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The effect of okadaic acid on non-adrenergic non-cholinergic contraction in guinea-pig isolated bronchus

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1 We have investigated the role of phosphatases in modulating contractile responses to electrical field stimulation (EFS), methacholine, substance P and capsaicin in guinea-pig isolated main bronchus by use of the phosphatase 1 and 2A inhibitor okadaic acid.

2 Non-adrenergic non-cholinergic (eNANC) contractile responses were elicited by EFS (3 Hz, 20 s, 0.5 ms max. voltage) in the guinea-pig isolated main bronchus in the presence of the non-selective muscarinic antagonist, atropine (1 μM), the non-selective β-adrenoceptor antagonist, propranolol (1 μM), the neutral endopeptidase inhibitor thiorphan (10 μM) and the cyclo-oxygenase inhibitor, indomethacin (5 μM). Okadaic acid significantly attenuated eNANC contractile responses (% inhibition) elicited by EFS (0.01 μM, 15.2$\pm$26.9%; 0.03 μM, 30.4$\pm$13.9%; 0.01 μM, 39.8$\pm$5.1%; 0.3 μM, 59.5$\pm$8.7%; 1 μM 77.8$\pm$7.8%; $P<0.05$, $n=4$). In contrast, the inactive analogue 1-Nor okadaone (0.3 μM) failed to attenuate significantly eNANC contractile responses (% inhibition elicited by 1-Nor okadaone, -1.25 ± 8.5% vs dimethylsulphoxide (DMSO), -13.5 ± 21.5%; $P>0.05$, $n=4$).

3 Cholinergic contractile responses were elicited by EFS (1–30 Hz, 10 s, 0.5 ms max. voltage) in guinea-pig isolated bronchus in the presence of the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 30 μM). Okadaic acid failed to attenuate significantly the contractile (% methacholine E_{max}) response elicited by EFS at all frequencies tested compared with the control (1 Hz, control, 22$\pm$7.9% vs okadaic acid, 18$\pm$7.7%; 3 Hz, control, 26$\pm$6.9% vs okadaic acid, 27$\pm$9.1%; 10 Hz, control, 36$\pm$7.6% vs okadaic acid, 33$\pm$8.9%; 30 Hz, control, 50$\pm$7.6% vs okadaic acid, 42$\pm$14%; $P>0.05$, $n=4$).

4 Okadaic acid (0.3 μM) failed to alter significantly the contractile potency (pD_2) to capsaicin (okadaic acid, 9.0$\pm$0.5, vs DMSO, 9.2$\pm$0.4; $P>0.05$ $n=6$), substance P (okadaic acid, 7.6$\pm$0.3 vs DMSO, 8.2$\pm$0.2; $P>0.05$ $n=7$) or methacholine (okadaic acid, 6.4$\pm$0.2 vs DMSO, 6.4$\pm$0.3; $P>0.05$ $n=4$).

5 Okadaic acid (0.01–1 μM) did not appear to reverse substance P-induced tone. The maximal relaxant response (% reversal of substance P-induced tone) mediated by okadaic acid (1 μM) was 33$\pm$11.7% ($n=4$), this was not significantly different from the DMSO (0.8%) or a time-dependent fall in tone of 34.3$\pm$23.1% ($n=4$) and 33$\pm$15.8% ($n=4$), respectively. Okadaic acid (0.3 μM) failed to augment isoprenaline-induced relaxation repsonses in substance P contracted bronchus (okadaic acid, 6.5$\pm$0.4 vs DMSO, 5.9$\pm$0.3; $P>0.05$, $n=9$).

6 These results indicate that protein phosphatases appear to regulate the release of sensory neuropeptides from airway sensory nerves in response to electrical field stimulation.

Keywords: Okadaic acid; phosphatase; bronchus; sensory nerves; airway smooth muscle

Introduction

Recently, it has been demonstrated that phosphatases play an important role in regulating the function of a number of cellular processes (Levitan 1985; Cohen *et al*., 1990; Greengard *et al*., 1993). Okadaic acid is a polyether monocarboxylic acid first isolated from *Halichondria okadaii* (marine sponge), and is a potent inhibitor of protein phosphatases 1 and 2A which dephosphorylate serine and threonine residues (Hescheler *et al*., 1988). Okadaic acid mediates both vascular and intestinal smooth muscle contraction independent of extracellular calcium (Ozaki *et al*., 1987; Obara *et al*., 1989; Hirano *et al*., 1989) as a consequence of the inhibition of myosin light chain phosphatase (Takai *et al*., 1987; Ishihara *et al*., 1989). In other studies, okadaic acid has been shown to induce relaxation of vascular and uterine smooth muscle (Karaki *et al*., 1989; Candenas *et al*., 1992), while in human airways both excitatory and inhibitory effects have been observed (Naline *et al*., 1994). The mechanism underlying the inhibitory effect of okadaic acid on smooth muscle function is not clear but may relate to a reduction in calcium entry (Naline *et al*., 1994) and/or to an increase in the open probability of calcium-dependent potassium channels (Kume *et al*., 1989).

Protein phosphatases may also regulate neurotransmission, since both okadaic acid and the protein phosphatase 1 and 2A inhibitor, calyculin A, have been shown to increase neurotransmitter release at the neuromuscular junction (Abdul-Ghani *et al*., 1991; Swain *et al*., 1991) and in the CA-1 region of rat hippocampus (Herron & Malenka, 1994). Similarly, okadaic acid increased the capsaicin-induced release of substance P from rat cultured dorsal root ganglion (DRG) neurones (Hingtgen *et al*., 1994), but attenuated the rolipram-induced prolongation of the prostaglandin E_2-mediated hyperalgesia in the skin (Ouseph *et al*., 1995). We and others have previously demonstrated that phosphodiesterase inhibitors attenuate non-adrenergic non-cholinergic (eNANC) but not cholinergic responses, in guinea-pig airways (Qian *et al*., 1994; Undem *et al*., 1994; Spina *et al*., 1995). This suggested that phosphorylation and dephosphorylation of various proteins may affect sensory nerve function in the airways. In the present study, we have therefore investigated whether phosphatases acting via a prejunctional mechanism can regulate eNANC contractile responses in guinea-pig isolated bronchus.

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Methods

Tissue preparation

Male Albino guinea-pigs (300–500 g) were killed by cervical dislocation and the lungs removed and placed in cold (4°C) Krebs-Henseleit solution containing the cyclo-oxygenase inhibitor, indomethacin (5 μM), and aerated with 95% O_2 and 5% CO_2 at 37°C. Tissues were allowed to equilibrate for 40 min before the viability and sensitivity of the preparation was confirmed by its response to methacholine (0.3 and 100 μM) added cumulatively to the bath. After the contractile response had reached a plateau, the tissues were washed 5 times over a 15 min period and allowed to equilibrate for a further 30 min.

Electrical field stimulation studies

eNANC Guinea-pig isolated main bronchi were placed between 2 platinum electrodes and electrically stimulated (3 Hz, 20 s, 0.5 ms pulse width at max. voltage) in the presence of atropine (1 μM), propranolol (1 μM) and thiorphan (10 μM). The tissues were then incubated for 20 min in okadaic acid (0.01–1 μM), 1-Nor okadaone (0.3 μM) or vehicle (dimethylsulphoxide, DMSO, 0.008%–0.8%) before a second electrical field stimulation (3 Hz).

Cholinergic Cholinergic nerve-mediated responses were generated by placing guinea-pig isolated main bronchi between 2 platinum electrodes and electrically stimulated (1–30 Hz, 10 s, 0.5 ms pulse width at max. voltage) in the presence of the NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 30 μM), propranolol (1 μM) and thiorphan (10 μM). The tissues were then incubated for 20 min with okadaic acid (0.3 μM) or vehicle (DMSO, 0.25%) before a second electrical field stimulation (1–30 Hz).

Spasmogen studies

Cumulative-concentration effect curves to capsaicin, substance P and methacholine were performed in the absence or 20 min after incubation of guinea-pig isolated bronchus with okadaic acid (0.3 μM) or vehicle (DMSO 0.25%). Thiorphan (10 μM) was present throughout the experiments.

Relaxation studies

Guinea-pig main bronchus was pre-contracted with substance P (10 nM) which induced a contractile response that was 50% of the methacholine E_{max}. Once the contractile response had reached a plateau, relaxation cumulative dose-response curves were superimposed to okadaic acid (0.1–1 μM). In other studies relaxation to isoprenaline was performed in substance P contracted tissues in the absence or presence of okadaic acid (0.3 μM). Thiorphan (10 μM) was present throughout the experiments.

Analysis of results

The arithmetic mean and s.e.mean were used throughout. In EFS studies, the contractile response in the presence of inhibitor or vehicle was expressed as a percentage of the contractile response in the absence of the inhibitor or vehicle. Contractile responses to substance P and capsaicin were expressed as a percentage of methacholine (100 μM). The contractile and relaxant potency ($pD_2 = -\log_{10} EC_{50}$) of agonists was calculated. Dose-response curves were analysed by ANCOVA and differences between mean values determined by Student's paired and non-paired t test and considered significant if $P<0.05$.

Drugs

N-acety-L-tryptophan3,5-*bis*(trifluoromethyl)-benzyl ester, atropine, capsaicin, dimethylsulphoxide (DMSO), indomethacin, methacholine (MCh), N^{ω}-nitro-L-arginine methyl ester (L-NAME), isoprenaline, substance P, thiorphan (Sigma); cyclo(Met-Asp-Trp-Phe-Dap-Leu)cyclo(2 beta-5β) (MEN 10627, Menarini); okadaic acid, 1-Nor okadaone (LC Laboratories). All drugs were dissolved in Krebs-Henseleit solution. Composition of Krebs-Henseleit solution was (mM): NaCl 117.6, KCl 5.4, $MgSO_4 7H_2O$ 0.57, KH_2PO_4 1.03, $NaHCO_3$ 25, glucose 11.1 and $CaCl_2 2H_2O$ 2.5. The stock concentration of indomethacin (0.01 M) was prepared in 0.5% Na_2CO_3. The stock concentration of capsaicin (0.01 M) was prepared in 100% ethanol. Stock concentration of substance P (0.6 mM) was prepared in 10% acetic acid and stored at −20°C. Stock concentration of okadaic acid (119 μM) and 1-Nor okadaone (119 μM) was prepared in 100% DMSO. The appropriate dilutions were then made in Krebs-Henseleit solution. All other drugs were prepared in Krebs-Henseleit solution.

Results

Electrical field stimulation

eNANC Electrical field stimulation (3 Hz) of guinea-pig isolated main bronchus induced a contractile response of $38 \pm 10.9\%$ MCh E_{max} ($n=5$; Figure 1). The contractile response to EFS was tetrodotoxin-sensitive (data not shown) and abolished by the NK_1 receptor antagonist, N-acety-L-tryptophan3,5-bis (trifluoromethyl)-benzyl ester (1 μM; $P<0.05$, $n=3$) and significantly attenuated by the NK_2 receptor antagonist MEN 10627 (1 μM) by $81 \pm 1.6\%$ ($n=3$, $P<0.05$). No significant change in the eNANC response was observed when consecutive responses were performed ($11 \pm 7\%$ inhibition; $P>0.05$, $n=5$). Similarly, no significant difference in the contractile response to EFS was observed in vehicle-treated (DMSO) compared with control preparations (Figure 2, $P>0.05$, $n=4-5$).

The protein phosphatase inhibitor okadaic acid inhibited the eNANC response (% inhibition) induced by EFS (Figures

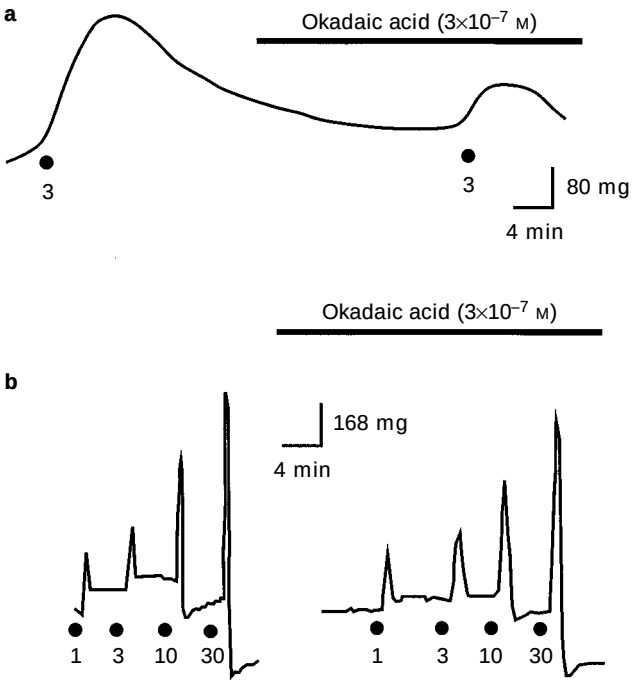

Figure 1 Representative line drawing of eNANC (a) and cholinergic (b) responses in guinea-pig airways to electrical field stimulation (●, Hz) in the absence or presence of okadaic acid (0.3 μM). Experiments were performed in the presence of indomethacin (5 μM), propranolol (1 μM) and thiorphan (10 μM) and together with L-NAME (1 μM) in (b).

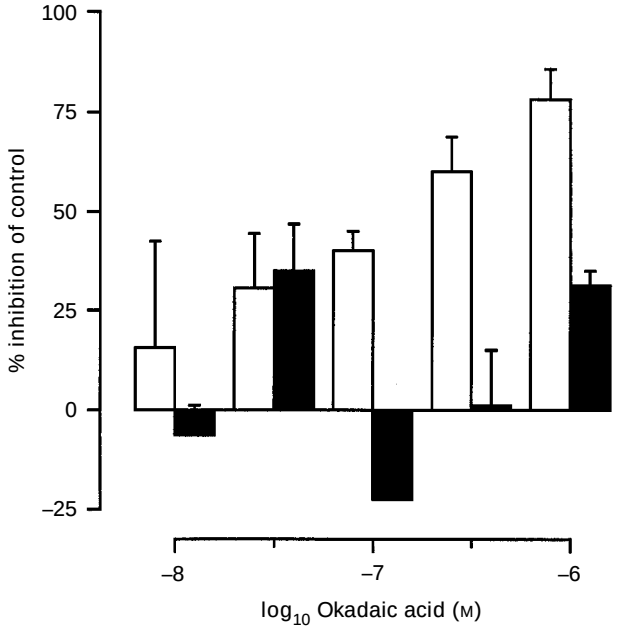

Figure 2 The effect of increasing concentrations of okadaic acid (open columns) and DMSO (solid columns) on electrical field stimulation (3 Hz, 20 s, 0.5 ms at max. voltage)-induced eNANC responses in guinea-pig isolated bronchus. Vertical lines represent s.e.mean ($n=4$). Experiments were performed in the presence of indomethacin (5 μM), propranolol (1 μM), atropine (1 μM) and thiorphan (10 μM).

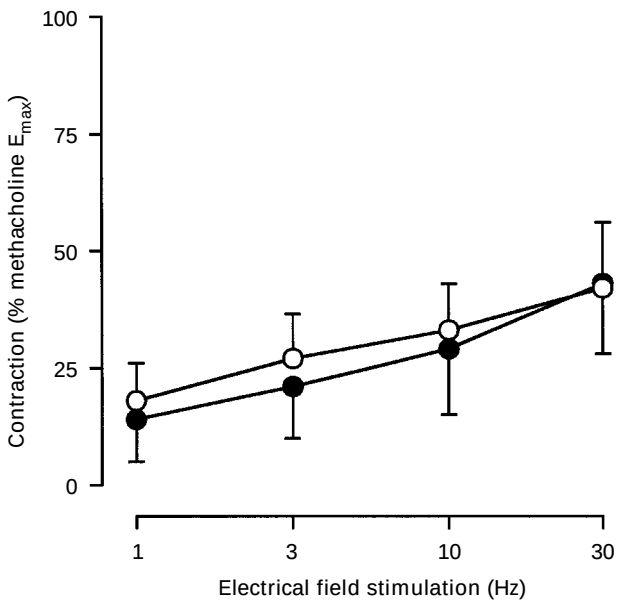

Figure 3 Cholinergic nerve-mediated contractions (1–30 Hz, 10 s, 0.5 ms at max. voltage) in vehicle (●, DMSO 0.25%)- and okadaic acid (○, 0.3 μM)-treated guinea-pig isolated bronchus. Each point represents the mean and vertical lines s.e.mean ($n=4$). Experiments were performed in the presence of indomethacin (5 μM), propranolol (1 μM), thiorphan (10 μM) and L-NAME (1 μM).

1 and 2). In contrast, 1-Nor okadaone (0.3 μM) failed to attenuate significantly the contractile response (% inhibition) to EFS (1-Nor-okadaone, $-1.3\pm8.5\%$ vs DMSO, $-13.5\pm21.5\%$; $P>0.05$, $n=4$).

Cholinergic In guinea-pig isolated main bronchus, cholinergic responses were elicited by EFS (1–30 Hz, 10 s, 0.5 ms pulse width at max. voltage). Atropine (1 μM) completely abolished the cholinergic response at all frequencies tested ($P<0.05$, $n=3$), data not shown). No significant reduction in the contractile response to EFS was observed in the presence of DMSO ($P>0.05$). The protein phosphatase inhibitor okadaic acid (0.3 μM) failed to inhibit significantly the cholinergic contractile response elicited by EFS compared with DMSO (Figure 3, $P<0.05$, $n=4$).

Spasmogens

Capsaicin, substance P and methacholine induced a concentration-dependent contraction of guinea-pig isolated main bronchus yielding a contractile potency (pD_2) of 9.1 ± 0.3 ($n=5$), 8.1 ± 0.3 ($n=6$) and 6.5 ± 0.1 ($n=12$), respectively. Okadaic acid (0.3 μM) failed to alter significantly the contractile potency to capsaicin (okadaic acid, 9.0 ± 0.5 vs DMSO, 9.2 ± 0.4; $P>0.05$, $n=6$, Figure 4a), substance P (okadaic acid, 7.6 ± 0.3 vs DMSO, 8.2 ± 0.2; $P>0.05$, $n=7$, Figure 4b) or methacholine (okadaic acid, 6.4 ± 0.24 vs DMSO, 6.4 ± 0.27; $P>0.05$, $n=4$, Figure 4c).

Relaxant responses

The protein phosphatase inhibitor okadaic acid failed to reverse significantly the substance P-induced contractile response. The maximal relaxant response (% reversal of substance P-induced tone) mediated by okadaic acid (1 μM) was $33\pm12.1\%$ ($n=4$). The substance P-induced contractile response also reversed in response to vehicle ($34.3\pm23.1\%$, $n=4$) and over the time course of the experiment ($33\pm16\%$, $n=4$).

Okadaic acid (0.3 μM) failed to alter significantly the relaxant potency to isoprenaline in substance P contracted tissue (okadaic acid, 6.5 ± 0.4 vs DMSO, 5.9 ± 0.3, $P>0.05$, $n=9$, Figure 5). The maximal relaxant response mediated by isoprenaline (100 μM) in the presence of okadaic acid (0.3 μM) was $86.7\pm6.8\%$, not significantly different from that observed in DMSO (0.25%)-treated preparations ($81.3\pm6.7\%$, $P>0.05$, $n=9$).

Discussion

We have demonstrated that the phosphatase type 1 and 2A inhibitor, okadaic acid inhibited the eNANC contractile response elicited by EFS. In contrast, okadaic acid failed to alter the contractile response to exogenously administered capsaicin, substance P, methacholine and to cholinergic nerve stimulation. This is consistent with the view that okadaic acid inhibited the eNANC contractile response in guinea-pig isolated bronchus by a prejunctional site of action in sensory nerves. Furthermore, protein phosphatases may be involved in modulating electrical rather than capsaicin-induced release of sensory neuropeptides from guinea-pig isolated airways. We also showed that the effect of okadaic acid on the eNANC contractile response was not attributable to a non-specific action of this agent, since 1-Nor okadaone, which is inactive as a phosphatase inhibitor, was without effect on the eNANC responses.

Okadaic acid inhibited the eNANC response but had no effect on the contractile response to capsaicin in guinea-pig bronchial preparations. Our results are in contrast with the observations of Hingtgen *et al.* (1994) that okadaic acid augmented the release of substance P from rat dorsal root ganglion (DRG) neurones in culture in response to capsaicin, potassium and bradykinin. The lack of effect of okadaic acid on capsaicin-induce contraction is consistent with previous findings demonstrating the lack of effect of galanin (Giuliani *et al.*, 1989a), neuropeptide Y (Giuliani *et al.*, 1989b), μ-opiods (Bartho *et al.*, 1987) and phosphodiesterase type IV isoenzyme inhibitors (Undem *et al.*, 1994; Spina *et al.*, 1995) on capsaicin-induced contraction in guinea-pig airways. This may reflect the

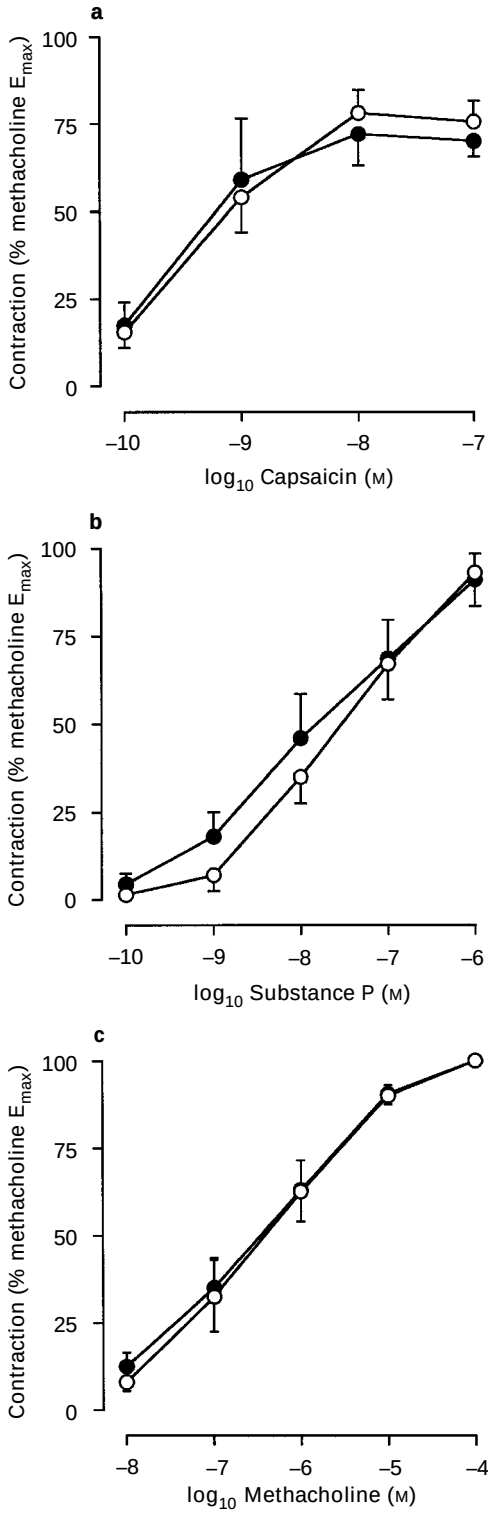

Figure 4 Concentration-effect curves in vehicle (●, DMSO 0.25%)- and okadaic acid (○, 0.3 μM)-treated guinea-pig isolated bronchus to (a) capsaicin ($n=6$), (b) substance P ($n=7$) and (c) methacholine ($n=4$). Each point represents the mean and vertical lines s.e.mean. Experiments were performed in the presence of indomethacin (5 μM), propranolol (1 μM) and thiorphan (10 μM).

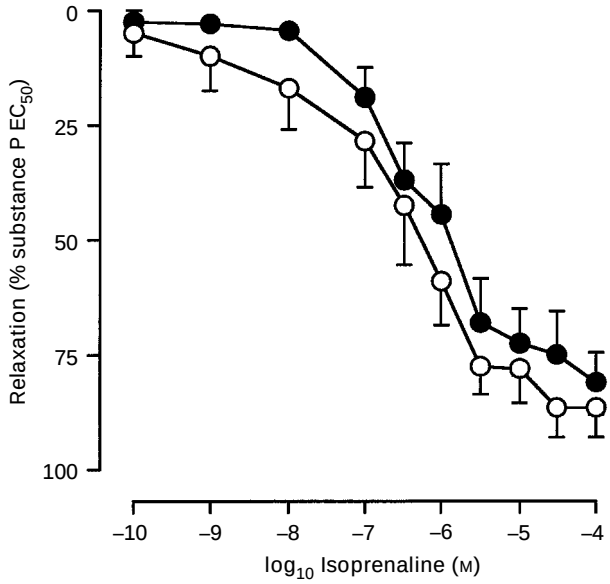

Figure 5 Relaxation concentration-effect curves to isoprenaline in vehicle (●, DMSO 0.25%)- and okadaic acid (○, 0.3 μM)-treated guinea-pig isolated bronchus contracted with substance P (EC_{50}). Each point represents the mean and vertical lines s.e.mean ($n=6$). Experiments were performed in the presence of indomethacin (5 μM), propranolol (1 μM) and thiorphan (10 μM).

different mechanism by which capsaicin mediates the release of neuropeptides from sensory neurones compared with electrical field stimulation (Maggi *et al*., 1988). Furthermore, okadaic acid which induces a rise of intracellular Ca^{2+} in rat sensory neurones, failed to alter the capsaicin-induced rise in Ca^{2+} (Cholewinski *et al*., 1993). The inhibitory effect of okadaic acid on the eNANC response cannot be attributable to a non-specific effect as 1-Nor okadaone, a close derivative of okadaic acid, but at least 2 orders of magnitude less potent as a protein phosphatase inhibitor (Nishiwaki *et al*., 1990), was without effect.

Okadaic acid had no effect on the cholinergic response in guinea-pig trachea, although okadaic acid has been shown to augment (Abdul-Ghani *et al*., 1991; Swain *et al*., 1991) or reduce (Van der Kloot & Molgo, 1993) neurotransmitter release from the neuromuscular junction. Similarly, the phosphatase 1 and 2A inhibitor, calyculin A, has been found to increase synaptic transmission in the rat hippocampus (Herron & Malenka, 1994). In contrast, okadaic acid decreases evoked, but not spontaneous, release of acetylcholine from rat hippocampus, but tends to increase spontaneous, but not evoked, release of glutamate (Vickroy *et al*., 1995). The effect of okadaic acid on acetylcholine release may be related to a reduction in the synthesis of acetylcholine in these tissues (Issa *et al*., 1996). These studies demonstrated that inhibition of protein phosphatases can have differential effects on neurotransmitter release that appears to be species- and cell type-dependent, and may reflect the different role played by protein phosphatases in the regulation of cell function in various tissues.

The lack of effect of okadaic acid on cholinergic responses and to responses to exogenously administered substance P and methacholine rule out a postjunctional mechanism of action. Okadaic acid failed to reverse significantly substance P-induced contractile response, nor did it augment isoprenaline-induced relaxation of guinea-pig bronchial preparations. This is consistent with the inability of okadaic acid alone, to alter phosphodiesterase type IV activity (Sette *et al*., 1994) or to elevate intracellular levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Schaefer *et al*., 1995). Together these findings suggest that okadaic acid does not directly alter airway smooth muscle tone in the guinea-pig.

The cellular targets of protein phosphatases in airway sensory nerves are not clear at present, although there are several possibilities. Okadaic acid can increase the open probability of calcium activated potassium channels (Kume *et al*., 1989; Carl

et al., 1991; Reinhart et al., 1991) and L-type calcium channels (Neumann et al., 1994; Sculptoreanu et al., 1995; Wiechen et al., 1995), but reduces the activity of N-type calcium channels (Sculptoreanu et al., 1995). It has previously been shown that a number of drugs known to elevate cyclic AMP, including β-adrenoceptor agonists (Aikawa et al., 1992; Verleden et al., 1993), phosphodiesterases type IV isoenzyme inhibitors (Qian et al., 1994; Undem et al., 1994; Spina et al., 1995), forskolin (Aikawa et al., 1992), prostaglandin E_1 (Aikawa et al., 1990) and the xanthines, theophylline and enprophylline (Aikawa et al., 1992; Barlinski et al., 1992; Meini et al., 1993), inhibit eNANC responses in guinea-pig isolated bronchus by a prejunctional mechanism of action. These studies provide circumstantial evidence that cyclic AMP may regulate sensory nerve function in the airways and this hypothesis is supported by observations that isoprenaline and theophylline can attenuate the release of neuropeptides from guinea-pig lung (Martins et al., 1991; Shah et al., 1996). However, it is unclear whether the effect of okadaic acid is attributable to a protein kinase A-dependent mechanism.

In conclusion, our results demonstrate the ability of the phosphatase inhibitor okadaic acid to modulate the eNANC response to EFS. However, okadaic acid does not appear to modulate the release of sensory neuropeptides mediated via activation of the capsaicin-receptor on airway sensory nerves. Protein phosphatases appear to play an important role in modulating neuropeptide release in the airways in response to EFS.

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