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European Journal of Pharmacology 394 (2000) 117–122

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Comparative effects of niflumic acid and nifedipine on 5-hydroxytryptamine- and acetylcholine-induced contraction of the rat trachea

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Received 30 November 1999; received in revised form 18 January 2000; accepted 25 January 2000

Abstract

The effects of niflumic acid, an inhibitor of Ca^{2+}-activated Cl^- ($Cl_{(Ca)}$) channels, were compared with those of the voltage-dependent Ca^{2+} channel (VDCC) blocker nifedipine on 5-hydroxytryptamine (5-HT)- and acetylcholine-induced contractions of the rat isolated trachea. Niflumic acid (3–100 μM) induced a concentration-dependent inhibition of 5-HT (10 μM)-induced contractions, with a reduction to 37.0 ± 9.5% of the control at the highest concentration. One micromolar nifedipine, which completely blocked 60 mM KCl-induced contractions, reduced the response to 5-HT similarly to 39.2 ± 11.5% of the control. The inhibition of the 5-HT response was not significantly different from that produced by the combined presence of nifedipine (1 μM) and niflumic acid (100 μM), suggesting that their effects were not additive. In contrast, neither niflumic acid (3–100 μM) nor nifedipine (1 μM) inhibited acetylcholine-induced contractions. The contraction to 5-HT (10 μM) in Cl^--free solution was decreased by more than ~85% of the control, whilst that of acetylcholine was reduced only by ~36%. Our data show that niflumic acid exerts selective inhibitory effects on 5-HT-induced contraction, and suggest that activation of $Cl_{(Ca)}$ channels may be a mechanism whereby 5-HT (but not acetylcholine) induces Ca^{2+} entry via VDCCs to elicit contraction.

Keywords: Niflumic acid; Trachea; Cl^- channel; $I_{Cl_{(Ca)}}$

1. Introduction

Previous studies have indicated the existence of Ca^{2+}-activated Cl^- ($Cl_{(Ca)}$) channels in respiratory smooth muscle (Janssen and Sims, 1992, 1993), whose activation may constitute a mechanism whereby neurotransmitters depolarise a variety of smooth muscle types (for review see Large and Wang, 1996). Neurotransmitters such as acetylcholine have been shown to depolarize single smooth muscle cells of the trachea, an action that may be at least partly mediated via the opening of small conductance Cl^- channels (Janssen and Sims, 1992). However, despite the detailed electrophysiological data produced from studies in single isolated respiratory smooth muscle cells, the putative functional relevance of $Cl_{(Ca)}$ channel activation induced by neurotransmitters to contraction in intact muscle has been comparatively neglected.

Recently niflumic acid, a relatively selective blocker of $I_{Cl_{(Ca)}}$ in smooth muscle cells (Pacaud et al., 1989; Janssen and Sims, 1992; Akbarali and Giles, 1993; Hogg et al., 1994; Lamb et al., 1994) has been shown to inhibit contractions induced by neurotransmitters in isolated blood vessels (Criddle et al., 1996, 1997; Yuan, 1997). We have previously demonstrated that niflumic acid reduces the amplitude of noradrenaline-induced contractions in rat aorta in a manner comparable to that of nifedipine, with no further increase in the presence of a combination of both drugs (Criddle et al., 1996). From these observations we proposed that activation of $Cl_{(Ca)}$ channels by neurotransmitters may lead to a depolarisation-induced entry of Ca^{2+}

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00089-3

into the cell via voltage-dependent Ca^{2+} channels (VDCCs) causing contraction of vascular smooth muscle (Criddle et al., 1996). Recent detailed studies using noradrenaline in rat aorta and endothelin in pulmonary artery have provided strong supportive data for this hypothesis (Hyvelin et al., 1998; Lamb and Barna, 1998).

We have previously found that 5-hydroxytryptamine (5-HT)-induced pressor responses are potently inhibited by niflumic acid in the mesenteric vascular bed of the rat (Criddle et al., 1997). In order to assess a possible influence of $Cl_{(Ca)}$ channels in the contractile response to 5-HT in non-vascular smooth muscle, we have compared the effects of niflumic acid and nifedipine on 5-HT- and acetylcholine-induced contraction of the rat isolated trachea.

2. Materials and methods

2.1. Isolated tracheal preparation

Male Wistar rats (250–350 g) were killed by a blow to the head and exsanguinated. The neck was opened and the trachea was carefully dissected and washed in fresh Tyrode's solution. Following this, rings (1 cm wide) were prepared and mounted vertically in an organ bath (10 ml capacity) containing Tyrode's solution bubbled with air (37°C, pH 7.4). Tissues were mounted under an initial resting tension of 1 g and left to equilibrate for a period of 1 h before starting the experimental protocol. Tension changes were recorded using isometric force transducers (Grass Model FT0.3, Quincy, MA., USA) connected to a Grass chart recorder (Model 5D).

The effects of niflumic acid were assessed on contractions induced by KCl (60 mM), 5-HT (10 μM) and acetylcholine (10 μM), with each agonist being evaluated in a separate experimental group. Following stable contractions to the contractile agent, niflumic acid was applied in increasing concentrations and further responses obtained (contact time of 15 min). Since dimethyl sulphoxide (DMSO) was used as a solvent for stock solutions of niflumic acid, the effects of this solvent at equivalent concentrations were assessed, using time-matched controls, on 5-HT-induced contraction of the trachea.

In separate experiments, the effect of extracellular Cl^- withdrawal from the bathing solution on the responses of the contractile agonists was assessed. Initially control contractions to 5-HT (10 μM) and acetylcholine (10 μM) were obtained. The tissues were then bathed in a modified Tyrode's solution ($0Cl^-$), in which all Cl^- salts had been substituted by their gluconate or acetate equivalents. Since application of $0Cl^-$ solution to the preparation caused a transient contraction, reapplication of 5-HT or ACh was carried out only when basal levels of tone were restored. In addition, the reversibility of any effect on agonist-induced contraction by $0Cl^-$ solution was assessed, by reintroducing normal (Cl^--containing) Tyrode's solution to the bath and repeating the application of 5-HT or ACh.

2.2.. Solutions and drugs

The following drugs were used: (5-HT), acetylcholine, niflumic acid and nifedipine (Sigma). Nifedipine stock solution was prepared in 70% ethanol under conditions of reduced illumination and all experiments with nifedipine were performed under similar conditions. Niflumic acid was prepared as a stock solution (10 mM) in DMSO and diluted on the day of the experiment in Tyrode's solution.

The bathing solution was a standard 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 K^+ concentration was raised (60 mM), the NaCl concentration was concomitantly reduced to maintain osmolarity. The Cl^--free solution was of the same composition as standard Tyrode's solution, except that all the Cl^- salts were replaced by their gluconate equivalents. The pH was always maintained constant throughout the experimental period at 7.4.

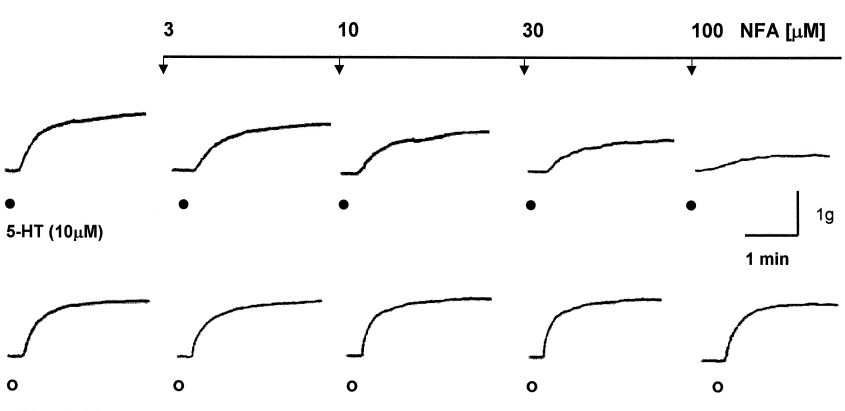

Fig. 1. Representative traces showing the comparative effects of niflumic acid (NFA, 3–100 μM) on the contractile responses to 5-HT (10 μM, ● *upper trace*) and acetylcholine (ACh; 10 μM, ○ *lower trace*) in the rat isolated trachea.

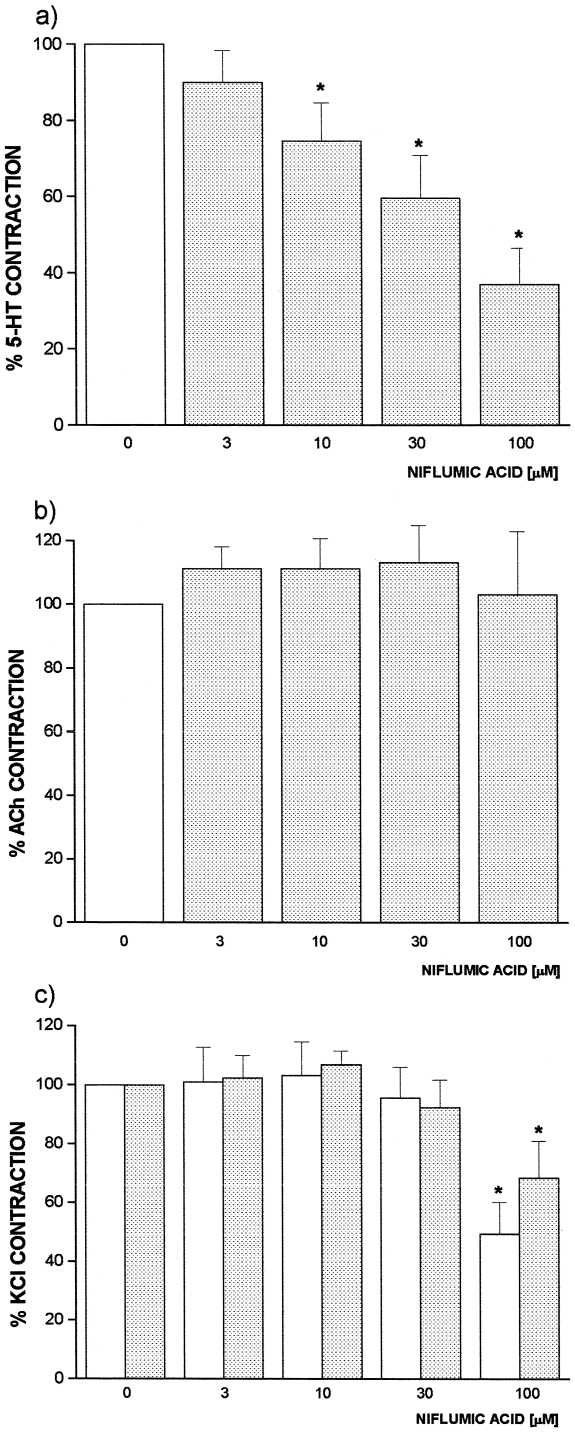

Fig. 2. Effects of niflumic acid (3–100 μM) on the contractile responses induced by (a) 5-HT (10 μM, $n = 7$), (b) acetylcholine (ACh; 10 μM, $n = 7$) and (c) KCl 60 mM (open bars, $n = 9$) and 40 mM (filled bars, $n = 6$) in the rat isolated trachea. Values are shown as the mean ± S.E.M. of n experiments, and are shown to differ significantly from the control when $^{*}P < 0.05$.

2.3. Analysis of data

Data are expressed as the mean of n observations ± S.E.M.. Inhibitory effects are expressed as percent of control responses in the absence of the drug. Statistical analysis was performed using a Student's t-test and values were taken to be significantly different when $P < 0.05$.

3. Results

3.1. Effects of niflumic acid on contractions induced by 5-HT, acetylcholine and KCl

5-HT (10 μM) elicited stable and reproducible contractions of the rat trachea of mean amplitude 0.95 ± 0.18 g (75.2 ± 13.2% of the control contraction to 60 mM KCl, $n = 7$). In the presence of niflumic acid (3–100 μM), the contractile response to 5-HT was inhibited concentration-dependently (Figs. 1 and 2) with a reduction to 37.0 ± 9.5% of the control value at the highest concentration tested ($P < 0.05$, $n = 7$). This effect was reversible on washout of the drug. In parallel control experiments, the solvent DMSO (at equivalent concentrations to those used to dissolve niflumic acid) did not inhibit the contractions produced by 5-HT throughout the course of the experimental period ($n = 7$).

Acetylcholine (10 μM) produced stable contractions of the isolated trachea of magnitude 0.96 ± 0.31 g (79.7 ± 7.0% of the control contraction to KCl, $n = 7$). In contrast to the effects on 5-HT, niflumic acid (3–100 μM) did not inhibit the contractions induced by acetylcholine (Figs. 1 and 2). In separate experiments, in concentrations up to 30 μM, niflumic acid did not inhibit the contractions induced by 60 or 40 mM KCl (control contractions of 1.2 ± 0.3 and 0.52 ± 0.09 g, respectively, $n = 6$–9), however, a concentration of 100 μM reduced these responses by 50.8 ± 10.9% and 41.7 ± 12.7% of the control responses (Fig. 1).

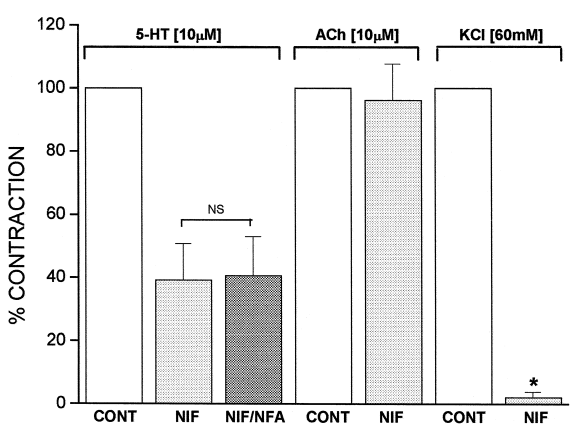

Fig. 3. Comparative effects of nifedipine (NIF, 1 μM) on contractions induced by 5-HT (10 μM, $n = 7$), acetylcholine (ACh; 10 μM, $n = 7$) and KCl (60 mM, $n = 9$) on the rat isolated trachea. Note that the effect of a combination of nifedipine (1 μM) and niflumic acid (NFA, 100 μM) on the contraction induced by 5-HT (10 μM) has also been included to show that the effects of these drugs were not additive. Values are shown as the mean ± S.E.M. of n experiments, and are shown to differ significantly from the control when $^{*}P < 0.05$.

3.2. Effects of nifedipine on contractions induced by 5-HT, acetylcholine and KCl

Nifedipine (1 μM), which completely blocked the contraction to 60 mM KCl ($n = 9$), reduced the contraction induced by 5-HT (10 μM) to 39.2 ± 11.5% of the control (Fig. 3; $P < 0.05$, $n = 7$). The inhibition of the 5-HT response was not significantly different from that observed in the combined presence of 1 μM nifedipine and 100 μM niflumic acid (40.7 ± 12.2% of the control response), suggesting that their effects were not additive (Fig. 3).

In contrast, nifedipine (1 μM) did not significantly reduce the contractile effect of acetylcholine (10 μM), the response being 96.3 ± 11.3% ($n = 7$) of the control value (Fig. 3).

3.3. Effects of 0Cl$^-$ solution on the contractile responses to 5-HT, Ach and KCl

Addition of 0Cl$^-$ (gluconate-substituted) Tyrode's solution to the tracheal preparation induced a transient contraction (mean amplitude of 0.42 ± 0.09 g, $n = 10$) that relaxed to basal levels of tone within several minutes. Following this return to resting values, the reapplication of 5-HT (10 μM) elicited a contraction which was 14.7 ± 7.3% of the control value in the presence of this solution in all preparations tested (Fig. 4 , $n = 8$). This inhibitory effect was reversible on re-exchange of the 0Cl$^-$ solution for normal Cl$^-$-containing Tyrode's solution. In contrast the contraction elicited by acetylcholine was much less sensitive to 0Cl$^-$ conditions, the response being reduced to only 63.4 ± 1.7% of the control response (Fig. 4, $n = 6$), whilst that of 60 mM KCl remained unchanged (Fig. 4, $n = 9$).

Additionally we compared the effects of Cl$^-$-free Tyrode's solution substituted with gluconate (relatively membrane-impermeable) on the response to 5-HT with Cl$^-$-free solution substituted with acetate (relatively membrane-permeable). The presence of Cl$^-$-free acetate-substituted external solution, inhibited the 5-HT response greatly, in a similar manner to that previously observed with gluconate-substituted solution, to 8.9 ± 6.6% of the control response (Fig. 4, $n = 5$).

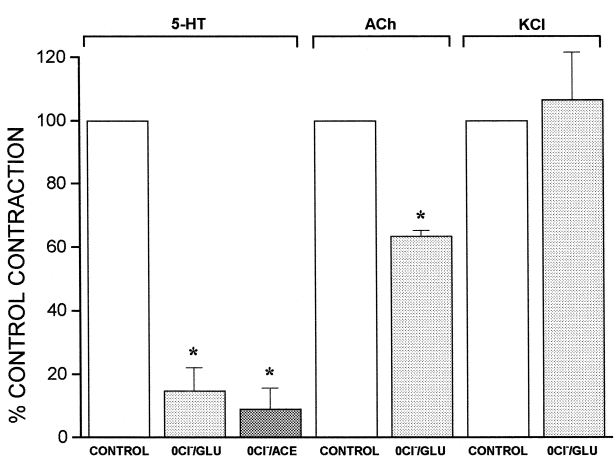

Fig. 4. Effects of Cl$^-$-free Tyrode's solution (substituted for gluconate; 0Cl$^-$/GLU) on the contractions of rat isolated trachea induced by 5-HT (10 μM, $n = 8$) and acetylcholine (ACh; 10 μM, $n = 6$) and 60 mM KCl ($n = 9$). Additionally the effect of Cl$^-$-free Tyrode's solution (substituted for acetate; 0Cl$^-$/ACE) on the contractions of rat isolated trachea induced by 5-HT (10 μM, $n = 5$) has been included. Values are shown as the mean ± S.E.M. of n experiments, and are shown to differ significantly from the control when $^* P < 0.05$.

4. Discussion

Our present study has shown that niflumic acid exerts selective inhibitory effects on 5-HT-induced contraction of the rat trachea, in concentrations previously shown to inhibit $I_{Cl_{(Ca)}}$ in a variety of smooth muscle cell types (Pacaud et al., 1989; Janssen and Sims, 1992; Akbarali and Giles, 1993; Hogg et al., 1994; Lamb et al., 1994). In accord with functional data in blood vessels (Criddle et al.,1996, 1997; Guibert et al., 1997; Hyvelin et al., 1998), we found that the magnitude of the inhibitory effect of niflumic acid in trachea was similar to that of nifedipine. Moreover, the effects of these drugs were not additive. This would suggest that niflumic acid and nifedipine, although apparently acting via different mechanisms, ultimately inhibit the same proportion of the 5-HT-induced contractile response. These results are thus supportive of the hypothesis that activation of $I_{Cl_{(Ca)}}$, leading to depolarisation and subsequent Ca^{2+} entry through VDCCs, constitutes a common mechanism whereby contractile agents may induce their mechanical responses (Criddle et al., 1996). A hypothetical model of the putative involvement of $Cl_{(Ca)}$ channels in the contractile action of 5-HT is shown in Fig. 5.

The present results indicate, however, that the contractile mechanisms utilized by acetylcholine and 5-HT in rat trachea are distinct, since only the effect of 5-HT was sensitive to niflumic acid and nifedipine. In accord with our present findings, the 5-HT-induced contraction in guinea-pig trachea, via the 5-HT_{2A} receptor subtype, is inhibited between 45%–60% by VDCC antagonists (Watts et al., 1994), whereas carbamylcholine-induced contraction is unaffected (Advenier et al., 1983; Lee et al., 1990; Watts et al., 1994). It has long been known that activation of muscarinic receptors produces depolarisation of airways smooth muscle (Suzuki et al., 1976; Baba et al., 1989; Janssen and Daniel, 1991) and previously acetylcholine has been shown to activate both $I_{Cl_{(Ca)}}$ and non-selective cation channels in guinea-pig and canine tracheal myocytes (Janssen and Sims, 1992; Sims, 1992). In the study of Janssen and Sims (1992) the depolarising current in-

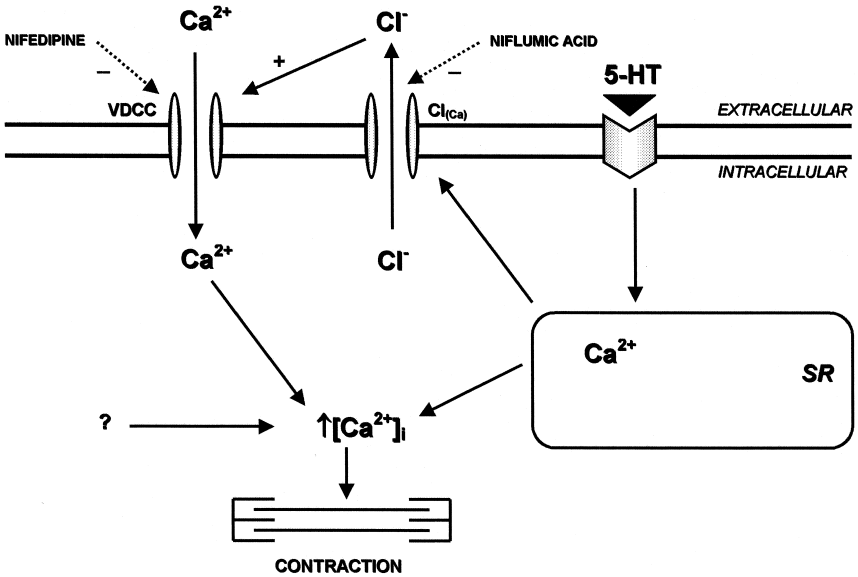

Fig. 5. Hypothetical working model outlining the inhibitory effects of niflumic acid and nifedipine on 5-HT-induced contraction of tracheal smooth muscle. Activation of plasmalemmal 5-HT receptors stimulates intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR), inducing an opening of $Cl_{(Ca)}$ channels. The subsequent flow of Cl^- ions depolarizes the cell membrane leading to opening of Ca^{2+} VDCCs and extracellular Ca^{2+} entry. The associated rise of the intracellular Ca^{2+} concentration stimulates contraction of the smooth muscle cell. This model predicts that a finite proportion of the contractile response to 5-HT is blocked by both niflumic acid and nifedipine (alone or in association) whilst other mechanisms are present that promote contraction independently of this pathway (designated ''?'').

duced by acetylcholine (I_{ACh}) was almost completely blocked by 100 μM niflumic acid, however, the authors concluded that activation of $I_{Cl_{(Ca)}}$ was unlikely to contribute to its contractile effect since full contraction of isolated myocytes was still present when I_{ACh} was almost completely eliminated by Cl^--channel blockers. In our functional study in rat trachea the same concentration of niflumic acid did not inhibit acetylcholine-induced contraction, additionally supporting such a conclusion in this tissue.

In contrast, the involvement of $I_{Cl_{(Ca)}}$ in the contractile response to 5-HT appears much more likely from the current data. Firstly, the 5-HT-induced contraction was reversibly inhibited in a concentration-dependent manner by niflumic acid, in agreement with previous observations in rat pulmonary artery (Yuan, 1997) and mesenteric vascular bed (Criddle et al., 1997). In addition, the fundamental importance of the cellular Cl^- gradient to the 5-HT-induced contraction in rat trachea was clearly evident, since this response was almost completely abolished under Cl^--free conditions, in contrast to that of acetylcholine which was only partially reduced. Thus the present data provide supportive evidence for an involvement of $I_{Cl_{(Ca)}}$ in the contractile response of 5-HT in rat trachea. Previously it has been shown that $I_{Cl_{(Ca)}}$ is activated in epithelial cells of the rat Cl^- plexus by 5-HT (Garner et al., 1993), and via stimulation of fundus 5-HT_{2B} receptors expressed in *Xenopus* oocytes (Foguet et al., 1992), although this action of 5-HT has yet to be demonstrated in isolated respiratory smooth muscle cells. Future detailed electrophysiological data in single cells from the rat trachea are required to provide a definitive answer to the putative involvement of $I_{Cl_{(Ca)}}$ in the contractile response to 5-HT.

Niflumic acid has been shown to possess actions in addition to blockade of $I_{Cl_{(Ca)}}$, such as the induction of K^+-currents and blockade of VDCCs when in concentrations above 50 μM (Hogg et al., 1994; Greenwood and Large, 1995). In our functional experiments the contractions induced by 60 mM KCl were not inhibited by niflumic acid in concentrations ≤ 30 μM, which substantially reduced the 5-HT-induced response, excluding the possibility of direct or indirect actions at VDCCs. These findings are in agreement with previous reports in rat aorta (Criddle et al., 1996), pulmonary artery (Guibert et al., 1997; Yuan, 1997; Hyvelin et al., 1998), mesenteric vascular bed (Criddle et al., 1997; He and Tabrizchi, 1997), rabbit colon (Patacchini et al., 1996), and stomach fundus (Criddle et al., 1999). However, we found that 100 μM niflumic acid inhibited KCl-induced contractions of the rat trachea, possibly indicating non-selective effects of the drug at this concentration, although interestingly the responses to acetylcholine remained unaffected apparently arguing against such a conclusion. Thus data obtained with 100 μM niflumic acid must be interpreted cautiously, albeit that many previous studies have used this high concentration of niflumic acid in single-cell and whole tissue experiments to evaluate $I_{Cl_{(Ca)}}$ (Janssen and Sims, 1992, 1993; Kirkup et al., 1996; Patacchini et al., 1996; Kamouchi et al., 1997; Nilius et al., 1997; Zegarra-Moran et al., 1997).

In conclusion, the present study has shown that niflumic acid concentration-dependently inhibits 5-HT-induced con-

traction of rat isolated trachea, which is in accord with our previous findings in vascular smooth muscle (Criddle et al., 1997). The current data suggest that activation of $Cl_{(Ca)}$ channels may constitute an excitatory mechanism whereby 5-HT, but not acetylcholine, might promote voltage-dependent Ca^{2+} entry and subsequent contraction.

Acknowledgements

This study was supported by the CNPq and FUNCAP.

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