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Effects of Phorbol 12,13-Diacetate and Its Influence on Spasmogenic Responses in Normal and Sensitized Guinea-pig Trachea

A. DE DIEGO, J. CORTIJO, V. VILLAGRASA, M. PERPIÑÁ, J. ESPLUGUES AND E. J. MORCILLO

Department de Farmacologia, Facultat de Medicina i Odontología, Universitat de València, València, Spain

Abstract

We have studied the effects of phorbol 12,13-diacetate (PDA) and its influence on a variety of spasmogenic responses in trachea isolated from normal and sensitized guinea-pigs.

Tracheal preparations were denuded of epithelium, treated with indomethacin (2·8 μM), and cooled to 20°C. In these experimental conditions, tracheal strips contracted to PDA (0·1 nM–1 μM). Contractions to PDA (1 μM) were greater in sensitized tissues. In normal trachea, contractions to PDA (0·1 μM) were depressed by H-7, 1-(5-isoquinolinyl-sulphonyl)-2-methylpiperazine, (50 μM), amiloride (10 μM), verapamil (10 μM) and Ca^{2+}-free exposure. Similar effects were obtained in sensitized trachea except that PDA-induced contraction was resistant to verapamil and Ca^{2+}-free exposure.

Cooling (20°C) of normal trachea substantially depressed the response to $CaCl_2$ (in K^+-depolarized tissues), KCl, histamine and 5-hydroxytryptamine without affecting the spasm induced by acetylcholine. This inhibitory effect of cooling was not observed in sensitized trachea. PDA (0·1 μM) did not affect spasmogenic responses at 37°C but counteracted the inhibitory effect of cooling in normal trachea. PDA had no effect on sensitized tissues. PDA (0·1–1 μM) did not alter Ca^{2+}-induced contraction of skinned normal and sensitized trachea.

These results support the hypothesis that intracellularly stored Ca^{2+} plays an important role in the activation of sensitized tracheal muscle.

Protein kinase C (PKC) is involved in the mechanical responses to agonist-induced activation of Ca^{2+}-mobilizing receptors (Nishizuka 1986). The finding that phorbol esters directly bind to, and activate, PKC (Castagna et al 1982) has provided a useful tool for investigating the complex role of this enzyme in intact tissues. Several authors have studied the effects of phorbol esters on the spontaneous tone of bovine (Park & Rasmussen 1985; Knox et al 1993), guinea-pig (Menkes et al 1986; Huang et al 1987; Jackson et al 1988; Hirst et al 1989; Souhrada & Souhrada 1989a; Morrison & Vanhoutte 1991; Cortijo et al 1994), rabbit (Schramm & Grunstein 1989), and swine (Baba et al 1989) tracheal smooth muscle. Both spasmogenic and relaxant responses to phorbol esters have been reported in the airway smooth muscle, and the underlying mechanisms include mobilization of Ca^{2+} from extracellular and intracellular sources, and altered Na^+ transport across the cell membrane (Park & Rasmussen 1985; Huang et al 1987; Schramm & Grunstein 1989; Souhrada & Souhrada 1989a; Knox et al 1993).

The multiplicity of actions ascribed to the activation of PKC by phorbol esters suggest that PKC can effectively influence agonist- and depolarization-induced contractile responses of trachealis muscle. However, although it has been shown that phorbol esters alter the responses to some spasmogenic drugs in airway smooth muscle (Menkes et al 1986; Schramm & Grunstein 1989; Morrison & Vanhoutte 1991), no systematic study has so far been made. The object of the present work was, therefore, to examine the influence of an active phorbol ester, phorbol 12,13-diacetate (PDA), on the responses to $CaCl_2$ (in a Ca^{2+}-free K^+-depolarizing solution), KCl, and structurally-specific spasmogens (acetylcholine, histamine and 5-hydroxytryptamine (5-HT)) in guinea-pig isolated trachealis. This investigation was extended to examine also the effects of PDA on the responses to the same spasmogens but obtained in cooled or sensitized trachea, two experimental situations which may be related to PKC activation and are considered of relevance to the pathogenesis of asthma (Huang et al 1987; Souhrada & Souhrada 1989b; Ortiz et al 1991). In addition, we have examined the effects of PDA on the contraction by Ca^{2+} acting on Triton X-100-skinned trachealis muscle obtained from normal and sensitized guinea-pigs.

Correspondence: J. Cortijo, Departament de Farmacologia, Facultad de Medicina, Av. Blasco Ibañez 17, 46010 Valencia, Spain.

Materials and Methods

Preparation of tissues and sensitization and cooling procedures

Adult male tricoloured guinea-pigs, 340–450 g, were randomly allocated to one of two groups, normal (non-sensitized) and sensitized. The sensitization procedure was as previously described (Ortiz et al 1989). Briefly, on day 0 the animals were injected subcutaneously with 0·25 mL Freund's complete adjuvant plus 1·25 μg g^{-1} bovine serum albumin (BSA) dissolved in 0·25 mL saline, and on day 2 and day 4 the animals received the same amount of Freund's complete adjuvant and BSA by the intramuscular route. The animals were used for experiments on days 21–25. The normal group was subjected to the same protocol but the

animals were not exposed to the antigen (Ortiz et al 1989). This sensitization procedure has been demonstrated to generate immunoglobulin G_1 (IgG_1) as the major homocytotrophic antibody mediating the immediate hypersensitivity response in this species (Regal 1984), whereas in man the major antibody is IgE. Despite this difference, the IgG_1 guinea-pig model is widely used as an animal model reflecting certain components of the asthmatic response (Undem et al 1985).

The animals were killed by stunning and bleeding to avoid PKC inhibition produced by barbiturates (Mikawa et al 1990). Tracheae were excised, cleaned of extraneous tissues, opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis, and divided into strips of about 3 mm width. To avoid the influence of the epithelium on tracheal responses to phorbols (Raeburn 1989; Souhrada & Souhrada 1991), the experiments were carried out in epithelium-denuded preparations. The epithelium was removed by gently rubbing the mucosal surface with a wet filter paper and the effectiveness of this procedure was confirmed by histology as previously reported (Iriarte et al 1990). Tracheal strips were suspended under a resting tension of 1 g in 10 mL tissue baths containing physiological salt solution (PSS, composition in mM: NaCl 118·4, KCl 4·7, $CaCl_2$ 2·5, $MgSO_4$ 0·6, KH_2PO_4 1·2, $NaHCO_3$ 25·0, and dextrose 11·1) bubbled with 95% O_2–5% CO_2 at 37°C (pH = 7·4). Tension was measured with isometric transducers (Grass FTO3) and recorded on a polygraph. Following an initial period of tissue equilibration of 60 min, indomethacin (2·8 μM) was added to the bath fluid to suppress the spontaneous tone of the preparation and to avoid the influence of endogenous prostanoids in the effects of PDA (Menkes et al 1986). Subsequent changes of bath fluid were carried out with PSS containing indomethacin (2·8 μM). In experiments that involve the cooling of the normal or sensitized tracheal preparations, the temperature of the bath solution was reduced from 37 to 20°C by means of a circulator (Selecta 398). Temperature changes were rapidly achieved (rate of cooling of about $-3°C\,min^{-1}$). Both the temperature and the pH of the bath solution were monitored as previously described (Ortiz et al 1991).

Assessment of the tracheal response to PDA and its modification by different pharmacological interventions

Following 60-min equilibration of normal or sensitized trachea with PSS and suppression of spontaneous tone by incubating (40 min) the tissue with PSS containing indomethacin (2·8 μM), the bath temperature was lowered to 20°C. Twenty minutes later, tissues were challenged with PDA (0·1 nM, 1 nM, 10 nM, 0·1 μM, 1 μM) and the responses followed for 60 min. In a separate group of experiments, responses to PDA. (0·1 to 1 μM) were obtained in indomethacin-treated normal trachea at 37°C. In subsequent experiments carried out in cooled (20°C), indomethacin-treated, normal or sensitized trachea, the response to PDA (0·1 μM) was obtained after incubation (30 min) and in the presence of verapamil (10 μM), amiloride (10 μM) or H-7 (50 μM). The response to PDA (0·1 μM) was also obtained in tissues exposed to Ca^{2+}-free PSS containing EGTA (0·1 mM). The period of exposure to the Ca^{2+}-free medium was for 30 min before, and during the generation of the PDA challenge. Time-matched control tissues were treated similarly but received no treatment before the challenge with PDA.

Assessment of the effects of PDA on tracheal responses to spasmogens

Following tissue equilibration (60 min) and suppression of spontaneous tone by incubating (40 min) the tracheal strips with PSS containing indomethacin (2·8 μM), two successive cumulative concentration–response curves were constructed for $CaCl_2$ (10 μM–20 mM), KCl (1–100 mM), acetylcholine (1 nM–1 mM), histamine (1 nM–1 mM) or 5-HT (0·1 nM–0·1 mM). The experiments with $CaCl_2$ as spasmogen were carried out in K^+-rich (55 mM KCl substituted for an equimolar concentration of NaCl), Ca^{2+}-free Tris solution as described previously (Sarriá et al 1989). After an initial concentration–response curve (first curve) for one of these spasmogens had been obtained, the tissues were randomly allocated in equal numbers to test groups or time-matched control groups, and a second concentration–response curve was obtained. Second curves in the test tissues were obtained in the presence of PDA (0·1 μM). The period of exposure to PDA was 30 min before and during the second curve. This experimental protocol (i.e. 1st and 2nd curves) was carried out in normal and sensitized tracheal preparations, and in both cases, the second curve for the spasmogens was obtained at either 37 or 20°C.

Normal and sensitized preparations were challenged with BSA ($1\,mg\,mL^{-1}$) to confirm the existence of an antigen-induced spasm in sensitized strips and the absence of response in normal tissues.

Assessment of the effects of PDA on skinned tracheal preparations

Segments of trachea from normal or sensitized guinea-pigs were skinned of their plasmalemmal membranes as previously described (Cortijo et al 1987). Tissue segments were incubated (4 h at 4°C) in a 1% (v/v) Triton X-100 solution which contained solutes as follows (mM): KCl 50, sucrose 150, EGTA 5, imidazole 20, and dithioerythritol 0·5 (pH = 7·4). After rinsing for 15 min in a similar solution but without Triton X-100, tissues were stored in a solution with the following composition (mM): EGTA 4, $MgCl_2$ 10, ATP 7·5, NaN_3 1, imidazole 20 and dithioerythritol 0·5 (pH = 6·7). The solution was stored with 50% glycerol at −20°C for up to 10 days. Under an imposed tension of 1 g, segments of skinned trachea were set up at 20°C for isometric recording of tension changes in 5 mL relaxing solution with the following composition (mM): EGTA 4, $MgCl_2$ 10, ATP 7·5, KH_2PO_4 6, NaN_3 1 and imidazole 20. The pH of the solution was adjusted to 6·7 with KOH. The relaxing solution did not contain added calmodulin. All tissues were allowed to equilibrate in this medium for 20 min before the construction of cumulative concentration–response curves for Ca^{2+}. The amount of $CaCl_2$ added to the relaxing solution to achieve the required bath concentration of free Ca^{2+} was calculated as previously reported (Cortijo et al 1987). Two successive concentration-response curves for Ca^{2+} (0·2–1 μM) were constructed. In test tissues, PDA (0·1 or 1 μM) was present from 30 min before, to the

end of production of the second curve for Ca^{2+}. Control tissues were treated similarly except that they were not exposed to PDA. At the end of each experiment the preparation was challenged with acetylcholine 100 μM.

Analysis of results

Spasmogenic responses are expressed as absolute (g or mg of force developed) or relative (% of control) values. The effective concentration producing half of the maximum effect (EC50) was estimated from each concentration–response curve and transformed into $-\log$ values for statistical purposes. Maximal effect (E_{max}) and $-\log$ EC50 were considered as estimates of the efficacy and potency of the spasmogens, or alternatively of the responsiveness and sensitivity of the preparation.

Data are presented as means ± s.e.m. Statistical analysis of the results was performed by analysis of variance followed by Duncan's test. Differences were considered significant for $P < 0.05$.

Drugs and solutions

Acetylcholine chloride, histamine hydrochloride, 5-HT, indomethacin, phorbol 12,13-diacetate and 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H-7) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Amiloride was from Merck, Sharp & Dohme (Madrid, Spain) and verapamil hydrochloride from Biosedra-Knoll (Madrid, Spain). Other chemicals used were of analytical grade (E. Merck, Darmstadt, Germany; Panreac, Barcelona, Spain). The stock solutions of indomethacin were prepared in absolute ethanol. PDA was dissolved initially in dimethylsulphoxide (Sigma), then diluted in distilled water and stored at −20°C. Other substances were dissolved in buffer solution just before use. Vehicle controls were run in parallel; no significant vehicle effects were observed. With the exception of KCl (where the stated concentration was that in excess of KCl provided by the buffer solution) drug concentrations are expressed as final bath concentrations of the active chemical.

Results

Response to PDA in normal, cooled and sensitized trachea and its modification by different pharmacological interventions

Fast cooling (20°C) resulted in a rapidly developing contraction of normal and sensitized, epithelium-denuded, trachea bathed in PSS containing indomethacin (2·8 μM). This contraction was not sustained and relaxation ensued to regain baseline values in about 15–20 min. In these conditions (epithelium-denuded, 20°C, indomethacin treatment), addition of PDA (0·1 nM–1 μM) resulted in a slowly developing and sustained, concentration-dependent, contraction of normal and sensitized trachea (Fig. 1). The contraction to PDA commenced after a lag of 5 to 10 min and reached a maximum 30–40 min later that was maintained to the end of 60 min of exposure. The contractile response to PDA (1 μM) is probably an under-estimation of its maximal response, as the curves do not appear to reach a plateau at that concentration. The contractile response to PDA (1 μM) in sensitized trachea at 20°C was significantly greater than that

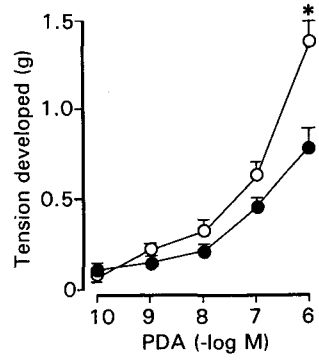

FIG. 1. The concentration–contraction curve to phorbol 12,13-diacetate (PDA) in epithelium-denuded, indomethacin (2·8 μM)-treated cooled (20°C) trachea from normal (●) and sensitized (○) guinea-pigs. Data are means ± s.e.m. of values from six experiments.

obtained in normal trachea at 20°C (Fig. 1). A separate group of experiments showed that contractions to PDA (0·1 and 1 μM) obtained in normal trachea at 20°C (0·48 ± 0·07 and 0·81 ± 0·09 g, respectively; n = 12) were significantly greater than those obtained at 37°C (0·19 ± 0·06 and 0·39 ± 0·07 g, respectively; n = 12; $P < 0.05$).

In subsequent experiments, the influence of different pharmacological interventions on the contractile response produced by PDA (0·1 μM) was studied at 20°C, in normal and sensitized, epithelium-denuded, indomethacin-treated preparations. This concentration of PDA was chosen because it is in the middle part of the concentration–response curve for PDA and is equi-effective in normal (0·51 ± 0·07 g, n = 14) and sensitized tissues (0·67 ± 0·16 g, n = 14; $P > 0.05$). Pretreatment with H-7 (50 μM) produced a marked inhibition of PDA-induced contraction in normal and sensitized tissues. Incubation with verapamil (10 μM) or with a Ca^{2+}-free PSS resulted in a partial inhibition of PDA-induced contraction in normal trachea but no inhibition was observed in sensitized trachea. Amiloride (10 μM) produced similar inhibition of contractions to PDA in normal and sensitized tissues (Table 1).

Influence of PDA on the spasmogenic responses of normal, cooled and sensitized trachea

In normal and sensitized, epithelium-denuded, indomethacin (2·8 μM)-treated trachea, control experiments at 37°C

Table 1. Contractile responses to PDA (0·1 μM) in epithelium-denuded, indomethacin (2·8 μM)-treated cooled (20°C) trachea from normal and sensitized guinea-pigs in the presence of different pharmacological interventions.

Experiment	Tension development (%)	
	Normal	Sensitized
H-7 (50 μM)	10 ± 5*	5 ± 2*
Verapamil (10 μM)	53 ± 9*	82 ± 7†
Ca^{2+}-free medium containing EGTA 0·1 mM	44 ± 9*	85 ± 5†
Amiloride (10 μM)	36 ± 12*	30 ± 9*

Values are means ± s.e.m., n = 5–6. *$P < 0.05$ compared with control, †$P < 0.05$ compared with normal tissue (i.e. non-sensitized).

Table 2. The effect of PDA (0·1 μM) on the responses to $CaCl_2$, KCl, acetylcholine, histamine and 5-HT in indomethacin (2·8 μM)-treated trachea from normal and sensitized guinea-pigs. Experiments were carried out at 37 and 20°C. Two successive concentration–response curves were generated. First curves (controls) are not shown. Second curves were obtained in the absence (time-matched controls; C) or presence of PDA (T).

		Normal (non-sensitized)				Sensitized			
		37°C		20°C		37°C		20°C	
		E_{max} (g)	pD_2	E_{max} (g)	pD_2	E_{max} (g)	pD_2	E_{max} (g)	pD_2
$CaCl_2$	C	1·55 ± 0·33	2·90 ± 0·12	0·57 ± 0·09*	2·60 ± 0·08	1·76 ± 0·29	3·02 ± 0·11	1·30 ± 0·22	2·65 ± 0·13
	T	1·17 ± 0·17	2·69 ± 0·11	0·89 ± 0·08*	2·89 ± 0·07*	1·48 ± 0·22	3·06 ± 0·13	1·58 ± 0·19	2·72 ± 0·11
KCl	C	3·31 ± 0·32	2·29 ± 0·08	1·21 ± 0·16*	2·27 ± 0·11	3·02 ± 0·37	2·48 ± 0·10	2·72 ± 0·41	2·49 ± 0·12
	T	3·32 ± 0·29	2·28 ± 0·09	1·84 ± 0·19*†	2·30 ± 0·09	3·12 ± 0·39	2·35 ± 0·11	2·46 ± 0·38	2·58 ± 0·13
Acetylcholine	C	3·58 ± 0·41	6·12 ± 0·14	3·19 ± 0·44	5·85 ± 0·13	3·62 ± 0·33	6·40 ± 0·16	3·16 ± 0·38	5·98 ± 0·15
	T	3·99 ± 0·42	5·96 ± 0·15	3·48 ± 0·47	6·10 ± 0·14	3·60 ± 0·39	6·33 ± 0·15	3·21 ± 0·39	5·80 ± 0·13
Histamine	C	4·25 ± 0·37	6·10 ± 0·18	1·67 ± 0·15*	6·02 ± 0·16	4·15 ± 0·44	6·01 ± 0·13	3·39 ± 0·40	6·20 ± 0·18
	T	3·40 ± 0·38	5·70 ± 0·19	2·36 ± 0·19*	6·12 ± 0·15	4·25 ± 0·48	6·03 ± 0·15	3·87 ± 0·42	5·97 ± 0·17
5-HT	C	3·14 ± 0·41	7·01 ± 0·10	1·90 ± 0·14*	6·80 ± 0·12	3·25 ± 0·38	6·95 ± 0·09	3·08 ± 0·32	6·80 ± 0·10
	T	3·16 ± 0·39	6·90 ± 0·08	2·35 ± 0·12*	6·95 ± 0·11	3·05 ± 0·37	6·60 ± 0·13	3·46 ± 0·37	6·90 ± 0·12

E_{max} is the maximal effect and pD_2 values are the $-\log$ of the molar concentration producing 50% of maximum. The data are means ± s.e.m. of six experiments for control and test tissues. *$P < 0·05$ compared with control. *$P < 0·05$ compared with 37°C. †$P < 0·05$ compared with test tissues at 37°C.

showed that log concentration–response curves for $CaCl_2$ (in depolarizing Ca^{2+}-free solution), KCl, acetylcholine, histamine and 5-HT (first curves; data not shown) did not change significantly when these plasmogens were retested following 30-min incubation in the appropriate solution (second curves; Table 2). The responsiveness and sensitivity of the tissues to the different spasmogens tested did not differ between normal and sensitized preparations. Pretreatment with PDA (0·1 μM) did not significantly alter responses to spasmogens obtained in normal and sensitized trachea at 37°C. Responsiveness to all spasmogens except for acetylcholine was depressed in normal trachea at 20°C compared with responses at 37°C, whereas sensitivity of the preparations was not diminished (Table 2). By contrast, cooling to 20°C did not significantly depress the spasmogenic responses produced in sensitized trachea. In normal trachea, pretreatment with PDA (0·1 μM) reversed the inhibitory effect of reduced temperature (20°C) on the response to $CaCl_2$, KCl (in this case the depressed response at low temperature was not fully reversed by PDA; see Table 2), histamine and 5-HT in normal trachea without altering that to acetylcholine which was not depressed by cooling to 20°C. In sensitized trachea, PDA (0·1 μM) did not significantly change the spasmogenic responses obtained at 20°C.

Effects of PDA on skinned tracheal preparations

Segments of skinned trachea prepared from normal and sensitized guinea-pigs contracted in a concentration-related manner to Ca^{2+}. Compared with the first concentration–response curve for Ca^{2+}, the second curve obtained in the same preparation did not change significantly ($-\log$ EC50 values: 6·48 ± 0·05 (n = 12) vs 6·52 ± 0·04 (n = 8) for normal tissues, and 6·62 ± 0·06 (n = 12) vs 6·57 ± 0·04 (n = 8) for sensitized tissues; E_{max} values are shown in Fig. 2). A 30-min incubation with PDA (0·1 or 1 μM) did not change the responsiveness and sensitivity of skinned normal and sensitized preparations. The $-\log$ EC50 values for the second curves in PDA-treated tissues were 6·50 ± 0·03 (normal) and 6·55 ± 0·04 (sensitized) for PDA 0·1 μM, and 6·49 ± 0·03 (normal) and 6·54 ± 0·04 (sensitized) for PDA 1 μM ($P > 0·05$; n = 9 in each group). The acetylcholine (100 μM) challenge terminating each experiment did not produce any contraction.

Discussion

Guinea-pig tracheal responses to PDA

Epithelium-denuded trachea isolated from normal or sensitized guinea-pigs and bathed in indomethacin-containing PSS at 20°C responded to PDA (0·1 nM–1 μM) with a sustained and concentration-dependent contraction instead of the suppression of spontaneous tone observed for intact trachea at 37°C in PSS without indomethacin (Menkes et al 1986; Cortijo et al 1994). This finding confirms and extends

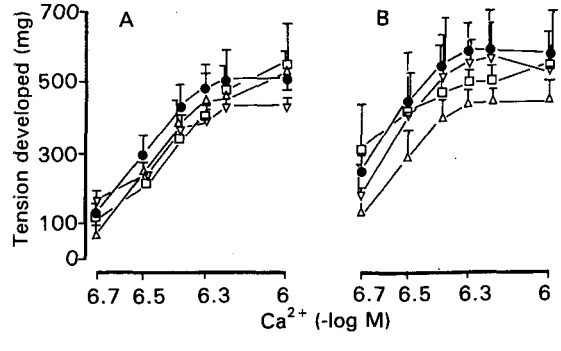

FIG. 2. Concentration–effect curves for Ca^{2+} in skinned trachea from normal (A) or sensitized (B) guinea-pigs. Two successive concentration–response curves were produced. Initial curves are shown (□). Second curves were obtained in the absence (time matched controls; ●) or presence of phorbol 12,13-diacetate 0·1 μM, ▽; or 1 μM, △). Points are means ± s.e.m. of nine experiments.

the previous observation of Huang et al (1987) that PDA produces a sustained contraction of unsensitized, untreated, intact guinea-pig trachea at low temperature (27, 22 and 17°C). The full expression of the contractile response to PDA was achieved only when the indomethacin-treated tissue was cooled (this study) as previously observed for other spasmogens (Small et al 1988). The effects of PDA in guinea-pig trachea have been previously ascertained to involve PKC (Menkes et al 1986). H-7, a purported PKC inhibitor (Hidaka et al 1984), markedly reduced or suppressed PDA (0·1 μM)-induced contraction of normal and sensitized trachea at 20°C (this study) at concentrations which neither alter tracheal contractions to acetylcholine (1 mM) under the same experimental conditions nor depress Ca^{2+} (20 μM)-induced contractions of guinea-pig skinned trachea (unpublished results from this laboratory). However, H-7 lacks the required selectivity for PKC over closely related protein kinases (Bradshaw et al 1993; Wilkinson & Hallam 1994).

Exposure of normal trachea to a Ca^{2+}-free EGTA-containing medium reduced, but did not abolish, the response to PDA (0·1 μM). This suggests dependence on both extracellular Ca^{2+} entry and intracellular Ca^{2+} release for PDA-induced contraction. Ca^{2+} influx through voltage-dependent Ca^{2+} channels is involved since verapamil (10 μM) partly impedes the contraction evoked by PDA (Huang et al 1987; this study). Contractions to PDA in cooled trachea did not significantly differ between normal and sensitized tissues except for the highest concentration tested (1 μM) that produced greater contractions of sensitized trachea. The precise mechanism underlying the hyper-reactivity to PDA under these experimental conditions is unknown. The existence of abnormalities in the homeostatic regulation of Ca^{2+} movements in sensitized airway smooth muscle cells (Triggle 1983) may translate into a greater Ca^{2+} availability for contraction, mainly from intracellular sources (Ortiz et al 1991). Contraction of sensitized trachea to PDA (0·1 μM) at 20°C was neither dependent on extracellular Ca^{2+} nor affected by verapamil, whilst a dependency was patent in normal trachea (see Table 1). This different pattern in the consequences of the exposure to Ca^{2+}-free medium and of the pretreatment with verapamil between normal and sensitized tissues was also observed for caffeine- and cooling-induced contraction of this preparation (Ortiz et al 1988, 1991) and gives further support to the notion that release of intracellular Ca^{2+} is decisive in the activation of sensitized tracheal muscle.

In the guinea-pig trachea, activation of PKC by phorbol esters is accompanied by Na^+ influx (Souhrada & Souhrada 1989a). Amiloride is an inhibitor of Na^+ entry channels, Na^+/H^+ and Na^+/Ca^+ exchange systems. Amiloride (10 μM) equally depressed PDA (0·1 μM)-induced spasm of normal and sensitized guinea-pig trachea at 20°C. In reducing spasm to PDA, amiloride could have interfered with Ca^{2+} entry through the Na^+/Ca^+ exchange process (an enhanced intracellular Na^+ concentration is reasonably expected under experimental conditions of low temperature and addition of PDA). However, the results with amiloride are difficult to interpret, since this agent is relatively non-selective and can also inhibit other systems such as PKC, and voltage-dependent Ca^{2+} channels (Knox et al 1993). The latter mechanism can be ruled out since amiloride (100 μM) did not alter guinea-pig tracheal responses to $CaCl_2$ (in depolarizing Ca^{2+}-free solution) and KCl (Cortijo et al 1992). The fact that the pattern of the effect of amiloride found in the present study is similar to that of H-7 suggests that amiloride may be acting as a PKC inhibitor in the airway smooth muscle, as indicated by others (Knox et al 1993).

Influence of PDA in the guinea-pig tracheal responses to spasmogens

The spasmogens tested in this study were selected to produce tracheal contraction through different mechanisms involving extracellular or intracellular Ca^{2+} release. Pharmacological manipulation via stimulation of PKC by PDA results in promotion of Ca^{2+} entry and intracellular Ca^{2+} release and could therefore affect tracheal responses to spasmogens. Although PDA is less potent than other phorbol esters in producing tracheal responses (Menkes et al 1986), the concentration of PDA used (0·1 μM) is probably sufficient to produce a marked activation of PKC. However, phorbols may act supraphysiologically and may also have other actions unrelated to PKC activation (Wilkinson & Hallam 1994). Therefore, interpretation of results obtained in phorbol-treated tissues as exclusively dependent of functionally relevant PKC activation must be cautious.

PDA (0·1 μM) did not significantly alter the responses to the different spasmogens tested in normal (i.e. non-sensitized) trachea at 37°C in the experimental conditions of this study (epithelium-denuded and indomethacin-treated preparations). In culture, canine tracheal mycocytes, phorbol 12-myristate 13-acetate (0·2 μM) blocks the histamine-induced release of intracellular Ca^{2+}, but no attempt was made to assess simultaneously the mechanical correlate of this effect (Kotlikoff et al 1987). Morrison & Vanhoutte (1991) reported that PDA (1 μM) caused a rightward displacement of the acetylcholine and 5-HT concentration–contraction curves in epithelium-denuded guinea-pig trachea. We found no shift of the concentration–response curve to the spasmogens tested. The reason for this discrepancy may be that in the study by Morrison & Vanhoutte (1991) the preparations had spontaneous tone which is inhibited by PDA. By contrast, in the experimental conditions of our study, no spontaneous tone remains in indomethacin-treated preparations and PDA produced only a sustained contraction. It is conceivable that PDA-induced relaxation might have contributed to the relatively small displacements of concentration–contraction curves reported by Morrison & Vanhoutte (1991).

Active sensitization of guinea-pigs results in an enhancement of tracheal responses to different spasmogens (Ortiz et al 1989). The precise mechanisms underlying this non-specific in-vitro hyper-responsiveness are not certain. In the experimental conditions of the present study (epithelium-denuded and indomethacin-treated preparations) the responsiveness and sensitivity of the sensitized trachea did not differ from that of normal tissues. The removal of the epithelium and the inhibition of the cyclo-oxygenase by suppressing the influence of mechanisms which modulate airway smooth muscle reactivity may contribute to approach the reactivity of normal and sensitized prepara-

tions as previously reported (Cortijo et al 1989). The tracheal responses to the spasmogens tested in sensitized, epithelium-denuded, indomethacin-treated preparations were not altered when tissues were treated with PDA (0·1 μM), as observed in normal trachea. These findings indicate that, at 37°C and under the experimental conditions of this study, activation of PKC by PDA (0·1 μM) did not affect the tracheal responses to a variety of spasmogens in either normal or sensitized tissues.

Cooling to 20°C substantially reduced the responsiveness of normal trachea to $CaCl_2$, KCl, histamine and 5-HT without altering its sensitivity. Responses to acetylcholine were resistant to reduced temperature. These results confirm and extend those previously reported (Ishii & Shimo 1986; Cortijo et al 1992). The mechanism by which cooling produced this anti-spasmogenic effect is reduction of Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Ishii & Shimo 1986) and interference with intracellular Ca^{2+} release triggered by certain spasmogens (Cortijo et al 1992). The cooling-induced inhibition of tracheal responses to $CaCl_2$, KCl, histamine and 5-HT was reduced in PDA (0·1 μM)-treated tissues, i.e. PDA restored to control values or enhanced the depressed responses obtained in normal trachea at 20°C. This enhancing effect of PDA at low temperature presumably involves extracellular as well as intracellular sources of Ca^{2+}, depending on the spasmogen (Cortijo et al 1992). When spasmogenic responses were elicited in sensitized trachea, cooling (20°C) of the preparations failed to depress the contractions produced by the agents tested in this study. We have previously reported that the active sensitization of guinea-pigs may result in alterations in Ca^{2+} mobilization even when hyper-reactivity to spasmogens was not present (Ortiz et al 1988), and that intracellularly stored Ca^{2+} is decisive in the contraction to cooling in sensitized airways (Ortiz et al 1991). The loss in sensitized trachea of both the inhibitory effect of cooling and the enhancing effect of PDA at reduced temperature may be explained as a consequence of the combined lesser dependence on Ca^{2+} influx and increased dependence on intracellular Ca^{2+}, to which the sensitized airway smooth muscle appears to be subjected.

Another mechanism by which active phorbol esters may enhance contractility is augmenting the sensitivity of the contractile apparatus to Ca^{2+}. This effect has been demonstrated for phorbol 12,13-dibutyrate in skinned vascular smooth muscle (Miller et al 1986). However, in the present study, PDA (0·1–1 μM) did not change the concentration–response curve for Ca^{2+} in skinned normal or sensitized trachea and hence, an effect on the intracellular contractile machinery is not apparently involved in the effects of PDA on tracheal contractility. This result confirms a previous finding obtained in normal skinned trachea (Cortijo et al 1992).

Accumulated experimental evidence suggests that inappropriate activation of PKC occurs in asthma (Bradshaw et al 1993). Pharmacological activation of PKC by using PDA produced a spasm of cooled, indomethacin-treated epithelium-denuded trachea but did not significantly alter the spasmogenic responses to a variety of agents obtained in sensitized trachea. Further investigation of the cellular processes involved in the airway smooth muscle contraction in conditions of cooling or sensitization will contribute to find new strategies for the pharmacological management of asthma.

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