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Effects of TMB-8, a calcium antagonist, on transport properties of the isolated canine tracheal epithelium

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The effects of the putative intracellular Ca^{2+} antagonist, TMB-8 (8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate), on the canine tracheal epithelium were examined. Luminal addition reduced rapidly, but reversibly, the transmucosal potential difference and increased the resistance across the open-circuited epithelium. Under short-circuit conditions, the drug reduced stimulation by prostaglandin E_2, forskolin, 8-bromo cyclic AMP, prostaglandin $F_{2\alpha}$ and A23187. Inhibition of prostaglandin E_2 responses were accompanied by reversal of net Cl^- fluxes produced by the agonist. The effects of TMB-8 were unaffected by increasing Ca^{2+} in the bathing solutions, and were not mimicked by procaine, nitrendipine, calmidazolium, compound 48/80 or trifluoperazine. W7 did, to a limited extent, produce similar responses, though the drug was more toxic, and the effects were irreversible.

Introduction

The central role played by Ca^{2+} and cyclic nucleotides in controlling cellular functions has been amply documented [1]. WIth particular reference to the airway epithelium, recent observations [2,3] have suggested that a defect in the control of Cl^- selective channels could occur in cystic fibrosis. The complex interactions that occur between Ca^{2+}, cyclic nucleotides, and endogenously produced eicosanoids could potentially be analysed by the use of pharmacological agents.

In this context, we reported recently [4] that the luminal addition of the putative intracellular Ca^{2+} antagonist, TMB-8, promptly reduced the transmucosal potential difference and increased resistance across the open-circuited canine tracheal epithelium, a widely-used model for airway epithelium [4–10]. These effects being similar to those seen with indomethacin, we tested the possibility that the drug acted as an inhibitor of cyclooxygenase. However, clear differences were seen between the two agents. Whereas prostaglandin E_2 markedly stimulated a tissue that had been treated with indomethacin, it failed to do so when added to one treated with TMB-8. Furthermore, indomethacin did not affect a tissue that had been stimulated with prostaglandin E_2, whereas TMB-8 promptly reversed the changes observed. This led us to suggest that TMB-8 acted at a step distal to prostaglandin production [4]. Since a tissue treated with TMB-8 still responded to added substance P, we raised the possibility that TMB-8 could be a useful pharmacological tool to study the actions of prostaglandins in this tissue. In the present report, we have characterised further the effects of TMB-8 in an attempt to define the specificity of the drug, as well as its possible mechanism of action.

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Materials and Methods

The isolated epithelium was obtained from mongrel dogs killed with sodium pentobarbital (100 mg/kg) and set up in conventional Ussing chambers for recording transmucosal potential differences and resistances (see Ref. 5). Bathing solutions on both sides were identical (composition mM): NaCl 116, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 1.2, glucose 10. Both solutions were bubbled continuously with 95% O_2/5% CO_2, maintaining a pH of 7.3–7.4 at 37 °C. An airlift system (MRA Corp.) was used for circulating the fluids. Potential differences were recorded with 3 M agar KCl bridges connected to a high impedance millivoltmeter (either Iowa Bioengineering Dual voltage Clamp or Physiologic Instruments Model VCC 600), and resistances were measured with periodic pulses of 50 μA currents passed across the tissue. Short-circuit currents were measured by passing sufficient current to keep the potential difference at zero, all appropriate corrections being made for solution resistances etc.

Transmucosal fluxes of ^{22}Na and ^{36}Cl were measured across matched pairs of tissues under short-circuiting conditions, using well established techniques [6,7]. Both isotopes were added to one side, their appearance on the opposite side being measured by periodic sampling. Care was taken to replace the volumes removed (1 ml). A 45–60 min period was allowed for isotopic equilibration before sampling was begun. 30-min flux periods were used to minimize sampling error. Fluxes of the isotopes were estimated by sequential counting of the samples, first in a well-type gamma spectrometer (LKB Rack Gamma), and then in a liquid scintillation spectrometer (Beckman LS 5801) (see 10).

ED_{50} values were estimated from the concentrations required to obtain half-maximal responses, and were obtained by linear regression of the data following probit transformations [5].

The prostaglandins, E_2 and $F_{2\alpha}$, were bought from Cayman Chemical Co. Sigma supplied TMB-8, forskolin, 8-bromo cyclic AMP, W7, calmidazolium, and quin2-AM. ^{22}Na and ^{36}Cl were purchased from New England Nuclear. All other chemicals were obtained from Fisher.

Results

(1) Luminal vs. serosal effects of TMB-8

TMB-8 ($5 \cdot 10^{-5}$ M) added to the luminal solutions bathing the isolated trachea reduced the transmucosal potential difference and increased the resistance as shown in Fig. 1. The onset was rapid, occurring within 30 s, a maximal effect being reached by 8–10 min. Serosal addition of TMB-8 had marginal effects at best. This suggested either that the drug had better access from the luminal side, or that it inhibited a luminally located process. It must be emphasised that the tissue recovered very rapidly following wash-out of the drug from the bathing solutions.

(2) Dose-response relationships

In the experiments reported, a fixed concentration of TMB-8 ($5 \cdot 10^{-5}$ M) had been used. In order to determine whether we were operating

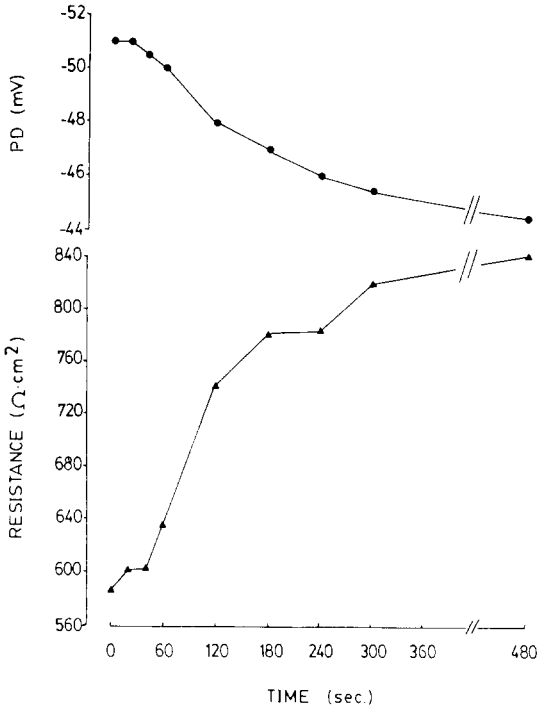

Fig. 1. Alterations in transmucosal potential differences (●), and resistance (▲) following luminal addition of TMB-8 ($5 \cdot 10^{-5}$ M). In eight such experiments, the potential differences decreased from 33.4 ± 3.5 mV to 25.5 ± 3.4 mV and transmucosal resistances increased from 395 ± 35.2 to 535 ± 59.9 $\Omega \cdot cm^2$.

with optimal concentrations of the drug, we evaluated the dose-response relations to cumulative luminal additions of TMB-8. Each addition was made after the previous response had attained a stable value; this generally took between 5 and 6 minutes. These experiments, done under open-circuited conditions (Fig. 2), show a mono-phasic decline in potential difference with a bi-phasic change in transmucosal resistance. There was an initial increase in resistance that reached peak values at $3 \cdot 10^{-4}$ M, and then decreased abruptly. With maximal concentrations tried, the resistance had fallen to 80% of control values, with potential differences near zero. Tissues treated with these concentrations never recovered, even after extensive washing. However, tissues treated with concentrations of TMB-8 that produced an increase in resistance, rapidly recovered after the drug was washed out. With the higher concentrations, there could have been irreversible structural damage to the tissues, responsible for the abrupt fall in resistance.

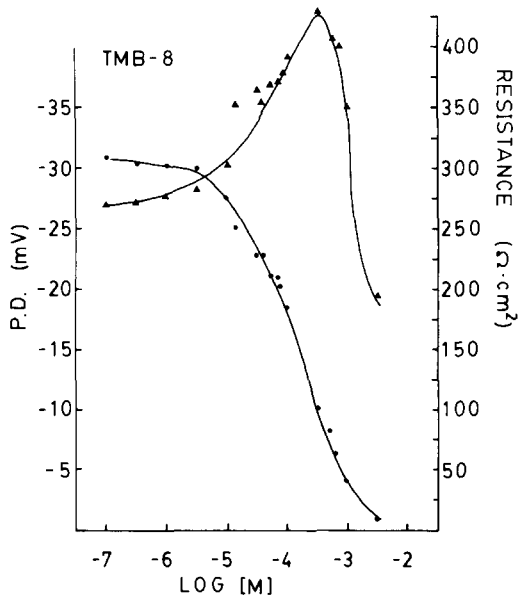

Fig. 2. A cumulative dose-response to luminal addition of TMB-8. Values are from a single experiment. In three such experiments, the peak increase in resistance was observed at a concentration of $3 \cdot 10^{-4}$ M, following which, the resistance rapidly declined as shown here. The changes in potential differences were monophasic. Resistance (▲); potential differences (●).

(3) Effects of TMB-8 on cumulative dose-responses to prostaglandin E_2

In the earlier report, we had shown that TMB-8 inhibited the responses to added prostaglandin E_2, but failed to affect the responses to substance P. To characterise further the inhibition produced by TMB-8, cumulative dose-responses were constructed, using prostaglandin E_2 as an agonist. Three such curves were constructed with each tissue. In the first instance, the tissues were treated with bilateral indomethacin ($5 \cdot 10^{-6}$ M) and once the short-circuit current had stabilised, a cumulative dose-response curve was constructed by adding successive increments of the agonist to the serosal solutions. The tissues were then extensively washed and a 60 min recovery period allowed. The tissues were then treated with bilateral indomethacin ($5 \cdot 10^{-6}$ M) followed by luminal TMB-8 ($5 \cdot 10^{-5}$ M) and once more a cumulative dose-response curve was constructed. Following this, the tissues were extensively washed and allowed to recover, and a third dose-response curve was constructed to assess the reversibility of the effects. The results of a representative experiment are shown in Fig. 3.

TMB-8 markedly reduced the maximal response observed with prostaglandin E_2 with a shift in concentration required to elicit a half-maximal response. Following washout of the antagonist, recovery was complete as far as maximal responses were concerned, but the altered ED_{50} persisted.

(4) Effect of TMB-8 on ion fluxes

We monitored changes in fluxes of ^{22}Na and ^{36}Cl across the short-circuited trachea stimulated initially by prostaglandin E_2, with subsequent inhibition by TMB-8. (see Fig. 4) When tissues were treated with bilateral indomethacin ($5 \cdot 10^{-6}$ M), there was a sharp decrease in short-circuit current which attained a steady value by 30 min. Addition of prostaglandin E_2 ($5 \cdot 10^{-6}$ M) to the luminal solution produced a rapid increase in current that attained a plateau within 5–7 min, and remained steady for 20 min. Addition of TMB-8 ($5 \cdot 10^{-5}$ M) to the luminal side elicited a prompt decrease in the current.

Samples were regularly removed, and estimates of ^{22}Na and ^{36}Cl fluxes (expressed as μequiv.·

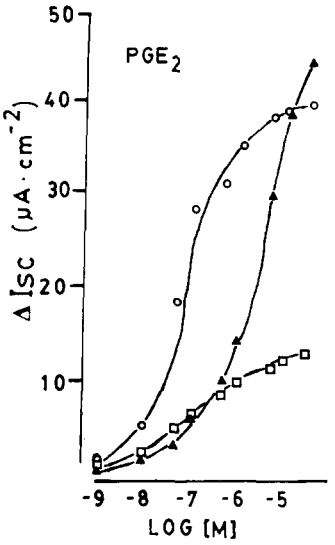

Fig. 3. Cumulative dose-response curves to serosal addition of prostaglandin E_2. Three dose-response curves were constructed: control (○), in the presence of TMB-8 (□), and following extensive washing (▲). Indomethacin ($5 \cdot 10^{-6}$ M) was added bilaterally 15 min prior to the addition of the agonist. The results are from a single representative experiment. Data from four such experiments, were as follows: Maximal responses, (percentages of control values, means ± S.E.), in the presence of TMB-8 (52.0 ± 11), after washout of the drug, (123.8 ± 52.1). ED_{50} values, control ($6.5 \cdot 10^{-8}$ M), with TMB-8 ($2.1 \cdot 10^{-7}$ M), following wash out ($3.5 \cdot 10^{-7}$ M).

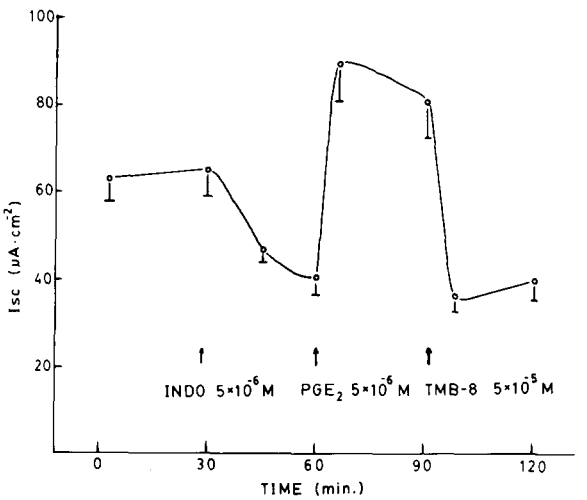

Fig. 4. Alterations in short-circuit current in response to prostaglandin E_2 and TMB-8. (Means ± S.E. from four experiments). Samples were removed at 30-min intervals for estimation of ion fluxes (details and results given in the text).

$cm^{-2} \cdot h^{-1}$) were calculated from one way fluxes of the two isotopes. The net Cl fluxes from four experiments (means ± S.E.) were as follows: (1) after indomethacin treatment, 0.74 ± 0.21,; (2) on stimulation with prostaglandin E_2, 1.84 ± 0.42, and (3) following TMB-8 treatment, 0.63 ± 0.21. The corresponding values for net Na fluxes in the same experiments were −1.23 ± 0.33 after indomethacin, −1.39 ± 0.42 following prostaglandin E_2 addition and −1.12 ± 0.36 on treatment with TMB-8. Thus, the trachea, under basal conditions, shows a net absorption of Na^+ and Cl^-. Stimulation with prostaglandin E_2 does not significantly affect Na^+ absorption, but markedly stimulates Cl^- secretion. These changes are promptly reversed by TMB-8.

(5) Effects of TMB-8 on responses to forskolin, 8-bromo cyclic AMP and IBMX

Stimulation of the canine trachea by prostaglandin E_2 is associated with increases in intracellular levels of cAMP [9,10]. TMB-8 could thus interfere with the activation of adenylate cyclase by prostaglandin E_2, or more distally by affecting the activity of the cAMP generated. To test these possibilities, we monitored the effects of TMB-8 on the responses of the trachea to forskolin (a stimulant of adenylate cyclase) and 8-bromo cyclic AMP, an analogue of cAMP. The protocol followed was identical to that used with prostaglandin E_2 viz. construction of three dose-response curves with each agonist – a control curve, one in the presence of TMB-8 and another following extensive washing of the tissue, demonstrating post-treatment recovery. As with the prostaglandin E_2 experiments, indomethacin was added to reduce stimulation of tissues by endogenous prostaglandins. The results are shown in Fig. 5.

The stimulation produced by both forskolin and 8-bromo cyclic AMP were reduced by TMB-8. Certain differences were, however, seen. The inhibition of forskolin was more pronounced and consistent, being noted in all five tissues tested. The inhibition of 8-bromo cyclic AMP, on the other hand, was less consistent. In 3 out of 10 experiments, no inhibition was observed, and in the other 7, inhibition ranged from 2 to 56%, with an average inhibition of 33%. Following washout

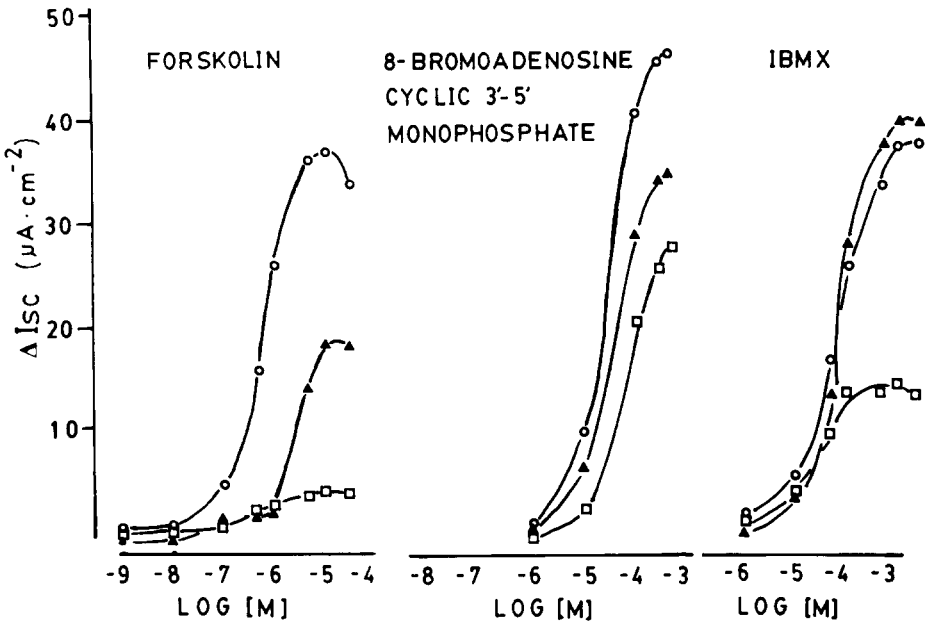

Fig. 5. Cumulative dose-response curves to forskolin, 8-bromo cyclic AMP and IBMX. In each instance, three dose-response curves were constructed: control (○), one in the presence of TMB-8 ($5 \cdot 10^{-5}$ M) (□), and a recovery curve following washing out of the drug (▲). In every instance, $5 \cdot 10^{-6}$ M indomethacin was added bilaterally prior to addition of either agonist or TMB-8. The figures are representative values from single experiments. The data from several such experiments are summarised below. In all cases, maximal responses are expressed as percentages of control values (means ± S.E.). (1) With forskolin ($n = 5$): maximal responses, following TMB-8 (55.7 ± 24.5), after washout, (75 ± 11.5). ED_{50} values: control ($5.2 \cdot 10^{-7}$ M), with TMB-8 ($1.59 \cdot 10^{-6}$ M), after washout ($1.7 \cdot 10^{-6}$ M). (2) With 8-bromocyclic AMP ($n = 7$): Maximal responses following TMB-8 (66.3 ± 7.7), after washout (173.2 ± 35.8). ED_{50} values: control ($2.4 \cdot 10^{-5}$ M), with TMB-8, ($5.5 \cdot 10^{-5}$ M), after washout ($3.3 \cdot 10^{-5}$ M). (3) With IBMX ($n = 4$): Maximal responses, following TMB-8 (49.4 ± 15), after washout (136 ± 18.6). ED_{50} values: control ($1.2 \cdot 10^{-5}$ M), after TMB-8 ($2.1 \cdot 10^{-5}$ M), after washout ($2.1 \cdot 10^{-5}$ M).

of the drug the recoveries were only partial in the forskolin treated tissues, neither the maximal responses nor the altered ED_{50} values, returning to control values. With 8-bromocyclic AMP, the inhibitions noted were more reversible. Although analogues of cyclic AMP are resistant to degradation by phosphodiesterases, enhancement of their responses by phosphodiesterase inhibitors have been noted, principally in cardiac tissue [11]. While testing the effects of IBMX, a commonly used phosphodiesterase inhibitor, we noted that the drug stimulated the epithelium by itself, this stimulation being reversibly inhibited by TMB-8 as shown in Fig. 5.

(6) Effects of TMB-8 on responses to prostaglandin $F_{2\alpha}$ and A23187

It has been shown that the agonist prostaglandin $F_{2\alpha}$ stimulates the canine tracheal epithelium without increasing the tissue levels of cyclic AMP. To test our hypothesis that TMB-8 interfered with the stimulation of Cl^- secretion by cyclic AMP, we assessed its effects on tissues stimulated by prostaglandin $F_{2\alpha}$. The protocol followed was identical to that used with the other agonists. As shown in Fig. 6, TMB-8 inhibited the responses to prostaglandin $F_{2\alpha}$ as well. Here again the inhibition was reversible as judged from the maximal responses which returned to control values, but the slight increase in ED_{50} persisted. This observation is difficult to reconcile with a unifying mechanism involving cAMP alone. A23187 is another agonist that stimulates the tissue without alterations in the level of cAMP. It was difficult to follow the protocol used for the other agonists, since it was difficult to obtain repeated dose-response curves to the drug. We have, however, shown (see Fig. 7) that TMB-8 added to a tissue stimulated with A23187 promptly reverses the associated increase in short circuit current.

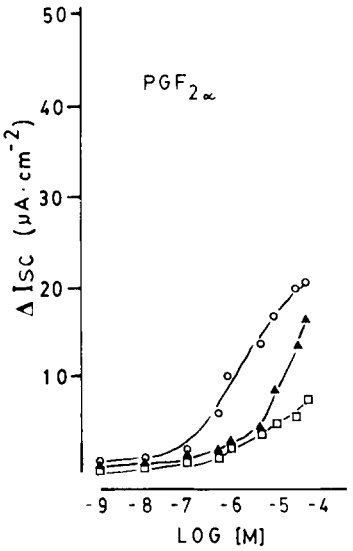

Fig. 6. A cumulative dose-response curve to prostaglandin $F_{2\alpha}$, and the inhibiting effects of TMB-8. The protocol followed was identical to that used to obtain results shown in Figs. 3 and 5. Symbols used are: control (○), with TMB-8 (□), and following wash-out (▲). Data from four experiments were: Maximal responses (% control), with TMB-8 (45.7±15.1), following washout (117.3±38). ED_{50} values: control ($1.75 \cdot 10^{-7}$ M), with TMB-8 ($3.3 \cdot 10^{-7}$ M), after washout of antagonist ($3.2 \cdot 10^{-7}$ M).

(7) Effects of high Ca^{2+} on hibition produced by TMB-8

If TMB-8 acted by preventing mobilization of intracellular Ca^{2+}, elevation of Ca^{2+} in the bathing solutions would be expected to antagonise, at least partially, the effects of TMB-8. An experiment to test this possibility is shown in Fig. 8. Forskolin was used as a stimulant, and the effects

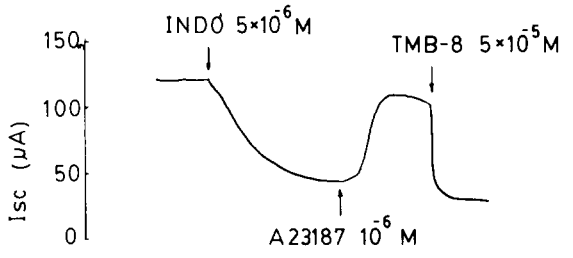

Fig. 7. The inhibiting effects of TMB-8 on a tissue stimulated by A23187. Indomethacin was added prior to addition of the agonist to reduce basal secretions. Actual trace shown. In three such experiments, TMB-8 reduced A23187 stimulated currents from 82.3±19.4 to 32.7±9.9 μA.

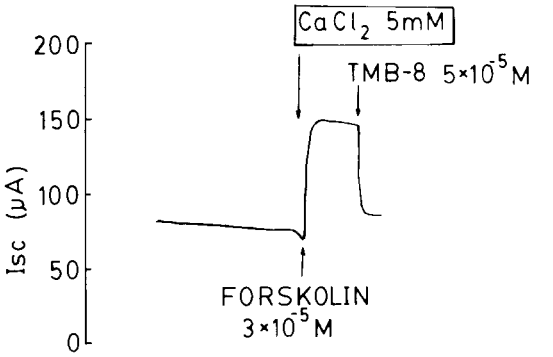

Fig. 8. Changes in short-circuit current following stimulation by forskolin in the presence of 5 mM $CaCl_2$. TMB-8, added subsequently, rapidly reduces the stimulation noted. Actual trace from a single experiment.

of TMB-8 were tested in the presence of 5 mM $CaCl_2$ or an equivalent isosmolar amount of NaCl. Under both conditions, TMB-8 rapidly reversed the stimulation seen in response to forskolin.

(8) Effect of quin2-AM

To test the possibility that the effects of TMB-8 resulted from complexing of intracellular Ca^{2+}, we incubated tissues with the ester quin2-AM ($3 \cdot 10^{-5}$ M). In two experiments, the initial average potential difference was 12.2 mV, and following incubation with the ester for 30–45 minutes, the potential difference was 11.6 mV. Thus, even after prolonged incubation, little change was observed. Luminal addition of TMB-8 promptly reduced potential differences and increased resistance. Quin2-AM is believed to enter cells readily, and be hydrolysed by cellular esterases to liberate quin2- which complexes intracellular Ca^{2+} [12]. The absence of an effect suggests that the ester does not enter the cells, is not hydrolysed by esterases, or that complexation of intracellular Ca^{2+} does not mimic the effects of TMB-8.

(9) Effects of calmodulin inhibitors

Since it has been suggested that TMB-8 could act as an inhibitor of calmodulin-dependent processes [13,14], we tested the effects of several well-known calmodulin inhibitors. Calmidazolium, Compound 48/80 and trifluoperazine had marginal effects. However, the compound W7, did mimic, to some extent, the effects of TMB-8 as shown in Fig. 9. It is, however, clear that in

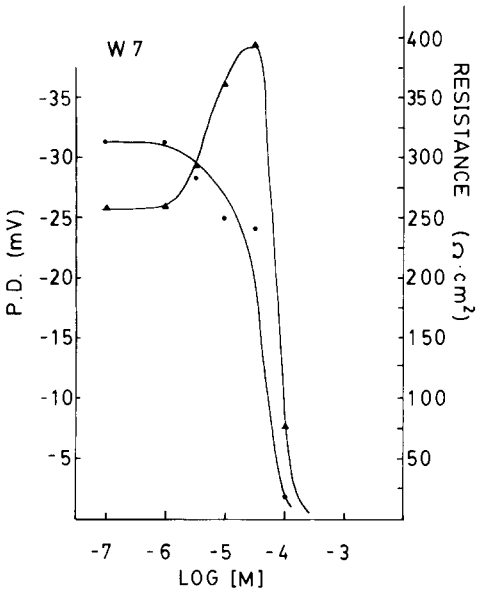

Fig. 9. Cumulative dose-response curve to luminal addition of the calmodulin antagonist W7. In three such experiments, the maximal increase in resistance was noted at a concentration of $5 \cdot 10^{-5}$ M, following which, the resistance rapidly declined. The changes in potential differences in all experiments followed the pattern shown above. Resistance (▲); potential differences (●).

comparison with TMB-8, the antagonist appears to have a narrower margin of safety (compare with Fig. 2). In keeping with this observation, tissues treated with W7 never recovered completely, even after the drug had been extensively washed out. This again contrasted with the rapid recovery following removal of TMB-8 from the bathing solutions.

Discussion

The present study sought to characterise further, the effects of the putative intracellular Ca^{2+} antagonist, TMB-8 on the isolated canine tracheal epithelium. Our earlier report showed that the drug acted as a reversible antagonist of prostaglandin E_2 on the epithelium. Since the agent was relatively ineffective against another agonist, substance P, this raised the possibility that it could prove to be a useful pharmacological tool to probe the effects of eicosanoids on this tissue. The present study sought answers to two questions: (a) Is TMB-8 a specific, reversible antagonist to prostaglandin E_2? and (b) What is its possible mechanism of action?

The answer to the first question is clearly in the negative. The drug reversibly inhibited a number of other stimulants besides prostaglandin E_2 – these included forskolin, 8-bromo cyclic AMP, IBMX and prostaglandin $F_{2\alpha}$. Cumulative dose-response curves to these agonists showed a clear inhibition of the maximal responses obtained with changes in potency. The responses to A23187 were also promptly reversed by TMB-8.

If one considers the three agonists, prostaglandin E_2, forskolin and IBMX, a common locus could be suggested – viz. stimulation of phosphodiesterase activity. Prostaglandin E_2 stimulates the canine tracheal epithelium by activating adenylate cyclase, and thus increasing intracellular levels of cAMP [9]; forskolin could be expected to do likewise. IBMX would increase intracellular cAMP by inhibiting phosphodiesterase [10]. Stimulation of phosphodiesterase activity could, in principle, reduce intracellular levels of cAMP, and thus reverse stimulation by the agonists tested. Analogues of cAMP are believed to be resistant to degradation by phosphodiesterases, though phosphodiesterase inhibitors do potentiate at least the inotropic effects of such derivatives [11]. Furthermore, eight substituted analogues do not appear to be enzymatically converted to cyclic AMP. Thus, the inhibition of the stimulation of 8-bromo cyclic AMP suggests a more distal locus for the inhibitory effects of TMB-8, perhaps on a cyclic AMP dependent protein kinase. Curiously, in the rat pancreatic islets, TMB-8 potentiates rather than inhibits the release of insulin by cyclic AMP, forskolin and IBMX [15].

The above hypothesis cannot, however, explain the inhibitory effects of the agent on stimulation produced by prostaglandin $F_{2\alpha}$. The agonist was found to stimulate tracheal epithelium without any significant alteration in levels of cellular cAMP [9], leading the authors to suggest that factors other than cAMP contribute to the stimulation of Cl^- secretion by prostaglandin $F_{2\alpha}$.

The precise mechanism of action of TMB-8 is uncertain. The compound was initially studied by Malagodi and Chiou [16] in smooth muscles where they showed that the drug inhibited, non-competitively, acetylcholine, norepinephrine, KCl etc.,

whereas it appeared to compete with $BaCl_2$. Increasing concentrations of Ca^{2+} antagonised the inhibitory responses of TMB-8, and the author suggested that the drug could produce its inhibitory effects by releasing Ca^{2+} from intracellular stores. Subsequently, the compound has been used in a variety of test systems, ranging from platelets [17], macrophages [18], neutrophils [19], adrenocortical cells [20], and the intestine [21]. Grossman [22] argued, for instance, that TMB-8 interfered with histamine release from isolated rat mast cells by interfering with oxidative metabolism, rather than by affecting intracellular calcium availability. The ready reversibility of its effects on the trachea make such a possibility less likely.

Recently, Donowitz et al. [21] studied the effects of TMB-8 on active Na^+ and Cl^- transport by rabbit ileum. They showed that the compound decreased the short-circuit current across the tissue, and increased Na^+ and Cl^- absorption, by increasing mucosal to serosal fluxes of Na^+ and Cl^-. In our experiments, prostaglandin E_2 increased net Cl^- fluxes across the short-circuited tissue, with negligible alterations of net Na^+ fluxes. These effects were rapidly reversed by TMB-8. However, in contrast to the observations of Donowitz et al. [21], increases in short-circuit current produced by forskolin were rapidly reduced by TMB-8, even in the presence of high Ca^{2+} (5 mM) in the bathing solutions. Furthermore, stimulation of the rabbit intestine by 8-bromo cyclic AMP was unaffected by TMB-8, which, however, clearly inhibited the response of the tracheal epithelium to the same agonist.

We used quin2-AM to determine whether chelation of intracellular Ca^{2+} would mimic the effects of added TMB-8. The absence of any effect suggests that either chelation of intracellular Ca^{2+} could not do so, or that the assumptions underlying the experiment, viz. ready penetrability of the ester with consequent hydrolysis to the base, were invalid in this tissue [12].

Several reports using test systems as disparate as the erythrocyte Ca^{2+}-ATPase [14] and the superfused guinea pig uterus [13], have suggested that the drug could interfere with calmodulin-dependent processes. The latter report by Poyser [13] noted that the effects of TMB-8 were similar to those seen with the calmodulin antagonist, W7. We accordingly tested the effects of several calmodulin inhibitors-calmidazolium, compound 48/80, trifluoperazine and the naphthalene sulfonamide, W7. Although the first two are reported to be relatively specific inhibitors of calmodulin [23–27], neither had any effect on the trachea; nor did trifluoperazine. W7 did, however, mimic, to a certain extent, the effects of TMB-8. On luminal addition, there was a distinct decrease in transmucosal potential difference and an increase in transmucosal resistance. The margin of safety was narrow, and tissues treated with W7 often failed to recover. A recent report by Schepp et al. [28] has shown that W7 inhibited the responses of isolated rat parietal cells to histamine, dibutyryl cAMP, forskolin and even high K^+. The action of the agonist followed non-competitive kinetics, and the effects, in contrast to those observed here, were reversible. Although it is difficult to explain why W7 alone mimicked the actions of TMB-8, and not the other calmodulin inhibitors tried, it is still reasonable to suggest that TMB-8 could act as a calmodulin inhibitor in this tissue. If it does, it appears to be more potent, and certainly more reversible than W7. It is interesting to note that after TMB-8 was washed out, the maximal responses to the diverse agonists quickly recovered, whereas the altered ED_{50} values appeared to persist.

The precise reasons are unclear but it is possible that the antagonist could have more than one effect, one perhaps at the level of agonist interaction with the receptors and another more distally. At present these comments must remain purely speculative.

To summarize, TMB-8 acts as a rapid, reversible antagonist to a variety of stimulants. The efficacy on luminal rather than serosal addition may suggest easier penetration, but more likely inhibition of a luminally located process. TMB-8 inhibited agonists whose effects are mediated by cAMP as well as those acting by Ca^{2+}-dependent mechanisms. Although this suggests a common locus, its precise nature is uncertain. The effects of cAMP could be mediated by Ca^{2+}, and vice versa, the precise pattern being tissue and species dependent [29,30]. To analyse further the effects of TMB-8, biochemical approaches are necessary. These would include, among others, an attempt to

phosphorylate membrane proteins and determine whether TMB-8 inhibits phosphorylation of any specific protein. It is possible that such approaches may not only help define the locus of the effects of TMB-8, but also give insight into the nature of the transporting systems involved.

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