British Journal of Pharmacology (1996) 119, 91-98

Adrenoceptor- and cholinoceptor-mediated mechanisms in the regulation of 5-hydroxytryptamine release from isolated tracheae of newborn rabbits

Anke Freitag, *Ignaz Wessler & [1]Kurt Racké

Institute of Pharmacology and Toxicology, University of Bonn, Reuterstr. 2b, D-53113 Bonn and *Department of Pharmacology, University of Mainz, Obere Zahlbacher Str. 67, D-55101 Mainz, Germany

1 Isolated tracheae of newborn rabbits were incubated *in vitro* and the outflow of 5-hydroxytryptamine (5-HT) was determined by h.p.l.c. with electrochemical detection. Evidence has previously been provided that this 5-HT outflow derives from neuroendocrine epithelial (NEE) cells of the airway mucosa.

2 Phenylephrine (1, 10 and 30 μM) enhanced the outflow of 5-HT by 80, 290 and 205%, respectively. 5-HT outflow evoked by 10 μM phenylephrine was not affected by the presence of the neurotoxin tetrodotoxin (1 μM).

3 Rauwolscine, ARC 239 (an α_{2B}-adrenoceptor preferring antagonist), yohimbine and prazosin antagonized the effect of 10 μM phenylephrine in a concentration-dependent manner with IC_{50} values of 150, 295, 300 and 1,700 nM, respectively. Comparison of the ratios (between all antagonists) of the present IC_{50} values with the corresponding ratios of K_i values obtained in binding studies for the α_{2A}-, α_{2B}-, α_{2C}- and α_{2D}-adrenoceptor subtypes strongly suggests the involvement of an α_{2B}-receptor.

4 5-HT outflow evoked by 10 μM phenylephrine was inhibited by 65% in the presence of 1 μM forskolin and abolished in the presence of 10 μM forskolin.

5 5-HT outflow evoked by 10 μM phenylephrine was inhibited by about 45 and 70% in the presence of 0.1 and 1 μM isoprenaline, respectively. The inhibitory effect of 1 μM isoprenaline was only marginally antagonized by 1 μM, but blocked by 10 μM propranolol.

6 5-HT outflow was not affected by the muscarine receptor agonist oxotremorine (10 μM), but was enhanced by 175% by 100 μM nicotine. The effect of nicotine was blocked by 100 μM hexamethonium and prevented by 1 μM tetrodotoxin or 1 μM yohimbine.

7 In conclusion, 5-HT release from NEE cells of the rabbit trachea is stimulated via α-adrenoceptors most likely of the α_{2B}-subtype localized directly at the NEE cells. Activation of β-adrenoceptors as well as direct activation of adenylyl cyclase by forskolin exert inhibitory effects on 5-HT release. Activation of nicotinic, but not of muscarinic receptors, also evokes the release of 5-HT. However, the effect of nicotine appears to be mediated indirectly via the release of noradrenaline.

Keywords: Trachea; airway mucosa; neuroendocrine epithelial cells; 5-HT secretion; α_{2B}-adrenoceptors; β-adrenoceptors; forskolin; adenylyl cyclase; nicotinic receptors

Introduction

The airway mucosa of different mammals contains a system of specialized epithelial cells which express a number of neuroendocrine markers, such as neurone-specific enolase, synaptophysin or chromogranins (e.g. Lee *et al.*, 1987; Balaguer & Romano, 1991; Adriaensen & Scheuermann, 1993; Bousbaa *et al.*, 1994). So far, these neuroendocrine epithelial (NEE) cells have been characterized primarily by morphological aspects and by their content of various peptides and amines (e.g Lauweryns *et al.*, 1972; 1982; Cutz *et al.*, 1975; 1981; Track & Cutz, 1982; Polak & Bloom, 1982; Scheuermann, 1987; Adriaensen & Scheuermann, 1993). 5-Hydroxytryptamine (5-HT) is an important amine in the NEE cells (Dey *et al.*, 1981; Lauweryns *et al.*, 1973; 1982) where it appears to be stored in dense core vesicles (i.e. secretory granules of the NEE cells) (Owman *et al.*, 1973; Cutz *et al.*, 1975; Scheuermann, 1987; Adriaensen & Scheuermann, 1993). 5-HT together with the different secretory peptides may be released from the NEE cells in response to specific, at present however, largely unknown stimuli.

Morphological studies demonstrating various nerve endings in the airway mucosa and in close proximity to NEE cells (see Adriaensen & Scheuermann, 1993; Jeffery, 1994) suggest that intrinsic neurotransmitters might be involved in the control of NEE cells. There is some evidence that cholinergic, nicotinic mechanisms may play a role in the regulation of the secretory activity of NEE cells (e.g. Lauweryns *et al.*, 1977; 1987; Tabassian *et al.*, 1990; Nylen *et al.*, 1990; 1993). In addition, NEE cells may be a target for local, paracrine mediators and also for various inhaled agents.

Very recently, we obtained evidence that the outflow of 5-HT from rabbit isolated tracheae reflects secretion from NEE cells (Freitag *et al.*, 1995). In agreement with the morphological observations that the density of NEE cells is particularly high during the perinatal period (Redick & Hung, 1984; Cho *et al.*, 1989), 5-HT outflow from tracheae of newborn animals was higher than that from tissue of adults. Moreover, it was observed that adrenaline, via activation of α-adrenoceptors, enhanced the outflow of 5-HT providing further evidence that receptor mediated control mechanisms may indeed be of significance in the modulation of the secretory activity of NEE.

In the present experiments the outflow of 5-HT from isolated tracheae of newborn rabbits was used as an *in vitro* model to study possible regulatory mechanisms which might play a role in the control of NEE cells. The aims of the present experiments were to characterize further the above described α-adrenoceptor-mediated stimulation of 5-HT release and to test whether β-adrenoceptors and/or muscarinic or nicotinic cholinoceptors may also participate in the regulation of 5-HT release from these NEE cells.

Preliminary accounts of part of the present results have been given (Freitag *et al.*, 1996a,b).

[1] Author for correspondence.

Methods

Animals

Newborn mongrel rabbits of either sex were used within 24 h after birth. Most of the animals were from Lammers (Euskirchen, Germany) except those for one series which were from Koch (Edingen, Germany) and this is indicated. Animals were treated humanly and the present study was approved by local authorities (Ordnungsamt Bonn).

Preparation and incubation of the tracheae

The animals were killed by stunning followed by exsanguination. The whole tracheae were dissected and freed from connective tissue. Single preparations were fixed in a glass organ bath and then incubated in 1.4 ml Krebs-HEPES solution of the following composition (mM): NaCl 118.5, KCl 5.7, $CaCl_2$ 1.25, $MgCl_2$ 1.2, Na_2EDTA 0.03, (+)ascorbic acid 0.06, HEPES 20.0 (adjusted to pH 7.4 using NaOH) and D-glucose 11.1. The medium was kept at 37°C and continuously gassed with 100% O_2. The bath fluid was changed every 10 min. The medium was collected in plastic tubes which contained 50 µl of 57 mM (+)ascorbic acid, 50 µl of 10 mM EDTA and 100 µl of 1 M perchloric acid (suprapure) to protect released 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) from spontaneous non-enzymatic degradation.

At the end of the incubation each preparation was blotted, weighed and extracted in 1 ml of 0.4 M $HClO_4$ for 2 h at 0–4°C. The supernatants were stored at 0–4°C and analysed within 3 days. No significant loss of 5-HT or 5-HIAA occurred within that time period.

Measurement of 5-HT and 5-HIAA

5-HT and 5-HIAA were measured by high pressure liquid chromatography (h.p.l.c.) with electrochemical detection as described previously (Schwörer *et al.*, 1987). The separation of 5-HT and 5-HIAA was achieved by a reverse phase column (length 250 mm, inner diameter 4.6 mm, prepacked with Shadon ODS-Hypersil, 5 µm) with a mobile phase of 0.1 M phosphate buffer (adjusted to pH 3.0), containing octane sulphonic acid sodium salt (160 mg l^{-1}), sodium EDTA (0.3 mM) and methanol (12%, v/v) with retention times of about 21 and 15 min, respectively. Quantitation was achieved with an electrochemical detector (Gynkothek M20) equipped with a glass carbon working electrode and an Ag/Ag reference electrode. The potential was set to +0.72 V. Portions of 200 µl of the incubation media or tissue extracts were directly injected into the h.p.l.c. column. The limit of detection was between 20 and 60 fmol for 5-HT and between 15 and 25 fmol for 5-HIAA per injection.

Calculations and statistical analysis

The outflow of 5-HT and 5-HIAA is expressed as pmol per g wet weight of tissue and per collection period (pmol g^{-1}, 10 min^{-1}) or as a percentage of the mean outflow observed from 40 to 50 min or from 50 to 60 min of incubation (depending on the experimental protocol, compare Figures 1 and 4) of the individual experiments (= *'initial outflow'*). The stimulation evoked outflow of 5-HT was calculated by summing the 5-HT outflow that exceeded the basal outflow. The respective basal outflow was extrapolated from the individual initial outflow assuming that the fractional decline would be similar to that observed in control experiments. In interaction experiments 5-HT outflow was expressed as percentage of the mean increase in 5-HT outflow evoked by the respective stimulus (phenylephrine or nicotine) in the absence of any further drug. Mean values are given ± s.e.mean of n experiments. The significance of differences was evaluated by ANOVA followed by Student's t test, by the computer programme Instat. When multiple comparisons were carried out, Bonferoni's correction was applied. Correlation coefficients and their statistical significance were also calculated by use of Instat.

Drugs and special chemicals

ARC 239 (2-(2,4-(O-methoxy-phenyl)-piperazine-1-yl)-ethyl-4,4-dimethyl-1,3-(2H,4H)-isoquinolindione, gift from Thomae, Biberach, Germany); forskolin (gift from Hoechst, Frankfurt, Germany); hexamethonium bromide (Serva, Heidelberg, Germany); isoprenaline hydrochloride (Boehringer Ingelheim, Germany); nicotine bitartrate, oxotremorine sesquifumarate, phenylephrine hydrochloride; (±)-propranolol hydrochloride, yohimbine hydrochloride (all Sigma, Deisenhofen, Germany); rauwolscine hydrochloride (Roth, Karlsruhe, Germany); prazosin hydrochloride (Pfizer, Karlsruhe, Germany); tetrodotoxin (Novabiochem, Bad Soden, Germany). Drugs were dissolved in distilled water, 1 mM HCl (phenylephrine, rauwolscine, yohimbine) or ethanol (75% or 100%) (forskolin, prazosin, tetrodotoxin). Ethanol alone at the maximum concentration (0.1%) did not affect spontaneous or phenylephrine-evoked 5-HT outflow.

Results

Spontaneous outflow and tissue levels of 5-HT and 5-HIAA

The mean spontaneous outflow of 5-HT from mucosa-containing tracheae of newborn rabbits in the absence of drugs (measured between 50 and 60 min of incubation) was 15.7 ± 1.1 and that of 5-HIAA 18.1 ± 1.5 pmol g^{-1} 10 min^{-1} (n = 61). Similar to previous observation (Freitag *et al.*, 1995), in control experiments the outflow of 5-HT (Figure 1) and that of 5-HIAA (not shown) continuously declined during the observation period. As the outflow of 5-HIAA changed only marginally in response to the various treatments described below the data on 5-HIAA outflow area are not documented in detail.

Tissue 5-HT at the end of incubation amounted to 641 ± 21 pmol g^{-1} and tissue 5-HIAA to 24 ± 1 pmol g^{-1} (n = 160). As none of the treatments caused significant effects on these tissue levels, pooled values are given.

α-Adrenoceptor-mediated stimulation of 5-HT outflow

Phenylephrine induced a marked, transient rise in the outflow of 5-HT (Figure 1), and the concentration-dependency of this

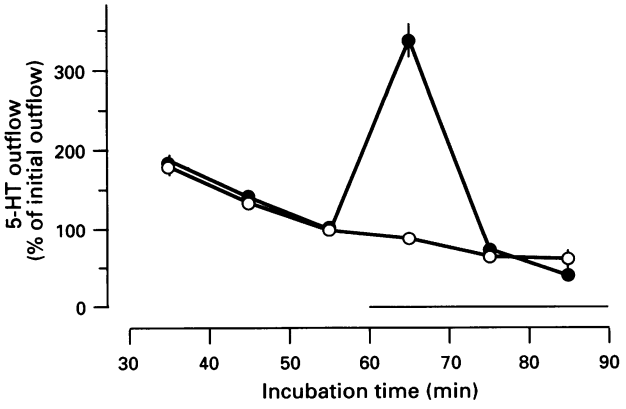

Figure 1 Effects of 10 µM phenylephrine (●) on the outflow of 5-HT from isolated tracheae of newborn rabbits. Phenylephrine was present from 60 to 90 min of incubation, as indicated by the horizontal bar. Ordinate scale: outflow of 5-HT, expressed as % of the initial outflow (50–60 min of incubation) in the respective individual experiment. Means of 7 (control, ○) or 22 (●) experiments are shown; vertical lines indicate s.e.mean.

effect is shown in Figure 2. A rise in 5-HT outflow by 80 and 290% was observed at 1 and 10 μM phenylephrine, respectively. However, after increasing the concentration of phenylephrine to 30 μM, the stimulating effect tended to decrease again, resulting in a bell-shaped concentration-response curve. In absolute terms, 5-HT released by 10 μM phenylephrine amounted to 32 ± 3.4 pmol g^{-1} ($n = 22$). The stimulating effect of 10 μM phenylephrine was antagonized by rauwolscine, ARC 239, yohimbine and prazosin in a concentration-dependent manner (Figure 3) with IC_{50} values of 150, 295, 300 and 1700 nM, respectively (Table 1). When the neurotoxin tetrodotoxin (1 μM) was present, the stimulating effect of 10 μM phenylephrine was not significantly affected (Figure 8).

β-Adrenoceptor-mediated inhibition of 5-HT outflow and role of cyclic AMP

Isoprenaline added 10 min before the phenylephrine (10 μM) stimulus reduced the evoked increase in 5-HT outflow in a concentration-dependent manner, by 46 and 72% at 0.1 and 1 μM, respectively (Figures 4 and 5). When isoprenaline was tested at a concentration of 10 μM, an impurity present in the isoprenaline solution produced a peak in the chromatogram close to 5-HT making it difficult to read exactly small 5-HT peaks. Therefore, it should only be mentioned that 10 μM isoprenaline 'appeared' to suppress almost completely the phenylephrine-evoked 5-HT outflow. The inhibitory effect of 1 μM isoprenaline was antagonized by propranolol in a concentration-dependent manner, but relatively high concentrations of propanolol were required (Figure 5). Isoprenaline also reduced the spontaneous outflow of 5-HT, maximally by about 40% (see Figure 4). However, as in other experiments the spontaneous outflow of 5-HT was close to the detection limit, inhibitory effects on spontaneous outflow are difficult to describe quantitatively. Therefore, effects on spontaneous outflow are not documented in detail.

Forskolin added 10 min before the phenylephrine (10 μM) stimulus also inhibited the evoked increase in 5-HT outflow, by 67% at 1 μM and completely at 10 μM (Figure 6). With the limitations just mentioned for effects on spontaneous outflow, forskolin also reduced the spontaneous outflow, to a similar extent as did isoprenaline (not shown).

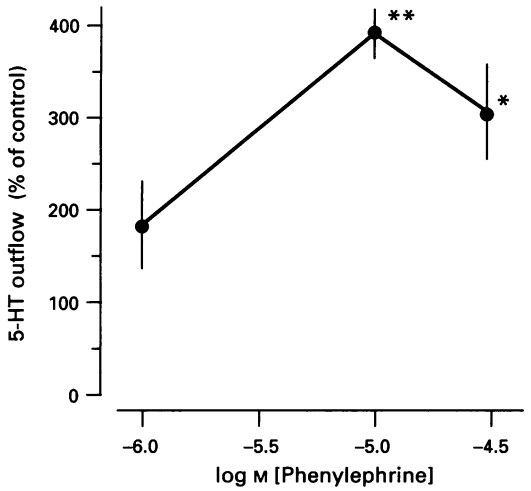

Figure 2 Concentration-dependent effects of phenylephrine on the outflow of 5-HT from isolated tracheae of newborn rabbits. Tracheae were incubated as described in Figure 1 and phenylephrine was added at the concentrations indicated on the abscissa scale. Ordinate scale: peak outflow of 5-HT, expressed as % of the mean basal outflow in control experiments (60–70 min of incubation, 89 ± 4% of initial outflow) in the absence of drugs. Means ± s.e.mean (vertical lines) of 4–22 experiments are shown. Significance of difference from the initial (= predrug) outflow: $*P < 0.05$; $**P < 0.01$.

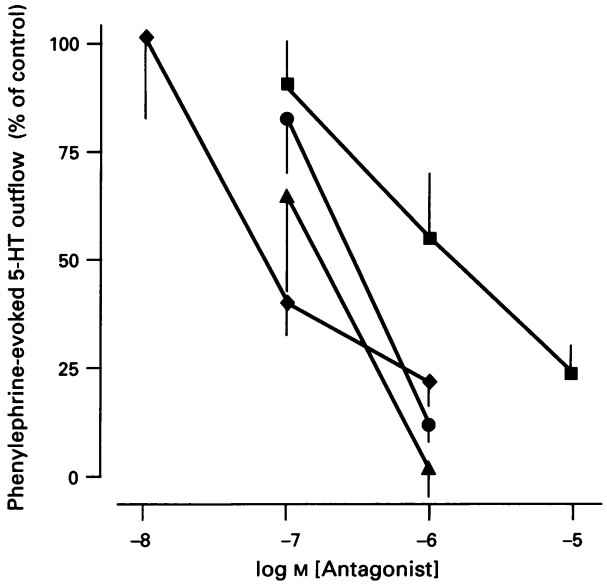

Figure 3 Concentration-dependent effects of ARC 239 (◆), rauwolscine (▲), yohimbine (●) or prazosin (■) on the outflow of 5-HT from isolated tracheae of newborn rabbits evoked by 10 μM phenylephrine. Tracheae were incubated as described in Figure 1, except that the antagonists at the concentrations indicated on the abscissa scale were present 60 min before phenylephrine. Ordinate scale: peak outflow of 5-HT, expressed as % of the mean peak outflow (60–70 min of incubation) evoked by 10 μM phenylephrine alone (control). Means ± s.e.mean (vertical lines) of 4–17 experiments are shown.

Table 1 Comparison of the IC_{50} values of rauwolscine, ARC 239, yohimbine and prazosin against the phenylephrine-evoked 5-HT outflow from neuroendocrine epithelial (NEE) cells in rabbit trachea with K_i values of these antagonists determined in radioligand binding studies

Antagonist	IC_{50} (nM) *NEE cells*	K_i (nM) α_{2A}	α_{2B}	α_{2C}	α_{2D}
Rauwolscine	150	10.9 ± 4.1	3.04 ± 0.92	0.61 ± 0.16	14.2 ± 3.5
ARC 239	295	615 ± 173	18.3 ± 11.4	48.9 ± 16.4	284 ± 79
Yohimbine	300	13.4 ± 5.1	4.82 ± 1.18	1.20 ± 0.32	11.8 ± 2.8
Prazosin	1700	1086 ± 202	52.5 ± 11.9	58.2 ± 11.9	376 ± 128
Correlation coefficient (r)		0.86	0.97*	0.70	0.76

Means ± s.e.mean of 6–26 values taken from the references given below. Signifcance of correlation between IC_{50} and K_i: $*P < 0.05$. Blaxall *et al.*, 1991; Bylund *et al.*, 1988; 1992; 1994; Cheung *et al.*, 1982; Devedijan *et al.*, 1994; Gleason & Hieble 1992; Laftipour *et al.*, 1982; Link *et al.*, 1992; MacKinnon *et al.*, 1992; Renouard *et al.*, 1994; Ruffolo *et al.*, 1991; 1993; Simonneaux *et al.*, 1991; Uhlen *et al.*, 1992; 1994; Weinshank *et al.*, 1990.

Cholinoceptor-mediated effects on 5-HT outflow

The muscarinic receptor agonist oxotremorine (10 μM) had no effect on 5-HT outflow (Figure 7). On the other hand, 5-HT outflow was increased by 175% by 100 μM nicotine, but not affected by 10 μM nicotine. The stimulating effect of 100 μM nicotine was blocked by 100 μM hexamethonium. In contrast to the stimulating effect of phenylephrine, that of nicotine was blocked in the presence of 1 μM tetrodotoxin (Figure 8). Most interestingly, yohimbine (1 μM) also prevented the stimulating effect of 100 μM nicotine (Figure 8).

However, it should be noted that in one series of experiments, for which the animals were supplied by another farm (Koch, Edingen), 100 μM nicotine caused only a marginal increase in 5-HT outflow (25.6±13.0%, $n=15$, $P>0.2$), although in this series phenylephrine evoked an outflow of 5-HT similar to that described above (data not shown).

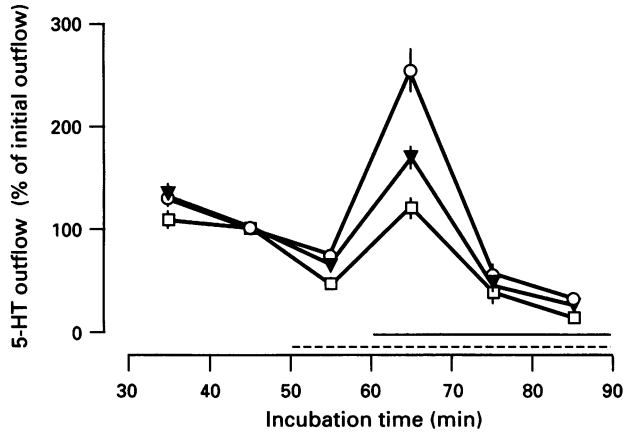

Figure 4 Effects of 10 μM phenylephrine, in the absence (○, $n=22$) or presence of isoprenaline (0.1 μM, ▲, $n=5$; or 1 μM, □, $n=6$) on the outflow of 5-HT from isolated tracheae of newborn rabbits. Phenylephrine was present from 60 to 90 min of incubation as indicated by the horizontal bar, isoprenaline was present from 50 to 90 min of incubation as indicated by the horizontal, dotted line. Ordinate scale: outflow of 5-HT, expressed as % of the initial outflow (40–50 min of incubation) in the respective individual experiment. Means±s.e.mean (vertical lines) of n experiments are shown.

Discussion

Previous experiments have shown that the *in vitro*, incubated isolated trachea of the rabbit, and particularly that of newborn animals, may be a useful preparation to study 5-HT release from airway NEE cells (Freitag *et al.*, 1995). Thus, it was shown that 5-HT outflow from that preparation depended on an intact mucosa and could be evoked by a depolarizing stimulus in a calcium-dependent manner, indicating, together with additional observations, that 5-HT outflow may reflect 5-HT secretion from NEE cells. It was further observed that adrenaline enhanced the outflow of 5-HT, an effect antagonized by phentolamine and prazosin, but not by propranolol which rather tended to enhance the stimulating effect of adrenaline (Freitag *et al.*, 1995).

Stimulant α_{2B}-adrenoceptors

In the present experiments another α-adrenoceptor agonist, phenylephrine effectively stimulated the outflow of 5-HT. The effect of phenylephrine remained unaffected in the presence of tetrodotoxin, a neurotoxin which blocks propagated neuronal activity (Catterall, 1980), excluding an indirect, neuronally mediated action and supporting the idea that stimulant α-adrenoceptors may be localized directly at the NEE cells. A

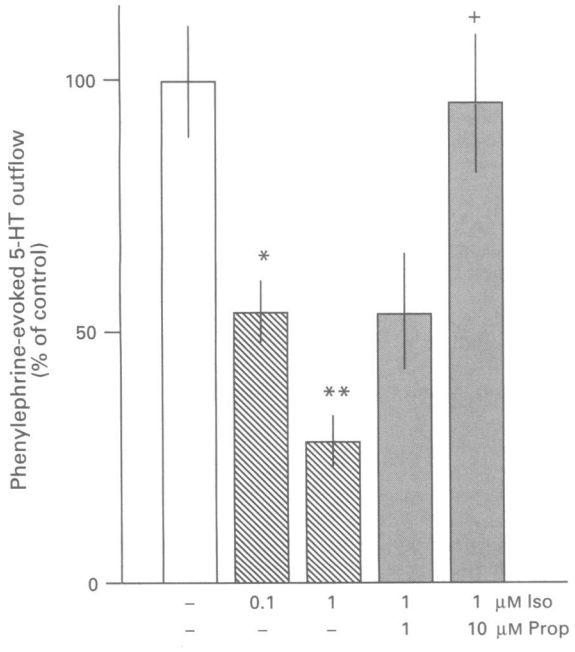

Figure 5 Concentration-dependent effects of isoprenaline (Iso) alone or in the presence of propranolol (Prop) on the outflow of 5-HT from isolated tracheae of newborn rabbits evoked by 10 μM phenylephrine. Tracheae were incubated and phenylephrine and isoprenaline were added as described in Figure 4. Propranolol was present from the onset of incubation. Height of columns: peak outflow of 5-HT, expressed as % of the mean peak outflow (60–70 min of incubation) evoked by 10 μM phenylephrine alone (control, open column). Means±s.e.mean of 5–22 experiments are shown. Significance of differences; $^{*}P<0.05$; $^{**}P<0.01$ from the control; $^{+}P<0.01$ from Iso 1 μM alone.

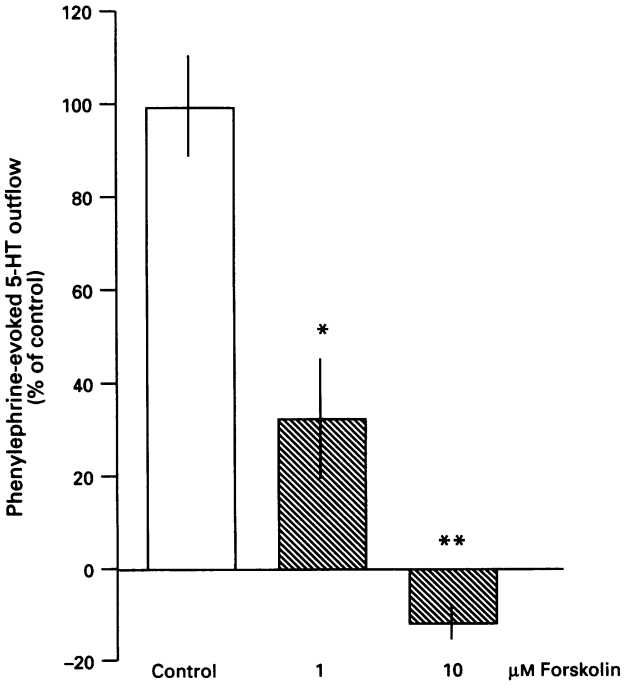

Figure 6 Effects of forskolin (at the concentrations indicated) on the outflow of 5-HT from isolated tracheae of newborn rabbits evoked by 10 μM phenylephrine. Tracheae were incubated as described in Figure 4 and forskolin was present 10 min before phenylephrine (as shown in Figure 4 for isoprenaline). Height of columns: peak outflow of 5-HT, expressed as % of mean peak outflow (60–70 min of incubation) evoked by 10 μM phenylephrine alone (control). Means ±s.e.mean of 4–22 experiments are shown. Significance of differences from the control: $^{*}P<0.05$; $^{**}P<0.01$.

non-neuronal, paracrine mechanism cannot, however, be excluded. Although phenylephrine is generally considered to be an α_1-adrenoceptor-selective agonist (see Hoffman & Lefkowitz, 1990), the present observation that the effect of phenylephrine was not only antagonized by prazosin, but also, and with even higher potencies, by the α_2-adrenoceptor selective antagonists rauwolscine and yohimbine (Figure 3 and Table 1), indicated that α_2-adrenoceptors mediated that stimulating effect on 5-HT secretion from NEE cells.

Currently, four subtypes of α_2-adrenoceptors, α_{2A}-, α_{2B}-, α_{2C}- and α_{2D}-receptors, can be differentiated by pharmacological and molecular biological techniques (Lomasney *et al.*, 1991; Bylund *et al.*, 1994). At the different α_2-adrenoceptor subtypes, prazosin shows a relatively high affinity for the α_{2B}- and α_{2C}-receptors. When the IC_{50} values of rauwolsine, ARC 239, yohimbine and prazosin obtained in the present experiments were correlated with the respective K_i values for the different α_2-adrenoceptor subtypes (given in Table 1), a significant correlation was obtained only for the α_{2B}-receptor ($r=0.97$, $P<0.05$). The availability of subtype selective antagonists useful for the characterization of α_2-adrenoceptor subtypes in functional experiments is still limited and particularly the pharmacological discrimination between α_{2B}- and α_{2C}-receptors is difficult. ARC 239 shows some preference for the α_{2B}-receptor (Bylund *et al.*, 1988; 1992) and its relatively high potency in the present experiments is in support of an α_{2B}-receptor. The comparison of ratios of K_i values of α_2-adrenoceptor antagonists obtained in radioligand binding studies with the respective ratios of functionally determined antagonistic potencies has been shown to be helpful for the differentiation of α_2-adrenoceptor subtypes involved in functional

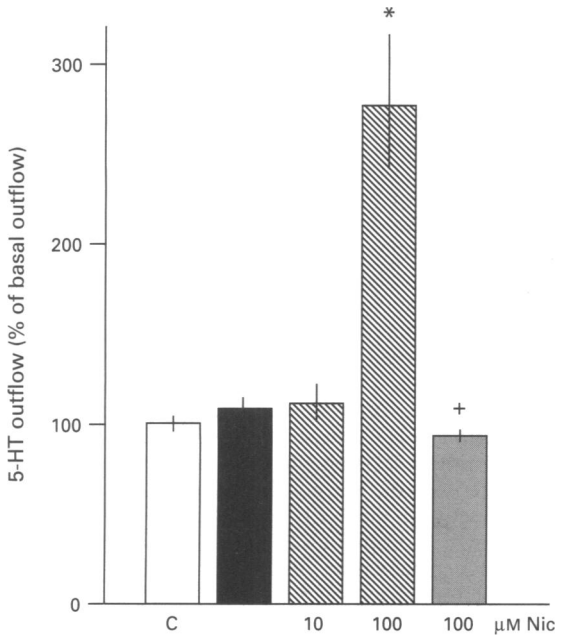

Figure 7 Effects of oxotremorine (10 μM, solid column) or nicotine (Nic, 10 or 100 μM) alone (hatched columns) or in the presence of 100 μM hexamethonium (cross-hatched column) on the outflow of 5-HT from isolated tracheae of newborn rabbits. Tracheae were incubated as described in Figure 1 except that nicotine or oxotremorine was used as test stimulus instead of phenylephrine. Hexamethonium was present 60 min before nicotine. Height of columns: peak outflow of 5-HT, expressed as % of the mean basal outflow in control (c) experiments (60–70 min of incubation, 89±4% of 'initial' outflow) in the absence of test drugs. Means±s.e.mean of 3–7 experiments are shown. Significance of differences: $^*P<0.01$ from controls (open column); $^+P<0.05$ from Nic 100 μM.

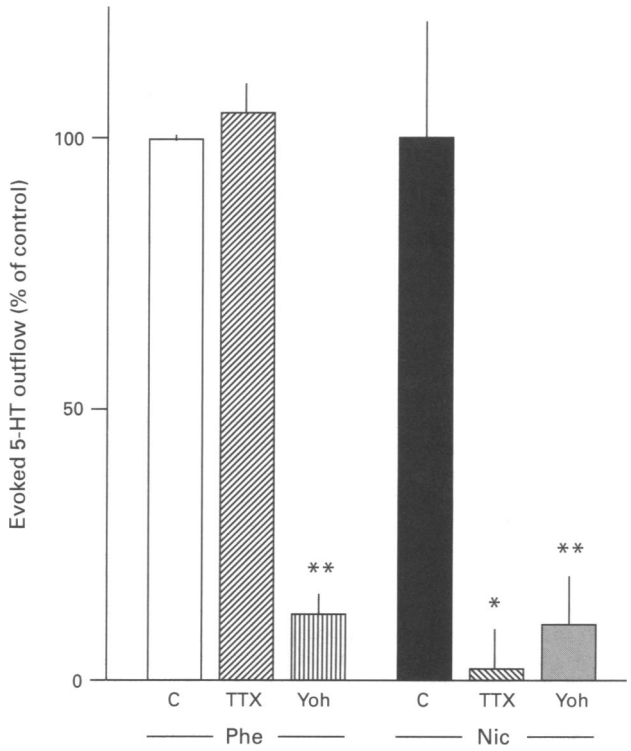

Figure 8 Effects of tetrodotoxin (TTX, 1 μM) or yohimbine (Yoh, 1 μM) on the outflow of 5-HT from isolated tracheae of newborn rabbits evoked by 10 μM phenylephrine (Phe) or 100 μM nicotine (Nic). Tracheae were incubated as described in Figure 1, except that either phenylephrine or nicotine was added as test stimulus. TTX or Yoh were present 60 min before the respective test stimulus. Height of columns: peak outflow of 5-HT expressed as % of mean peak outflow (60–70 min of incubation) evoked by the respective test stimulus (Phe or Nic) alone (C). Means±s.e.mean of 4–22 experiments are shown. Significance of differences from the respective control (C). Stimulus alone: $^*P<0.05$, $^{**}P<0.01$.

Table 2 Comparison of ratios of the IC_{50} values of ARC 239, prazosin, rauwolscine and yohimbine determined against the phenylephrine-evoked 5-HT outflow from rabbit neuroendocrine epithelial (NEE) cells (Table 1) with the respective ratios of K_i values of these antagonists in radioligand binding studies

	NEE cells	α_{2A}	α_{2B}	α_{2C}	α_{2D}
Prazosin/rauwolscine	11.3	481±73	24.9±5.8	182±37	25.3±3.3
Prazosin/yohimbine	5.7	461±93	11.2±3.4	52±9.5	40.1±21.8
Yohimbine/rauwolscine	2.0	1.35±0.15	2.66±0.27	3.89±0.75	1.1±0.27
Prazosin/ARC 239	5.8	1.87±0.24	4.07±0.68	1.36±0.19	1.69±1.13
ARC 239/rauwolscine	2.0	260±45	4.14±0.54	198±56	30±10.8
ARC 239/yohimbine	1.0	230±58	3.71±2.22	55±14	–
Correlation coefficient (r)		0.52	0.91*	0.30	0.21

Means±s.e.mean of 5–26 ratios. Each individual ratio was formed from K_i values determined in the same study and same tissue. Siginificance of correlation between the ratios for NEE cells and those for the α_2-adrenoceptor subtypes: $^*P=0.01$.

response (Simonneaux *et al.*, 1991; Lawhead *et al.*, 1992; Limberger *et al.*, 1992; Molderings & Göthert, 1995). Such a comparison for the present observations is summarized in Table 2. Again, a significant correlation of the data for NEE cells was found only for the α_{2B}-receptor ($r=0.91$, $P=0.01$). In addition, the absolute values of all ratios for NEE cells were close to those for the α_{2B}-receptor. These observations strongly support the contention that the α_2-adrenoceptors on NEE cells belong to the α_{2B}-subtype.

As already mentioned, phenylephrine is generally considered to be an α_1-adrenoceptor selective agonist. Indeed, functional experiments carried out long before the differentiation of α_2-adrenoceptors into four subtypes was known, showed phenylephrine to be only a weak agonist in eliciting α_2-adrenoceptor-mediated responses (see Starke, 1981). In binding studies, however, the affinity constants of phenylephrine for α_1-adrenoceptors were even somewhat lower than those for α_2-adrenoceptors (see Starke, 1981), suggesting low intrinsic activity of phenylephrine at α_2-adrenoceptors. On the other hand, nothing is known about possible differences in the activity of phenylephrine at the different α_2-adrenoceptor subtypes. From the present observations it appears that phenylephrine is an effective agonist at the α_{2B}-subtype. Here, it should be emphasized that at present only very few data about the functional role of α_{2B}-receptors are available. We are aware of only one study in which sufficient evidence supported the conclusion that a functional response (inhibition of sensory neurotransmission) was mediated via α_{2B}-receptors (Fuder & Selbach, 1993).

Inhibitory β-adrenoceptors and role of cyclic AMP

The spontaneous as well as the phenylephrine-evoked outflow of 5-HT was inhibited by isoprenaline. The effect of isoprenaline was antagonized by propranolol indicating the involvement of specific β-adrenoceptors. However, the potencies of both, the agonist isoprenaline and the antagonist propranolol, were relatively low. This suggests that an atypical β-adrenoceptor (possibly a β_3-adrenoceptor) mediated that effect, since these are characteristics of β_3-adrenoceptors. However, more detailed pharmacological studies will be necessary for a clear characterization of the present β-adrenoceptor. Generally, β-adrenoceptors mediate the activation of adenylyl cyclase (Lefkowitz *et al.*, 1983) and such an effect may also be involved in the present inhibition of 5-HT release from NEE cells. At least forskolin, which is known to activate directly adenylyl cyclase (Seamon *et al.*, 1981), also inhibited both the spontaneous and evoked 5-HT outflow.

Although at present little is known about the functional significance of 5-HT release from NEE cells of the airways, there is some evidence that 5-HT could be involved in the development of broncho-obstructive symptoms (see Cazzola *et al.*, 1995). Since β-adrenoceptor agonists play an important role in the treatment of broncho-obstructive diseases, it is an interesting idea that NEE cells might be an additional target for β-adrenoceptor agonists in the airways.

Role of cholinoceptors

Muscarinic receptor-mediated effects appear not to play a role in the control of NEE cells of the rabbit trachea, as the muscarinic receptor agonist oxotremorine, even at the high concentration of 10 μM, failed to affect the outflow of 5-HT. On the other hand, nicotine caused an increase in 5-HT release which was blocked by hexamethonium indicating that specific nicotine receptors mediated that effect. In contrast to the effect of phenylephrine, the stimulation of 5-HT release by nicotine was prevented by tetrodotoxin. Thus, in the rabbit trachea nicotine appears not to act directly at the NEE cells, but may induce the release of a stimulating neurotransmitter. Since yohimbine also blocked the effect of nicotine, it is concluded that nicotine may stimulate the release of noradrenaline which may activate the above described α_{2B}-adrenoceptors on the NEE cells. These observations demonstrate, in addition, that these α_{2B}-adrenoceptors are indeed a target for the endogenous neurotransmitter.

In contrast to the present observations which do not indicate the presence of nicotinic receptors on the NEE cells of rabbit trachea there are several studies which suggest that nicotinic receptors might be present on lung NEE cells of other species, either in primary culture (Nylen *et al.*, 1993) or in different neuroendocrine cell lines derived from small cell lung cancer (Schuler, 1989; Tarroni *et al.*, 1992; Catteneo *et al.*, 1993; Quik *et al.*, 1994). Whether this discrepancy reflects species differences or cell specific differences (NEE of upper versus lower airways) remains to be established. The present finding that large differences in the magnitude of the effect of nicotine were observed dependent on the source of the animals suggests a particular variability of nicotinic effects. Whether environmental factors or whether strain differences account for the variations observed in our study is unclear. On the other hand, it should be pointed out that the α-adrenoceptor-mediated stimulation of 5-HT release did not show such variability.

In conclusion, 5-HT release from NEE cells of the trachea of newborn rabbit is stimulated via α-adrenoceptors which have characteristics of the α_{2B}-subtype and which appear to be localized directly at the NEE cells. Activation of β-adrenoceptors, for which propranolol showed relatively low affinity, as well as direct activation of adenylyl cyclase by forskolin induced inhibitory effects on 5-HT release and opposed the α_2-adrenoceptor-mediated facilitation. Activation of nicotinic receptors, but not of muscarinic receptors also evoked the release of 5-HT. However, the effect of nicotine appears to be mediated indirectly via the release of noradrenaline which acts via the described α_{2B}-adrenoceptors on the NEE cells.

This study was supported by the VERUM foundation. This paper contains parts of the Dr. rer. nat. thesis of A.F.

References

ADRIAENSEN, D. & SCHEUERMANN, D.W. (1993). Neuroendocrine cells and nerves of the lung. *Anat. Rec.*, **236**, 70–85.

BALAGUER, L. & ROMANO, J. (1991). Solitary neuroendocrine cells and neuroepithelial bodies in the lower airways of embryonic, fetal, and postnatal sheep. *Anat. Rec.*, **231**, 333–338.

BLAXALL, H.S., MURPHY, T.J., BAKER, J.C., RAY, C. & BYLUND, D.B. (1991). Characterization of the alpha-2C adrenergic receptor subtype in the opossum kidney and in the OK cell line. *J. Pharmacol. Exp. Ther.*, **259**, 323–329.

BOUSBAA H., PORON, F. & FLEURY-FEITH, J. (1994). Changes in chromogranin A-immunoreactive guinea-pig pulmonary neuroendocrine cells after sensitization and challenge with ovalbumin. *Cell Tissue Res.*, **275**, 195–199.

BYLUND, D.B., BLAXALL, H.S., IVERSEN, L.J., CARON, M.G., LEFKOWITZ, R.J. & LOMASNEY, J.W. (1992). Pharmacological characteristics of α_2-adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol. Pharmacol.*, **42**, 1–5.

BYLUND, D.B., EIKENBERG, D.C., HIEBLE, J.P., LANGER, S.Z., LEFKOWTIZ, R.J., MINNEMAN, K.P., MOLINOFF, P.B., RUFFOLO, R.R. JR. & TRENDELENBURG, U. (1994). IV. International union of pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.*, **46**, 121–136.

BYLUND, D.B., RAY-PRENGER, C. & MURPHY, T.J. (1988). Alpha-2A and alpha-2B adrenergic receptor subtypes: antagonist binding in tissues and cell lines containing only one subtype. *J. Pharmacol. Exp. Ther.*, **245,** 600–607.

CATTENEO, M.G., CODIGNOLA, A., VICENTINI, L.M., CLEMENTI, F. & SHER, E. (1993). Nicotine stimulates a serotonergic autocrine loop in human small-cell lung carcinoma. *Cancer Res.*, **53,** 5566–5568.

CATTERALL, W.A. (1980). Neurotoxins that act on voltage sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.*, **20,** 15–43.

CAZZOLA, M., MATERA, M.G., D'AMATO, G. & ROSSI, F. (1995). Effects of serotonin on airways: recent developments. *Allergy*, **50,** 1–10.

CHEUNG, Y.-D., BARNETT, D.B. & NAHORSKI, S.R. (1982). [^{3}H]Rauwolscine and [^{3}H]yohimbine binding to rat cerebral and human platelet membranes: possible heterogenity of α_2-adrenoceptors. *Eur. J. Pharmacol.*, **84,** 79–85.

CHO, T., CHAN, W. & CUTZ, E. (1989). Distribution and frequency of neuro-epithelial bodies in post-natal rabbit lung: Quantitative study with monoclonal antibody against serotonin. *Cell Tissue Res.*, **255,** 353–362.

CUTZ, E., CHAN, W. & TRACK, N.S. (1981). Bombesin, calcitonin and leuenkephalin immunoreactivity in endocrine cells of human lung. *Experientia*, **37,** 765–767.

CUTZ, E., CHAN, W., WONG, V. & CONEN, P.E. (1975). Ultrastructure and fluorescence histochemistry of endocrine (APUD-type) cells in tracheal mucosa of human and various animal species. *Cell Tissue Res.*, **158,** 425–437.

DEVEDIJAN, J.-C., ESCLAPEZ, F., DENIS-POUXVIEL, C. & PARIS, H. (1994). Further characterization of human α_2-adrenoceptor subtypes: [^{3}H]RX821002 binding and definition of additional selective drugs. *Eur. J. Pharmacol.*, **252,** 43–49.

DEY, R.D., ECHT, R. & DINERSTEIN, R.J. (1981). Morphology, histochemistry and distribution of serotonin-containing cells in tracheal epithelium of adult rabbit. *Anat. Rec.*, **199,** 23–91.

FREITAG, A., WESSLER, I. & RACKÉ, K. (1995). Characterization of 5-hydroxytryptamine release from isolated rabbit and rat trachea: the role of neuroendocrine epithelial cells and mast cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353,** 55–63.

FREITAG, A., WESSLER, I. & RACKÉ, K. (1996a). Characterization of adrenergic and cholinergic mechanisms in the control of 5-HT release from neuroepithelial cells of newborn rabbit trachea. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **353,** R79.

FREITAG, A., WESSLER, I. & RACKÉ, K. (1996b). Adrenoceptor-mediated modulation of 5-hydroxytryptamine (5-HT) release from neuroepithelial cells of trachea of newborn rabbits. *Br. J. Pharmacol.*, **117,** 197P.

FUDER, H. & SELBACH, M. (1993). Characterization of sensory neurotransmission and its inhibition via α_{2B}-adrenoceptors and via non-α-receptors in rabbit iris. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **347,** 394–401.

GLEASON, M.M. & HIEBLE, J.P. (1992). The α_2-adrenoceptors of the human retinoblastoma cell line (Y79) may represent an additional example of the α_{2C}-adrenoceptor. *Br. J. Pharmacol.*, **107,** 222–225.

HOFFMAN, B.B. & LEFKOWITZ, R.J. (1990). Catecholamines and sympathomimetic drugs. In *Godman and Gilman's The Pharmacological Basis of Therapeutics*. ed. Gilman A,G., Rall, T.W., Nies, AIS. & Taylor P. pp. 187–220, New York: Pergamon Press.

JEFFERY, P.K. (1994). Innervation of the airway mucosa: Structure, function and changes in airway disease. In *Handbook of Immunopharmacology: Immunopharmacology of Epithelial Barriers*. ed. Goldie, R.G. pp. 85–118, London: Academic Press.

LAFTIPOUR, J., JONES, S.B. & BYLUND, D.B. (1982),. Characterization of [^{3}H]-yohimbine binding to putative alpha-2 adrenergic receptors in neonatal rat lung. *J. Pharmacol. Exp. Ther.*, **223,** 606–611.

LAUWERYNS, J.M., COKELAERE, M. & THEUNYNK, P. (1972). Neuroepithelial bodies in the respiratory mucosa of various mammals. A light optical, histochemical and ultrastructural investigation. *Z. Zellforsch.*, **135,** 569–592.

LAUWERYNS, J.M., COKELAERE, M. & THEUNYNCK, P. (1973). Serotonin-producing neuroepithelial bodies in rabbit respiratory mucosa. *Science*, **180,** 410–413.

LAUWERYNS, J.M., COKELAERE, M., DELEERSNYDER, M. & LIEBENS, M. (1977). Intrapulmonary neuroepithelial bodies in newborn rabbits. Influence of hypoxia, hyperoxia, hypercapnia, nicotine, reserpine, L-dopa and 5-HT. *Cell Tissue Res.*, **182,** 425–440.

LAUWERYNS, J.M., DEBOCK, V., VERHOFSTAD, A.A.J. & STEINBUSCH, H.W.M. (1982). Immunohistochemical localization of serotonin in intrapulmonary neuro-epithelial bodies. *Cell Tissue Res.*, **226,** 215–223.

LAUWERYNS, J.M., DE BOCK, V. & DECRAMER, M. (1987). Effects of unilateral vagal stimulation on intrapulmonary neuroepithelial bodies. *J. Appl. Physiol.*, **63,** 1781–1787.

LAWHEAD, R.G., BLAXALL, H.S. & BYLUND, D.B. (1992). α-2A is the predominant α-2 adrenergic receptor subtype in human spinal cord. *Anesthesiology*, **77,** 983–991.

LEE, I., GOULD, V.E., MOLL, R., WIEDENMANN, B. & FRANK, W.W. (1987). Synaptophysin expressed in the bronchopulmonary tract: neuroendocrine cells, neuroepithelial bodies, and neuroendocrine neoplasms. *Differentiation*, **34,** 115–125.

LEFKOWITZ, R.J., STADEL, J.M. & CARON, M.G. (1983). Adenylate cyclase-coupled beta-adrenergic receptors: structure and mechanism of activation and desensitization. *Ann. Rev. Biochem.*, **52,** 159–186.

LIMBERGER, N., TRENDELENBURG, A.U. & STARKE, K. (1992). Pharmacological characterization of presynaptic α_2-autoreceptors in rat submaxillary gland and heart atrium. *Br. J. Pharmacol.*, **107,** 246–255.

LINK, R., DAUNT, D., BARSH, G., CHRUSCINSKI, A. & KOBILKA, B. (1992). Cloning of two mouse genes encoding α_2-adrenergic receptor subtypes and identification of a single amino acid in the mouse α_2-C10 homolog responsible for an interspecies variation in antagonist binding. *Mol. Pharmacol.*, **42,** 16–27.

LOMASNEY, J.W., COTECCHIA, S., LEFKOWITZ, R.J. & CARON, M.J. (1991). Molecular biology of α-adrenergic receptors: implications for receptor classification and for structure-function relationship. *Biochim. Biophys. Acta*, **1095,** 127–139.

MACKINNON, A.C., KILPATRICK, A.T., KENNY, B.A., SPEDDING, M. & BROWN, C.M. (1992). [^{3}H]-RS-15385-197, a selective and high affinity radioligand for α_2-adrenoceptors: implications for receptor classification. *Br. J. Pharmacol.*, **106,** 1011–1018.

MOLDERINGS, G.J. & GÖTHERT, M. (1995). Subtype determination of presynaptic α_2-autoreceptors in the rabbit pulmonary artery and human saphenous vein. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **352,** 483–490.

NYLEN, E.S., BECKER, K.L., SNIDER, R.H., TABASSIAN, A.R., CASSIDY, M.M. & LINOILA, R.I. (1993). Cholinergic-nicotinic control of growth and secretion of cultured pulmonary neuroendocrine cells. *Anat. Res.*, **236,** 129–135.

NYLEN, E.S., SNIDER, R.H., MOORE, C.F. & BECKER, K.L. (1990). Modulation of pulmonary bombesin by nicotine and vagotomy. *Life Sci.*, **47,** 213–218.

OWMAN, C., HÅKANSON, R. & SUNDLER, F. (1973). Occurrence and function of amines in endocrine cells producing polypeptide hormones. *Fed. Proc.*, **32,** 1785–1791.

POLAK, J.M. & BLOOM, S.R. (1982). Regulatory peptides and neuron-specific enolase in the respiratory tract of man and other mammals. *Exp. Lung Res.*, **3,** 313–328.

QUIK, M., CHAN, J. & PATRICK, J. (1994). alpha-Bungarotoxin blocks the nicotinic receptor mediated increase in cell number in a neuroendocrine cell line. *Brain Res.*, **655,** 161–167.

REDICK, M.L. & HUNG, K.-S. (1994). Quantitation of pulmonary neuroepithelial bodies in pre- and postnatal rabbits. *Cell Tissue Res.*, **238,** 583–587.

RENOUARD, A., WIDDOWSON, P.S. & MILLAN, M.J. (1994). Multiple alpha$_2$ adrenergic receptor subtypes. I. Comparison of [^{3}H]RX821002-labeled rat $R_{alpha\text{-}2A}$ adrenergic reecptors in cerebral cortex to human $H_{alpha2A}$ adrenergic receptor and other populations of alpha-2 adrenergic subtypes. *J. Pharmacol. Exp. Ther.*, **270,** 946–957.

RUFFOLO, R.R., JR., NICHOLS, A.J., STADEL, J.M. & HIEBEL, J.P. (1991). Structure and function of α-adrenoceptors. *Pharmacol. Rev.*, **43,** 475–505.

RUFFOLO, R.R., JR., NICHOLS, A.J., STADEL, J.M. & HIEBEL, J.P. (1993). Pharmacologic and therapeutic applications of α_2-adrenoceptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **32,** 243–279.

SCHEUERMANN, D.W. (1987). Morphology and cytochemistry of the endocrine epithelial system in the lung. *Int. Rev. Cytol.*, **106,** 35–88.

SCHULER, H.M. (1989). Cell type specific, receptor-mediated modulation of growth kinetics in human lung cancer cell lines by nicotine and tobacco-related nitrosamines. *Biochem. Pharmacol.*, **38,** 3439–3442.

SCHWÖRER, H., RACKÉ, K. & KILBINGER, H. (1987). Spontaneous release of endogenous 5-hydroxytryptamine and 5-hydroxyindoleacetic acid from the isolated vascularly perfused ileum of the guinea-pig. *Neuroscience*, **21**, 297–303.

SEAMON, K.B., PADGETT, W. & DALY, J.W. (1981). Forskolin: Unique diterpene activator of adenylate cyclase in membranes and intact cells. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 3363–3367.

SIMONNEAUX, V., EBADI, M. & BYLUND, D.B. (1991). Identification and characterization of α_{2D}-adrenergic receptors in bovine pineal gland. *Mol. Pharmacol.*, **40**, 235–241.

STARKE, K. (1981). Alpha-adrenoceptor subclassification. *Rev. Physiol. Biochem. Pharmacol.*, **88**, 199–236.

TABASSIAN, A.R., NYLEN, E.S., LUKACS, L., CASSIDY, M.M. & BECKER, K.L. (1990). Cholinergic regulation of hamster pulmonary neuroendocrine cell calcitonin. *Exp. Lung Res.*, **16**, 267–277.

TARRONI, P., RUBBOLI, F., CHINI, B., ZWART, R., OORTIGIESEN, M., SHER, E. & CLEMENTI, F. (1992). Neuronal-type nicotinic receptors in human neuroblastoma and small cell lung carcinoma cell lines. *Fed. Eur. Biochem. Soc.*, **312**, 66–70.

TRACK, N.S. & CUTZ, E. (1982). Bombesin-like immunoreactivity in developing human lung. *Life. Sci.*, **30**, 1553–1556.

UHLEN, S., PORTER, A.C. & NEUBIG, R.R. (1994). The novel alpha-2 adrenergic radioligand [^{3}H]-MK912 is alpha-2C selective among human alpha-2A, alpha-2B and alpha-2C adrenoceptors. *J. Pharmacol. Exp. Ther.*, **271**, 1558–1565.

UHLEN, S., XIA, Y., CHHAJLANI, V., FELDER, C.C. & WIKBERG, J.E.S. (1992). [^{3}H]-MK 912 binding delineates two α_2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d α_2-adrenoceptor. *Br. J. Pharmacol.*, **106**, 986–995.

WEINSHANK, R.L., ZGOMBICK, J.M., MACCHI, M., ADHAM, N., LICHTBLAU, H., BRANCHEK, T.A. & HARTIG, P.R. (1990). Cloning, expression, and pharmacological characterization of a human α_{2B}-adrenergic receptor. *Mol. Pharmacol.*, **38**, 681–688.

(Received February 2, 1996
Revised May 8, 1996
Accepted June 3, 1996)