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1   It has been shown previously that nordihydroguaiaretic acid (NDGA) inhibits endothelin-1 (ET-1)-induced contractions in rat isolated tracheal smooth muscle. To investigate the underlying mechanisms, this study examined the effects of NDGA on various aspects of the ET$_A$ and ET$_B$ receptor-effector systems which mediate ET-1-induced contractions in this preparation.

2   NDGA inhibited contractions induced by each of the isoforms of ET (ET-1, ET-2 and ET-3) but not those induced by the ET$_B$ receptor-selective agonist, sarafotoxin S6c, the cholinoceptor agonist, carbachol or the depolarizing spasmogen, KCl.

3   Quantitative autoradiographic studies of [^{125}I]-ET-1 binding to rat tracheal smooth muscle indicated that NDGA was not an ET receptor antagonist.

4   NDGA inhibited the ET$_A$ receptor-mediated, intracellular Ca^{2+}-dependent contractions induced by 100 nM ET-1 in Ca^{2+}-free solution (by 75%, $P<0.01$). Furthermore, NDGA markedly inhibited the contractions induced by ryanodine and cyclopiazonic acid; contractions purportedly due to Ca^{2+} release from intracellular stores.

5   Like NDGA, the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitors cyclopiazonic acid and thapsigargin inhibited contractions to ET-1, but not carbachol or KCl. However, cyclopiazonic acid, but not NDGA, also (a) induced transient contractions in rat trachea, (b) potentiated contractions induced by KCl, and (c) potentiated the extracellular Ca^{2+}-dependent phase of ET-1-induced contractions, indicating that NDGA did not inhibit ET-1-induced contractions through Ca^{2+}-ATPase inhibition and depletion of sarcoplasmic reticular Ca^{2+}.

6   In control preparations, ET-1 induced a slowly developing, sustained contraction. However, in the presence of NDGA or the ET$_A$ receptor antagonist, BQ123, ET-1-induced contractions resembled the transient contractions induced by sarafotoxin S6c. In nominally Ca^{2+}-free solution, ET$_A$ receptor-mediated contractions induced by ET-1 developed very slowly and were inhibited by NDGA.

7   Additional studies indicated that the inhibitory effects of NDGA on endothelin-1-induced contractions were not the result of any significant actions of NDGA on lipoxygenase, cytochrome P$_{450}$, L- or T-type Ca^{2+}-channels, Na$^+$-channels or protein kinase C.

8   In summary, NDGA selectively inhibited ET-1-induced contractions in rat tracheal smooth muscle via a lipoxygenase-independent mechanism involving inhibition of the ET$_A$ but not the ET$_B$, receptor-effector system. NDGA did not appear to inhibit the initial events in the ET$_A$ signal transduction pathway, such as receptor binding and protein kinase C activation. However, NDGA inhibited the intracellular Ca^{2+}-dependent component of ET-1-induced contraction, possibly by inhibiting mobilisation of intracellular Ca^{2+}. As an apparent direct consequence of inhibiting the ET$_A$ receptor-effector system, NDGA markedly changed the time course of ET-1-induced contractions; from a slowly developing and sustained contraction into a transient contraction resembling that induced by sarafotoxin S6c.

Keywords: Endothelin-1; sarafotoxin S6c; nordihydroguaiaretic acid; cyclopiazonic acid; staurosporine; ryanodine; rat trachea; endothelin receptors; airway smooth muscle

Introduction

Endothelin-1 (ET-1) is a potent spasmogen of airway smooth muscle in many animal species including man, and may contribute to the elevated bronchomotor tone of asthma (Uchida *et al.*, 1988; Advenier *et al.*, 1990; Henry *et al.*, 1990b; Springall *et al.*, 1991). ET-1 initiates contraction by stimulating high affinity receptors located on the surface membrane of the airway smooth muscle cells (Turner *et al.*, 1989; Henry *et al.*, 1990b; Mattoli *et al.*, 1991). At least two ET receptor subtypes have recently been identified, termed ET$_A$ and ET$_B$, and both have been implicated in mediating ET-1-induced contraction of airway smooth muscle. For example, functional experiments using the ET$_A$ receptor-selective antagonist, BQ123 and the ET$_B$ receptor-selective

agonist, sarafotoxin S6c, indicate that ET-1-induced contraction of airway smooth muscle is mediated primarily by ET$_A$ receptors in some species, such as the sheep (Noguchi *et al.*, 1992) and by ET$_B$ receptors in other species including the guinea-pig (Hay, 1992). In addition, recent autoradiographic, biochemical and functional studies indicate that ET-1-induced contractions in rat trachea appear to be mediated by both ET$_A$ and ET$_B$ receptor-effector systems (Henry, 1993). These latter studies showed that ET-1-induced contractions induced by the stimulation of ET$_A$ receptors appear to have resulted from activation of the phosphoinositide pathway and the mobilisation of intracellular Ca^{2+}, whereas those induced by the stimulation of ET$_B$ receptors seem to have resulted from the influx of extracellular Ca^{2+}.

Nordihydroguaiaretic acid (NDGA), a drug routinely used as a nonspecific inhibitor of lipoxygenase, has recently been

shown to inhibit ET-1-induced contractions in rat isolated tracheal smooth muscle (Henry *et al.*, 1992). However, it appears unlikely that products of arachidonic acid metabolism by 5-lipoxygenase, such as the leukotrienes, contributed significantly to ET-1-induced contractions in this preparation. Firstly, leukotrienes are poor spasmogens of rat isolated tracheal smooth muscle (Chand *et al.*, 1990). Secondly, the leukotriene receptor antagonist, SKF104353, did not inhibit ET-1-induced contractions (Henry *et al.*, 1992). Finally, in the current study, NDGA was the only lipoxygenase inhibitor tested that inhibited ET-1-induced contractions. Thus, NDGA appears to inhibit ET-1-induced contractions via a lipoxygenase-independent but as yet undefined, pathway. With respect to this pathway, NDGA has been reported to modulate the activity of other factors which may participate in smooth muscle contraction. For example, NDGA has been shown to inhibit enzymes such as protein kinase C, cyclo-oxygenase, guanylate cyclase and cytochrome P_{450} in various cell and tissue systems (Clark & Linden, 1986; Rondeau *et al.*, 1990; Force *et al.*, 1991). Furthermore, NDGA, inhibits Ca^{2+} currents in some cell systems (Korn & Horn, 1990) and has some structural similarities with the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, cyclopiazonic acid.

Thus, the principle purpose of this study was to investigate the mechanisms through which NDGA inhibits ET-1-induced contractions in rat isolated tracheal smooth muscle. Functional and autoradiographic techniques were used to examine the effects of NDGA on various aspects of the recently elucidated ET_A and ET_B receptor-effector systems, which mediate ET-1-induced contractions in this preparation.

Methods

Preparation of tracheal segments

Male Wistar rats (10–12 weeks) and SR/C Tricolor guinea-pigs (6–8 weeks) were stunned and killed by cervical dislocation and exsanguination. The trachea was excised, placed in cold Krebs-bicarbonate solution (KBS) and cleaned of adhering connective tissue. The composition of KBS was (in mM): NaCl 117, KCl 5.36, NaHCO$_3$ 25.0, KH$_2$PO$_4$ 1.03, MgSO$_4$·7H$_2$O 0.57, CaCl$_2$·2H$_2$O 2.5 and glucose 11.1. Eight tracheal ring segments (2 mm long) were obtained from each trachea and denuded of epithelium (Goldie *et al.*, 1986). Four preparations were used immediately and the remainder stored in KBS at 4°C and used within 3 h. Tracheal segments were suspended under a resting tension of 0.5 g and placed in organ baths containing 3 ml of KBS at 37°C, bubbled continuously with 5% CO$_2$ in O$_2$. Changes in isometric tension were measured with a Model 7D Polygraph via FTO3 force-displacement transducers (Grass Instruments). Tracheal segments were allowed to equilibrate for 45 min before exposure to the cumulative addition of 0.3 and 10 µM carbachol. Upon reaching contraction plateau the preparations were washed for 15 min with drug-free KBS.

Functional studies using tracheal segments

Concentration-effect curves to ET-1, carbachol, and KCl were constructed in the presence and absence of inhibitors of lipoxygenase (NDGA, phenidone, BW755C, eicosatetraynoic acid (ETYA)), cytochrome P_{450} (proadifen, metyrapone), Ca^{2+}- and Na^+-channels (verapamil, nicardipine, NiCl$_2$, amiloride), sarcoplasmic reticulum Ca^{2+}-ATPase (cyclopiazonic acid, thapsigargin) and protein kinase C (staurosporine). Unless otherwise stated, tracheal preparations were exposed for 20 min to an inhibitor or its solvent (paired control preparations) and then to cumulative additions of the spasmogens ET-1 (1–300 nM), carbachol (30 nM–100 µM) or KCl (15–90 mM). The Ca^{2+}-ATPase inhibitors induced transient contractions in these preparations (see below). Consequently, the pretreatment period of these drugs was extended

to 30 min to allow the contraction to return to baseline levels of tone. In all experiments, only one concentration-effect curve was constructed on each preparation. Spasmogen-induced contractions were plotted as a percentage of the initial contraction produced by 10 µM carbachol (C_{max}) and the concentration of spasmogen producing 50%C_{max} was estimated by fitting the concentration-effect data to a logistic function using computer-assisted non-linear least squares regression analysis.

To determine the relative contribution of intracellular and extracellular Ca^{2+} to ET-1-induced contractions, contractile responses to 100 nM ET-1 were determined in nominally Ca^{2+}-free KBS (see Figure 3a for experimental protocol). In this series of experiments, preparations were washed four times during a 5 min period with Ca^{2+}-free KBS containing 10 µM EGTA and equilibrated for 20 min in Ca^{2+}-free KBS (without EGTA). Preparations were then exposed to 100 nM ET-1. When the ET-1-induced contraction had reached plateau, CaCl$_2$ was added to the bath at a final concentration of 2.5 mM and the subsequent contraction recorded until it had reached plateau. To establish the influence of NDGA and cyclopiazonic acid on these responses, preparations were incubated with 20 µM NDGA or 10 µM cyclopiazonic acid at the beginning of the 20 min equilibration period and for the remainder of the experiment (see Figure 3).

To determine the effect of NDGA on protein kinase C activity, concentration-effect curves to the protein kinase C activator, phorbol 12,13-dibutyrate were completed in the presence and absence of NDGA. Under conditions of basal tone, phorbol 12,13-dibutyrate-induced contractions were small (less than 15% C_{max} at 10 µM). It has been reported previously that phorbol ester-induced contractions can be markedly enhanced by pre-contracting the preparation with KCl (Menkes *et al.*, 1986; Huang *et al.*, 1987; Ozaki *et al.*, 1990). Thus, the effect of NDGA on phorbol 12,13-dibutyrate-induced contractions was determined in preparations pre-contracted with KCl to about 35% C_{max}. In all studies with phorbol 12,13-dibutyrate, concentration-effect curves to the inactive phorbol ester, 4α-phorbol 12,13-didecanoate (4α-Pdd), were concomitantly completed in paired preparations.

Autoradiography

The effects of NDGA on the binding of ET-1 to its receptor was investigated by use of quantitative autoradiography. Autoradiographs of [^{125}I]-ET-1 binding to rat tracheal sections were prepared as described previously (Henry *et al.*, 1990b). Each slide contained two tracheal sections (non-serial) from each of 4 rats. Slide-mounted tracheal sections were incubated with 0.5 nM [^{125}I]-ET-1 (specific activity; 674 Ci mmol^{-1}) in the presence of 10 µM NDGA or 1% ethanol (control). This concentration of [^{125}I]-ET-1 is close to the dissociation constant (K_d) of specific [^{125}I]-ET-1 binding determined previously in rat tracheal smooth muscle (0.43 nM; Henry *et al.*, 1990b). Non-specific binding was determined by use of 1 µM unlabelled ET-1 in the presence of 10 µM NDGA or vehicle (1% ethanol). Autoradiographic grain densities over the tracheal smooth muscle band were determined with an automated grain detection and counting system (Henry *et al.*, 1990a). Four separate fields (3 over smooth muscle and one background measurement over a non-tissue area) were viewed from each tracheal section and quadruplicate total and non-specific slides were analysed. Thus, a total of 512 fields were analysed [(4 fields per section) × (2 sections per rat trachea) × (4 rat tracheae per slide) × (4 slides per treatment) × (4 treatments)]. Autoradiographic grain densities were expressed as grains per 1000 µm^2 (grains 1000 µm^{-2}).

Drugs

Drugs used were; ET-1, ET-2, ET-3, [^{125}I]-ET-1, sarafotoxin S6c, BQ-123 (cyclo (D-Trp,D-Asp,L-Pro,D-Val,L-Leu); Auspep,

Melbourne, Australia), carbamylcholine chloride (carbachol), metyrapone, (±)-verapamil hydrochloride, nicardipine hydrochloride, amiloride hydrochloride, nordihydroguaiaretic acid (NDGA), phorbol 12,13-dibutyrate, 4α-phorbol 12,13 didecanoate (4α-Pdd), phenidone, cyclopiazonic acid, thapsigargin, ryanodine, TMB-8 (3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester), EGTA (Sigma Chemical Company, St. Louis, U.S.A.), eicosatetraynoic acid (ETYA, ICN Biomedicals, Sydney, Australia), staurosporine (Boehringer Mannheim), proadifen (SKF525A, SmithKline Beechams Laboratories), BW755C (3-amino-1-[m-(trifluoromethyl)-phenyl]-2-pyrazoline Wellcome Research Laboratories, UK). Staurosporine, phorbol esters, cyclopiazonic acid, thapsigargin and ETYA were dissolved in dimethylsulphoxide, and NDGA and metyrapone were dissolved in ethanol. All other drugs were made up in saline. Drugs were stored on ice and protected from light. In Ca^{2+}-free KBS, CaCl$_2$ was omitted.

Statistical analyses

In each preparation, contractile potency is expressed in terms of the concentration of drug required to produce 50% of the maximum response to 10 μM carbachol (50% C$_{max}$). Data are presented as mean [− log (concentration of drug producing 50% C$_{max}$)] ± s.e.mean from n experiments (i.e. n different animals) and differences between treatment means assessed by analysis of variance followed by a modified t statistic (Wallenstein et al., 1980). P values less than 0.05 were considered to be statistically significant.

Results

Functional studies

Selective inhibition of ET-1-induced contractions by NDGA As shown previously (Henry et al. 1990b; 1992), ET-1 was a potent spasmogen in rat isolated tracheal smooth muscle preparations (Figure 1a). The concentration of ET-1

Figure 1 Mean concentration-effect curves to (a) ET-1, (b) carbachol, (c) KCl and (d) sarafotoxin S6c in the absence (O) or presence of NDGA (3 μM, □; 10 μM, △; 20 μM, ●) in rat isolated tracheal smooth muscle preparations. Note that the curve obtained to ET-1 in the presence of 10 μM NDGA is obscured by the curve obtained in the presence of 20 μM NDGA. Shown are the mean ± s.e.mean responses obtained from 5 or 6 separate experiments. For abbreviations, see text.

required to induce a contraction of 50% C$_{max}$ was 29 nM (95% confidence limits, 17–51 nM, $n = 6$). ET-1-induced contractions were concentration-dependently inhibited by NDGA (Figure 1a). For example, in the presence of 20 μM NDGA, the concentration of ET-1 required to induce a contraction of 50% C$_{max}$ was 85 nM (95% confidence limits, 58–125 nM), 3 fold greater than in control preparations ($P < 0.01$). NDGA (20 μM) similarly inhibited contractions to ET-2 (concentration required to induce 50% C$_{max}$ was 26 nM (13–52 nM) in the absence of NDGA versus 93 nM (36–240 nM) in the presence of NDGA; $n = 6$; $P < 0.05$) and to ET-3 (46 nM (34–62 nM) in the absence of NDGA versus 165 nM (105–265 nM) in the presence of NDGA; $n = 6$; $P < 0.05$). In contrast, 20 μM NDGA did not inhibit contractions to the ET$_B$ receptor-selective agonist sarafotoxin S6c, the cholinoceptor agonist, carbachol or the depolarizing spasmogen, KCl (Figure 1).
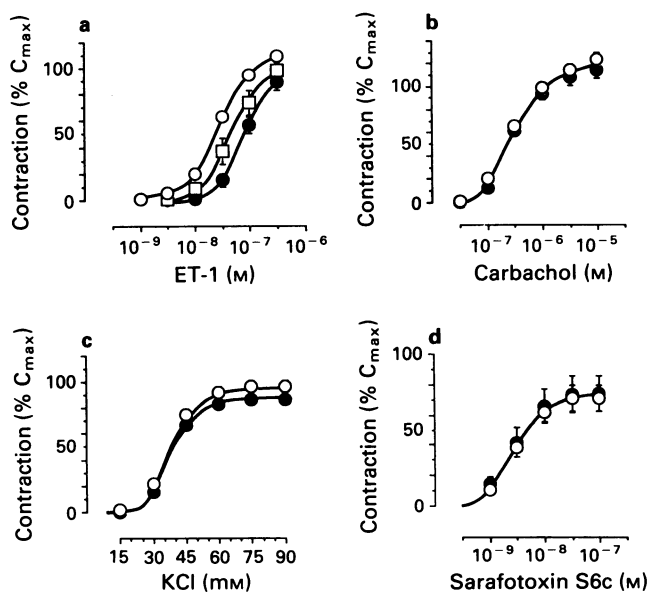
Despite the inhibitory effects of NDGA, ET-1-induced contractions were not inhibited by any of the other lipoxygenase inhibitors tested (phenidone, BW755C, ETYA) (Table 1). Furthermore, inhibitors of cytochrome P$_{450}$ (proadifen, metyrapone), Ca^{2+}-channels (NiCl$_2$, verapamil, nicardipine) and Na$^+$ transport (amiloride) did not attenuate ET-1-induced contractions (Table 1).

Effects of NDGA on the time course of ET-1-induced contraction In addition to inhibiting the magnitude of the contractile response to ET-1, NDGA also changed the time course of contraction (Figure 2). In control preparations, contractile responses to 100 nM ET-1 were slow to develop (peak response of 91.3 ± 5.8% C$_{max}$ after 20 min) and sustained (79.7 ± 5.1% C$_{max}$ after 60 min). However, in the presence of NDGA, contractile responses to 100 nM ET-1 peaked earlier (53.3 ± 4.3% C$_{max}$ after 10 min) and were not sustained (12.5 ± 4.1% C$_{max}$ after 60 min) (Figure 2b). A similar effect was produced in the presence of the ET$_A$ receptor antagonist, BQ-123 (peak response of 55.8 ± 5.0% C$_{max}$ after 8 min reduced to 1.7 ± 2.2% C$_{max}$ after 60 min) (Figure 2b). Indeed, in the presence of NDGA or BQ123, contractile responses to 100 nM ET-1 resembled the transient contractions induced by the ET$_B$ receptor-selective agonist, sarafotoxin S6c (Figure 2c). In contrast, the ET$_A$ receptor-mediated contractile responses to ET-1 in Ca^{2+}-free KBS were very slow to develop and sustained (Figure 2c).

Effect of NDGA on intracellular and extracellular Ca^{2+}-dependent contractions to ET-1 As shown previously (Henry, 1993), ET-1-induced contractions in rat isolated tracheal smooth muscle used both intracellular and extracellular Ca^{2+} (Figure 3). The intracellular Ca^{2+}-dependent contraction to 100 nM ET-1, produced in Ca^{2+}-free KBS, was significantly inhibited by 20 μM NDGA (53.5 ± 8.2% C$_{max}$ versus 13.3 ± 3.5% C$_{max}$ respectively, $n = 6$, $P < 0.01$). In contrast, the extracellular Ca^{2+}-dependent contraction to 100 nM ET-1, induced following the addition of 2.5 mM Ca^{2+}, was not inhibited by 20 μM NDGA (54.6 ± 5.8% C$_{max}$ versus 56.0 ± 5.4% C$_{max}$, respectively). In additional experiments, the intracellular Ca^{2+}-dependent phase of contraction to 100 nM ET-1 (51.5 ± 3.9% C$_{max}$, $n = 6$) was significantly inhibited by TMB-8 (100 μM, 5.2 ± 0.9% C$_{max}$) and ryanodine (10 μM, 23.8 ± 3.7% C$_{max}$).

NDGA and modulators of intracellular Ca^{2+} Under conditions of basal tone, 10 μM ryanodine did not induce any contraction in rat tracheal smooth muscle preparations ($n = 5$). However, in preparations pre-contracted with KCl (concentration-range, 17.5–20 mM producing 10.6 ± 1.6% C$_{max}$, $n = 14$) 10 μM ryanodine induced an additional, slowly-developing contraction (36.7 ± 3.2% C$_{max}$, $n = 6$, Figure 4a). The ryanodine-induced contraction was markedly inhibited by both 10 μM and 20 μM NDGA (Figure 4).

The Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, induced transient, concentration-dependent contractions of rat

Table 1 Effects of various drugs on the mean [− log (concentration of drug producing 50% C_{max})] for endothelin-1, carbachol and KCl in rat isolated trachea

Drug	[Drug] (μM)	Endothelin-1	Endothelin-1 + drug	Carbachol	Carbachol + drug	KCl	KCl + drug
Amiloride	10	7.50 ± 0.06	7.43 ± 0.07	6.63 ± 0.08	6.23 ± 0.10**	1.37 ± 0.07	1.22 ± 0.04*
Nickel chloride	330	7.63 ± 0.15	7.56 ± 0.14	6.48 ± 0.10	6.24 ± 0.10	1.35 ± 0.05	1.30 ± 0.08
Verapamil	10	7.61 ± 0.16	7.48 ± 0.12	6.60 ± 0.13	6.20 ± 0.12*	1.35 ± 0.05	NC[a]
Nicardipine	1	7.62 ± 0.15	7.38 ± 0.10	6.50 ± 0.10	6.24 ± 0.09	1.31 ± 0.03	NC[b]
NDGA	20	7.53 ± 0.09	7.07 ± 0.06**	6.64 ± 0.03	6.58 ± 0.03	1.57 ± 0.02	1.59 ± 0.02
Phenidone	100	7.67 ± 0.09	7.47 ± 0.08	6.89 ± 0.07	6.61 ± 0.10*	ND	ND
BW755C	50	7.71 ± 0.03	7.82 ± 0.19	6.47 ± 0.07	6.60 ± 0.10	ND	ND
ETYA	50	7.65 ± 0.17	7.59 ± 0.10	6.65 ± 0.07	6.66 ± 0.13	ND	ND
Proadifen	25	7.61 ± 0.15	7.67 ± 0.05	6.74 ± 0.04	5.70 ± 0.09***	ND	ND
Metyrapone	300	7.61 ± 0.15	7.52 ± 0.14	6.74 ± 0.04	6.47 ± 0.08**	ND	ND
Staurosporine	0.01	7.71 ± 0.03	7.32 ± 0.17*	6.47 ± 0.07	6.29 ± 0.08	ND	ND
	0.1	7.71 ± 0.03	7.10 ± 0.09***	6.47 ± 0.07	5.92 ± 0.14**	ND	ND

Each value is presented as the mean [− log (concentration of drug producing 50% C_{max})] ± s.e.mean and represents data obtained using preparations from each of 5 to 6 different animals.
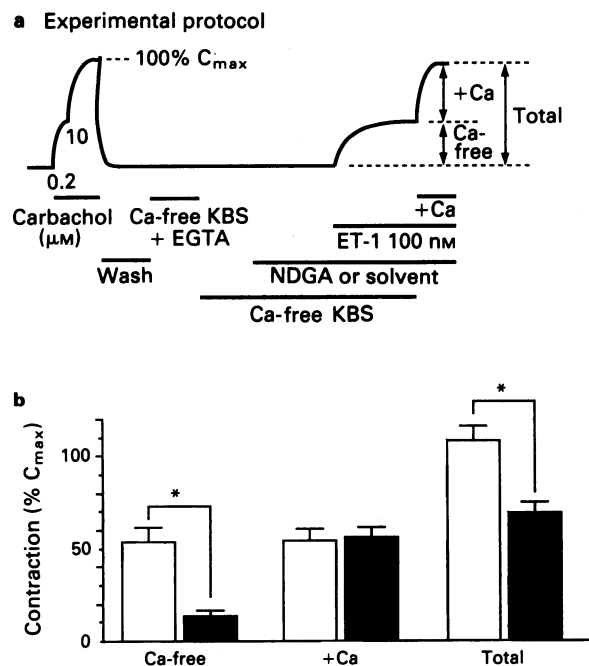
$*P < 0.05$, $**P < 0.01$, $***P < 0.001$; indicate that in the presence of the drug, the mean [− log (concentration of drug producing 50% C_{max})] for the spasmogen were statistically different from than those values obtained in the absence of the drug.

ND; not determined. NC; not calculable because maximum response less than 50% C_{max}, NC[a]; maximum response to KCl was 19.5 ± 4.1% C_{max} in the presence of 10 μM verapamil, NC[b]; maximum response to KCl was 38.8 ± 5.5% C_{max} in the presence of 1 μM nicardipine.
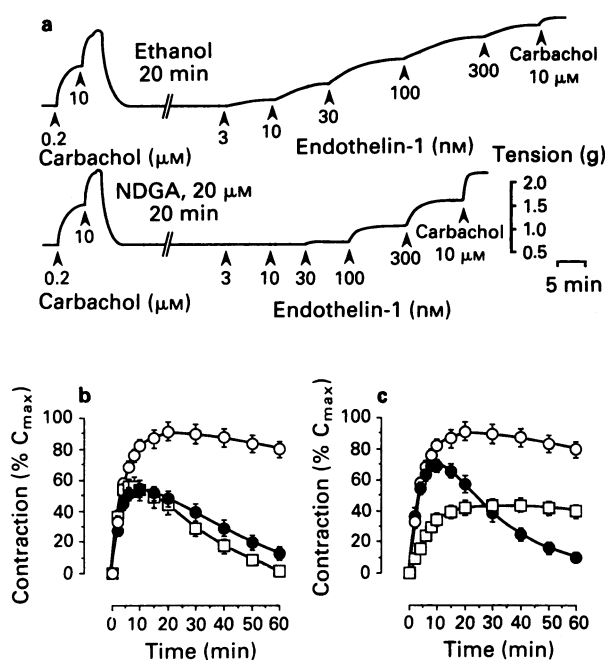


Figure 2 (a) Representative traces illustrating the effects of 20 μM NDGA on contractile responses of rat tracheal smooth muscle preparations to ET-1. In the presence of NDGA (lower trace) the contractile potency of ET-1 was reduced. Furthermore, the time required for each ET-1-induced contraction to reach plateau response was also reduced in the presence of NDGA. (b) Time course of contractions to 100 nM ET-1 in the absence (O) and presence of 20 μM NDGA (●) or the ET$_A$ receptor-selective antagonist, BQ123 (10 μM, □). Shown are the mean ± s.e.mean of 6 experiments. (c) Time course of contractions to 100 nM ET-1 in normal (O) and Ca^{2+}-free (□) KBS and to the ET$_B$ receptor-selective agonist, sarafotoxin S6c (●) in normal KBS. Shown are the mean ± s.e.mean responses of 6 experiments.

Figure 3 (a) Experimental protocol for experiments in Ca^{2+}-KBS (see Methods for details). (b) Contractile responses to 100 nM ET-1 produced in the absence (open columns) or presence of 20 μM NDGA (solid columns) in Ca^{2+}-free KBS ('Ca-free') and after the addition of 2.5 mM Ca^{2+} ('+ Ca'), according to the experimental protocol described in (a). The total contraction produced by 100 nM ET-1 (i.e. 'Ca-free' plus '+ Ca') is also presented ('Total'). Shown are the mean ± s.e.mean responses of 6 experiments.

isolated tracheal smooth muscle preparations (Figure 5a). Cyclopiazonic acid-induced contractions were not maintained and within 30 min of peak response, the contraction had usually returned to baseline levels of tone. The peak contractions induced by cyclopiazonic acid were markedly inhibited by 20 μM NDGA (Figure 5).

Contractions induced by ET-1 in preparations pretreated with 10 μM cyclopiazonic acid for 30 min were significantly attenuated compared to control contractions (Figure 6a). For example, in the presence of 10 μM cyclopiazonic acid, the concentration of ET-1 required to produce a 50% C_{max} contraction was 3.5 fold greater than in control preparations (1.2–10.4 fold, $n = 6$, $P < 0.05$). Experiments in Ca^{2+}-free KBS revealed that cyclopiazonic acid inhibited the intracellular Ca^{2+}-dependent phase of ET-1-induced contractions, but potentiated the extracellular Ca^{2+}-dependent phase of
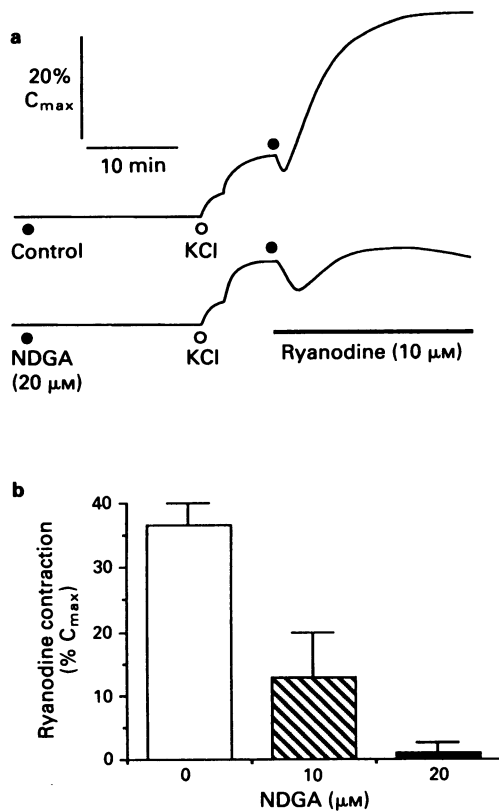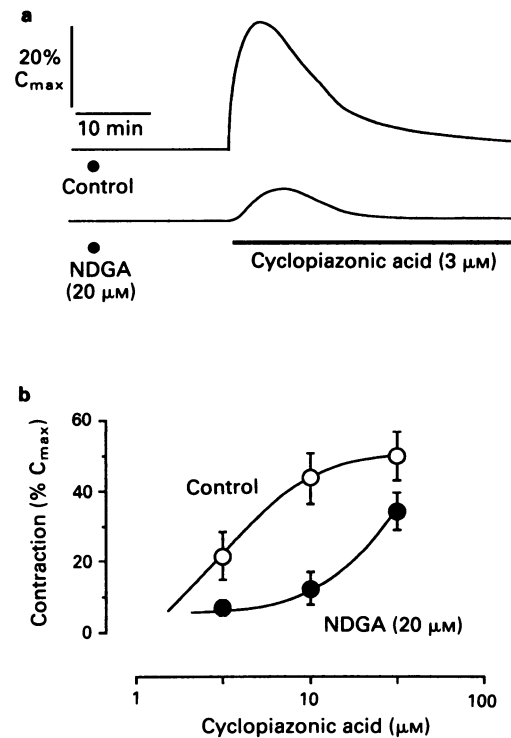
Figure 4 (a) Isometric tension recordings obtained simultaneously in paired rat tracheal smooth muscle preparations (precontracted with KCl) showing the contraction induced by ryanodine (control, upper trace) and its sensitivity to inhibition by 20 μM NDGA (lower trace). (b) Mean contractions induced by ryanodine in KCl-contracted preparations in the absence (open column, $n = 6$) and presence of 10 μM NDGA (hatched columns, $n = 4$) and 20 μM NDGA (solid columns, $n = 4$). Shown are the mean ± s.e.mean responses.

Figure 5 (a) Isometric tension recordings obtained simultaneously in paired rat tracheal smooth muscle preparations showing the transient contraction induced by cyclopiazonic acid (control, upper trace) and its sensitivity to inhibition by 20 μM NDGA (lower trace). (b) Mean concentration-effect curves to cyclopiazonic acid in the absence (O) and presence (●) of 20 μM NDGA. Contractions induced by cyclopiazonic acid were transient and hence cumulative concentration-effect curves were not performed. Each data point represents the mean (± s.e.mean) peak contractile response obtained to a single dose of cyclopiazonic acid (3, 10 or 30 μM) in 6–7 different preparations.
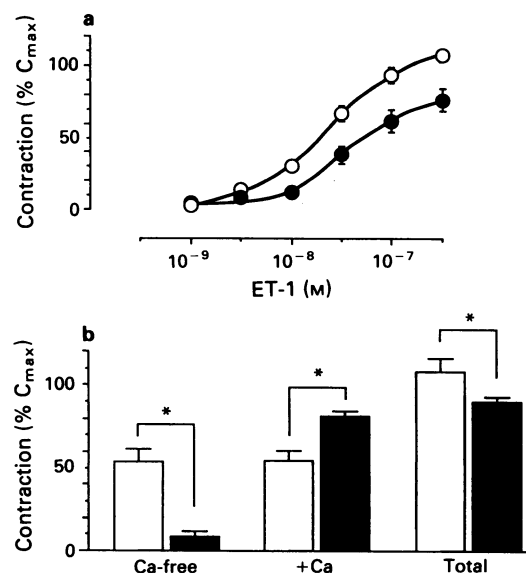
contraction (Figure 6b). Whereas cyclopiazonic acid inhibited contractile responses to ET-1, it was inclined to potentiate responses to carbachol (concentration required to produce 50% C_{max}; 165 nM (150–180 nM) in control preparations versus 90 nM (25–320 nM) in the presence of cyclopiazonic acid; $n = 3$, NS) and to KCl (39 mM (34–48 mM) in control preparations versus 31.5 mM (24–42 mM) in the presence of cyclopiazonic acid; $n = 3$, NS). Thapsigargin (3 μM), a structurally dissimilar inhibitor of Ca^{2+}-ATPase also inhibited contractions to ET-1 (3.1 (1.45–6.6) fold greater concentration of ET-1 required to produce 50% C_{max} in the presence of thapsigargin compared with control, $n = 7$, $P < 0.05$) but not those to carbachol ($n = 4$) or KCl ($n = 4$).

NDGA, ET-1 and protein kinase C ET-1-induced contractions were inhibited by the protein kinase C inhibitor staurosporine (Table 1). In the presence of 10 nM staurosporine, a 2.45 fold (1.1–5.6 fold, $n = 6$, $P < 0.05$) higher concentration of ET-1 was required to produce a 50% C_{max} contraction. This concentration of staurosporine had no significant inhibitory effect on contractile responses to carbachol (Table 1).

In rat isolated tracheal preparations precontracted with KCl (33.0 ± 2.1% C_{max}, $n = 10$), phorbol 12,13-dibutyrate induced concentration-dependent contractions (the concentration of phorbol 12,13-dibutyrate that induced a 50% C_{max} contraction, above the KCl-induced contraction, was 0.48 μM (0.062–3.7 μM) and the magnitude of contraction induced by 10 μM phorbol 12,13-dibutyrate was 66.0 ± 3.7% C_{max} above the KCl-induced contraction, $n = 5$). These phorbol 12,13-dibutyrate-induced contractions were not inhibited by 20 μM

Figure 6 (a) Mean concentration-effect curves to ET-1 in the absence (O) or presence (●) of 10 μM cyclopiazonic acid. (b) Contractile responses to 100 nM ET-1 in the presence (solid columns) or absence (open columns) of 10 μM cyclopiazonic acid in Ca^{2+}-free KBS ('Ca-free') and after the addition of 2.5 mM Ca^{2+} ('+ Ca'), according to the experimental protocol described in Figure 3a (NDGA replaced by cyclopiazonic acid). The total contraction produced by 100 nM ET-1 (i.e. 'Ca-free' plus '+ Ca') is also presented ('Total'). The mean ± s.e.mean responses of 6 experiments is shown.

NDGA (in the presence of NDGA, the concentration of phorbol 12,13-dibutyrate that induced a 50% C_{max} contraction was 0.124 μM (0.021–0.75 μM) and the magnitude of contraction induced by 10 μM phorbol 12,13-dibutyrate was 71.2 ± 6.6% C_{max} above the KCl-induced contraction, $n = 5$). The inactive phorbol ester, 4α-phorbol didecanoate had no significant effect on KCl-induced tone in these preparations.

Effect of NDGA on guinea-pig isolated tracheal responses to ET-1   NDGA had no significant inhibitory effect on ET-1-induced contractions in epithelium-denuded, guinea-pig isolated tracheal preparations (concentration of ET-1 that produced 30% C_{max} was 14 nM (7.3–26 nM, $n = 5$) in control preparations and 16 nM (7.2–33 nM) in paired preparations exposed to 20 μM NDGA; the magnitude of contraction induced by 300 nM ET-1 was 56.1 ± 2.4% C_{max} in control preparations and 56.9 ± 2.3% C_{max} in preparations exposed to NDGA). Similarly, NDGA did not inhibit contractions induced by 10 μM ryanodine in KCl-contracted, guinea-pig isolated tracheal preparations (ryanodine-induced contractions; 60.7 ± 5.8% C_{max} and 59.3 ± 5.8% C_{max} in the absence and presence of 20 μM NDGA ($n = 7$), respectively). Furthermore, cyclopiazonic acid did not inhibit contractile responses to ET-1 in guinea-pig isolated tracheal preparations (concentration of ET-1 required to produce 30% C_{max}; 6.8 nM (1.8–25 nM, $n = 4$) in control preparations versus 6.1 nM (3.2–12 nM, $n = 4$) in the presence of 3.3 μM cyclopiazonic acid).

Quantitative autoradiography

In rat isolated tracheal sections incubated with 0.5 nM [^{125}I]-ET-1, light microscopic autoradiography revealed high densities of autoradiographic grains over the tracheal smooth muscle band. Over 90% of these autoradiographic grains were over specific [^{125}I]-ET-1 binding sites (total grain density, 331 ± 28 grains 1000 μm^{-2}; non-specific grain density, 26 ± 2 grains 1000 μm^{-2}; specific grain density, 305 ± 29 grains 1000 μm^{-2}, $n = 4$ rat trachea). NDGA (10 μM) had no significant effect on the density of autoradiographic grains over specific [^{125}I]-ET binding sites (312 ± 37 grains 1000 μm^{-2} in the presence of 10 μM NDGA versus 305 ± 29 grains 1000 μm^{-2} in the absence of NDGA, $n = 4$ rat trachea) or non-specific [^{125}I]-ET binding sites (32 ± 4 grains 1000 μm^{-2} in the presence of 10 μM NDGA versus 26 ± 2 grains 1000 μm^{-2} in the absence of NDGA, $n = 4$ rat trachea) in rat tracheal smooth muscle.

Discussion

NDGA and selective inhibition of the ET_A receptor-effector system

In rat tracheal smooth muscle, ET-1 can induce contractions by activating ET_A and/or ET_B receptor-effector systems (Henry, 1993). Thus, the findings in this study that NDGA inhibited the contractions induced by ET-1, but not those induced by the ET_B receptor selective agonist, sarafotoxin S6c, indicate that NDGA selectively attenuated the ET_A receptor-effector system. The marked similarities in the inhibitory effects of NDGA and the ET_A receptor-selective antagonist BQ-123 on the time-course of ET-1-induced contractions provides additional evidence that NDGA selectively inhibited the ET_A, but not the ET_B, receptor-effector system.

NDGA and BQ-123 inhibited ET-1-induced contractions in rat isolated tracheal smooth muscle. However, it is unlikely that NDGA acted as an ET_A-receptor antagonist because autoradiographic studies revealed that unlike BQ-123 (Henry, 1993), NDGA did not inhibit the binding of [^{125}I]-ET-1 to rat isolated tracheal smooth muscle in the current study. This is consistent with our previous studies showing

that ET-1-induced accumulation of [^{3}H]-inositol phosphates in this tissue is inhibited by BQ-123 (Henry, 1993), but not by NDGA (Henry et al., 1992). These latter findings agree with recent reports that NDGA (50–70 μM) did not inhibit phospholipase C activity in rat glomerular cells (Force et al., 1991) or human T-lymphocytes (Mire-Sluis et al., 1989). Thus, the site of action of NDGA in the ET_A receptor-effector system appears to be distal to ET_A receptor activation and the generation of inositol phosphates.

NDGA and modulators of intracellular Ca^{2+}

In rat tracheal smooth muscle, ET-1-induced contractions utilise intracellular and extracellular Ca^{2+} (Henry, 1993). The intracellular Ca^{2+}-dependent contractions induced by ET-1 in Ca^{2+}-free solution are mediated by ET_A receptors (Henry, 1993) and were inhibited in the current study by agents that deplete intracellular Ca^{2+} stores (ryanodine and cyclopiazonic acid) or that act as intracellular Ca^{2+} antagonists (TMB-8). Of particular interest was the finding that NDGA also selectively inhibited the intracellular Ca^{2+}-dependent component of ET-1-induced contraction.

One explanation for these findings is that NDGA inhibited the release of Ca^{2+} from intracellular stores such as the sarcoplasmic reticulum. Support for this explanation is provided by the findings that NDGA significantly inhibited contractions induced by both ryanodine and cyclopiazonic acid; contractions purportedly dependent upon Ca^{2+} release from the sarcoplasmic reticulum. Ryanodine is an agonist for the Ca^{2+}-release channel in the sarcoplasmic reticulum and thereby stimulates the release of Ca^{2+} from the sarcoplasmic reticulum, elevates cytosolic Ca^{2+} levels and may promote smooth muscle contraction (Low et al., 1992; Missiaen et al., 1992). The amount of Ca^{2+} in the sarcoplasmic reticulum is determined by a balance between the depletion of Ca^{2+} via passive outward leak or agonist-induced leak and the repletion of Ca^{2+} via the Ca^{2+}-ATPase pump (Low et al., 1991). Thus, in the presence of a Ca^{2+}-ATPase inhibitor such as cyclopiazonic acid, the passive outward leak of sarcoplasmic reticular Ca^{2+} is not balanced by repletion of Ca^{2+} into the sarcoplasmic reticulum and the resultant rise in cytosolic Ca^{2+} levels initiates smooth muscle contraction (Groeger et al., 1988; Seidler et al., 1989; Shima & Blaustein, 1992). Hence, the inhibitory effects of NDGA on the contractions induced by ryanodine, cyclopiazonic acid and ET-1 may be explained by proposing that NDGA inhibited the release of Ca^{2+} from the sarcoplasmic reticulum and thereby prevented the rise in cytosolic Ca^{2+} levels that precedes contraction.

Little is presently known of the direct actions of NDGA on the release of Ca^{2+} from the sarcoplasmic reticulum, although NDGA has been shown to inhibit Ca^{2+}-channel activity in some cell systems. For example, in ArT-20 and GH_3 anterior pituitary cell lines, NDGA inhibited Ca^{2+}-channel activity by partitioning into the membrane and interacting either with the channel protein directly or with another membrane-bound Ca^{2+}-channel modulator, independently of actions on L-type Ca^{2+}-channels and of arachidonic acid metabolism (Korn & Horn, 1990). Thus, the findings in the current study that NDGA inhibited contractions to ryanodine and cyclopiazonic acid provides some evidence that NDGA may inhibit Ca^{2+} release from intracellular stores in rat isolated tracheal smooth muscle. Inhibition of agonist-induced Ca^{2+} mobilisation may likewise explain the attenuating actions of NDGA on ET-1-induced contractions.

An alternative, but less likely, explanation for the finding that NDGA inhibited the intracellular Ca^{2+}-dependent component of ET-1-induced contractions is that NDGA depleted the sarcoplasmic reticular stores of Ca^{2+}. With respect to this possibility, it is relevant to note that (a) NDGA has some structural resemblance to the sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, an agent that depletes sarcoplasmic reticular stores of Ca^{2+} and (b) NDGA and

cyclopiazonic acid both inhibited ET-1-induced contractions. However, despite these similarities, many of the characteristic actions of Ca^{2+}-ATPase inhibitors were not exhibited by NDGA. For example, incubation with Ca^{2+}-ATPase inhibitors such as cyclopiazonic acid and the structurally unrelated agent thapsigargin, (a) induced marked transient contractions of tracheal smooth muscle preparations, (b) potentiated contractions induced by KCl and (c) potentiated the extracellular Ca^{2+}-dependent component of ET-1-induced contraction. Each of these effects is consistent with the actions of Ca^{2+}-ATPase inhibitors and have been previously explained on the basis that inhibition of Ca^{2+} uptake into the sarcoplasmic reticulum either reduces the buffering action that the sarcoplasmic reticulum normally exerts on rises in intracellular Ca^{2+} and/or enhances the plasma membrane permeability to Ca^{2+} (Mason et al., 1991; Demaurex et al., 1992; Shima & Blaustein, 1992; Shimamoto et al., 1992). None of the actions was exhibited by NDGA and thus, on balance, it appears unlikely that NDGA inhibited ET-1-induced contractions by inhibiting sarcoplasmic reticulum Ca^{2+}-ATPase and depleting intracellular Ca^{2+} stores.

Recent studies report that ET-1-induced contractions in rat and guinea-pig tracheal smooth muscle involve, at least partly, stimulation of the phosphoinositide pathway and mobilisation of intracellular Ca^{2+} (Hay, 1990; Henry et al., 1992). However, the findings in the current study that NDGA inhibited the contractile responses to ET-1 and ryanodine in the rat, but not the guinea-pig, indicate that significant differences exist between the species with respect to the mechanism of ET-1-induced contraction in airway smooth muscle. The reasons for these species differences are not yet clear. At present it is not known whether ET-1-induced contractions in human airway smooth muscle are affected by NDGA, although it is interesting to note that ET-1-induced contractions in human bronchial smooth muscle, like rat tracheal smooth muscle, appear to be dependent upon the mobilisation of intracellular Ca^{2+} stores (McKay et al., 1991). Preliminary data from human vascular smooth muscle suggest that ET-1-induced contractions in some blood vessels (Resink et al., 1989) although not all (Miyauchi et al., 1990) may be susceptible to inhibition by NDGA.

Other possible mechanisms of NDGA action

Many other cellular processes involved in the regulation of smooth muscle tone can be modulated by NDGA and/or ET-1 including lipoxygenase, protein kinase C, guanylate cyclase, Na$^+$/H$^+$ exchange the L- and T-type Ca^{2+} channels. The possibility that NDGA may have inhibited ET-1-induced contraction via an action at one or other of these sites is addressed below.

The concentration-range for NDGA that selectively inhibited ET-1-induced contractions in the current study (3 to 20 μM) is similar to that routinely used to inhibit lipoxygenase activity. However, NDGA was the only lipoxygenase inhibitor tested that exerted any inhibitory effect on ET-1-induced contractions. Neither the acetylenic analogue of arachidonic acid, ETYA, nor lipoxygenase inhibitors having antioxidant activity (phenidone, BW755C) inhibited ET-1-induced contractions. Thus, it is unlikely that inhibition of lipoxygenase activity contributed significantly to the inhibitory effects of NDGA on ET-1-induced contractions.

The possibility that NDGA may have attenuated ET-1-induced contractions by inhibiting protein kinase C activity was examined in the light of a recent report that NDGA inhibited protein kinase C activity in cell cultures (Rondeau et al., 1990) and that in airway smooth muscle cells ET-1 stimulates the generation of diacylglycerol, the proposed endogenous activator of protein kinase C (Mattoli et al., 1991). Evidence supporting the involvement of protein kinase C in the spasmogenic actions of ET-1 in rat isolated tracheal smooth muscle is provided by the findings in this study that an inhibitor of protein kinase C, staurosporine, attenuated

ET-1-induced contractions in this tissue. However, it should be noted that staurosporine is not a specific inhibitor of protein kinase C (Ruegg & Burgess, 1989) and that activation of protein kinase C by ET-1 remains to be shown using more direct methods. Nevertheless, it is unlikely that inhibition of protein kinase C activity contributed significantly to the inhibitory effects of NDGA on ET-1-induced contractions since NDGA had no inhibitory effects on contractions induced by a direct activator of protein kinase C such as phorbol 12, 13 dibutyrate in this preparation.

Activation of soluble guanylate cyclase, by agents such as nitric oxide, can inhibit the development of spasmogen-induced contraction in smooth muscle. Thus, although NDGA has previously been shown to attenuate guanylate cyclase activity (Clark & Linden, 1986) inhibition of guanylate cyclase by NDGA cannot explain the inhibitory effects of NDGA on the development of ET-1-induced contractions observed in rat tracheal smooth muscle.

Activation of the Na$^+$/H$^+$ exchanger has been implicated in ET-1-induced contractions in airway smooth muscle (Battistini et al., 1991). At present the role of the Na$^+$/H$^+$ exchanger in ET-1-induced contractions and its susceptibility to inhibition by NDGA in rat tracheal smooth muscle is not known. However, it is unlikely that inhibition of the Na$^+$/H$^+$ exchanger can account for the inhibitory effects of NDGA on ET-1-induced contraction observed in the current study. For example, the findings that ET-1-induced contractions in guinea-pig tracheal smooth muscle are attenuated by inhibitors of the Na$^+$/H$^+$ exchanger (Battistini et al., 1991), but not by NDGA (current study) suggest that NDGA does not inhibit the Na$^+$/H$^+$ exchanger in guinea-pig tracheal smooth muscle. These findings, together with those showing that ET-1-induced contractions in rat trachea were not inhibited by amiloride are not compatible with NDGA-induced inhibition of the Na$^+$/H$^+$ exchanger.

Finally, the findings that NDGA, but not inhibitors of L- and T-type Ca^{2+}-channels, inhibited ET-1-induced contractions is entirely consistent with the view that these Ca^{2+}-channels play no significant role in ET-1-induced contraction of rat tracheal smooth muscle (Turner et al., 1989; Henry, 1993) and moreover that NDGA-induced inhibition of contractions to ET-1 in this preparation occur independently of plasmalemma Ca^{2+} channels.

NDGA and the time-course of ET-1-induced contractions

ET-1-induced contractions in vascular and airway smooth muscle are characteristically slow to develop, sustained and resistant to reversal by washout. Thus, it was of particular interest to find that NDGA significantly altered the time-course of ET-1-induced contractions; in the presence of NDGA, ET-1-induced contractions reached peak response more quickly and were not sustained. Subsequent experiments revealed that qualitatively similar effects on ET-1-induced contractions were produced by the ET$_A$ receptor antagonist, BQ-123. Furthermore, the transient contractions induced by ET-1 in the presence of NDGA or BQ-123 closely resemble those induced by the ET$_B$ receptor agonist, sarafotoxin S6c. The simplest interpretation of these findings is that stimulation of the ET$_A$ and ET$_B$ receptor-effector systems induce contractions with different temporal profiles. Stimulation of ET$_A$ receptors induces a contraction that develops slowly and is sustained whereas stimulation of ET$_B$ receptors induces a contraction that develops relatively quickly but is not sustained. Thus, by selectively inhibiting the slowly developing contraction induced by the ET$_A$ receptor-effector system, NDGA and BQ-123 transformed the characteristically slow and sustained contraction of ET-1 into a transient contraction similar to that induced by the ET$_B$ receptor-selective agonist, sarafotoxin S6c. At present it is not clear which events in the ET$_A$ receptor effector system cause the slowly developing phase of contraction. However, it is of interest to note that ryanodine-induced contractions, which

were inhibited by NDGA, also developed very slowly in this preparation. Furthermore, the findings in the current study that NDGA inhibits ET-1-induced contractions via an action at the level of intracellular Ca^{2+}-mobilisation concur with the proposal of Marsault and coworkers (1991) that the rate limiting step for the contractile action of ET-1 is a post-receptor event distal to the early changes in intracellular Ca^{2+} levels.

Conclusions

In summary, the major finding of this study in rat isolated tracheal smooth muscle is that NDGA dose-dependently attenuates ET-1-induced contractions by selectively inhibiting the ET_A receptor-effector system, probably at the level of

intracellular Ca^{2+} mobilisation. These effects do not involve an action of NDGA on arachidonic acid metabolism, ET receptors, inositol phosphate generation, protein kinase C, L- or T-type Ca^{2+}-channels or the ET_B receptor-effector pathway. As a further consequence of inhibiting the ET_A receptor-effector system, NDGA converts the ET-1-induced contraction from the characteristically slowly developing and sustained contraction into a transient contraction resembling that induced by the ET_B receptor agonist, sarafotoxin S6c.

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