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Relaxation by Calcium Antagonists of Potassium-contracted Trachea from Normal and Sensitized Guinea-pigs: Influence of Epithelium and the Surface of Drug Entry

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Abstract—A technique by which drug access was restricted to either the mucosal or the adventitial surface of tracheal rings, isolated from normal (unsensitized) or sensitized guinea-pigs, was used to study the role of the epithelium in the relaxation produced by calcium antagonists (verapamil, nifedipine, cinnarizine and flunarizine) of K^+-induced contraction. In trachea from normal guinea-pigs, the relaxation to verapamil for unrestricted or mucosal drug entry was reduced in the absence of epithelium, whereas the relaxation produced by nifedipine, cinnarizine or flunarizine was unchanged. In sensitized trachea, the relaxation elicited by the calcium antagonists tested was similar in intact and epithelium-denuded tracheal rings irrespective of the surface of drug entry. These results confirm that the epithelium influences the relaxation to verapamil. This modulatory effect is absent in sensitized trachea and is not shared by other calcium antagonists.

Findings from in-vitro pharmacomechanical and bioassay experiments indicate that the respiratory epithelium modulates airway smooth muscle reactivity (Cuss & Barnes 1987; Fedan et al 1988). Mechanical removal of the epithelium enhances the contractile responses of isolated airway preparations to a variety of agonists. Hence, it has been suggested that bronchial hyper-reactivity in asthmatics may be related to epithelial damage (Laitinen et al 1985). While most of the studies have addressed the influence of the epithelium on the responses to spasmogens, less attention has been paid to the consequences of epithelium removal on the effects of relaxant agents (Farmer et al 1986; Goldie et al 1986). The impairment of relaxation mechanisms in epithelium-denuded airways may contribute to bronchial hyper-reactivity and may decrease the clinical efficacy of bronchodilator drugs.

Calcium antagonists reduce the contractile effects of several spasmogens in animal and human airways (Drazen et al 1983; Advenier et al 1984; Foster et al 1984; Ahmed et al 1985). However, currently available calcium antagonists provide few benefits in the clinical management of asthma (Barnes 1985). The effect of verapamil on rabbit and human airway smooth muscle contraction (Raeburn et al 1986a,b) was reduced in epithelium-denuded preparations but information is lacking with respect to other calcium antagonists.

The effects of epithelium removal on airways reactivity is best studied in circumstances where drugs enter the airway wall from either the mucosal or the adventitial surface alone (Munakata et al 1988; Iriarte et al 1990; Small et al 1990).

In the present study, we have investigated the influence of the epithelium and of the surface of drug entry in the relaxant effect of verapamil, nifedipine, cinnarizine and flunarizine, on the K^+-induced contraction of trachea isolated from normal and sensitized guinea-pigs.

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Materials and Methods

Isolated preparations and sensitization procedure

Guinea-pigs, 350–450 g, were randomly allocated to one of two groups: normal (unsensitized) and sensitized. The sensitization procedure was as previously described (Ortiz et al 1989). On day 0, the animals were injected subcutaneously with 0·25 mL of Freund's complete adjuvant plus 1·25 $\mu g\ g^{-1}$ of bovine serum albumin (BSA) dissolved in 0·25 mL 0·9% NaCl (saline). 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 to 25. The normal group was subjected to the same protocol but received only saline.

Animals were killed by stunning and bleeding. Tracheae were excised, dissected free of extraneous tissue and cut into rings of about 4 mm width. At least 6 rings were obtained from each trachea. Rings were randomly allocated to provide paired preparations serving as epithelium-intact and epithelium-denuded preparations for either unrestricted or selective (luminal or adventitial) drug entry. The method to select the surface of drug entrance to the tracheal wall was as previously described (Iriarte et al 1990). In brief, vaseline was applied to cover completely either the adventitial or the mucosal surface of the ring, thus restricting drug entry to the opposite surface. The epithelium was removed by gently rubbing the mucosal surface with a wet filter paper. Rings were suspended under an applied load of 2 g in tissue baths containing Krebs bicarbonate solution (mM): NaCl 118·0, KCl 4·6, $CaCl_2$ 2·5, KH_2PO_4 1·15, $MgSO_4$ 1·15, $NaHCO_3$ 25·0 and glucose 5·5 bubbled with 95% O_2–5% CO_2 at 37°C (pH 7·4). The applied load of 2 g was found by preliminary experiments to allow optimal responses to agonists (Iriarte et al 1991). An equilibration period of 60 min was permitted with changes of the Krebs solution at 15 min intervals. Tension was measured with Grass FT03 isometric transducers and recorded on a model 7 Grass polygraph.

Experimental protocol

After the equilibration period, indomethacin (1 μM) was added to the bath to suppress the spontaneous tone of the preparation. The tone was raised to a stable plateau of contraction by changing the Krebs solution to an iso-osmotic K^+ Krebs solution (mM): KCl 122·6, $CaCl_2$ 2·5, $MgSO_4$ 1·15, $NaHCO_3$ 25·0, KH_2PO_4 1·15, glucose 5·5 containing indomethacin (1 μM).

Cumulative concentration-response curves to verapamil (0·1–10 μM), nifedipine (5 nM–0·3 μM), cinnarizine (1 nM–10 μM) and flunarizine (1 nM–10 μM) were obtained in indomethacin-treated tracheal rings when a steady tension to potassium (124 mM) had developed. Relaxant responses are expressed as percent decrease of the pre-existing tension generated by potassium (124 mM).

Tracheal rings from actively sensitized guinea-pigs were challenged, once the experiment was terminated, with BSA (1 mg mL^{-1}) to confirm the existence of an antigen-induced contraction. Efficacy (E_{max}) and potency ($-\log EC50$) of the relaxant agents was calculated from individual concentration-effect curves. The results are given 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

The following chemicals were used: bovine serum albumin (Sigma, Madrid, Spain), Freund's complete adjuvant (Difco, Detroit, USA), cinnarizine and flunarizine (Dr Esteve Laboratory, Barcelona, Spain), nifedipine (Bayer, Madrid, Spain), verapamil hydrochloride (Knoll Ibérica, Madrid, Spain). KCl was of analytical grade. Drugs were prepared and diluted in distilled water and added to the tissue bath in small volumes. Nifedipine, cinnarizine and flunarizine were prepared in absolute ethanol. Previous experiments demonstrated that addition of the vehicle to the bath did not alter drug-induced responses (data not shown). Drug concentrations are presented as final bath concentrations.

Results

Relaxation by calcium antagonists in normal trachea contracted by K^+

The Krebs solution containing K^+ (124 mM) produced an immediate (phasic) contraction followed by a sustained tonic contraction which remained stable for 2–3 h. The four calcium antagonists tested, verapamil, nifedipine, cinnarizine, and flunarizine, relaxed, in a concentration-related manner, the K^+-contracted tracheal rings under the different experimental conditions studied. The potency ($-\log EC50$) and efficacy (E_{max}) of these agents is shown in Tables 1 and 2, respectively. The rank order of potencies for unrestricted drug entry in intact tracheal rings was nifedipine (8·17) > flunarizine (6·83), verapamil (6·46) > cinnarizine (5·54). The concentration-response curves to verapamil with either unrestricted or mucosal entry in epithelium-denuded rings were displaced to the right of those obtained in intact rings (Fig. 1). The degree of rightward displacement produced after epithelium removal was greater for mucosal access for verapamil (log concentration ratio of 0·29 ± 0·04) than for unrestricted drug entry (log concentration ratio of 0·13 ± 0·4; $P<0·05$). When verapamil entry was restricted to the adventitial surface of the trachea, its concentration-response curve did not significantly differ in the absence or presence of epithelium. The concentration-response curves to the other calcium antagonists tested showed no significant differences when generated in the absence or presence of epithelium, irrespective of the surface of drug entry.

The concentration-response curves for verapamil and nifedipine entering from the adventitial surface were displaced to the right of those obtained for unrestricted entry: differences in potencies are shown in Table 1.

The maximal relaxant effect of verapamil for unrestricted and mucosal entry was reduced in epithelium-denuded preparations compared with intact rings. The maximal relaxation produced by cinnarizine or flunarizine tended to be smaller than that elicited by verapamil or nifedipine (Table 2).

Relaxation by calcium antagonists in sensitized trachea contracted by K^+

The potencies and efficacies of verapamil, nifedipine, cinnarizine and flunarizine as derived from their concentration-response curves, are shown in Tables 1 and 2. The rank order of potencies (expressed as $-\log EC50$) for unrestrictive drug entry in intact tracheal rings was different from that observed in normal preparations, nifedipine (8·32) > flunarizine (7·82), > cinnarizine (6·41) = verapamil (6·02). In intact (unrestricted drug entry) preparations from sensitized guinea-pigs, verapamil was less potent compared with normal trachea under similar experimental conditions, cinnarizine was more potent, flunarizine tended to be more potent but failed to reach statistical significance, and nifedipine showed no difference (Table 1). The maximal effects did not differ between normal and sensitized tissues with the exception of verapamil for unrestricted entry in intact rings (Table 2). The epithelium had no influence on the responses to calcium antagonists in sensitized tracheal rings irrespective of the conditions for drug entry.

Discussion

Responses in intact normal trachea for unrestricted drug entry

Contraction to potassium is due to membrane depolarization and subsequent Ca^{2+} entry through voltage-operated Ca^{2+} channels (Foster et al 1983) and is thus suitable for assessing relaxation to calcium antagonists (Hof & Vuorela 1983). The antagonism produced by the calcium antagonists tested in the present study appeared to be qualitatively similar. These results confirm and extend those obtained in other studies (Advenier et al 1984; Foster et al 1984; Ahmed et al 1985). Although verapamil, nifedipine, cinnarizine and flunarizine did not significantly differ in their maximal relaxant effects for unrestricted drug entry, there were significant differences in their potencies. The rank order of potencies as measured by their $-\log EC50$ values (nifedipine > flunarizine = verapamil > cinnarizine) is in agreement with those reported in the literature for respiratory, vascular and intestinal smooth muscles (Godfraind et al 1986).

Table 1. Potency of verapamil, nifedipine, cinnarizine and flunarizine as relaxants of potassium (124 mM)-induced contraction of normal and sensitized guinea-pig trachea. The potency is expressed as −log EC50 values for unrestricted or selective (mucosal or adventitial) drug entry in intact (E+) or epithelium-denuded (E−) tracheal rings.

	Normal			Sensitized		
	Unrestricted	Mucosal	Adventitial	Unrestricted	Mucosal	Adventitial
Verapamil						
E+	6·46±0·04	6·44±0·04	6·05±0·05[bc]	6·02±0·11[d]	6·00±0·06	5·74±0·11[d]
E−	6·33±0·04[a]	6·15±0·04[ab]	5·95±0·08[b]	6·03±0·06[d]	6·07±0·11	5·92±0·07
Nifedipine						
E+	8·17±0·02	8·24±0·12	7·84±0·04[bc]	8·32±0·15	8·27±0·17	7·84±0·20
E−	8·19±0·02	8·12±0·02	7·93±0·06[bc]	8·05±0·15	8·10±0·10	7·85±0·13
Cinnarizine						
E+	5·54±0·18	5·97±0·22	6·41±0·35	6·41±0·29[d]	6·79±0·34	6·80±0·26
E−	6·06±0·28	6·15±0·23	6·45±0·38	6·21±0·29	6·24±0·21	7·10±0·25
Flunarizine						
E+	6·83±0·35	6·48±0·35	7·16±0·28	7·82±0·36	8·06±0·30	8·07±0·33
E−	6·89±0·36	6·71±0·29	7·06±0·34	7·96±0·33	7·64±0·43	7·93±0·35

Data are means±s.e.m. of at least 6 experiments in each group. [a]$P<0·05$ from rings with epithelium. [b]$P<0·05$ from values for unrestricted drug entry. [c]$P<0·05$ from values for mucosal drug entry. [d]$P<0·05$ from values in normal trachea.

Table 2. Maximal effect of verapamil, nifedipine, cinnarizine and flunarizine as relaxants of potassium (124 mM)-induced contraction of normal and sensitized guinea-pig trachea. The maximal effect is expressed in g and the values correspond to unrestricted or selective (mucosal or adventitial) drug entry in intact (E+) or epithelium-denuded (E−) tracheal rings.

	Normal			Sensitized		
	Unrestricted	Mucosal	Adventitial	Unrestricted	Mucosal	Adventitial
Verapamil						
E+	1·92±0·13	1·86±0·16	1·48±0·17	1·46±0·14[b]	1·66±0·31	1·28±0·21
E−	1·41±0·09[a]	1·42±0·10[a]	1·04±0·17	1·76±0·29	1·29±0·26	1·22±0·21
Nifedipine						
E+	1·92±0·22	1·51±0·15	1·73±0·08	1·90±0·20	1·28±0·13	1·74±0·13
E−	1·58±0·19	1·76±0·30	1·62±0·20	1·54±0·22	1·21±0·37	1·68±0·16
Cinnarizine						
E+	0·82±0·08[c]	0·89±0·14[c]	0·86±0·10[c]	0·91±0·13[c]	0·78±0·15[c]	0·77±0·09[c]
E−	1·02±0·09[c]	1·13±0·11	0·72±0·09[c]	1·02±0·16	1·02±0·09	0·79±0·08[c]
Flunarizine						
E+	1·19±0·09[c]	1·11±0·14	1·02±0·14[c]	1·03±0·17[c]	1·11±0·16	1·10±0·09[c]
E−	1·27±0·10	1·12±0·12	0·90±0·11[c]	1·35±0·18	1·28±0·13	1·12±0·10[c]

Data are means±s.e.m. of at least 6 experiments in each group. [a]$P<0·05$ from rings with epithelium. [b]$P<0·05$ from values in normal trachea. [c]$P<0·05$ from their corresponding values for nifedipine.

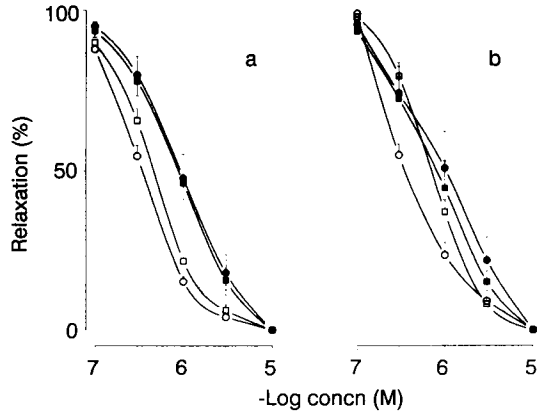

FIG. 1. Log concentration-response curves to verapamil as relaxant of depolarization (K^+ 124 mM)-induced contraction of guinea-pig isolated trachea. Responses were obtained for unrestricted (a) or mucosal (b) entry of drugs, in intact (○,●) or epithelium-denuded (□,■) preparations from normal (open symbols) or sensitized (closed symbols) animals. Data are expressed as percentage of maximal responses and are shown as means of at least 6 experiments for each group; s.e.m. shown by vertical bars.

Influence of epithelium and surface of drug entry on the responses to calcium antagonists in normal trachea

The effects of epithelium removal on tracheal responses to relaxant agonists has been studied to a lesser extent than that to contractile agents (Cuss & Barnes 1987; Fedan et al 1988). Raeburn et al (1986b) reported that verapamil (1 μM) inhibited methacholine-, histamine- and K^+-induced contractions of rabbit airways. In secondary bronchus for methacholine and histamine, and in primary bronchus for KCl, the antagonistic effect of verapamil (1 μM) on the maximum response was reduced in denuded preparations. The inhibitory effect of verapamil on methacholine-induced contraction in human isolated trachea was also reduced by epithelium removal (Raeburn et al 1986a). No information is available relating to the influence of epithelium on the effects of other calcium antagonists. In the present study, the role of epithelium has been confirmed for the inhibition by verapamil of the guinea-pig tracheal contraction to potassium. Both the efficacy and potency of verapamil were reduced in epithelium-denuded preparations. By contrast, the other

calcium antagonists tested, nifedipine, cinnarizine and flunarizine, showed no epithelium dependency for their antagonistic effect on KCl-induced contraction. The mechanism underlying this preferential influence of epithelium for verapamil is presently unknown. Removal of the epithelium produced a greater rightward displacement of the concentration-response curves to verapamil entering from the mucosal surface than that observed in preparations where drug entry was unrestricted. An explanation of this difference may be suggested as follows. For unrestricted entry, the drug enters through both surfaces of the preparation; hence, a part of the response observed is produced by adventitial entry which is independent of the presence of the epithelium. It is therefore understandable that the loss of sensitivity resulting from epithelium removal was most pronounced in those preparations where drug entry through the adventitial surface was prevented. That the epithelium was mainly responsible for limiting the potency of verapamil entering from the mucosal surface is also suggested by the finding that epithelium removal rendered the tissue equisensitive to verapamil entering via the adventitial or mucosal surfaces.

The reduced maximal response to verapamil entering from the mucosal surface in epithelium-stripped rings strengthens the hypothesis that the epithelium modulates relaxation to verapamil. These findings are compatible with verapamil-invoked release of an epithelium-derived inhibitory factor.

The results obtained with the other calcium antagonists tested indicate that their mechanism of action as relaxants of K^+-induced spasm is independent of the epithelium integrity. The differences observed in potencies of verapamil and nifedipine for unrestricted vs selective entries were small in magnitude and are probably related to the concentration gradient created by drug diffusion through the tracheal wall as discussed in other studies (Iriarte et al 1990).

Influence of epithelium and surface of drug entry on the responses to calcium antagonists in sensitized trachea

Verapamil, nifedipine, cinnarizine and flunarizine, each inhibited the potassium contracture in a concentration-related fashion. The potency of verapamil was significantly reduced in sensitized rings compared with normal tissues, the potency of nifedipine did not change, whereas the potency of cinnarizine and flunarizine was greater in sensitized tissues, although it failed to reach statistical significance for flunarizine. These findings are in agreement with previous publications from this laboratory (Perpiñá et al 1987) indicating a different rank order of potencies for calcium antagonists in sensitized trachea compared with normal trachea.

As in the normal trachea, contraction of sensitized preparations to KCl was not altered by epithelium removal irrespective of whether KCl entry was from either the mucosal or the adventitial surface (Iriarte et al 1991). The effect of verapamil was not changed by epithelium removal in sensitized trachea for either unrestricted or selective drug entry. This finding is in contrast with the results in normal trachea and suggests that the enhancing effect of epithelium on verapamil-induced relaxation is absent in sensitized tissues. The loss of the epithelium modulatory effect on the verapamil response following sensitization may be due to epithelial damage. Antigen challenge elicits the pulmonary recruitment of leucocytes in guinea-pigs and some of their products cause epithelial injury. Thus, a greater number of epithelial cells is present in the bronchoalveolar lavage fluid of sensitized guinea-pigs compared with normal animals (Motojima et al 1989). However, the situation appears to be different for spasmogens. The concentration ratio (with and without epithelium) values for histamine and acetylcholine in sensitized trachea tended to be smaller when compared with those obtained in normal trachea, but differences did not reach statistical significance (Iriarte et al 1990, 1991). In this case, immunization did not result in a loss of the modulatory role of the epithelium in an animal model of asthma (Iriarte et al 1991).

Verapamil has shown disappointingly small effects in the treatment of asthma (Barnes 1985). This lack of effectiveness could be related to the epithelial destruction observed in the airways of asthmatic patients (Laitinen et al 1985). However, the clinical results obtained with nifedipine, which produces tracheal relaxation independently of the epithelium, are not superior to those of verapamil. Therefore, the poor response to calcium antagonists in asthmatics cannot be explained by mucosal damage in the asthmatic airway.

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