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Stimulation of airway sensory nerves by cyclosporin A and FK506 in guinea-pig isolated bronchus

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1 We have investigated the contractile property of cyclosporin A and FK506 in guinea-pig isolated bronchus.

2 Cyclosporin A (10 μM) failed to significantly attenuate the excitatory non-adrenergic non-cholinergic (eNANC) and cholinergic contractile response (per cent methacholine E_{max}) induced by electrical field stimulation (EFS). In contrast, eNANC responses were significantly attenuated by both the neurokinin (NK)-1 and (NK)-2 receptor antagonists, N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl and SR48968, respectively.

3 Cyclosporin A and FK506 caused a concentration-dependent contraction in guinea-pig isolated bronchus, which was significantly attenuated by NK-1 and NK-2 receptor antagonists. The capsaicin receptor antagonist, capsazepine (10 μM) significantly reduced the contractile response to cyclosporin A and capsaicin, but not to FK506.

4 The N-type calcium channel blocker, ω-Conotoxin (ωCTX: 10 nM), significantly reduced the contractile response to FK506 and the eNANC response following EFS. In contrast, ω-CTX failed to significantly reduce the contractile potency to capsaicin or cyclosporin A.

5 In bronchial preparations desensitized by repeated application of capsaicin (1 μM), the contractile responses to both cyclosporin A (100 μM) and FK506 (100 μM), were significantly reduced. In contrast, the contractile responses to substance P and neurokinin A (10 μM) were not altered. Furthermore, repeated application of cyclosporin A (100 μM) significantly inhibited the contractile response to capsaicin (1 μM).

6 The findings from this study would indicate that cyclosporin A and FK506 mediate contraction of guinea-pig isolated bronchus secondary to the release of neuropeptides from airway sensory nerves. However, the release of sensory neuropeptides appears to be mediated *via* different mechanisms for cyclosporin A and FK506, the former by stimulation of the vanilloid receptor and the latter *via* opening of N-type calcium channels.

Keywords: Cyclosporin A; FK506; sensory neuropeptides; bronchial smooth muscle; neurotransmitter release; neurokinin-1 and neurokinin-2 receptor antagonists; sensory nerves; capsaicin; capsazepine

Introduction

Cyclosporin A is derived naturally from a fungal metabolite of *Tolypocladium inflatum* (Borel *et al*., 1977), and tacrolimus (FK506) an antifungal natural product macrolide (Liu, 1993). They are both immunosuppressants which are used in the treatment of transplantation to prevent allograft rejection (Shevach, 1985), of which FK506 has been shown to be 10 to 100 times more potent than cyclosporin A *in vivo* (Waschulewski *et al*., 1993). Cyclosporin A binds to and inhibits a family of basic cytosolic receptor proteins termed cyclophilins which exhibit cis-trans peptidyl-propyl isomerase activity. FK506 binds to the rotamase site and inhibits the basic cytosolic protein FK506 binding protein (FKBP). Furthermore, both the cyclosporin A-cyclophilin and the FK506-FKBP complex binds to, and inhibits, the activity of the calcium-calmodulin dependent serine/threonine protein phosphatase 2B, calcineurin (Liu, 1993; Ho *et al*., 1996). Therefore, immunosuppression is a consequence of inhibition of calcineurin and the signal transduction pathways which lead to the activation of specific transcription factors (e.g. nuclear factor of activated T cells, NF-AT) involved in IL-2 gene transcription in lymphocytes (Liu, 1993; Ho *et al*., 1996).

Both cyclosporin A and FK506 have also been shown to modulate the function of other cell types. Thus, IgE-dependent degranulation of mast cells and basophils (Hultsch *et al*., 1991; Stellato *et al*., 1992; Marone *et al*., 1993), exocrine secretion from pancreas (Waschulewski *et al*., 1993; Groblewski *et al*., 1994), nitrite/nitrate production by vascular smooth muscle cells (Akita *et al*., 1994; Marumo *et al*., 1995) and expression of inducible nitric oxide synthetase (Marumo *et al*., 1995) are all inhibited by cyclosporin A and FK506 which is attributed to inhibition of calcineurin. There are further suggestions that ion channel function is regulated by calcineurin, which is inhibited by both the cyclosporin A-cyclophilin and FK506-FKBP complexes (Chad & Eckert, 1986). FK506 (50 μM) increased synaptic transmission under basal conditions and following tetanic stimulation of *Helix* neurones (Wang & Kelly, 1996). Furthermore, cyclosporin A has recently been shown to inhibit capsaicin-evoked desensitization of dorsal root ganglion neurones from rats (Docherty *et al*., 1996).

However, cyclosporin A has been reported to mediate effects that are unrelated to inhibition of calcineurin. Cyclosporin A inhibited the binding of radiolabelled substance P to membrane homogenates of guinea-pig lung, indicative of neurokinin-1 antagonism (Gitter *et al*., 1995). This is thought to account for the ability of cyclosporin A to inhibit the secretion of IL-6 and phosphatidylinositol turnover from U-

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373 MG astrocytoma cells induced by substance P (Gitter *et al*., 1995). Similarly, the ability of substance P to stabilize IL-2 mRNA in Jurkat cells is inhibited by cyclosporin A (Calvo, 1994). In contrast, cyclosporin A failed to inhibit histamine release from human skin mast cells induced by substance P (Stellato *et al*., 1992).

We have previously demonstrated that the protein phosphatase 1 and 2A inhibitor, okadaic acid, attenuated contraction induced by stimulation of eNANC nerves in guinea-pig isolated main bronchus (Harrison *et al*., 1997), suggesting that protein phosphatases regulate the release of neuropeptides from airway sensory nerves. However, the role of protein phosphatase 2B in this response is not clear. We have therefore examined the role of cyclosporin A and FK506 in modulating neuropeptide release from airway sensory nerves.

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. Bronchial rings were suspended under a resting tension of 0.5 g in Krebs-Henseleit solution and aerated with 95% O_2 and 5% CO_2 at 37°C, containing the cyclo-oxygenase inhibitor indomethacin (5 μM) and the non-selective β-antagonist propranolol (1 μM). Tissues were allowed to equilibrate for 40 min before methacholine (0.3 and 100 μM) was added cumulatively to the bath to assess the sensitivity of bronchial preparations. After the contractile response had reached plateau, the tissues were washed five times over a 15 min period and allowed to equilibrate for a further 30 min.

Electrical field stimulation studies

eNANC Platinum electrodes were placed either side of guinea-pig isolated bronchi and stimulated electrically (3 Hz, 20 s, 0.5 ms pulse width at maximum voltage) in the presence of the non-selective muscarinic antagonist, atropine (0.1 μM) and the neutral endopeptidase inhibitor, thiorphan (10 μM). After the contractile response had returned to baseline, bronchial preparations were incubated for 20 min prior to electrical field stimulation (EFS, 3 Hz, 20 s, 0.5 ms maximum voltage) with cyclosporin A (10 μM), the neurokinin-1 receptor antagonist, N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM), the neurokinin-2 receptor antagonist, SR48968 (0.1 μM), the N-type calcium channel blocker, ω-conotoxin (10 nM) or vehicle.

Cholinergic In other experiments, cholinergic responses were obtained in guinea-pig isolated main bronchi by EFS (1–30 Hz, 10 s, 0.5 ms pulse width at maximum voltage) in the presence of the nitric oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME, 30 μM) and the neutral endopeptidase inhibitor, thiorphan (10 μM). The tissues were then incubated for 20 min with cyclosporin A (10 μM) or vehicle, then stimulated electrically for a second time.

Spasmogen studies

Cumulative-concentration effect curves to cyclosporin A, FK506 and capsaicin were performed in the absence, or 20 min after incubation of guinea-pig isolated bronchus with capsazepine (10 μM), N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM), SR48968 (0.1 μM), ω-conotoxin (10 nM) or vehicle.

In other studies, concentration-effect curves to substance P or neurokinin A were performed in the absence, or 20 min after incubation of guinea-pig isolated bronchus with cyclosporin A (10 μM), N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM), SR48968 (0.1 μM) or vehicle.

In further studies, cumulative-concentration effect curves to cyclosporin H were performed in guinea-pig isolated bronchus.

Desensitisation studies

Guinea-pig bronchial preparations were contracted with capsaicin (1 μM), then washed repeatedly with Krebs-Henseleit solution until the response had returned to baseline. This procedure was repeated again and bronchial preparations were allowed to equilibrate for a further 30 min before concentration response curves to cyclosporin A, FK506, substance P or neurokinin A (0.01–100 μM) were performed. Control preparations were treated with repeated doses of vehicle (0.1% ethanol) before the dose response curve to cyclosporin A, FK506, substance P or neurokinin A were performed. In other experiments, the effect of cyclosporin A (100 μM) on the contractile response to substance P in capsaicin-desensitized tissues was studied.

In some experiments, guinea-pig bronchial preparations were contracted with cyclosporin A (100 μM), the tissues were washed repeatedly with Krebs-Henseleit solution until the response had returned to baseline. This procedure was repeated again and tissues were allowed to equilibrate for a further 30 min before commencement of the dose response curves to capsaicin (0.01–1000 nM). Control preparations were treated with repeated doses of vehicle (0.1% ethanol) before the dose response curve to capsaicin was performed.

Analysis of results

Results were expressed as arithmetic mean$\pm$s.e.mean. In studies involving EFS, the contractile response was expressed as a percentage of the contractile response in the absence of the inhibitor or vehicle (per cent inhibition). Contractile responses to capsaicin, cyclosporin A, FK506, neurokinin A and substance P were expressed as a percentage of methacholine E_{max} (100 μM). The effect of drug treatment on cumulative concentration-effect curves was assessed using Analysis of Covariance and contractile potency was expressed as the logarithm of the concentration of agonist which produced a contractile response of 25% of the methacholine E_{max} ($-\log_{10} EC_{25}$). In the desensitization studies, the maximum response to cyclosporin A and FK506 (100 μM) was compared to the response observed in vehicle control preparations. The difference between mean values was determined with Student's paired and non-paired *t*-test and considered significant if $P<0.05$.

Drugs

N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (neurokinin-1 receptor antagonist), atropine, capsaicin, ω-conotoxin (GVIA), dimethylsulphoxide (DMSO), indomethacin, isoprenaline, methacholine, neurokinin A, Nω-nitro-L-arginine methyl ester (L-NAME), substance P, thiorphan (Sigma) and capsazepine (Alexis). Cyclosporin A and cyclosporin H were kindly donated by Dr K.H. Buchheit

(Novartis, AG Basle, Switzerland) and dissolved in ethanol:-water:tween-80 (50 : 48 : 2 v v^{-1}). FK506 was kindly donated by Dr K. Murato (Fujisawa, GmbH München) and dissolved in ethanol and further dilutions dissolved in ethanol:water: tween-80 [50 : 48 : 2 v v^{-1}]. Composition of Krebs-Henseleit solution was (mM): NaCl 117.6, KCl 5.4, $MgSO_47H_2O$ 0.57, KH_2PO_4 1.03, $NaHCO_3$ 25, glucose 11.1 and $CaCl_22H_2O$ 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 concentrations of neurokinin A and substance P (0.6 mM) were prepared in 10% acetic acid and stored at $-20^{\circ}C$. The appropriate dilution's were then made 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} ($n=5$). We have previously reported that contraction induced by EFS of eNANC nerves was tetrodotoxin-sensitive.

Cyclosporin A (10 μM) did not alter the eNANC response (per cent inhibition) induced by EFS (cyclosporin A, 12.3 ± 1.6 vs control, 5.5 ± 5.5; $P>0.05$, $n=4$). In contrast, N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM) and SR48968 (0.1 μM) significantly attenuated (per cent inhibition) the eNANC response induced by EFS (NK-1 antagonist, 89.5 ± 3.3 vs control, 1.2 ± 1.3; $P<0.05$, $n=4$: NK-2 antagonist, 93.5 ± 4.3 vs control, 6.3 ± 0.2; $P<0.05$, $n=4$). ω-Conotoxin (10 nM), an N-type calcium channel blocker, significantly attenuated the eNANC response induced by EFS (ωCTX, 76.7 ± 4.4 vs control, 8.0 ± 8.8, $P<0.05$, $n=4$).

Cholinergic response

In guinea-pig isolated main bronchus, contractile responses (per cent methacholine E_{max}) to cholinergic nerve stimulation (1–30 Hz, 10 s, 0.5 ms pulse width at maximum voltage) were not altered by cyclosporin A (10 μM) (1 Hz, cyclosporin A, 7.7 ± 2.9 vs control, 9.0 ± 4.6; 3 Hz, cyclosporin A, 8.1 ± 2.9 vs control, 10.3 ± 4.4; 10 Hz, cyclosporin A, 10.8 ± 3 vs control, 14.3 ± 6.8; 30 Hz, cyclosporin A, 20.9 ± 5.4 vs control, 24.9 ± 12.6; $P>0.05$, $n=4$).

Spasmogen experiments

Cyclosporin A, FK506, capsaicin and substance P all induced a concentration-dependent contraction of guinea-pig isolated main bronchus yielding a contractile potency ($-\log_{10} EC_{25}$) of 5.9 ± 0.5 ($n=15$), 7.1 ± 0.2 ($n=16$), 9.5 ± 0.4 ($n=18$) and 8.3 ± 0.2 ($n=17$), respectively. Cyclosporin H induced a concentration-dependent contraction of guinea-pig isolated main bronchus yielding a contractile potency of 3.9 ± 0.1 ($n=4$) and a maximum response (0.3 mM) of $49.8 \pm 11.3\%$ methacholine E_{max} ($n=4$). Vehicle (0.1%) for cyclosporin A and FK506 had no significant contractile response ($2.5 \pm 1.0\%$ methacholine E_{max}, $n=4$).

Capsazepine (10 μM) significantly reduced the contractile (ANCOVA $P<0.05$), and maximum (per cent methacholine E_{max}) response, to cyclosporin A (capsazepine, 12 ± 13 vs control, 83 ± 8.9; $P<0.05$, $n=5-6$; Figure 1a) and contractile potency ($-\log_{10} EC_{25}$) to capsaicin (capsazepine, 5.9 ± 0.3 vs control, 9.8 ± 0.2; $P<0.05$, $n=4$; Figure 1b). In contrast, capsazepine had no effect on the contractile potency to FK506 (capsazepine, 6.5 ± 0.6 vs control, 6.5 ± 0.2; $P>0.05$, $n=4$; Figure 1c).

The neurokinin-1 and neurokinin-2 receptor antagonist, N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM) and SR48968 (0.1 μM) respectively, reduced the contractile potency ($-\log_{10} EC_{25}$) to cyclosporin A (NK-1 antagonist, 4.2 ± 0.1 vs vehicle, 5.3 ± 0.4; $P<0.05$, $n=4$: NK-2 antagonist, 4.6 ± 0.2 vs control, 6.6 ± 0.5; $P<0.05$, $n=5$; Figure 2a), FK506 (NK-1 antagonist, 4.3 ± 0.1 vs control,

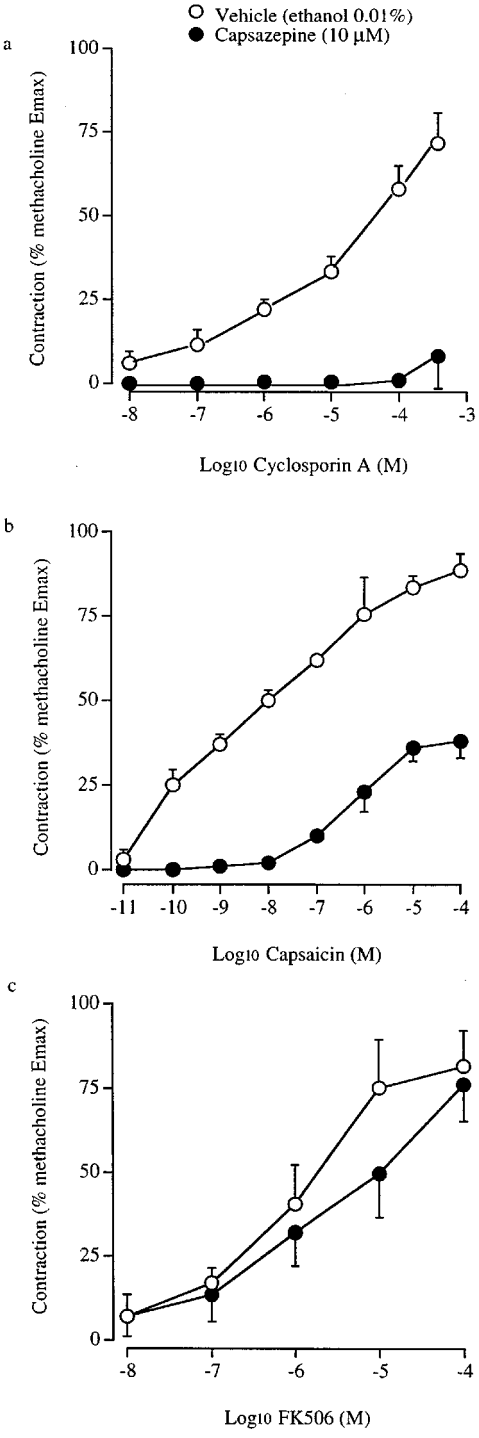

Figure 1 Concentration-effect curves to (a) cyclosporin A, (b) capsaicin, (c) FK506 in the absence or presence of capsazepine (10 μM, $n=4-7$). Vertical lines represent s.e.mean.

7.2±0.2; $P<0.05$, $n=4$: NK-2 antagonist, 5.4±0.2 vs control 6.6±0.5; $P<0.05$, $n=4$; Figure 2b) and substance P (NK-1 antagonist, 7.2±0.4, vs control, 8.7±0.1; $P<0.05$, $n=4$; Figure 3a). However, SR48968 failed to alter the contractile potency to substance P (NK-2 antagonist, 8.0±0.2 vs control, 7.6±0.2; $P>0.05$, $n=4$; Figure 3a). In the presence of N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM) the contractile potency to capsaicin was not significantly altered (NK-1 antagonist 8.8±0.7 vs control, 9.8±0.5, $P>0.05$, $n=4$). In contrast, the NK-2 antagonist SR48968 (0.1 μM), significantly reduced the contractile potency to capsaicin (NK-2 antagonist, 7.3±0.2 vs control, 8.7±0.1, $P<0.05$, $n=4$; Figure 3b).

Cyclosporin A (10 μM) failed to significantly alter the contractile potency ($-\log_{10}EC_{25}$) to substance P (cyclosporin A, 8.0±6.5 vs control, 8.1±0.7; $P>0.05$, $n=5-6$; Figure 4a) and neurokinin A (cyclosporin A, 6.6±0.3 vs control, 7.0±0.2, $P>0.05$, $n=4$; Figure 4b).

ω-Conotoxin (10 nM) significantly reduced the contractile potency ($-\log_{10}EC_{25}$) to FK506 (ωCTX, 4.3±0.1 vs control 6.8±0.6, $P<0.05$, $n=4$; Figure 5a). In contrast, ω-conotoxin (10 nM) failed to significantly reduce the contractile potency to both capsaicin (ωCTX, 8.6±0.4 vs control 9.5±0.5, $P>0.05$, $n=6$; Figure 5b) and cyclosporin A (ωCTX, 6.8±0.3 vs control 5.9±0.5, $P>0.05$, $n=4$; Figure 5c).

Desensitization studies

Capsaicin (1 μM) contracted (per cent methacholine E_{max}) guinea-pig bronchial preparations (46.8±1.6, $n=7$) but the response to a second application of capsaicin (1 μM) was significantly inhibited (27±5, $n=7$, $P<0.05$). In the capsaicin-desensitized preparations, the contractile response to cyclosporin A (100 μM) (capsaicin-treated, 8.3±2.8 vs ethanol control, 37±8.4 vs time control 59.3±12.3, $P<0.05$, $n=7$) and FK506 (100 μM) (capsaicin-treated, 14.8±4.4 vs ethanol control, 58.8±4.4 vs time control, 84.3±4.8, $P<0.05$, $n=4$) was significantly reduced compared with ethanol control. However, the contractile response to substance P (10 μM) (capsaicin-treated, 84.5±2.3 vs control, 92.3±4.6, $P>0.05$, $n=4$) and neurokinin A (10 μM) (capsaicin-treated, 72.3±9.5 vs control, 98.5±1.5, $P>0.05$, $n=4$) were not altered. Cyclosporin A (100 μM) which failed to significantly increase tone in capsaicin desensitized tissues, had no effect on the

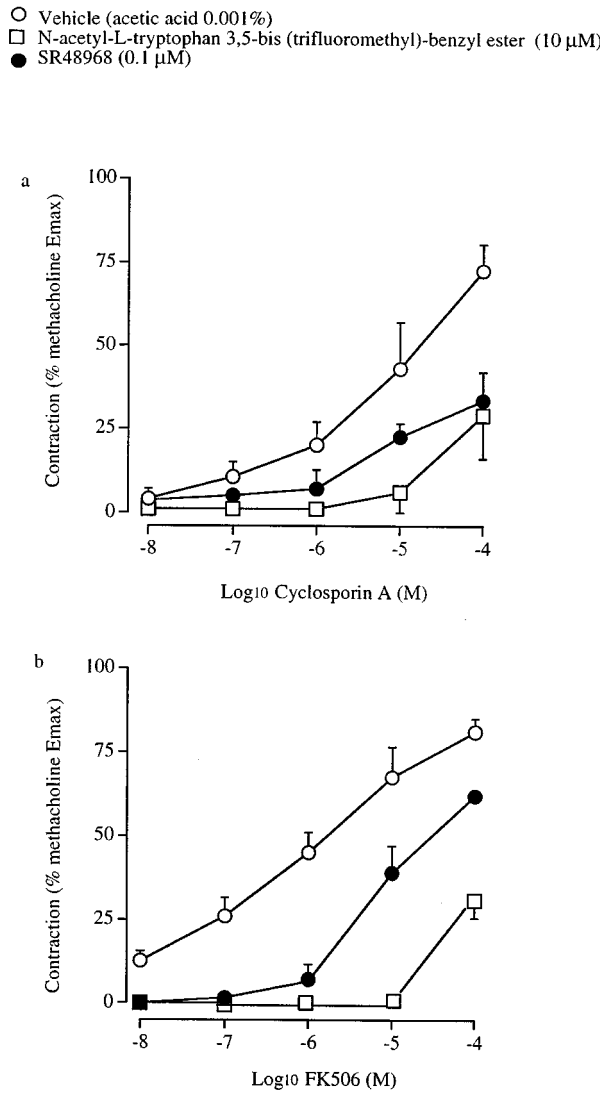

Figure 2 Concentration-effect curves to (a) cyclosporin A and (b) FK506 in the absence or presence of N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM, $n=4-5$) or SR48968 (0.1 μM, $n=4-5$). Vertical lines represent s.e.mean.

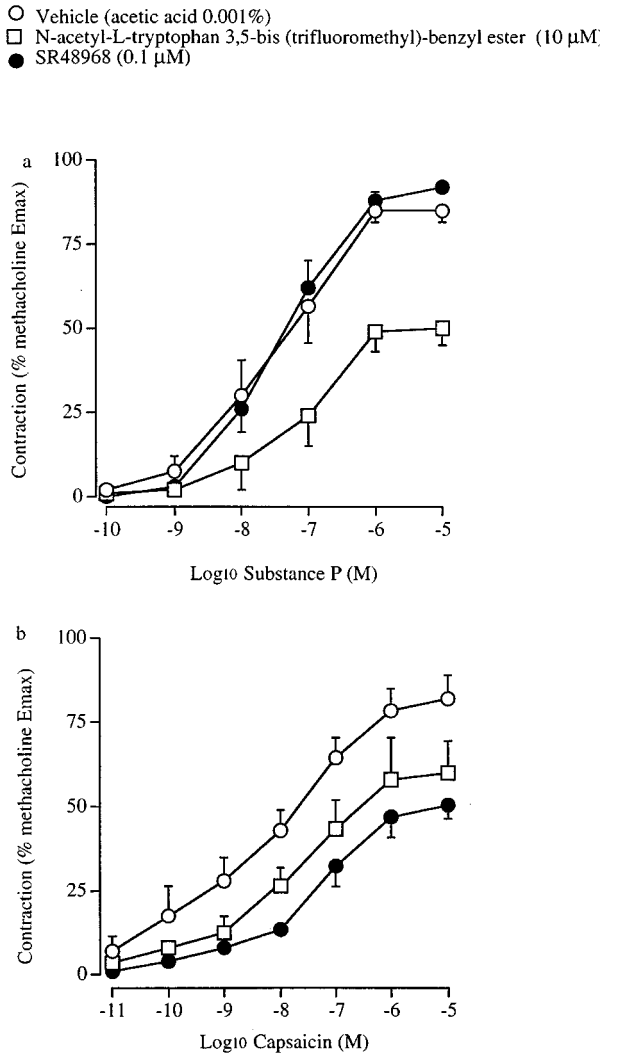

Figure 3 Concentration-effect curves to (a) substance P and (b) capsaicin in the absence or presence of N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester (10 μM, $n=4$) or SR48968 (0.1 μM, $n=4$). Vertical lines represent s.e.mean.

contractile potency ($-\log_{10}EC_{25}$) to substance P (cyclosporin A, 7.3 ± 0.7 vs control 7.4 ± 0.7, $P>0.05$, $n=4$).

Cyclosporin A (100 μM) contracted guinea-pig bronchial preparations ($29.5\pm6.5\%$ methacholine E_{max}, $n=4$) but the response to a second application of cyclosporin A (100 μM) was significantly inhibited (9.3 ± 2.9, $n=4$, $P<0.05$). In cyclosporin A treated preparations, the contractile response to capsaicin (1 μM) was also significantly reduced (cyclosporin A-treated, 9.5 ± 3.5 vs control, 37 ± 11, $P<0.05$, $n=4$) compared to the control experiment.

Discussion

We report the novel finding that cyclosporin A and FK506 caused a concentration-dependent contraction in guinea-pig isolated bronchus. The neurokinin-1 and neurokinin-2 receptor antagonists, N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester and SR48968 respectively, significantly attenuated the contractile response to cyclosporin A and FK506. Moreover, capsaicin-induced desensitization of the tissue significantly reduced the contractile potency of cyclosporin A and FK506 and when tissues were treated with cyclosporin A, cross-desensitization to capsaicin was also evident. Collectively, these findings indicate that cyclosporin A and FK506 contracted guinea-pig bronchus secondary to the release of neuropeptides from airway sensory nerves. Capsazepine, a capsaicin receptor antagonist, significantly attenuated the contractile response to both capsaicin and cyclosporin A, but was ineffective against the contractile response to FK506. In contrast, the N-type calcium channel blocker, ω-conotoxin, significantly attenuated the contractile response to EFS and FK506, but not to cyclosporin A or capsaicin. The mechanism of contraction appears to be

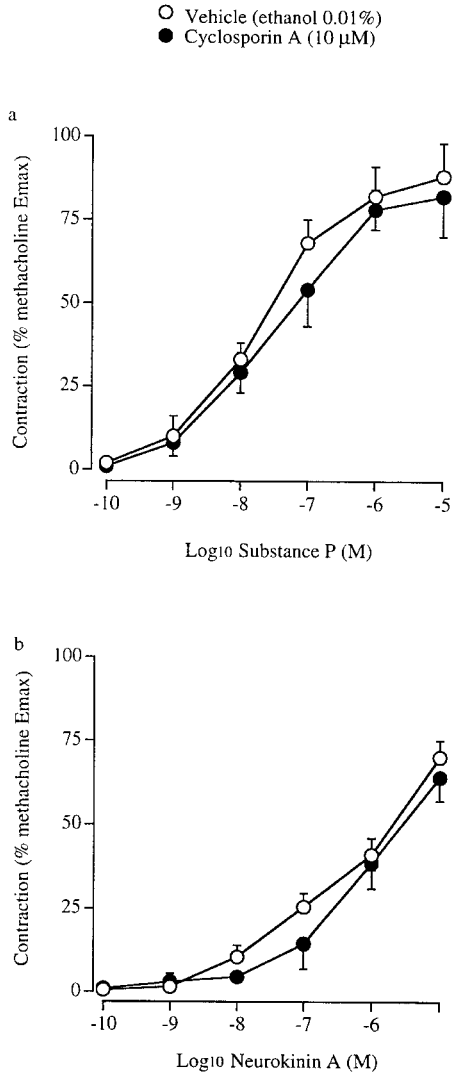

Figure 4 Concentration-effect curves to (a) substance P and (b) neurokinin A in the absence or presence of cyclosporin A (10 μM, $n=4-6$). Vertical lines represent s.e.mean.

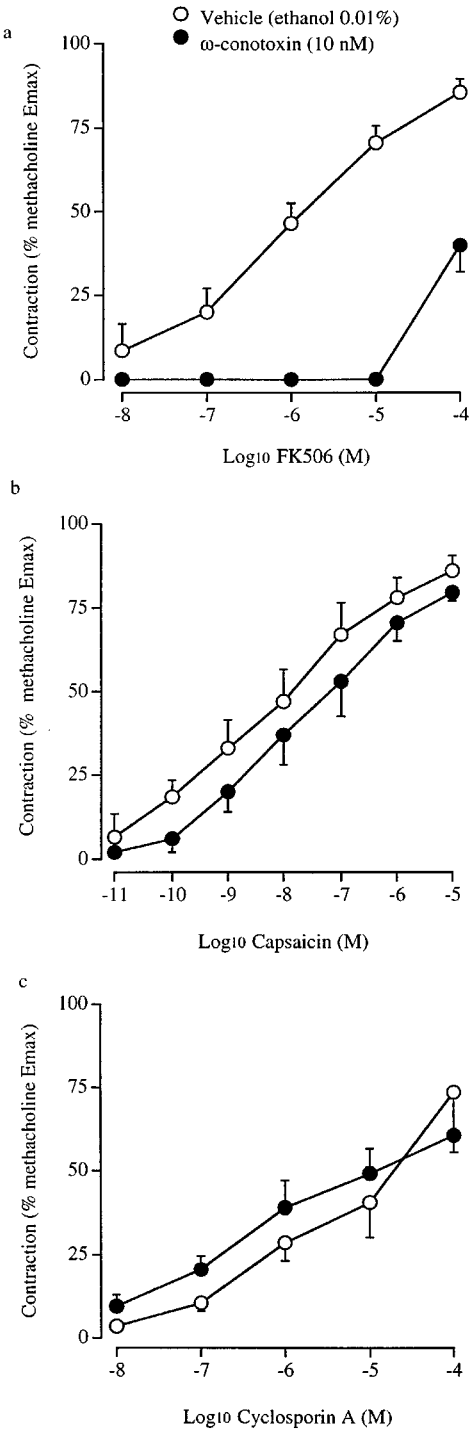

Figure 5 Concentration-effect curves to (a) FK506 (b) capsaicin and (c) cyclosporin A in the absence or presence of ω-conotoxin (10 nM, $n=4-6$). Vertical lines represent s.e.mean.

capsazepine-sensitive in the case of cyclosporin A, and ω-conotoxin sensitive in the case of FK506.

It is well established that cyclosporin A-cyclophilin and the FK506-FKBP complex inhibits the calcium/calmodulin dependent protein phosphatase, calcineurin (Marks, 1996). The protein and mRNA for cyclophilins and calcineurin is localized to nerve cells and is distributed widely throughout the brain and spinal cord (Steiner *et al*., 1992; Dawson *et al*., 1994; Chen *et al*., 1995). Moveover, cyclophilin and calcineurin appear to be co-localized in neurones (Steiner *et al*., 1992; Dawson *et al*., 1994). Several studies have suggested that synaptic activity may be modulated by calcineurin, including synpatic depression (Mulkey *et al*., 1994) and down regulation of long-term potentiation in the hippocampus (Wang & Kelly, 1996). Cyclosporin A complexed with cyclophilin inhibited the ability of capsaicin to induce desensitization of rat dorsal root ganglion (DRG) neurones (Docherty *et al*., 1996). Similarly, cyclosporin A promoted phosphorylation of sodium channels in rat brain (Chen *et al*., 1995), inhibited spontaneous action potentials in rat cortical neurones (Victor *et al*., 1995), promoted dephosphorylation of dynamin in rat brain terminals (Nichols *et al*., 1994), increased glutamate release *via* a presynaptic mechanism of action (Nichols *et al*., 1994; Victor *et al*., 1995) and inhibited voltage-dependent potassium channels in lymphocytes (Panyi *et al*., 1996).

In the present study, relatively high concentrations of cyclosporin A and FK506 evoked contractile responses that were inhibited by N-acetyl-L-tryptophan 3,5-bis (trifluoromethyl)-benzyl ester, which has been shown to selectively inhibit the binding of substance P to cloned human neurokinin 1 receptor expressed in CHO cells (IC_{50} 3.8 μM, MacLeod *et al*., 1993), and by SR48968 (neurokinin 2 receptor antagonist) which inhibits binding of neurokinin A to guinea-pig lung parenchyma (IC_{50} 0.617 μM, McKee *et al*., 1993). Both antagonists significantly inhibited the contractile response to cyclosporin A and FK506, indicating that the contractile response to these agonists is dependent on the release of neuropeptides from sensory nerves in guinea-pig isolated bronchus. The neurokinin receptor antagonists used in this study attenuated the eNANC response following EFS of bronchial tissue. This confirms previous studies which have examined the inhibitory effect of NK-1 (CP96,345: 0.01 – 1 μM) and NK-2 (SR48968: 10 – 0.1 nM) receptor antagonists (Martin *et al*., 1992; Lou *et al*., 1993) against the eNANC response. Although NK-2 receptors appear to mediate the eNANC response (Maggi *et al*., 1991), NK-1 receptors are also involved in this response particularly following inhibition of enzymes which degrade endogenously released neuropeptides. Thus both NK-1 and NK-2 receptors are involved in eNANC contractions in guinea-pig isolated bronchus induced by EFS.

In guinea-pig isolated bronchus, cyclosporin A is at least three orders of magnitude less potent than capsaicin at mediating contraction. Capsaicin induces cough (O'Connell *et al*., 1994) and bronchoconstriction (Hathaway *et al*., 1993) in asthmatic subjects. In contrast, cough but not bronchoconstriction is observed in transplantation patients following inhalation of capsaicin (0.03 – 0.15 mg ml^{-1}) (Hathaway *et al*., 1993) presumably due to a loss in afferent innervation in the transplanted lung (Springall *et al*., 1990). Consistent with these findings, cyclosporin A (62.5 mg ml^{-1}) has no bronchoconstrictor action in heart/lung transplant patients (Iacono *et al*., 1997), although cough was reported in three of nine subjects, despite pretreatment with lidnocaine and β_2-agonists. In healthy subjects, nebulized cyclosporin A-dilauroylphosphatidylcholine (5 mg ml^{-1}) caused a 30% fall in FEV in one subject and caused cough in eight of ten normal (non-smoker) subjects (Alvarez *et al*., 1997). Clearly the cough response to cyclosporin A is less than for capsaicin, which is consistent with the difference in spasmogen potency observed in our *in vitro* study.

The vanilloid receptor antagonist, capsazepine has been shown to selectively inhibit a number of functional responses elicited by capsaicin. Thus, contraction of guinea-pig airway (Belvisi *et al*., 1992; Ellis & Undem, 1994), excitation of C-fibres *in vitro* (Fox *et al*., 1995), calcium uptake and rubidium efflux from rat DRG neurones (Bevan *et al*., 1992; Cholewinski *et al*., 1993) and stimulation of ion currents (Bevan *et al*., 1992) by capsaicin are inhibited by capsazepine. We have shown that capsazepine inhibited the contractile response to both capsaicin and cyclosporin A, while acute desensitization with capsaicin abolished the contractile response to cyclosporin A. Furthermore, tissues exposed to cyclosporin A were less responsive to capsaicin suggesting cross-desensitization. The effect was unrelated to antagonism of post-junctional neurokinin receptor, since cyclosporin A failed to inhibit the contractile response to exgenously administered substance P and neurokinin A. The data suggest that cyclosporin A may stimulate the vanilloid receptor, although radioligand binding studies are required to confirm this. In contrast, the contractile response to FK506 was unaffected by capsazepine, yet was inhibited by the N-type calcium channel blocker, ω-conotoxin. Moreover, ω-conotoxin had no effect on the contractile response to capsaicin or cyclosporin A, consistent with the view that multiple mechanisms mediate the release of sensory neuropeptides (Maggi *et al*., 1988).

Lowering pH can evoke the release of calcitonin gene related peptide (CGRP) from isolated perfused heart (Franco Cereceda *et al*., 1993; 1994), trachea (Hua *et al*., 1995) and soleus muscle (Santicioli *et al*., 1993), while capsazepine attenuated the release of CGRP induced by capsaicin (Franco Cereceda *et al*., 1993, 1994; Santicioli *et al*., 1993; Hua *et al*., 1995) and low pH (Santicioli *et al*., 1993; Franco Cereceda *et al*., 1993, 1994). In contrast, capsazepine did not inhibit CGRP release from rat trachea (Hua *et al*., 1995), rubidium efflux from rat DRG neurones (Bevan *et al*., 1992) and electrical activity of whole guinea-pig vagus (Fox *et al*., 1995) following lowering of pH. Recently, cDNA encoding of the capsaicin receptor from rat DRG neurones has been expressed in HEK239 cells. Both capsaicin and noxious heat evoked currents in HEK239 cells was inhibited by capsazepine and ruthenium red, respectively. In contrast, low pH failed to evoke currents in HEK239 cells, yet augmented the response to capsaicin (Caterina *et al*., 1997). The mechanism by which low pH can stimulate sensory nerves in these studies, in light of the recent findings in HEK239 cells remain to be established, but might be a consequence of the release of prostacyclin (Franco Cereceda *et al*., 1994) or to the release of unidentified endogenous ligand(s) (Fox *et al*., 1995). However, it is unlikely that prostaglandin's play a role in the contractile response to cyclosporin A or FK506, since indomethacin was present in all the experiments. It also remains to be established whether cyclosporin A induces the release of a non-prostanoid ligand which binds to the vanilloid receptor. The role of inhibition of calcineurin in modulating neuropeptide release in the airway remains to be established, particularly in view of the finding that cyclosporin H, which has poor affinity for cyclophilins, also induced a concentration-dependent contractile response, albeit to a lesser extent than cyclosporin A and FK506.

We have previously reported that the protein phosphatase 1 and 2A inhibitor, okadaic acid, inhibited the contractile response following stimulation of eNANC nerves but not to capsaicin in guinea-pig isolated bronchus (Harrison *et al*.,

1997). This suggested that protein phosphorylation/dephosphorylation regulates neuropeptide release from airway sensory nerves following activation of voltage-dependent calcium channels. Previous studies have shown that okadaic acid facilitated neuropeptide release (Hingtgen & Vasko, 1994), but had no effect on calcium entry (Cholewinski *et al*., 1993) or desensitization (Docherty *et al*., 1996) induced by capsaicin in rat DRG neurones. The latter findings are consistent with a lack of effect of okadaic acid on capsaicin-induced contractile responses in the guinea-pig (Harrison *et al*., 1997).

In conclusion, we have demonstrated that cyclosporin A and FK506, at concentrations above that necessary for immunosuppression can stimulate airway sensory nerves *in vitro*, the former by a capsazepine and the latter an ω-conotoxin sensitive mechanism.

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