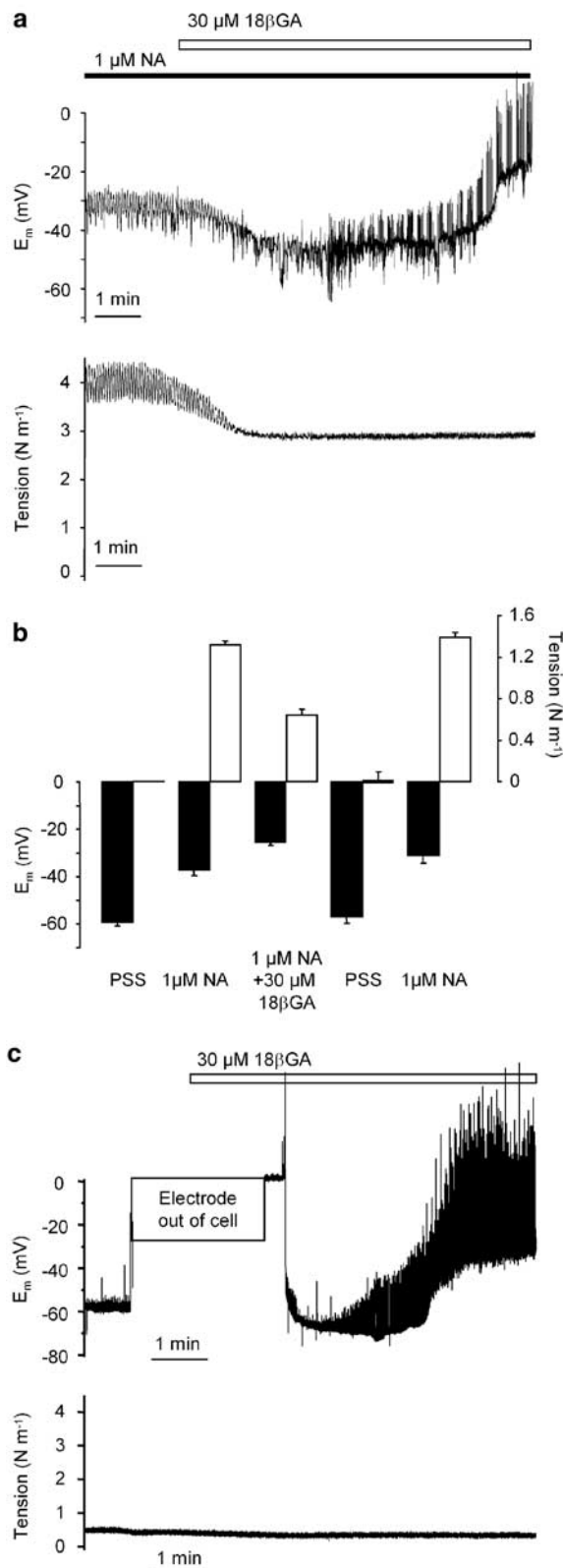

Figure 3 Effects of 18β-glycyrrhetinic acid on membrane potential, cell input resistance (rapid deflections) and tension. (a) Original recordings of simultaneous measurements of membrane potential and isometric tension of isolated mesenteric small arteries activated with noradrenaline (NA). (b) Bars illustrate the reversibility of 18β-glycyrrhetinic acid responses on membrane potential and tension: arteries ($n = 7$) were stimulated with NA, superfused with 18β-glycyrrhetinic acid, rinsed for 15 min and stimulated with NA again. No significant difference between first and second NA stimulation was found. (c) Effects of 18β-glycyrrhetinic acid on membrane potential and cell input resistance in isolated mesenteric small arteries under resting conditions. 18β-glycyrrhetinic acid was present as indicated.
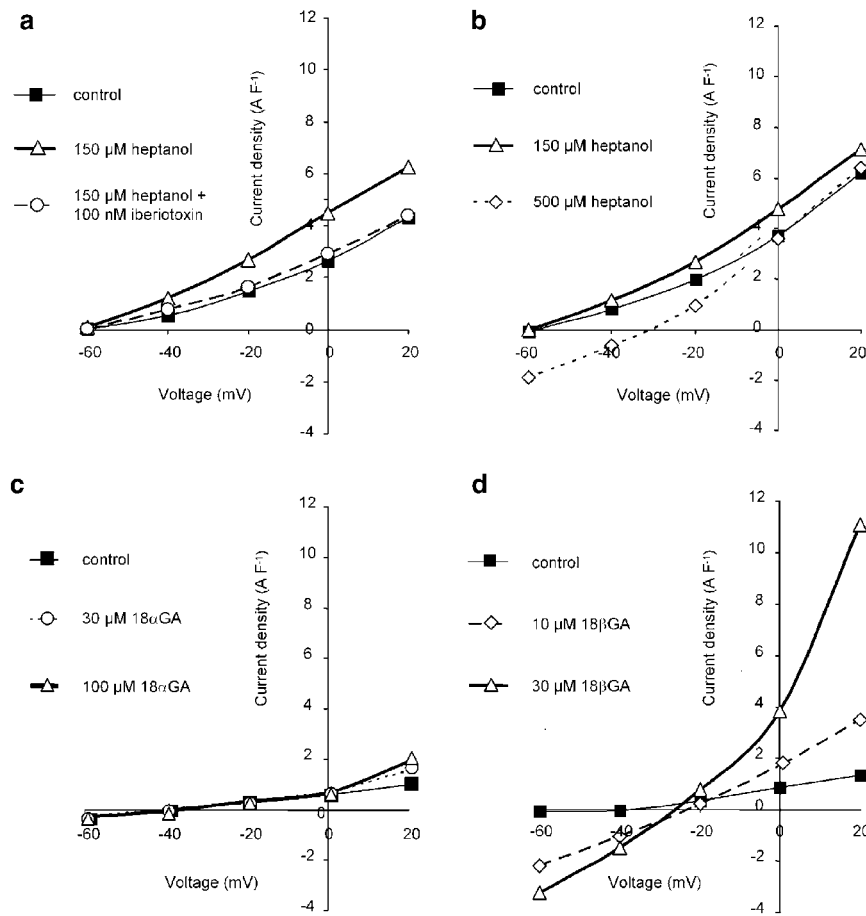
Figure 4 Current–voltage relations obtained in isolated smooth muscle from rat mesenteric small arteries. (a) I/V relation under control conditions, in the presence of 150 μM heptanol and in the presence of 150 μM heptanol and 100 nM iberiotoxin. (b) I/V relation under control conditions, in the presence of 150 μM heptanol and in the presence of 500 μM heptanol. (c) I/V relation under control conditions, in the presence of 30 μM 18α-glycyrrhetinic acid and in the presence of 100 μM 18α-glycyrrhetinic acid. (d) I/V relation under control conditions, in the presence of 10 μM 18β-glycyrrhetinic acid and in the presence of 30 μM 18β-glycyrrhetinic acid.

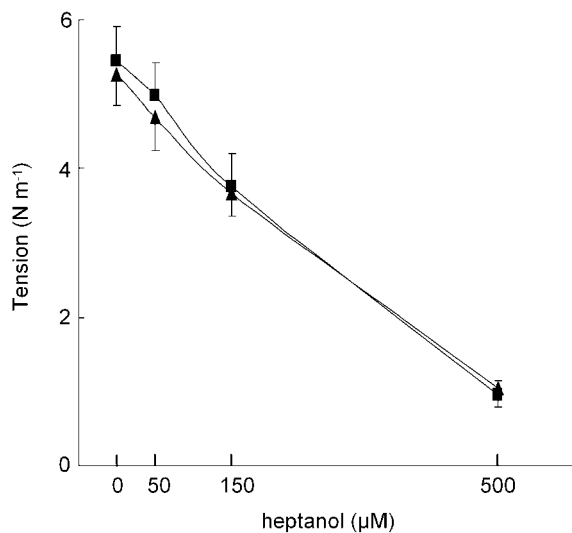**Figure 5** Concentration–tension relation for heptanol in arteries activated with 10 μM noradrenaline (triangles) and with 10 μM noradrenaline in the presence of 100 nM iberiotoxin (squares), $n = 4$. Vertical bars indicate s.e.m.

et al., 1996; Chaytor *et al.*, 1997; 1998; Yamamoto *et al.*, 1998; Murai *et al.*, 1999), 18αGA (Chaytor *et al.*, 2000; Schuster *et al.*, 2001; Tare *et al.*, 2002) and 18βGA (Davidson & Baumgarten, 1988; Santicioli & Maggi, 2000; Coleman *et al.*, 2001; 2002; Tare *et al.*, 2002) has often been questioned but there have been few direct measurements of their specificity in the intact artery wall (Hashitani & Suzuki, 1997; Yamamoto *et al.*, 1998; Murai *et al.*, 1999; Tare *et al.*, 2002). The present study demonstrates that heptanol and 18βGA increase input resistance and prevent synchronisation between single smooth muscle cells in the arterial wall – observations consistent with inhibition of gap junctions – whereas 18αGA is without significant effect. Most importantly, it also demonstrates nonjunctional effects of heptanol and 18βGA on ion currents, $[Ca^{2+}]_i$ and force that are present at concentrations lower than those required for significant inhibition of gap junctions.

We showed that 150 μM heptanol causes hyperpolarisation, decreases $[Ca^{2+}]_i$ and tension, but has no significant effect on input resistance. At higher concentrations input resistance increased, oscillation in $[Ca^{2+}]_i$ became desynchronised and vasomotion stopped – all effects consistent with blockade of gap junctions. The most likely reason for the hyperpolarising
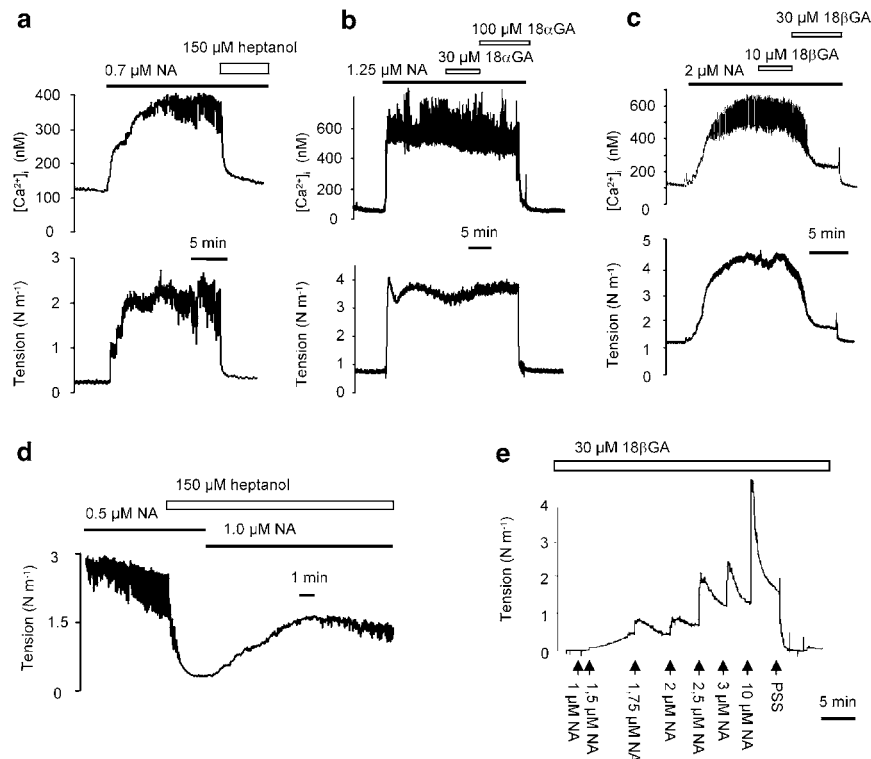
Figure 6 Original recordings of the effects of heptanol (a), 18αGA (b) and 18βGA (c) on tension and $[Ca^{2+}]_i$ in arteries stimulated with a submaximal concentration of noradrenaline (NA). (d) Addition of 150 μM heptanol relaxes the artery and inhibits NA-induced oscillations, but force can be recovered by increasing the NA concentration; oscillation reappeared, but with reduced amplitude. (e) In the presence of 30 μM 18βGA NA evokes only tonic contraction.
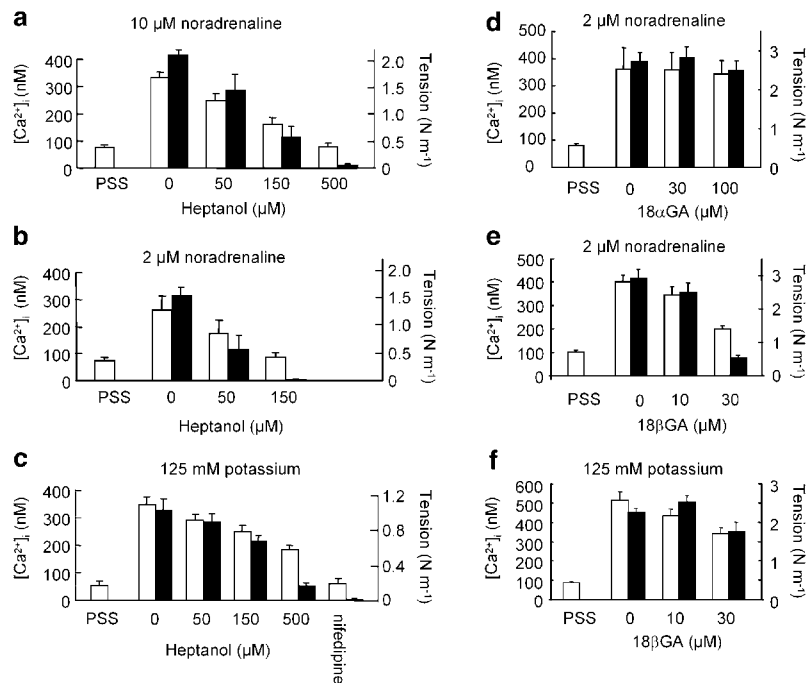

Figure 7 Concentration-dependent effects of heptanol (a–c), 18αGA (d) and 18βGA (e–f) on $[Ca^{2+}]_i$ (open bars) and tension (filled bars). Arteries were activated with 10 μM noradrenaline, $n = 5$ (a), 2 μM noradrenaline, $n = 4$ (b), 125 mM potassium, $n = 5$ (c), 2 μM noradrenaline, $n = 7$ (d), 2 μM noradrenaline, $n = 10$ (e) or 125 mM potassium, $n = 9$ (f). Vertical bars indicate s.e.m.
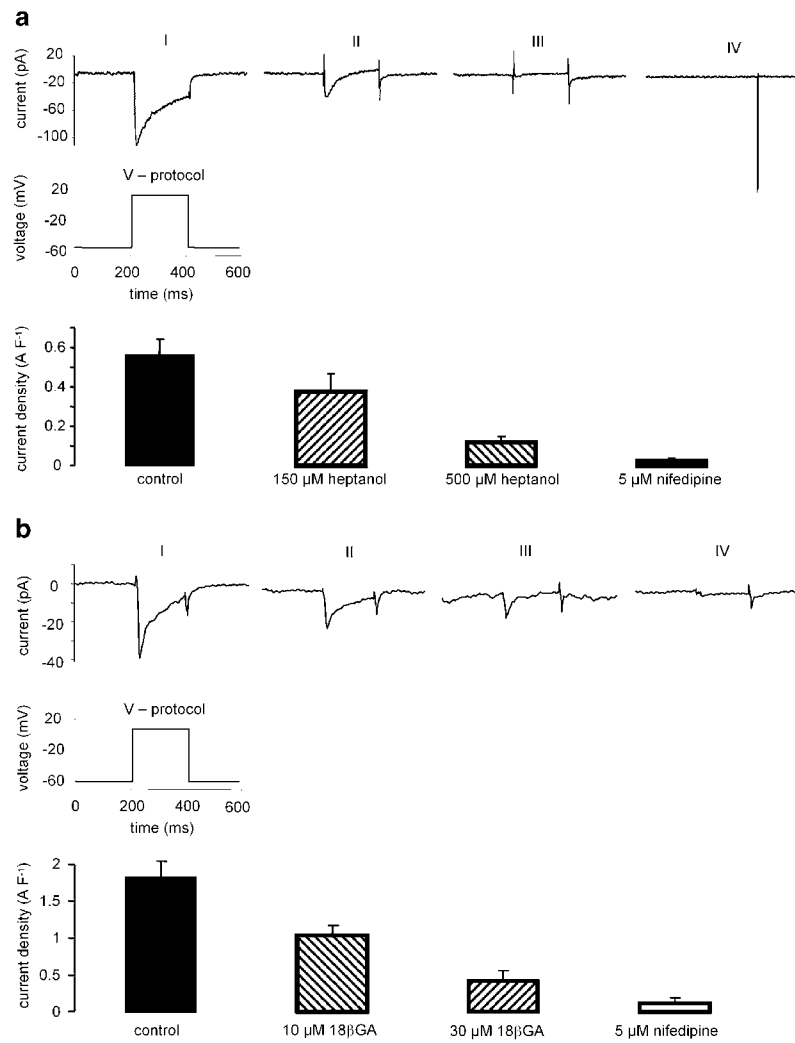
Figure 8 Whole-cell calcium currents from smooth muscle cells isolated from rat mesenteric small arteries. Traces in (a) show (I) control current, with (II) 150 μM and (III) 500 μM heptanol and with (IV) 5 μM nifedipine; (V) shows the voltage protocol applied. $n = 5$. Traces in (b) show (I) control current, with (II) 10 μM and (III) 30 μM 18βGA and (IV) 5 μM nifedipine; (V) shows the voltage protocol applied; $n = 4$. Vertical bars indicate s.e.m.
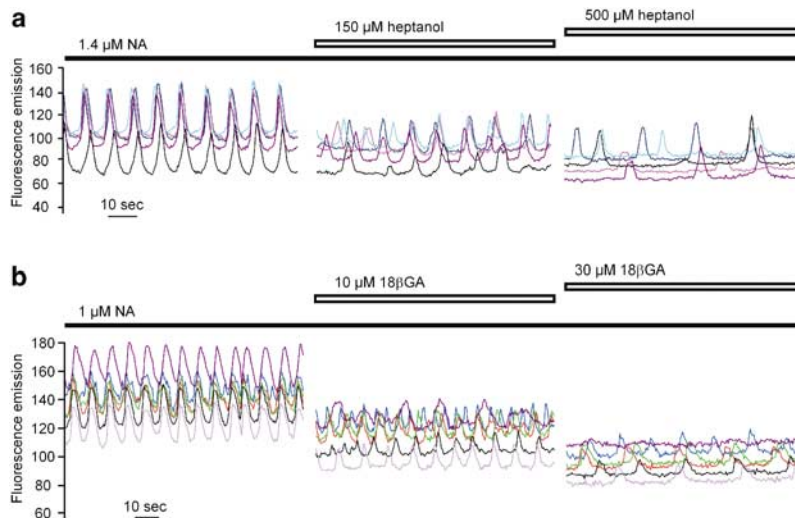

Figure 9 Confocal recordings of $[Ca^{2+}]_i$ in regions of interest (ROI, see Methods). Each trace represents one ROI corresponding to a single smooth muscle cell in the arterial wall. (a) Effects of 150 and 500 μM heptanol on an artery activated with noradrenaline (NA). (b) Effects of 10 and 30 μM 18βGA.

effect of 150 μM heptanol is the opening of K_{Ca} and possibly K_{ATP} channels, although other mechanisms may contribute. In non-vascular smooth muscle heptanol has been reported to lack effect on membrane potential (Blennerhassett & Garfield, 1991; Manchanda & Venkateswarlu, 1997), or to cause a depolarisation at concentrations $\geqslant$200 μM (Huizinga *et al.*, 1988). Despite recent findings that 1 mM heptanol hyperpolarises VSM cells in cerebral arteries (Lagaud *et al.*, 2002) and is without effect on the membrane potential in submucosal arterioles (Hashitani & Suzuki, 1997), we found that in concentrations $\geqslant$500 μM heptanol depolarises mesenteric small arteries. It therefore seems likely that the nonjunctional effects of heptanol are concentration-dependent and may vary between preparations. 18βGA at 5–10 μM increased input resistance and partly desynchronised the calcium transients in smooth muscle cells, consistent with inhibition of gap junctions (Chaytor *et al.*, 1997; Dhein, 1998; Saez *et al.*, 2003). At these concentrations it also depolarised the membrane, reduced $[Ca^{2+}]_i$ and relaxed the arteries. A depolarising effect of 18βGA on smooth muscle cells has been reported previously in submucosal arterioles (Imaeda *et al.*, 2000; Coleman *et al.*, 2001) and in tail artery (Tare *et al.*, 2002). Consistent with the depolarisation, we found both heptanol and 18βGA to be associated with an increase of a net inward (depolarising) current in isolated cells at physiological membrane potentials. This increase of membrane conductance may be due to the lipophilic nature of heptanol (Burt & Spray, 1989; Bastiaanse *et al.*, 1993; Guan *et al.*, 1997; Yamamoto *et al.*, 1998) and 18βGA (Davidson & Baumgarten, 1988; Itoh *et al.*, 1989; Yamamoto *et al.*, 1998; Coleman *et al.*, 2001) but cannot be due to an effect on intercellular communication as increased membrane conductance was seen in isolated smooth muscle cells.

It seems unlikely that potassium channel activation is the only mechanism responsible for the relaxation to 150 μM heptanol since iberiotoxin was without effect on the relaxation and because 150 μM heptanol also reduced $[Ca^{2+}]_i$ in potassium-activated arteries. These findings could suggest a direct action of heptanol on Ca^{2+} channels, which is consistent with our patch-clamp measurements, showing inhibition of Ca^{2+} channels by heptanol. The same principle could apply to the observation with 18βGA. Therefore, we suggest that blockade of Ca^{2+} channels is a common mechanism by which these compounds relax depolarised arteries.

In contrast to heptanol and 18βGA, 100 μM 18αGA did not have any detectable effect on smooth muscles. 18αGA has been suggested previously to be a specific gap junction blocker at concentrations up to 100 μM (Davidson *et al.*, 1986; Davidson & Baumgarten, 1988) where it effectively blocks myoendothelial gap junctions (Taylor *et al.*, 1998). In preliminary experiments we also found 18αGA to attenuate acetylcholine-induced responses, indicating possible gap junction block between endothelial and smooth muscle cells (data not shown). These findings are in accordance with previous reports describing 18αGA as myoendothelial uncoupler (Taylor *et al.*, 1998; Kagota *et al.*, 1999; Hill *et al.*, 2000). Although 18αGA has been used to block communication between smooth muscle cells in pressurised rat middle cerebral arteries (Lagaud *et al.*, 2002), this contrasts with many others previous reports (Taylor *et al.*, 1998; Chaytor *et al.*, 2000; Schuster *et al.*, 2001) as well as with the present study where 100 μM 18αGA did not change vascular tone and $[Ca^{2+}]_i$ in the smooth muscle. 18αGA failed to affect the oscillation in tone and $[Ca^{2+}]_i$ observed in intact arteries nor did it affect the ionic currents in isolated smooth muscle cells, which is consistent with a lack of effect on membrane potential observed in guinea pig coronary artery smooth muscle (Tare *et al.*, 2002). Thus, 18αGA seems to have neither junctional nor nonjunctional effects on mesenteric small arteries smooth muscle.

In conclusion, these findings suggest that the vasodilator effects of heptanol and 18βGA can be mediated through several mechanisms unrelated to an effect on gap junctions. These nonjunctional effects appear at concentrations lower than those required to significantly increase input resistance (which signifies the inhibition of gap junctional communication). Our data suggest that 18αGA cannot be used to block gap junctions between smooth muscle cells in mesenteric small arteries (although it might be effective in inhibiting myoendothelial communication), and that caution is needed when interpreting the effects of using heptanol and 18βGA in vascular preparations. Ascribing the tension reduction seen with these compounds solely as a response to gap junction inhibition is not justified in the absence of more direct evidence, and this should be kept in mind when interpreting data obtained with these compounds.

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