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Neuroregulation of mucus secretion by opioid receptors and K_{ATP} and BK_{Ca} channels in ferret trachea *in vitro*

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1 Opioid agonists inhibit neurogenic mucus secretion in the airways. The mechanism of the inhibition is unknown but may be via opening of potassium (K^+) channels. We studied the effect on neurogenic secretion in ferret trachea *in vitro* of the OP_1 receptor (formerly known as δ opioid receptor) agonist [D-Pen^{2,5}]enkephalin (DPDPE), the OP_2 receptor (formely κ) agonist U-50,488H, the OP_3 receptor (formerly μ) agonist [D-Ala^2, N-Me-Phe, Gly-ol^5]enkephalin (DAMGO), the ATP-sensitive K^+ (K_{ATP}) channel inhibitor glibenclamide, the large conductance calcium activated K^+ (BK_{Ca}) channel blocker iberiotoxin, the small conductance K_{Ca} (SK_{Ca}) channel blocker apamin, the K_{ATP} channel opener levcromakalim, a putative K_{ATP} channel opener RS 91309, and the BK_{Ca} channel opener NS 1619. Secretion was quantified by use of $^{35}SO_4$ as a mucus marker.

2 Electrical stimulation increased tracheal secretion by up to 40 fold above sham-stimulated levels. DAMGO or DPDPE (10 μM each) significantly inhibited neurogenic secretion by 85% and 77%, respectively, effects which were reversed by naloxone. U-50,488H had no significant inhibitory effect on neurogenic secretion, and none of the opioids had any effect on ACh-induced or [Sar^9]substance P-induced secretion.

3 Inhibition of neurogenic secretion by DAMGO or DPDPE was reversed by iberiotoxin (3 μM) but not by either glibenclamide or apamin (0.1 μM each). Iberiotoxin alone did not affect the neurogenic secretory response.

4 Levcromakalim, RS 91309 or NS 1619 (3 nM – 3 μM) inhibited neurogenic secretion with maximal inhibitions at 3 μM of 68%, 72% and 96%, respectively. Neither levcromakalim nor RS 91309 at any concentration tested significantly inhibited acetylcholine (ACh)-induced secretion, whereas inhibition (60%) was achieved at the highest concentration of NS 1619, a response which was blocked by iberiotoxin.

5 Inhibition of neurogenic secretion by levcromakalim (3 μM) or RS 91309 (30 nM) was inhibited by glibenclamide but not by iberiotoxin. In contrast, inhibition by NS 1619 (30 nM and 3 μM) was blocked by iberiotoxin but not by glibenclamide.

6 We conclude that, in ferret trachea *in vitro*, OP_1 or OP_3 opioid receptors inhibit neurogenic mucus secretion at a prejunctional site and that the mechanism of the inhibition is via opening of BK_{Ca} channels. Direct opening of BK_{Ca} channels or K_{ATP} channels also inhibits neurogenic mucus secretion. In addition, opening of BK_{Ca} channels inhibits ACh-evoked secretion of mucus. Drugs which open BK_{Ca} channels may have therapeutic anti-secretory activity in bronchial diseases in which neurogenic mechanisms and mucus hypersecretion are implicated in pathophysiology, for example asthma and chronic bronchitis.

Keywords: Airways; K^+ channel; K^+ channel opener; mucus; mucus secretion; nerves; opioids; potassium channels; potassium channel openers; NS1619

Introduction

Secretion of mucus in the airways is a protective response to inhalation of airborne irritants and physiological stress and is controlled by humoral and neuronal mechanisms. In mammalian airways, the predominant neural control is cholinergic with a minor adrenergic component (Rogers, 1997). Capsaicin-sensitive 'sensory-efferent' nerves also contribute to neural control of secretion, although the magnitude of their contribution varies with species (Ramnarine & Rogers, 1994). Suppression of airway neurogenic mucus secretion is of interest, because neural mechanisms and mucus hypersecretion are implicated in the pathophysiology of a number of severe airway conditions, most notably asthma and chronic bronchitis (Barnes, 1986; Rogers, 1997).

Opioid agonist drugs are neural suppressants and have been shown to inhibit neurotransmission in the airways (Barnes *et al*., 1990). For example, with reference to the topic of the present study, neurogenic goblet cell secretion in guinea-pig trachea is inhibited by activation of opioid OP_3 receptors (formerly known as μ-receptors; Dhawan *et al*., 1996) or OP_1 receptors (formerly δ-receptors), but not by OP_2 receptors (formerly κ-receptors) (Kuo *et al*., 1992a). Opening of potassium (K^+) channels is a mechanism mediating opioid inhibitory activity (North *et al*., 1987). Potassium channels sensitive to intracellular concentrations of adenosine 5′-triphosphate (K_{ATP} channels) inhibit a number of neurogenic responses in the airways (Ichinose & Barnes, 1990; Burka *et al*., 1991; Kuo *et al*., 1992b; Lei *et al*., 1993), but do not appear to be the mediators of opioid inhibition in the lung. Rather, a K^+ channel blocked by charybdotoxin, a scorpion venom extract which acts on calcium-activated and voltage-dependent K^+ channels (BK_{Ca} and K_V respectively), appears to be the endogenous mediator, at least of OP_3 opioid receptor inhibition of neurogenic bronchoconstriction (Stretton *et al*.,

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1992; Miura *et al*., 1992). The K^+ channel mediating OP_1 and OP_3 opioid receptor inhibition of neurogenic mucus secretion has not been determined.

In the present study, we investigated the involvement of K^+ channels in opioid inhibition of neurogenic mucus secretion in ferret trachea *in vitro*. Firstly, we determined the opioid receptor type mediating inhibition of secretion with the OP_1 receptor agonist [D-Pen^{2,5}]enkephalin (DPDPE) (Mosberg *et al*., 1983), the OP_2 receptor agonist U-50,488H (Lahti *et al*., 1982), and the OP_3 opioid receptor agonist [D-Ala^2, N-Me-Phe, Gly-ol^5]enkephalin (DAMGO) (Gillan & Kosterlitz, 1982). We then determined the class of K^+ channel involved in the inhibition by use of the K_{ATP} channel inhibitor glibenclamide (Niki *et al*., 1989; Schmid-Antomarchi *et al*., 1990), the blocker of K_{Ca} channels of small conductance (SK_{Ca} channels) apamin (Banks *et al*., 1979), and the large conductance K_{Ca} (BK_{Ca}) channel blocker iberiotoxin (Galvez *et al*., 1990; Garcia *et al*., 1991). Finally, we investigated the effect on neurogenic secretion of three K^+ channel openers, namely an established K_{ATP} channel opener levcromakalim (formely known as BRL 38227) (Hamilton *et al*., 1993), a new putative K_{ATP} channel opener RS 91309, and the BK_{Ca} channel opener NS 1619 (Olesen, 1994; Olesen *et al*., 1994). We used $^{35}SO_4$ as a marker for mucus (Gashi *et al*., 1987; Davis *et al*., 1990).

Methods

Tissue preparation

Male ferrets (Regal Rabbits, Great Bookham, Surrey) weighing 1.0–2.0 kg were used and were housed 'free range' with free access to food and water. They were killed with pentobarbitone sodium (Sagatal; 60 mg kg^{-1}, i.p.). Our preparation of ferret tracheal segments for examination of neurogenic mucus secretion has been described in detail previously (Ramnarine *et al*., 1994). Briefly, tracheae were excised, cleared of surrounding tissue and bathed in warm (37°C) aerated (95% O_2 + 5% CO_2) Krebs-Henseleit solution of final composition (mM): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_2$ 25.5 and glucose 5.05, pH 7.4 after aeration. Each trachea was cut longitudinally through the smooth muscle band of the dorsal membrane, opened flat and cut transversely into four segments. Each segment was pinned and clamped across the aperture separating the two halves of perspex Ussing-type chambers so that the tissue divided the chamber into a 'luminal' (i.e. mucus producing) and submucosal side. The exposed surface area of each segment was 1.12 cm^2. Each side of the tissue was bathed with 9 ml warmed, oxygenated Krebs-Henseleit solution (see above) which was circulated by use of gas-lift pumps. The tracheal tissue segments could be subjected to a unipolar electrical current to stimulate excitable tissue (for example nerves) via two pairs of pins which pierced the tissue on either side and were connected through outlet wires from the chambers to a pulse generator (Multi-Stim System D330, Digitimer Ltd., Welwyn-Garden-City, Herts.). Stimulation was at 10 Hz, 50 V, 0.5 ms for the first five minutes of a 15 min incubation period. We have previously found that these stimulation parameters are optimal for inducing neurogenic mucus secretion in ferret trachea (Ramnarine *et al*., 1994).

We have previously established the optimal conditions under which collections are taken in order to define baseline secretion and to maximize detection of the secretory response to drug addition and electrical stimulation (Meini *et al*., 1993; Ramnarine *et al*., 1994). In order to label newly-synthesized intracellular mucus, at time 0 h, 0.1 mCi $Na_2{}^{35}SO_4$ (Amersham International plc, Aylesbury, Bucks.) was added to the submucosal half of each chamber, where it remained throughout the duration of the experiment. At unit time intervals, the fluid in the luminal half chamber (containing secretions) was collected and replaced with fresh Krebs-Henseleit solution. Baseline stability of spontaneous output of $^{35}SO_4$-labelled macromolecules was reached 2.5 h after addition of radiolabel, during which time six collections were taken: four after 30 min incubation periods followed by two after 15 min incubation periods. These collections were discarded. After stabilization of secretion, drugs were added and the tissues were electrically- or agonist-stimulated.

Measurement of $^{35}SO_4$-labelled macromolecule secretion

Luminal fluid, approximately 9 ml and comprising secretions in Krebs-Henseleit solution, was collected into tubes containing 5 g guanidine hydrochloride to solubilize the blebs of collected mucus. The final concentration of guanidine hydrochloride in the fluid was 6 M. Following this, each sample was exhaustively dialyzed against distilled water containing excess Na_2SO_4 and sodium azide (10 mg l^{-1}), by transferring the samples from the collection tubes into separate bags of cellulose tubing (Medicell International Ltd., London), which allowed molecules of 12–14 kDa or less to pass through. Sodium azide was used to limit bacterial contamination. Dialysis in this way was carried out in order to displace non-covalently bound sulphate. The samples were recovered after at least six changes of distilled water or until the radioactive count of the water was the same after dialysis as before dialysis (~17 disintegrations per minute (d.p.m.)). The recovered samples were weighed and the remaining radioactivity in 1 ml duplicates of each sample mixed with 2 ml scintillant (Ultima Gold XR, Canberra Packard Ltd., Pangbourne) and determined by scintillation spectrometry (model 1900CA Spectrophotometer, Canberra Packard Ltd.). The total radioactivity of each sample was determined by multiplying the radioactivity present in a 1 ml aliquot of that sample by the total weight of the sample (assuming a 1 ml sample weighs 1 g).

Protocols for secretory studies

To examine the effects of opioid agonists on stimulated mucus secretion, each agonist was applied to the luminal side of the tissue for the 15 min incubation period before the induction of secretion (by electrical stimulation, ACh, or the selective tachykinin receptor agonist [Sar^9, Met(O$_2$)^{11}] substance P (SP); [Sar^9]SP; Regoli *et al*., 1988), where they remained present for the duration of the 15 min stimulation incubation period. Concentrations of opioid agonists (0.1 μM and 10 μM) were near maximal or maximal for inhibition of airway neural contractile responses (Miura *et al*., 1992; Stretton *et al*., 1992). Concentrations of ACh (0.1 μM) or [Sar^9]SP (1 μM) were submaximal for inducing secretion in ferret trachea *in vitro* (Meini *et al*., 1993; Ramnarine *et al*., 1994). For each chamber, two possible pieces of information are obtained, for example the effect of drug on baseline secretion followed by its effect on stimulated secretion. Thus, for one trachea, a possible eight combinations of pieces of information can be gained. Collections for four groups of treatment were obtained, namely: (1) sham stimulation (i.e. collections in the absence of electrical stimulation, but at the same time point as other tissues were electrically-, ACh-, or [Sar^9]SP-stimulated), (2) electrical, ACh or [Sar^9]SP stimulation alone, (3) electrical

stimulation in the presence of opioid agonist, (4) ACh or [Sar^9]SP stimulation in the presence of opioid agonist.

For the studies involving the effects of the K^+ channel openers on stimulus or agonist-evoked secretion, each opener (RS 91309, levcromakalim or NS 1619; each at 3 nM, 30 nM or 3 μM) was added to the luminal half chamber for a 30 min incubation period before the electrical stimulation (i.e. present for two 15 min collection periods). K^+ channel openers remained present in the baths for the duration of the stimulation incubation period. To determine the selectivity of the K^+ channel openers, the K^+ channel inhibitors glibenclamide (3 μM), apamin (0.1 μM) or iberiotoxin (0.1 μM) were added to the luminal half chamber 45 min before stimulation (i.e. 15 min before addition of the opener), and remained present in the baths along with K^+ channel openers for the duration of the stimulation incubation period. Near-maximally effective concentrations of K^+ channel blockers were chosen from data in the literature (see Introduction). Responses to six groups of treatment were obtained, namely: (1) sham-stimulation (see above), (2) electrical stimulation alone, (3) electrical stimulation in the presence of a K^+ channel opener, (4) electrical stimulation in the presence of a K^+ channel blocker, (5) electrical stimulation in the presence of K^+ channel blockers and opener, (6) electrical stimulation in the presence of K^+ channel blocker and opioid agonist.

Drugs and chemicals

The following drugs were used: acetylcholine chloride, apamin, DAMGO, dimethyl sulphoxide (DMSO) and glibenclamide (Sigma Chemical Company Ltd., Poole); naloxone hydrochloride (Du Pont Ltd., Stevenage, Herts.); pentobarbitone sodium B.P. (Sagatal; R.M.B. Animal Health Ltd., Dagenham); [Sar^9, Met(O$_2$)^{11}]SP ([Sar^9]SP) and DPDPE (Bachem U.K. Ltd., Saffron Walden, Essex). U-50,488H (*trans*-(+)-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl) cyclohexyl]-benzeneacetamide methane sulphonate) was a gift from the Upjohn Company (Kalmazoo, U.S.A.). Levcromakalim, RS 91309 (N-[[4-(1,2-dihydro-2-oxo-1-pyridyl)-2,2-dimethyl-6-trifluoromethyl-2H-1-benzopyran-3-yl]methyl]-N-hydroxy-acetamide) and NS 1619 (1-(2′-hydroxy-5′-trifluoromethylphenyl)-5-trifluoromethyl-2(3*H*)benzimidazolone) were gifts from Syntex Research Centre (Edinburgh; courtesy Dr Peter Hicks). Iberiotoxin samples were gifts from Syntex (as above) and Merck Frosst Canada Inc. (courtesy Dr Ian Rodger).

The following drugs were prepared as stock solutions and stored at -20°C: K^+ channel openers, 10 mM in 5% ethanol; [Sar^9]SP, 10 mM in distilled water; glibenclamide, 1 mM in DMSO; apamin and IbTX, 1 mM in distilled water. Drugs were diluted appropriately from stock on each day of experimentation. Concentrations of drugs are final concentrations in the Ussing chambers.

Data analysis

Apart from baseline radioactivity, data in Results are the arithmetic mean and s.e.mean, with n values the number of animals. Data for baseline radioactivity (d.p.m.) are presented as median and range to illustrate the variability in this parameter. Because baseline d.p.m. displayed considerable variability between tracheal segments, responses obtained from individual segments were calculated to give percentage changes in radiolabel output for the differences between response to drug or electrical stimulation and the proceeding collection. Significance of changes in secretion pre- and post-drug or electrical stimulation were assessed by use of the Wilcoxon sign-rank sum test. The significance of differences between groups was assessed by the Mann-Whitney U-test. The null hypothesis was rejected at $P<0.05$ (two-tailed). Inhibition of stimulated secretion by a K^+ channel opener was considered complete when the value for stimulation with activator was significantly different from the value for stimulation without opener and not significantly different from sham-stimulated values.

Results

Median baseline radioactivity in the experiments described below was of the order of 50 d.p.m. (range 10–60 d.p.m.). Median stimulated radioactivity was of the order of 90 d.p.m. (range 40–400 d.p.m.).

Effect of opioid receptor agonists on secretion

In this series of experiments, electrical stimulation resulted in a 30 fold increase in (neurogenic) $^{35}SO_4$ output above sham-stimulated levels (Figure 1). At 10 μM, both DAMGO and DPDPE significantly inhibited neurogenic $^{35}SO_4$ output by 85% and 77%, respectively (Figure 1), whereas at 0.1 μM neither drug gave significant inhibition (mean electrically-stimulated increase of 125±24% in $^{35}SO_4$ output in the presence of DAMGO and 130±21% increase in the presence of DPDPE, compared with 147±38% increase for electrically-stimulated control group). At neither concentration did U50,488H inhibit neurogenic $^{35}SO_4$ output (126±31% at

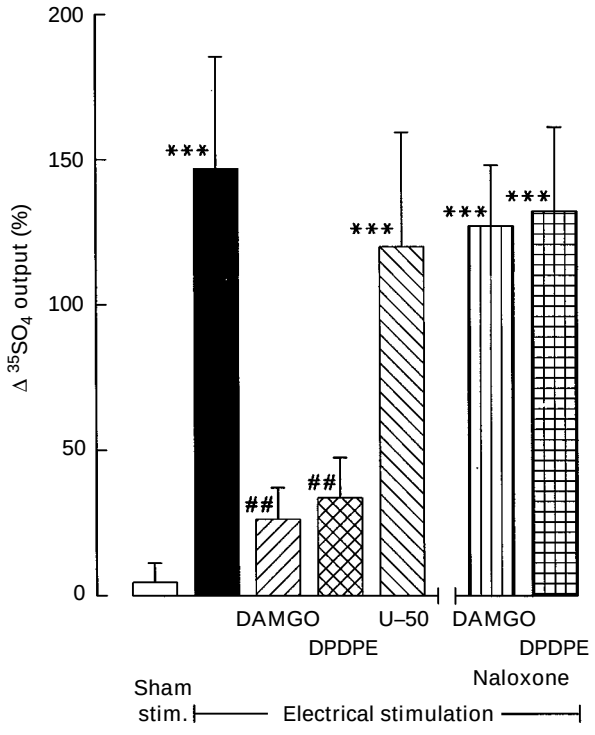

Figure 1 Opioid inhibition of neurogenic mucus secretion of ferret trachea *in vitro*. Open column, sham stimulation; solid column, electrical stimulation (50 V, 10 Hz, 0.5 ms, 5 min); hatched columns, OP_3 opioid receptor agonist DAMGO, OP_1 receptor agonist DPDPE, OP_2 receptor agonist U 50,488H (all at 10 μM) and effect of naloxone on response to DAMGO or DPDPE. Data are mean % changes in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–8 animals per group; vertical lines show one s.e.mean. ***$P<0.001$ compared with sham stimulated group; ##$P<0.01$ compared with electrical stimulation.

0.1 μM; Figure 1 for 10 μM). In these experiments, neither DAMGO, DPDPE nor U50,488H had any significant effect on baseline $^{35}SO_4$ output. In a separate group of animals, naloxone reversed the inhibitions of the neurogenic secretory response observed with DAMGO or DPDPE (Figure 1). Naloxone had no significant effect on the lack of inhibition by U50,488H.

ACh administration resulted in a 32 fold increase in $^{35}SO_4$ output (mean increase of $161 \pm 34\%$, $n=5$, compared with sham stimulated levels of $5 \pm 7\%$, $n=7$; $P<0.01$). This response was unaffected in the presence of either DAMGO, DPDPE or U50,488H (all at 10 μM): $132 \pm 18\%$ increase with DAMGO, $145 \pm 32\%$ with DPDPE, and $178 \pm 29\%$ with U50,488H (none significantly different from ACh-stimulated output). Similarly, DAMGO (10 μM) did not significantly inhibit $^{35}SO_4$ output induced by [Sar^9]SP (1 μM): $143 \pm 28\%$ increase with [Sar^9]SP ($n=5$), a 31 fold increase above sham levels, compared with $102 \pm 15\%$ increase with DAMGO with [Sar^9]SP ($n=9$).

Effect of K^+ channel blockers on opioid-induced inhibition of secretion

In this series of experiments, electrical stimulation resulted in a 22 fold increase in mucus secretion above the sham-stimulated group. Iberiotoxin (3 μM) reversed the inhibitions by DAMGO or DPDPE of neurogenic $^{35}SO_4$ output (Figure 2). Iberiotoxin alone had no significant effect on neurogenic $^{35}SO_4$ output ($188.7 \pm 20.4\%$ increase in output, $n=4$, for stimulation, compared with $191.0 \pm 18.0\%$, $n=4$, for stimulation in the presence of 3 μM iberiotoxin). In contrast to iberiotoxin, neither glibenclamide (0.1 μM; Figure 2) nor apamin (0.1 μM) significantly reversed the inhibitions of secretion by the opioid agonist drugs. With apamin, DAMGO still inhibited neurogenic secretion by 94% ($n=7$, $P<0.01$ compared with stimulated levels) and DPDPE still inhibited secretion by 81% ($n=7$, $P<0.01$ compared with stimulated levels).

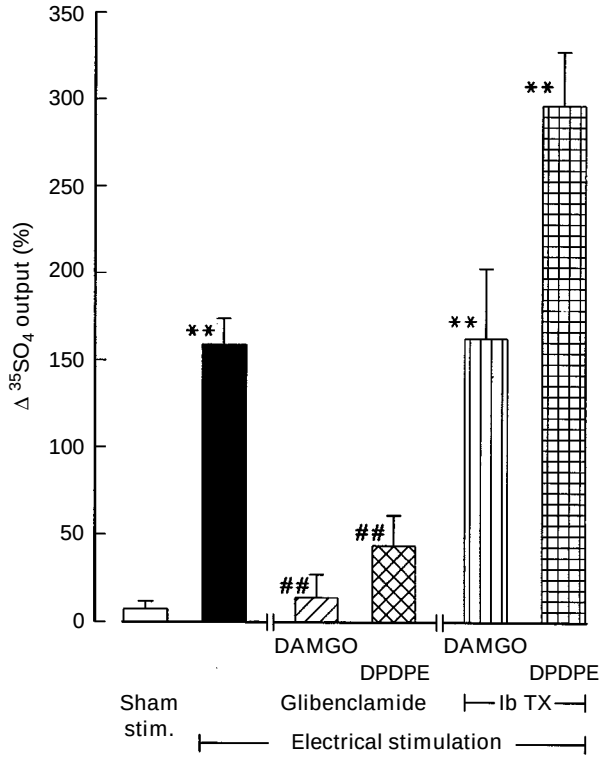

Figure 2 Effect of K^+ channel blockers on opioid inhibition of neurogenic mucus secretion of ferret trachea *in vitro*. Open column, sham stimulation; solid column, electrical stimulation (50 V, 10 Hz, 0.5 ms, 5 min); hatched columns, effect of glibenclamide (0.1 μM) on inhibition by DAMGO (OP_3 opioid receptor agonist) or DPDPE (OP_1 receptor agonist) (both at 10 μM), and effect of iberiotoxin (IbTX, 3 μM) on inhibition by DAMGO or DPDPE. Data are mean % changes in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–7 animals per group; vertical lines show s.e.mean. $^{**}P<0.01$ compared with sham stimulated group; $\#\#P<0.01$ compared with electrical stimulation.

Effect of K^+ channel openers on secretion

In this series of experiments, electrical stimulation resulted in a 38 fold increase in $^{35}SO_4$ output above sham levels (Figure 3). Levcromakalim, RS 91309 and NS 1619 all inhibited neurogenic secretion, although with different potencies and to varying degrees. Levcromakalim (3 nM, 30 nM and 3 μM) inhibited neurogenic secretion only at a concentration of 3 μM (68% inhibition). In contrast, RS 91309 significantly inhibited neurogenic secretion at 30 nM (68% inhibition) and at 3 μM (72% inhibition). Similarly, NS 1619 inhibited neurogenic secretion in a concentration-dependent manner with inhibitions of 82% at 30 nM and 96% at 3 μM (Figure 3). Sham-stimulated secretion changed by $8.1 \pm 3.8\%$ ($n=7$) compared with the previous unstimulated incubation period and neither levcromakalim ($n=4-5$), RS 91309 ($n=4-7$) nor NS 1619 ($n=4-5$) at any of the concentrations tested produced any significant deviation from this value.

Administration of ACh (10 μM) resulted in a 19 fold increase in $^{35}SO_4$ output over control values (Figure 3). Neither levcromakalim nor RS 91309 (3 nM–3 μM, $n=6$ for each drug) had any significant effect on this response. In contrast, although NS 1619 at 3 or 30 nM had no significant effect on ACh-induced secretion, at 3 μM NS 1619 partially inhibited ACh-induced secretion by 60% (Figure 3). In the following studies on the effect of K^+ channel blockers on K^+ opener

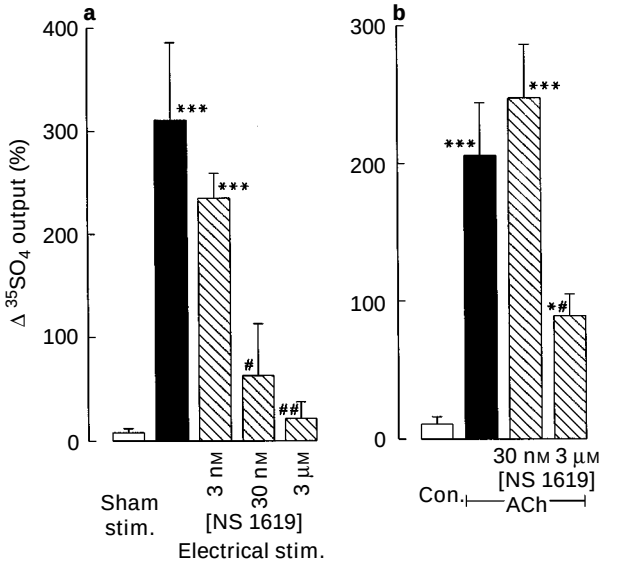

Figure 3 Effect of BK_{Ca} channel activator NS 1619 on neurogenic (a) or acetylcholine (ACh)-induced (b) mucus secretion in ferret trachea *in vitro*. Open columns, sham stimulation or control; solid columns, electrical stimulation (50 V, 10 Hz, 0.5 ms, 5 min) or ACh (10 μM); hatched columns, effect of NS 1619 on stimulated secretion. Data are mean % changes in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–9 animals per group; vertical lines show s.e.mean. $^{***}P<0.001$ compared with sham stimulated group; $\#P<0.05$, $\#\#P<0.01$ compared with electrical stimulation.

inhibition of neurogenic secretion, 3 μM levcromakalim was used (sole effective concentration) and 30 nM RS 91309 was used as the concentration giving effective inhibition of neurogenic secretion. For NS 1619, both 30 nM and 3 μM were used.

Effect of K^+ channel blockers on K^+ channel opener inhibition of secretion

In this series of experiments, electrical stimulation resulted in a 17 fold increase in $^{35}SO_4$ output over basal levels. Levcromakalim (3 μM) inhibited neurogenic secretion by 81%, and this was reversed by glibenclamide (0.1 μM) but not by IbTX (3 μM) (Figure 4). Similarly, RS 91309 (30 nM) resulted in an 82% inhibition of the neurogenic response which was inhibited by glibenclamide (mean 232% increase in secretion above unstimulated levels, comparable to the control stimulation of 267% in the absence of drugs) but was unaffected by IbTX (86% inhibition). In contrast. 30 nM NS 1619 inhibited neurogenic secretion by 86% which was unaffected by glibenclamide but was reversed by IbTX (Figure 5). Inhibition by 3 μM NS 1619 was also blocked by iberiotoxin (3 μM) ($188.7 \pm 20.4\%$ for the simulated $^{35}SO_4$ output, $n=4$; $85.4 \pm 18.7\%$ for stimulation in the presence of NS 1619, $n=5$, $P<0.05$ compared with stimulated value; $195 \pm 31.1\%$ for stimulation in the presence of iberiotoxin and NS 1619, $n=6$, $P<0.05$ compared with value in the presence of NS 1619).

Administration of ACh (0.1 μM) resulted in a mean $245 \pm 83\%$ increase in $^{35}SO_4$ output above control levels ($n=5$, $P<0.05$). The inhibition of this response by 3 μM NS 1619 (see previous section) was inhibited by IbTX (mean $176 \pm 33\%$ increase in secretion, $n=5$: not significantly different from ACh response).

Discussion

In the present study, electrical stimulation of ferret trachea *in vitro* markedly increased $^{35}SO_4$ output which, in this preparation, is a marker for mucus secretion by submucosal glands. The major source of mucus in the preparation is the submucosal glands because ferret trachea has few surface epithelial goblet cells but large numbers of glands (Robinson *et al*., 1986; Meini *et al*., 1993). By autoradiography, under basal conditions there is selective uptake and retention of $^{35}SO_4$ by tracheal submucosal glands, rather than epithelium, in cat (Davies *et al*., 1990) and ferret (Gashi *et al*., 1987), with some labelling of cartilage. Stimulation of ferret trachea *in vitro* with neuromimetic drugs increased radioactive counts in the incubation medium with concomitant loss of autoradiographic grains from the glands, compared with untreated or antagonist-blocked glands (Gashi *et al*., 1987): neither cartilage nor epithelium showed loss of grains. In the cat, tracheal washings labelled with $^{34}SO_4$ have a molecular weight and buoyant density consistent with a typical mucus molecule (Davies *et al*., 1990). Thus, the increased output of $^{35}SO_4$

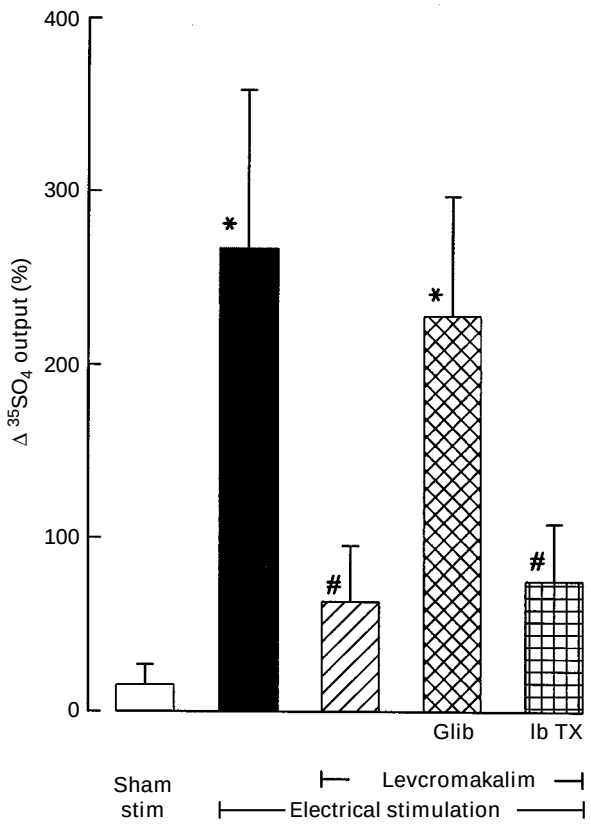

Figure 4 Effect of K^+ channel blockers on levcromakalim inhibition of mucus secretion in ferret trachea *in vitro*. Open column, sham stimulation; solid column, electrical stimulation (50 V, 10 Hz, 0.5 ms, 5 min); hatched columns, inhibition by levcromakalim (3 μM) and effect of glibenclamide (Glib; 0.1 μM) or iberiotoxin (IbTX, 3 μM). Data are mean % changes in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–9 animals per group; vertical lines show s.e.mean. *$P<0.05$ compared with sham stimulated group; #$P<0.05$ compared with control electrical stimulation group.

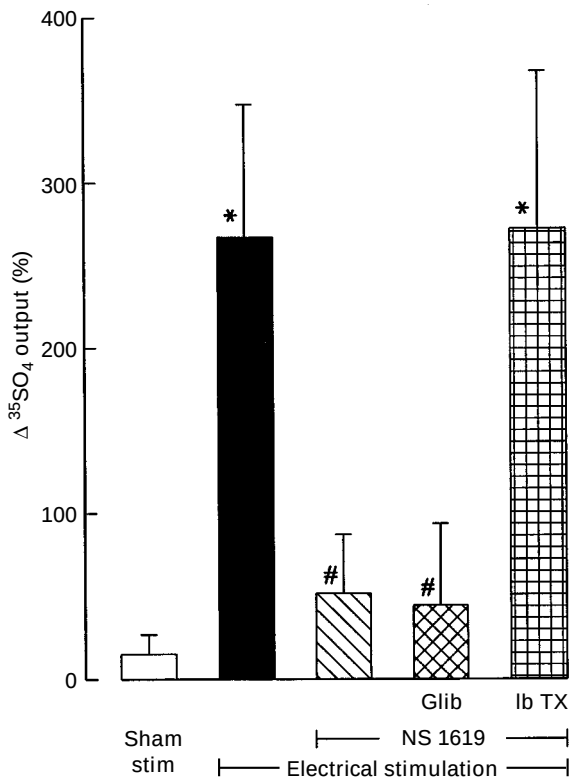

Figure 5 Effect of K^+ channel blockers on NS 1619 inhibition of mucus secretion in ferret trachea *in vitro*. Open column, sham stimulation; solid column, electrical stimulation (50 V, 10 Hz, 0.5 ms, 5 min); hatched columns, inhibition by NS 1619 (30 nM) and effect of glibenclamide (Glib; 0.1 μM) or iberiotoxin (IbTX, 3 μM). Data are mean % changes in output of macromolecules labelled *in situ* with $^{35}SO_4$ (representing mucus) for 5–9 animals per group; vertical lines show s.e.mean. *$P<0.05$ compared with sham stimulated group; #$P<0.05$ compared with control electrical stimulation group.

observed herein is indicative of an increase in tracheal submucosal gland mucus secretion.

We have characterized previously the neural pathways involved in induction of mucus secretion by electrical stimulation of ferret trachea *in vitro* (Ramnarine *et al*., 1994): cholinergic nerves account for approximately 55% of the response and sensory-efferent nerves account for approximately 35% of the response. The identity of the minor remaining neural pathway(s) is unknown, but may comprise adrenergic nerves and/or release of neuropeptides including vasoactive intestinal peptide (VIP) and neuropeptide tyrosine (NPY) (Ramnarine & Rogers, 1994). In any event, tetrodotoxin blocks the complete neurogenic secretory response (Ramnarine *et al*., 1994). Thus, in the present study, activation of nerves, predominantly cholinergic and sensory-efferent combined, accounts for the marked increase in tracheal mucus secretion with electrical stimulation.

We found herein that opioid agonists selective for the OP_1 or OP_3 receptor types inhibited neurogenic mucus secretion. The inhibitions were reversed by naloxone, which indicates that the inhibitory effect of the agonists was via interaction with opioid receptors. Opioid inhibition did not extend to secretion induced by either ACh or [Sar^9]SP. A similar lack of inhibition has been observed for ACh-induced contraction *in vitro* of bronchi of guinea-pig (Belvisi *et al*., 1990) or man (Belvisi *et al*., 1992), and for ACh- or substance P-induced bronchoconstriction *in vivo* (Belvisi *et al*., 1988). The latter observations and our present data indicate that opioid inhibition is at a prejunctional site on the nerves. The neuroregulation may be via inhibition of neurotransmitter release because OP_3 receptors mediate inhibition of release of substance P-like immunoreactivity (SP-LI) from rat trachea (Ray *et al*., 1991) and, in a preliminary study, release of [^{3}H]-choline, a marker for acetylcholine, from guinea-pig and human trachea (Belvisi *et al*., 1993). OP_1 receptors did not appear to be involved in inhibition of neurotransmitter release in either of the latter two studies. Formal release studies in ferret trachea need to be carried out to determine whether OP_1 and OP_3 opioid receptors mediate inhibition of neurotransmitter release from cholinergic and sensory-efferent nerves controlling secretion.

The opioid agonists herein virtually blocked the total neurogenic secretory response, which indicates inhibition of both cholinergic and sensory-efferent nerve activity. Opioid inhibition of cholinergic and sensory-efferent neural activity has been observed previously in the airways, including inhibition of cholinergic bronchoconstriction in guinea-pig and human airways (Belvisi *et al*., 1990; 1992), cholinergic and sensory-efferent induction of neurogenic goblet cell secretion in guinea-pig trachea (Kuo *et al*., 1992a), sensory-efferent induction of plasma exudation in guinea-pig airways (Belvisi *et al*., 1989), and sensory-efferent induced cough and reflex bronchoconstriction in guinea-pigs (Karlsson *et al*., 1990). The involvement of OP_1 or OP_3 receptor types in inhibition in the present study is consistent with our previous observation that both receptor types are involved in inhibition of neurogenic goblet cell secretion in guinea-pig trachea *in vivo* (Kuo *et al*., 1992a). In contrast, inhibition of cholinergic bronchoconstriction in guinea-pigs *in vivo* (Belvisi *et al*., 1988) or sensory-efferent-induced airway smooth muscle contraction *in vitro* (Belvisi *et al*., 1990) is mediated predominantly via the OP_3 receptor. The reasons for the discrepancy are unclear but, from the discussion above, would appear to be related more to differences in the nerves involved in control of airway secretion compared with smooth muscle contraction rather than to a species difference or to differences between *in vitro* and *in vivo* investigation. In the present study, the OP_2 receptor agonist U 50,488H had no significant inhibitory effect on neurogenic mucus secretion, which is consistent with its lack of effect in a number of other *in vitro* and *in vivo* airway preparations (Belvisi *et al*., 1988; 1990; Kuo *et al*., 1992a), and in inhibiting SP-LI release from rat trachea (Ray *et al*., 1991). However, it should be noted that, at similar concentrations to those used here, U 50,488H inhibits substance P release from cultured primary sensory neurones (Chang *et al*., 1989), and reduces neurogenic plasma exudation (Cox, 1988). In addition, circumstantial evidence indicates that inhibition of cough and reflex bronchoconstriction in guinea-pigs is mediated via OP_2 and OP_3 receptors (Karlsson *et al*., 1990). Thus, the results indicate that for opioid inhibition in the airways, the OP_3 receptor is ubiquitous and that the involvement of other receptors, and their type, is dependent upon the experimental system being used.

Opioid receptors are coupled, through G-proteins, to K^+ channels (North *et al*., 1987; Ikeda *et al*., 1995). In the present study, we found that the inhibitory effect on neurogenic secretion of the OP_1 and OP_3 receptor agonists was reversed by the selective BK_{Ca} channel blocker iberiotoxin (Galvez *et al*., 1990; Garcia *et al*., 1991; Vantapour & Harvey, 1995). Inhibition was not reversed by the SK_{Ca} channel blocker apamin (Banks *et al*., 1979) or the K_{ATP} channel inhibitor glibenclamide (Niki *et al*., 1989; Schmid-Antomarchi *et al*., 1990). These data indicate that the inhibitory effects of OP_1 and OP_3 receptors in ferret trachea are mediated via BK_{Ca} channels. However, since electrical stimulation of neurones increases intracellular calcium, BK_{Ca} channels would be anticipated to open and dampen the response. Inhibition of these channels could produce a functional antagonism of any inhibitory response, irrespective of its underlying mechanism. This is unlikely to be the case in the present study because iberiotoxin alone did not affect the neurogenic secretory response. The lack of inhibition by apamin or glibenclamide of opioid-inhibition of neurogenic secretion is unlikely to be due to use of too high concentrations of opioids because the concentrations used were not supramaximal and both naloxone and iberiotoxin effectively blocked their inhibitory effects (see above). In addition, levcromakalim effectively inhibited a marked neurogenic secretory response, and this was inhibited by glibenclamide. Thus, the concentration (0.1 μM) of glibenclamide used in the present study is effective against marked inhibition of the secretory response.

The suggestion that BK_{Ca} channels mediate the OP_1 and OP_3 receptor-inhibition of neurogenic mucus secretion observed herein extends previous observations that charybdotoxin reverses opioid inhibition of cholinergic airway contraction in guinea-pig, human and dog airways (Miura *et al*., 1992; Tagaya *et al*., 1995) and of sensory-efferent neural contraction of guinea-pig bronchi (Stretton *et al*., 1992). Charybdotoxin is a blocker of BK_{Ca} channels (Miller *et al*., 1985) which has activity at other K^+ channels, for example other K_{Ca} channels and K_V channels (Deutsch *et al*., 1991; Garcia *et al*, 1991; Vantapour & Harvey, 1995). Our data here with iberiotoxin indicate that opioid inhibition of neurogenic airway contraction (Miura *et al*., 1992; Stretton *et al*., 1992; Tagaya *et al*., 1995) was via activation of BK_{Ca} channels.

Our present data, discussed above, indicate that opening of BK_{Ca} channels rather than SK_{Ca} or K_{ATP} channels is the endogenous mechanism for OP_1 and OP_3 opioid receptor inhibition of neurogenic mucus secretion in ferret trachea *in vitro*. Consistent with this was our observation that the BK_{Ca} channel opener NS 1619 (Olesen, 1994; Olesen *et al*., 1994) inhibited neurogenic mucus secretion. NS 1619 has also been

shown to inhibit evoked firing of airway nerve fibres and neurogenic bronchoconstriction and cough; responses reversed by iberiotoxin (Fox *et al*., 1997). Although NS 1619 opens BK_{Ca} channels in a number of smooth muscle and neuronal preparations, it also inhibits L-type calcium channels (Edwards *et al*., 1994; Sellers & Ashford, 1994; Macmillan *et al*., 1995; Holland *et al*., 1996). Concentrations of NS 1619 which have been demonstrated to open native or cloned BK_{Ca} channels are in the μM range (usually 3–30 μM; Olesen, 1994; Dworetzky *et al*., 1996). In the present study, the inhibition of neurogenic mucus secretion by a low concentration of NS 1619 may be an indication that inhibition is not due to an action on BK_{Ca} channels but rather on neuronal calcium channels. Since the latter channels are voltage-sensitive, functional antagonism by iberiotoxin could occur since iberiotoxin would depolarize the neuron and stimulate the opening of additional voltage-sensitive calcium channels. However, functional antagonism may not be involved in the effects of iberiotoxin in the present study because it blocked the inhibitory effects of NS 1619 at all concentrations tested, including the concentration of 3 μM which opens BK_{Ca} channels (see above). In addition, inhibition here by NS 1619 of tracheal neurogenic secretion was not blocked by glibenclamide which precludes a non-selective action of NS 1619 on K_{ATP} channels.

In addition to the inhibitory effect of NS 1619, we found in the present study that the channel K_{ATP} openers levcromakalim and RS 91309 also inhibited neurogenic mucus secretion. Levcromakalim (formerly BRL 38227) is the active enantiomer of the racemate cromakalim which is an established K_{ATP} channel opener (Hamilton *et al*., 1993). Inhibition herein by levcromakalim or RS 91309 of neurogenic mucus secretion was reversed by glibenclamide but not by iberiotoxin, which is consistent with an action of these two drugs on K_{ATP} channels in ferret trachea. K_{ATP} channel opener drugs have been shown previously to inhibit a number of airway neurogenic responses, including bronchoconstriction and goblet cell secretion (Ichinose & Barnes, 1990; Kuo *et al*., 1992b).

In the present study, we found that NS 1619, at a concentration of 3 μM, but not at the lower concentrations tested, inhibited secretion induced by ACh and that the effect was reversed by iberiotoxin. This observation suggests that there are BK_{Ca} channels on the mucus secretory cells of the ferret trachea which, when activated, inhibit secretion. The mechanism of the inhibition is unexplored in the present study, but may be related to the mechanisms underlying inhibition of secretion of neurotransmitter(s) by nerve cells. In contrast, neither levcromakalim nor RS 91309 inhibited ACh-induced secretion. This is consistent with the observation that levcromakalim had no effect on ACh- or SP-induced goblet cell secretion in guinea-pig trachea *in vivo* (Kuo *et al*., 1992b). However, it is in contrast to the inhibition by levcromakalim and other K_{ATP} channel openers of sustained secretion of lysozyme from submucosal gland serous cells in the *in vitro* ferret whole trachea preparation (Griffin, 1995). It would be of interest to determine whether different classes of K^+ channel have a different distribution over serous and mucous gland cells and regulate different aspects of the airway secretory process.

In summary, we have shown that, in ferret trachea *in vitro*, opioid drugs which interact with OP_1 or OP_3 receptors inhibit neurogenic mucus secretion at a prejunctional site and that the mechanism of the inhibition is probably due to the opening of BK_{Ca} channels. Similarly, drugs which directly open BK_{Ca} channels or K_{ATP} channels also inhibit neurogenic mucus secretion. In addition, opening of BK_{Ca} channels inhibits ACh-evoked secretion of mucus. Thus, drugs which open BK_{Ca} channels may have therapeutic anti-secretory activity in bronchial diseases in which neurogenic mechanisms and mucus hypersecretion are implicated in pathophysiology, for example in asthma and chronic bronchitis (Rogers, 1996).

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