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Effects of chloride channel blockers on hypotonicity-induced contractions of the rat trachea

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1 We have investigated the inhibitory effects of blockers of volume-activated (Cl_{vol}) and calcium-activated (Cl_{Ca}) chloride channels on hypotonic solution (HS)-induced contractions of rat trachea, comparing their effects with those of the voltage-dependent calcium channel (VDCC) blocker nifedpine.

2 HS elicited large, stable contractions that were partially dependent on the cellular chloride gradient; a reduction to $41.45 \pm 7.71\%$ of the control response was obtained when extracellular chloride was removed. In addition, HS-induced responses were reduced to $26.8 \pm 5.6\%$ of the control by 1 μM nifedipine, and abolished under calcium-free conditions, indicating a substantial requirement for extracellular calcium entry, principally *via* VDCCs.

3 The established Cl_{vol} blockers tamoxifen ($\leqslant 10\,\mu$M) and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (1–100 μM), at concentrations previously reported to inhibit Cl_{vol} in smooth muscle, did not significantly inhibit HS-induced contractions.

4 In contrast, the recognized Cl_{Ca} blocker niflumic acid (NFA; 1–100 μM) produced a reversible, concentration-dependent inhibition of HS responses, with a reduction to $36.6 \pm 6.4\%$ of control contractions at the highest concentration. The mixed Cl_{vol} and Cl_{Ca} blocker, 5-nitro 2-(3-phenylpropylamine) benzoic acid (NPPB; 10–100 μM) also elicited concentration-related inhibition of HS-induced contractions, producing a decrease to $35.9 \pm 11.3\%$ of the control at 100 μM.

5 Our results show that HS induces reversible, chloride-dependent contractions of rat isolated trachea that were inhibited by NFA and NPPB, while exhibiting little sensitivity to recognized blockers of Cl_{vol}. The data support the possibility that opening of calcium-activated chloride channels under hypotonic conditions in respiratory smooth muscle may ultimately lead to VDCC-mediated calcium entry and contraction.

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Abbreviations: 0Ca, calcium-free solution; $0Cl^-$, chloride-free solution; Cl_{Ca}, calcium-activated chloride channel; Cl_{vol}, volume-activated chloride channel; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; DMSO, dimethyl sulphoxide; E_{Cl}, equilibrium potential for chloride; HS, hypotonic solution; $HS0Cl^-$, chloride-free hypotonic solution; 5-HT, 5-hydroxytryptamine; NFA, niflumic acid; NIF, nifedipine; NMDG, *N*-methyl-D-glucamine; NPPB, 5-nitro 2-(3-phenylpropylamine) benzoic acid; TAM, tamoxifen; TEA, tetraethylammonium; VDCC, voltage-dependent calcium channel

Introduction

The role of chloride in the contractility of smooth muscle has received increasing interest (for reviews, see Large & Wang, 1996; Chipperfield & Harper, 2000), with much research recently directed towards understanding of both the elusive identity (Jentsch *et al*., 2002) and physiological role(s) of volume-activated chloride channels (Cl_{vol}). These channels are widely encountered in a variety of vascular and nonvascular smooth muscle cell types, including guinea-pig antrum (Xu *et al*., 1997), canine pulmonary and renal arteries (Yamazaki *et al*., 1998), canine colon (Dick *et al*., 1998; 1999) and rabbit portal vein (Greenwood & Large, 1998). The consensus of current evidence obtained from single-cell electrophysiological studies in smooth muscle indicates that Cl_{vol} channels are relatively selectively inhibited by substances such as 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) and tamoxifen (TAM), while exhibiting little or no sensitivity to established blockers of calcium-activated chloride channels (Cl_{Ca}) such as niflumic acid (NFA) (Greenwood & Large,

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1998; Dick *et al*., 1999; Jentsch *et al*., 2002). An exception to the general trend has been the study of Xu *et al*. (1997), which demonstrated a clear inhibition of Cl_{vol} channels by 10 μM NFA.

Previous investigations have shown that chloride conductances are activated by hypotonic solutions (HSs) in myocytes from rat portal vein (Greenwood & Large, 1998), guinea-pig antrum (Xu *et al*., 1997) and rabbit myocardium (Hagiwara *et al*., 1992), however, little is known about the functional consequence of channel activation in whole tissues. A prior study in vascular smooth muscle has suggested that application of extracellular HS may activate anion channels, leading to a depolarization-mediated opening of voltage-dependent calcium channels (VDCC) and subsequent contraction of aorta (Lang *et al*., 1995). As yet, however, to our knowledge, there have been no functional studies specifically examining the pharmacology of Cl_{vol} in respiratory smooth muscle.

Recently, we have demonstrated the selective inhibitory effects of NFA on 5-hydroxytryptamine (5-HT)-induced chloride-dependent contractions of the rat trachea (Teixeira *et al*., 2000), supporting a role for Cl_{Ca} in agonist-induced contractile responses. In the present study, we decided to perform a pharmacological investigation of the effects of commercially available blockers of Cl_{vol} and Cl_{Ca}, on HS-induced contractions in this tissue, comparing their effects with those of the VDCC blocker nifedpine (NIF). Preliminary data have been presented to the XVI Latinamerican Congress of Pharmacology, São Paulo, Brazil (Coelho *et al*., 2000).

Methods

Isolated tracheal preparation

Institutional approval for experimental protocols used in this study was obtained. Male Wistar rats (250–350 g) were killed by stunning and exsanguination. The neck was opened, the trachea carefully dissected and rings (1 cm wide) were prepared and mounted vertically in an organ bath containing Tyrode's solution bubbled with air (37°C, pH 7.4). Tissues were stabilized under an initial resting tension of 1 *g* for a period of 1 h before starting the experimental protocols. Tension changes were recorded using a computerized data-aquisition system (Windaq v1.65; DATAQ Instruments, Inc., U.S.A.).

The effects of NFA (1–100μM), TAM (1–100 μM), DIDS (1–100 μM), 5-nitro 2-(3-phenylpropylamine) benzoic acid (NPPB, 1–100 μM) and NIF (0.01–10 μM) were assessed on contractions induced by HS (Tyrode's solution with 50% NaCl). In each experimental protocol, two HS-induced contractions (interval of ten minutes) were initially obtained as controls during the experiment. The data are expressed as percentages of these initial responses. Following stable control contractions to HS, NFA, NIF, DIDS, NPPB, TAM (pre-exposure time of 10 min) were applied to the organ bath in increasing concentrations and further responses to HS were obtained. Since dimethyl sulphoxide (DMSO) was used as a solvent for stock solutions of NFA, the effects of this solvent at equivalent concentrations were assessed on HS-induced contraction of the trachea as time-matched controls.

In separate experiments, the effect of extracellular chloride withdrawal from the bathing solution on the responses of HS was assessed. Initially, two control contractions to HS were obtained. The tissues were then bathed in a modified Tyrode's solution ($0Cl^-$), in which all chloride salts had been substituted by their gluconate equivalents. Since application of $0Cl^-$ solution to the preparation caused a transient contraction, reapplication of HS without chloride (HS $0Cl^-$) was performed only when basal levels of tone had been restored. In addition, the reversibility of any effect on HS-induced contraction by $0Cl^-$ solution was assessed, by reintroducing normal (Cl^--containing) HS to the bath. Similarly, substitution of Na^+ was carried out using *N*-methyl-D-glucamine (NMDG) and the effects of HS (50% NMDG) were assessed accordingly. Additionally, in order to evaluate whether the contractile response was due to hypotonicity *per se* or due to a reduction of ionic strength, experiments were performed in which 50% of NaCl was taken away and mannitol added to maintain an iso-osmolar external solution (Greenwood & Large, 1998).

To assess the participation of extracellular calcium on the contraction induced by HS, a Tyrode's solution without $CaCl_2$ (10 mM EGTA) was used (0Ca). Initially, two HS-induced control contractions were obtained. Following this, the tissues were bathed in a modified 0Ca Tyrode's solution for 10 min and 60 mM KCl solution was applied to verify that any membrane-bound calcium had been removed by washing. Following washout of the KCl, tissues were then exposed to HS in nominally calcium-free solution. Finally, the tissues were bathed with calcium-containing Tyrode's solution and HS reapplied to verify the recuperation of the tissue response.

Solutions and drugs

The bathing solution was a modified Tyrode's solution of the following composition (mM): NaCl 136, KCl 5, $MgCl_2$ 0.98, $CaCl_2$ 2, NaH_2PO_4 0.36, $NaHCO_3$ 11.9, glucose 5.5. In solutions in which the potassium concentration was raised (60 mM), the NaCl concentration was concomitantly reduced to maintain osmolarity of the solution. The HS was of the same composition as modified Tyrode's solution, except with a 50% reduction of NaCl. The chloride-free (0Cl) and calcium-free (0Ca) solutions were of the same composition as the modified Tyrode's solution, except that all the chloride salts were replaced by their gluconate equivalents, and $CaCl_2$ was omitted with addition of EGTA (10 mM), respectively. The pH was always maintained constant throughout the experimental period at 7.4.

The following drugs were used: NFA, nifedipine (NIF), TAM, DIDS, NPPB, acetazolamide and bumetanide. NIF stock solution was prepared in 70% ethanol under conditions of reduced illumination, and all experiments with NIF were performed under similar conditions. NFA, TAM, DIDS and NPPB were prepared as a 10^{-2} M stock solutions in DMSO, and diluted on the day of the experiment in fresh Tyrode's solution. All the reagents were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.), Merck (Darmstadt, Germany) or Reagen (Rio de Janeiro, RJ, Brazil).

Analysis of data

Data are expressed as the mean of n observations $\pm$ s.e.m. Inhibitory effects are expressed as % of control responses in the absence of the drug. Statistical analysis was performed using ANOVA and a Bonferroni *post hoc* test, with values taken to be significantly different from controls when $P<0.05$.

Results

Contractile effects of HS on rat trachea

Exposure of the rat tracheal rings to HS, by progressively reducing the normal NaCl content of the Tyrode's solution by 25, 50, 75 and 100%, induced reproducible, dose-dependent contractions, whose maximum response was 0.89 ± 0.10 g ($n = 15$, Figure 1). HS (50%), which induced a submaximal contractile response, was selected for use in subsequent experimental protocols, also in accordance with a previous electrophysiological study (Greenwood & Large, 1998).

Chloride and calcium dependency of the HS-induced contractile responses

In experiments designed to assess the contribution of the cellular chloride gradient to the contraction induced by HS, addition of zero chloride (gluconate-substituted) Tyrode's solution to the tracheal preparation induced a transient contraction of 0.40 ± 0.11 g ($n = 6$) that returned to resting levels within several minutes (Figure 2a). The possible basis for this contraction was briefly assessed by performing paired experiments in the presence and absence of bumetamide (10 μM) and acetazolamide (1 mM), inhibitors of the $Na^+K^+2Cl^-$ cotransporter and Pump III in smooth muscle, respectively (Chipperfield & Harper, 2000). Bumetanide did not alter the contraction induced by $0Cl^-$/gluconate (100.6 ± 23.6% of the control, $n = 5$), however, acetazolamide reduced this response to 50.1 ± 13.8% of the control value ($n = 5$).

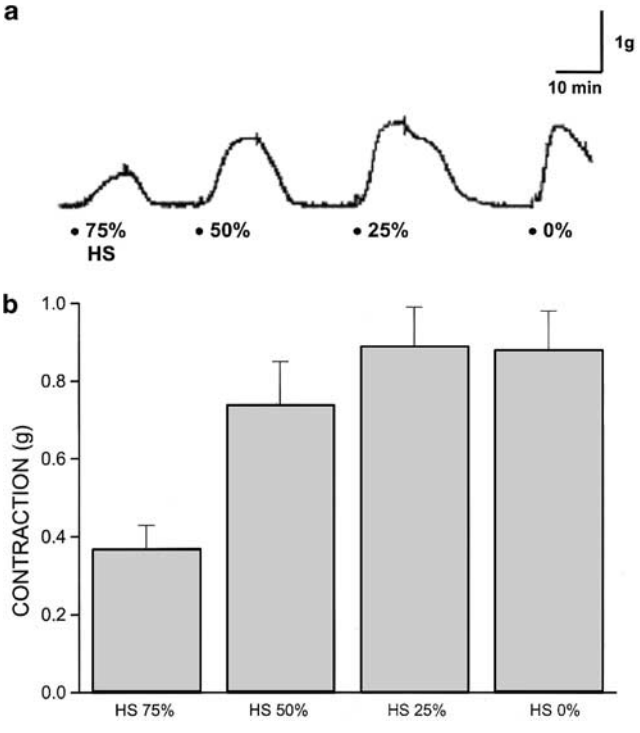

Figure 1 (a) Typical trace and (b) mean data showing contractile responses induced by HS in the rat isolated trachea. HS percentage values indicate NaCl content of the normal Tyrode's solution (e.g. HS 75%; NaCl content reduced by 25% of the normal value). Data are presented as mean $\pm$ s.e.m. ($n = 15$).

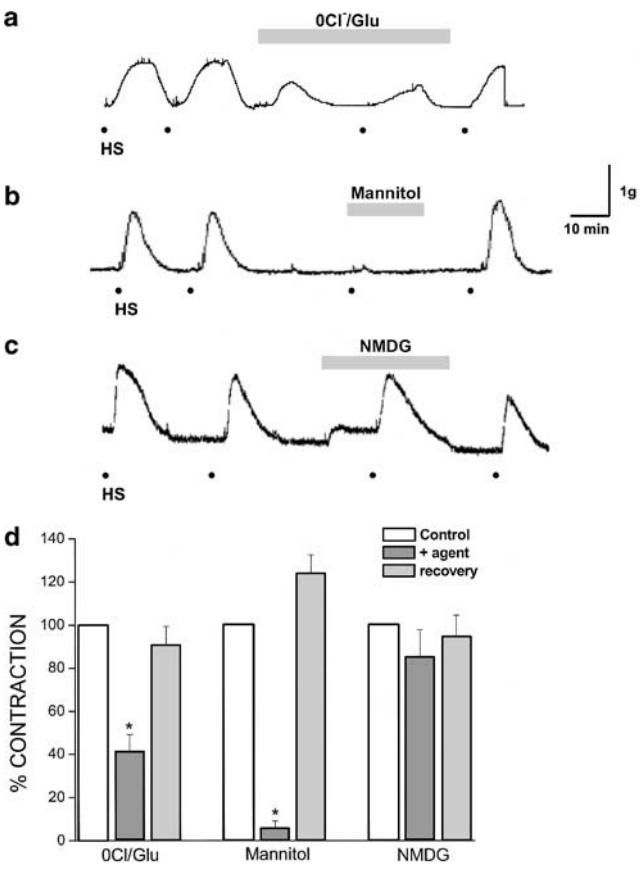

Figure 2 Representative traces showing the effects of (a) $0Cl^-$/Glu (gluconate-substituted), (b) mannitol (to maintain iso-osmolarity) and (c) $0Na^+$/NMDG (NMDG-substituted) on the contractile responses to HS in the rat isolated trachea. (d) Mean data from these experiments. Values are shown as the mean $\pm$ s.e.m. of 5–9 experiments, and are shown to differ significantly from controls when $P < 0.05$ (*).

Following a spontaneous return to basal tonus upon withdrawal of extracellular chloride, subsequent application of HS $0Cl^-$ (50% sodium gluconate) elicited a contraction that was noticeably slower in onset and smaller in magnitude (41.45 ± 7.71% of the control) than the HS-induced response in chloride-containing solution (Figures 2a and d, $n = 6$), indicating a dependency on the transmembrane chloride gradient.

In order to verify that HS-induced contractions were due to a reduction in osmolarity and not simply due to a decrease in ionic strength, experiments were performed in which mannitol was added to the HS in order to maintain isosmotic conditions. In this experimental situation, there was no contraction elicited by reduction of the sodium chloride content of the external physiological solution (Figures 2b and d), indicating that hypotonicity alone was responsible for the HS-induced responses.

Additionally, to evaluate the possible involvement of nonselective cation channels in the HS-induced contractions, experiments were performed in which NMDG was used to substitute for Na^+ in a similar manner as previous experiments with Na-gluconate. Complete substitution of Na^+ for NMDG in the physiological solution produced a

small sustained contraction of the tracheal preparation; however, when hypotonic conditions were imposed (by removal of 50% extracellular NMDG), a contraction not significantly different to controls was obtained (Figures 2c and d, $n=9$).

Under calcium-free conditions, HS control contractions obtained in the presence of extracellular calcium (mean amplitude 0.99 ± 0.28 g, $n=4$) were completely abolished. Following reintroduction of calcium-containing Tyrode's solution, further addition of HS induced a contraction that was 96.4 ± 6.07% of HS control contraction ($n=4$), demonstrating the reversibility of the inhibition (Figure 3a).

In separate experiments, NIF (0.01–1 μM) concentration-dependently inhibited HS-induced contractions (mean control response of 0.70 ± 0.14 g, $n=5$), with a reduction to 26.8 ± 5.6% of the control at 1 μM, a concentration which also completely abolished the contractile response of the trachea to 60 mM KCl (Figure 3b). The IC_{50} value for inhibition of the HS-induced response was 0.29 ± 5.9 μM ($n=5$).

Effects of TAM and DIDS on contractions induced by HS

To test the hypothesis that HS-induced contractions occurred *via* activation of Cl_{vol}, the effects of two recognized blockers of this channel, TAM and DIDS (Greenwood & Large, 1998) were evaluated. Control contractions to HS (mean amplitude of 0.78 ± 0.16 g, $n=6$) were unaltered by TAM ($\leqslant10$ μM), although modest reductions to 80.8 ± 6.04 and 71.9 ± 5.13% of the control was observed in the presence of 30 and 100 μM, respectively (Figures 4a and b, $n=6$). These inhibitory effects were not reversible, however, after a period of 45 min washout.

In separate experiments, HS induced stable contractions (mean amplitude of 0.76 ± 0.17 g) that were not significantly inhibited by DIDS (1–100 μM) (Figure 4c, $n=9$).

Effects of NFA and NPPB on contractions induced by HS

In the absence of marked inhibitory effects of Cl_{vol} blockers, the effects of the Cl_{Ca} blocker NFA were evaluated. HS produced stable control contractions of 0.79 ± 0.06 g, that were inhibited in a concentration-dependent manner by NFA (1–100 μM, Figures 5a and b). A significant ($P<0.05$) reduction of the contractile response to 36.6 ± 6.4% of control was observed at the highest concentration used, with an IC_{50} value of 31.6 ± 6.2 μM ($n=12$). In contrast to the action of TAM, the inhibitory effects of NFA were fully reversible on washout of

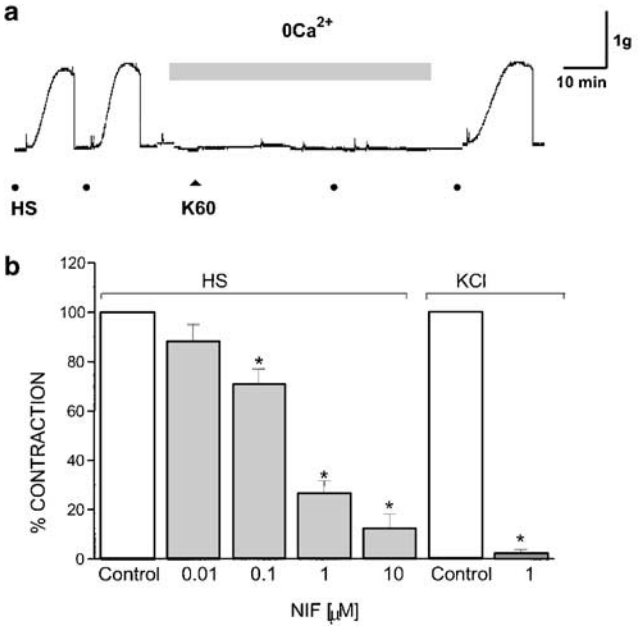

Figure 3 (a) Inhibitory effect of 0Ca solution on the contractile responses to HS in the rat isolated trachea. KCl (60 mM) was added to verify the removal of any membrane-bound calcium. (The chart recording was stopped on washout of the HS.) (b) Inhibitory effects of NIF (0.01–10 μM) on the contractile responses induced by HS ($n=5$) and KCl (60 mM, $n=9$) on the rat isolated trachea. Values are shown as the mean$\pm$s.e.m. of n experiments, and are shown to differ significantly from the control when $P<0.05$ (*).

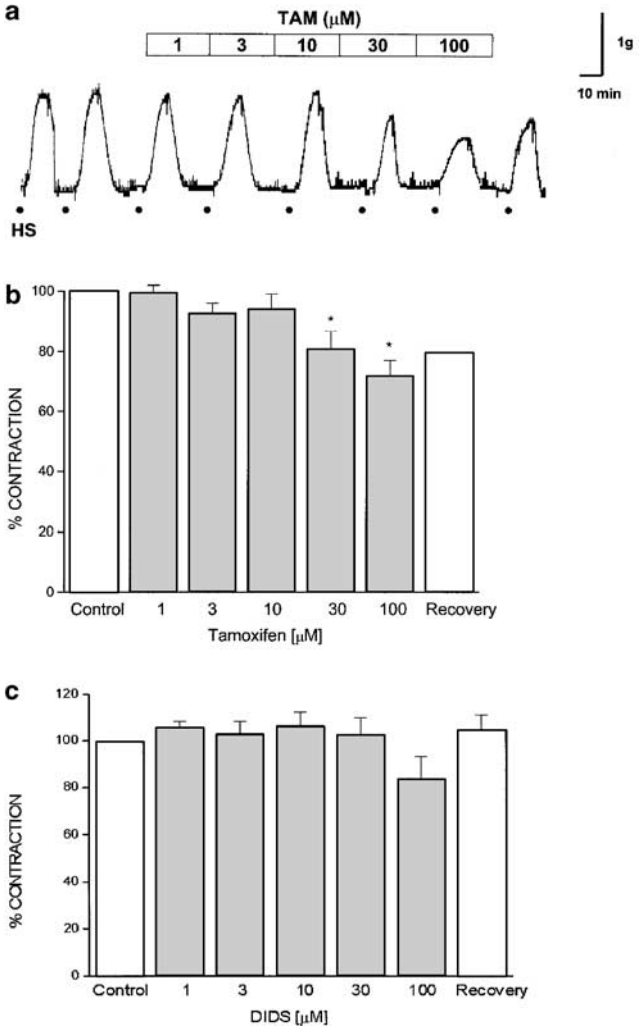

Figure 4 (a) Representative trace and (b) mean data showing the effects of TAM (1–100 μM) on the contractile responses to HS in the rat isolated trachea. Values are shown as the mean$\pm$s.e.m. of six experiments, and are shown to differ significantly from the control when $P<0.05$ (*). (c) Effects of DIDS (1–100 μM) on the contractile responses to HS in the rat isolated trachea. Values are shown as the mean$\pm$s.e.m. of nine experiments, and are shown to differ significantly from the control when $P<0.05$ (*).

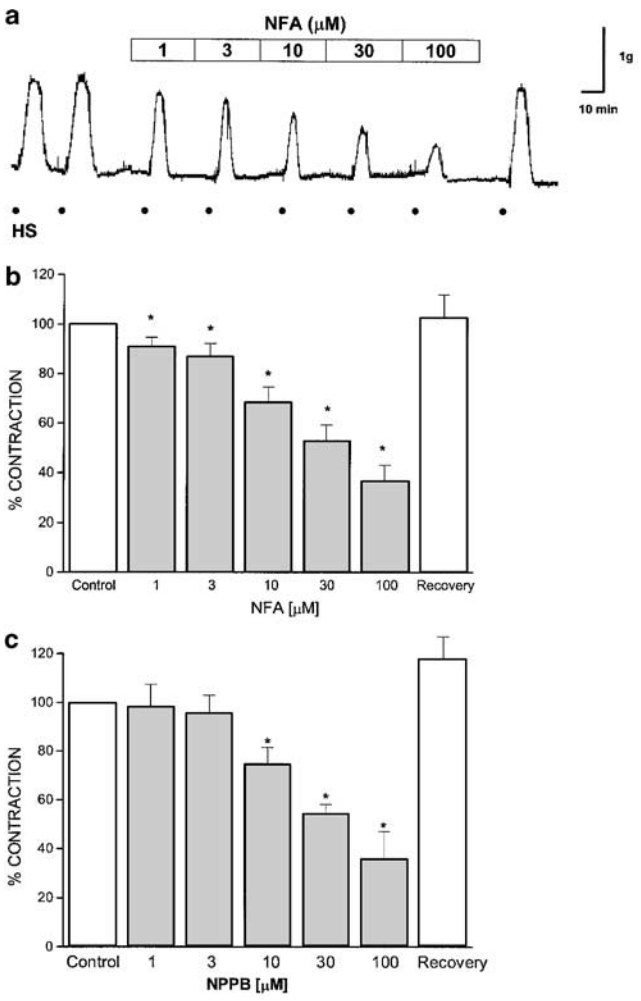

Figure 5 (a) Representative trace and (b) mean data showing the reversible inhibitory effects of NFA (1–100 μM) on the contractile responses to HS in the rat isolated trachea. Values are shown as the mean$\pm$s.e.m. of 12 experiments, and are shown to differ significantly from the control when $P<0.05$ (*). (c) Effects of NPPB (1–100 μM) on the contractile responses to HS in the rat isolated trachea. Values are shown as the mean$\pm$s.e.m. of five experiments, and are shown to differ significantly from the control when $P<0.05$ (*).

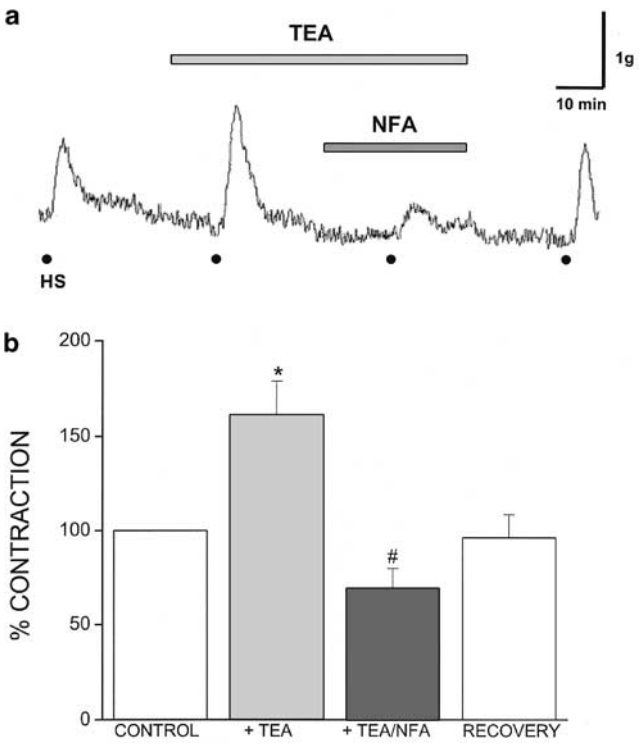

Figure 6 (a) Representative trace and (b) mean data showing the reversible inhibitory effects of NFA (30 μM) on the contractile responses to HS in the rat isolated trachea in the absence and presence of 5 mM TEA. Values are shown as the mean$\pm$s.e.m. of six experiments.

the drug. In parallel control experiments, the solvent DMSO (at equivalent concentrations to those used to dissolve NFA) did not inhibit the contractions produced by HS ($n=4$). In addition, we have previously shown that NFA ($\leqslant 30\,\mu$M) does not inhibit either 40 mM or 60 mM KCl-induced contractions in this preparation (Teixeira *et al*., 2000), suggesting that it does not act by opening of K^+-channels or direct blockade of VDCCs. Furthermore, 30 μM NFA was able to substantially inhibit the contraction elicited by HS in the presence of the nonselective potassium channel blocker tetraethylammonium (TEA) (5 mM), indicating that it was not acting by opening of such ion channels under the present experimental conditions (Figure 6a and b).

The mixed Cl_{vol} and Cl_{Ca} blocker NPPB (10–100 μM) also significantly reduced the contractions induced by HS in a concentration-dependent manner, with a decrease of the response to 35.9$\pm$11.3% of the control at a concentration of 100 μM (Figure 5c, $n=5$). This inhibitory effect was also completely reversible on washout of the drug. The IC_{50} value for inhibition of the HS-induced contractile response was 33.2$\pm$7.5 μM ($n=5$).

Discussion

The present study has shown that HSs induce stable, reproducible contractions of isolated rat trachea, the magnitude of which was related to the reduction of osmolarity. When mannitol was used to maintain iso-osmolarity, there was no contraction evident upon reduction of external NaCl, supporting the hypothesis that the stimulus for contraction was hypotonicity *per se* and not an alteration of ionic strength. It has been recognized for some time that application of HSs induces *in vitro* contraction of isolated human airways (Jongejan *et al*., 1990), while inhalation of nebulized hypotonic saline leads to bronchoconstriction in asthmatics (Schoeffel *et al*., 1987). In our study, HS-induced contractions were completely dependent on extracellular calcium entry, being abolished under nominally calcium-free conditions, coherent with the study of Jongejan *et al*. (1990) in human tissue. Furthermore, in our present study, the HS-induced responses were greatly inhibited by NIF, suggesting a substantial involvement of VDCCs in these contractions, in agreement with a previous study in vascular tissue in which D-600 inhibited both hypotonicity-induced vasoconstriction in aortic strips and increase in intracellular calcium concentration in isolated aortic cells (Lang *et al*., 1995).

Our data indicate that the contraction induced by HS is dependent on the cellular chloride gradient. Previously, chloride-dependent depolarizations have been reported in human aortic cells exposed to hypotonic extracellular solution (Lang *et al*., 1995), whilst chloride conductances are activated by HSs in myocytes from rat portal vein (Greenwood & Large, 1998), canine renal and pulmonary arteries (Yamazaki *et al*., 1998) and guinea-pig antrum (Xu *et al*., 1997). The equilibrium potential for chloride (E_{Cl}) is approximately -25 mV in smooth muscle cells (Aickin & Brading, 1982), and opening of volume-sensitive membrane chloride channels would produce a depolarization of the plasmalemma that could open VDCCs, allowing calcium influx into the cell and subsequent contraction (Lang *et al*., 1995; Nelson *et al*., 1997). However, it should also be noted that HSs may activate other types of ion channels including nonspecific cation channels (Waniishi *et al*., 1997; Welsh *et al*., 2000) that may promote smooth muscle depolarization. That the contraction induced by HS was not significantly altered when NMDG was employed to substitute NaCl would appear to suggest that such channels do not play a major role in rat trachea under the present experimental conditions.

In the present study, we found that HS-induced contractions were relatively insensitive to DIDS and TAM, previously characterized as blockers of volume-sensitive chloride currents in isolated smooth muscle cell studies (Greenwood & Large, 1998; Dick *et al*., 1999; Jentsch *et al*., 2002), when applied within an appropriate concentration range. In isolated smooth muscle cell studies, it is apparent that myocyte swelling due to application of HS occurs within seconds, followed by the development of a calcium-independent chloride-mediated current within 30–60 s that is distinct from Cl_{Ca} (Xu *et al*., 1997; Dick *et al*., 1998; Greenwood & Large, 1998). TAM ($\leqslant 10\,\mu$M), which completely blocked I_{Clvol} in rat portal vein (Greenwood & Large, 1998), elicited no alteration of HS-induced contractions in rat trachea, although we also observed that higher concentrations (30–100 μM) induced a weak but irreversible inhibition. This may indicate an action on Cl_{vol}, however, at this elevated concentration, TAM can also inhibit ligand-gated cation channels (Allen *et al*., 1998) and VDCCs in colonic smooth muscle (Dick *et al*., 1999), complicating the interpretation of these functional data. Similarly DIDS (1–100 μM), which completely abolished Cl_{vol} in portal vein and colonic myocytes (Greenwood & Large, 1998; Dick *et al*., 1999), was without significant inhibitory effects in our study.

In contrast, NFA elicited concentration-dependent inhibitory effects on HS-induced contractions, at concentrations previously shown to inhibit Cl_{Ca} in single smooth muscle cells (Pacaud *et al*., 1989; Janssen & Sims, 1992; Akbarali & Giles, 1993; Hogg *et al*., 1994; Lamb *et al*., 1994). Additionally, NPPB, which has been reported to block both Cl_{Ca} and Cl_{vol} channels (Jentsch *et al*., 2002), inhibited HS-induced contractions over a similar concentration range. HS-induced contractions were completely blocked under calcium-free conditions in our study, supporting a possible activation of Cl_{Ca} as an integral transduction step, although other calcium-dependent processes may be involved. Previously, we have proposed that activation of Cl_{Ca} is a mechanism utilized by agonists to depolarize blood vessels, leading to the opening of VDCCs and subsequent contraction (Criddle *et al*., 1996; 1997), a hypothesis supported by subsequent studies in both vascular and nonvascular smooth muscle (Guibert *et al*., 1997; Hyvelin *et al*., 1998; Lamb & Barna, 1998; Scarparo *et al*., 2000; Teixeira *et al*., 2000). Interestingly, our current data demonstrate that both NFA and NPPB reduced the HS-induced contractions to a comparable level to that of the VDCC blocker NIF. Thus, the most likely explanation for our present data would appear to be that elevation of intracellular calcium by HSs, *via* an undefined mechanism, may activate Cl_{Ca} causing voltage-dependent calcium entry and contraction. Such an *indirect* activation of VDCCs in respiratory smooth muscle *via* chloride channel-mediated membrane depolarization would be in direct agreement with the previous study of Lang *et al*. (1995) in vascular tissue.

However, it should be emphasized that conclusions based on purely functional data should be treated cautiously in the absence of detailed electrophysiological experiments in isolated respiratory smooth muscle cells, which were beyond the scope of our present study. The structurally diverse chloride channel blockers may exert nonspecific effects in vascular smooth muscle (Doughty *et al*., 1998; Kato *et al*., 1999) and blockade of HS-induced contractions in the present study may not constitute an absolute indication of Cl_{Ca} involvement. However, we found that TEA did not block the inhibitory effects of NFA on HS-induced contractions, in agreement with our previous data in rat trachea, suggesting that the inhibitory effects of NFA are not mediated *via* opening of K^+ channels or a direct inhibition of VDCCs (Teixeira *et al*., 2000). Furthermore, Remillard *et al*. (2000) have demonstrated that NFA inhibited phenylephrine-induced vasoconstriction in pressurized rabbit mesenteric arterioles, without affecting the myogenic tone or KCl-induced responses, additionally suggesting that this compound does not directly inhibit the contractile apparatus. Thus, it would appear that the effects of this blocker within the present functional protocols are likely to be mediated *via* an interaction with Cl_{Ca} channels. However, 10 μM NFA has also been reported to inhibit Cl_{vol} channels in gastric smooth muscle (Xu *et al*., 1997), a concentration that we found to induce significant inhibition of HS-induced contractile responses in isolated trachea. Thus, the possibility exists that the HS-induced contractions of rat trachea observed in our functional tests might alternatively involve the activation of a NFA-sensitive Cl_{vol} channel.

In conclusion, we have shown that HSs induce large, reversible and chloride-dependent contractions of rat isolated respiratory smooth muscle. These effects are inhibited by NFA and NPPB, while exhibiting little sensitivity to recognized blockers of Cl_{vol}. Since the questionable selectivity of the structurally diverse chloride channel blockers remains a controversial area in smooth muscle research, coupled with current interest in the development of more potent and selective agents (Large & Wang, 1996; Kozlowski, 1999; Criddle *et al*., 2002), further detailed electrophysiological and functional studies are clearly necessary in a variety of tissues to understand more fully the basic pharmacology of these antispasmodic agents.

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