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# The influence of age on isolated tracheal responsiveness to spasmogens

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**Abstract**

The influence of animal age was examined on the responses of guinea-pig (birth–156 weeks) and rat (4–136 weeks) isolated tracheal tissue to the spasmogens histamine, 5-hydroxytryptamine (5-HT) and potassium ions ($K^+$) using functional and biochemical techniques. Over the ages birth–12 weeks in the guinea-pig, $K^+$ potency decreased 1.5 fold whereas histamine potency increased 2-fold between the ages of birth–2 weeks and then declined to original levels by age 20 weeks. 5-HT potency declined over the entire age range examined, resulting in a 25.1 fold decrease between the ages of 1 and 156 weeks. In the rat, 5-HT potency remained unchanged and a small but progressive increase in $K^+$ potency was observed with respect to animal age. Significant age-related changes in inositol phosphate accumulation were observed in both unstimulated and histamine-stimulated isolated guinea-pig tracheal smooth muscle which did not correlate with the functional changes observed in response to spasmogenic stimulation. The results describe disparate age-related changes between two species of different spasmogenic agonists with the majority of age-related changes occurring during the maturation phase of growth of the guinea-pig. © 1998 Elsevier Science B.V.

*Keywords:* Aging; Airway; Maturation; Histamine; $K^+$; 5-HT (5-hydroxytryptamine, serotonin); Inositol phosphate

## 1. Introduction

Histamine, 5-hydroxytryptamine (5-HT) and potassium ions ($K^+$) cause a concentration-dependent contraction of airway smooth muscle. However, the influence of aging over a broad range on these responses is not documented. In studies examining both in vivo and in vitro responsiveness to histamine in guinea-pig airway tissue, it has been shown that tracheal tissue, but not bronchial tissue from older animals is less sensitive to histamine than that from younger animals (Brink et al., 1980; Douglas et al., 1984). Studies of pig isolated tracheal tissue have shown a similar decrease in histamine potency, with a concurrent fall in maximal contractile activity (Mitchell and Nayler, 1986). Contractile responses to 5-HT in the isolated guinea-pig trachea have also been shown to be decreased in older animals compared with younger animals (Bayol et al., 1985).

Although numerous subtypes of histamine and 5-HT receptor subtypes are known, it is the histamine $H_1$ receptor subtype which mediates histamine-induced smooth muscle contraction in the airways, most likely via the generation of inositol phosphates (Hall and Hill, 1988; Hill, 1990). Of the many 5-HT receptor subtypes known, current evidence suggests that airway smooth muscle contraction is mediated by the 5-hydroxytryptamine $5\text{-HT}_2$ receptor in the guinea-pig (Baumgartner et al., 1990; Buckner et al., 1991). This subtype elicits responses in brain and other non-airway tissues via generation of inositol phosphates (Zifa and Fillion, 1992).

This study examined the possibility that age is a significant determinant of airway smooth muscle responsiveness to 5-HT and histamine and that altered capacity to generate inositol phosphates was the cause of such modulation.

## 2. Methods

### 2.1. Functional studies

Male guinea-pigs (SR/C tricolour) and male Wistar rats aged birth–156 weeks and 4–136 weeks, respectively, were used for this study. Guinea-pigs were sacrificed by cervical dislocation and exsanguinated, whilst rats were sacrificed by a blow to the head followed by exsanguina-

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tion. Tracheal tissue was removed and placed in ice-cold Krebs–bicarbonate solution of the following composition (mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, $KH_2PO_4$ 1.03, $MgSO_4 \cdot 7H_2O$ 0.57, $CaCl_2 \cdot 2H_2O$ 2.5 and glucose 11.1. Tracheal rings 3–4 mm in length were obtained from the entire length of the trachea and suspended in Krebs–bicarbonate solution in water-jacketed organ baths maintained at 37°C under 500 mg weight tension and continuously gassed with 95% $O_2$ and 5% $CO_2$. The tissue preparations were allowed to equilibrate for 1 h before testing with spasmogens. During the equilibration period, the incubation medium was changed regularly and changes in tension were adjusted back to 500 mg weight. Cumulative concentration–effect curves were then constructed to histamine, 5-HT and $K^+$ on alternate ring preparations so that responses to each of the spasmogens was obtained from rings obtained from all regions of the trachea.

### 2.2. Inositol phosphate generation

Tracheal tissue from 3 animals of similar age was obtained as described above and 3–4 rings grouped together for determination of inositol phosphate generation following stimulation with agonist. Preparations were blot dried onto filter paper and weighed into glass vials. In some studies, the smooth muscle and cartilage components of the tracheal rings were dissected and examined separately. The tissue was then pre-incubated in oxygenated Krebs–bicarbonate solution in a shaking water bath for 30 min at 37°C. The solution was then replaced with 1 ml of oxygenated *myo*[2-$^3$H]inositol (5 $\mu$Ci) in which the tissues were incubated for 3 h at 37°C with continuous agitation. The incorporation of *myo*[2-$^3$H]inositol was then halted by twice washing the tissue preparations with 5 ml oxygenated Krebs–bicarbonate solution at 37°C for 15 min, followed by a third wash for a further 15 min. The Krebs solution was then replaced with 1 ml LiCl (5 mM) for 15 min to prevent the breakdown of inositol phosphates to inositol. Addition of agonists (or Krebs solution to determine basal levels) in a volume of 20 $\mu$l followed and the stimulation reaction halted by the addition of 1.5 ml chloroform/methanol (1:2 v/v) for a further 15 min. Separation of the aqueous and lipid phases was achieved by addition of 0.5 ml distilled water and 0.5 ml chloroform for 15 min. The entire upper layer or aqueous phase was then removed and applied to an anion-exchange column of Dowex AG1-X8 resin which had been converted to the formate form in the presence of excess formic acid for 30 min and then washed 3 times for 10 min with distilled water. Inositol was eluted by passing 10 ml distilled water through the column and the glycerophosphoinositol was eluted with 15 ml buffer consisting of 5 mM sodium tetraborate and 60 mM sodium formate. Finally, inositol phosphates were eluted with 10 ml of a buffer consisting of 0.1 M formic acid and 0.75 M ammonium formate. This eluent was collected and dispensed into 1 ml aliquots to which 10 ml of scintillant (5.8 g $l^{-1}$ 2,5-diphenyloxazol (PPO) in Triton X100/toluene (1:2)) was added and the radiation counted in a Tri-Carb liquid scintillation counter (Packard, Model 1500). Total *myo*[2-$^3$H]inositol phosphate was calculated as dpm per mg tissue.

### 2.3. Data analysis

For functional experiments, the $pD_2$ and $E_{max}$ values for each cumulative concentration–effect curve were estimated after curve fitting by an iterative non-linear least squares regression analysis computer program written in our laboratory. The potency for each agonist was determined using the equation: $pD_2 = -\log_{10}(EC_{50})$ where $EC_{50}$ is the concentration of agonist required to elicit 50% of maximal contractile response. The potency of each spasmogen was calculated for each tracheal ring from each animal in each age group. The mean potency for each animal was then obtained and a final mean representing animals within an age group was calculated. The animal means were compared using Analysis of Variance (ANOVA) to determine the influence of age on spasmogen potency. Due to the increase in tracheal size and thus smooth muscle mass with animal age, the maximal tension (mg weight) generated in response to spasmogen stimulation was determined and normalised against $K^+$-induced contractile tension (Kong and Stephens, 1984; Dunn et al., 1989) obtained in the same tracheal ring. Inositol phosphate generation was expressed in terms of dpm per mg tissue and the mean values for animals within each age group were examined for age-related changes using ANOVA. Data were deemed to be significantly different when $P < 0.05$.

### 2.4. Drugs

The drugs used in this study included: histamine dihydrochloride, 5-hydroxytryptamine creatinine sulphate complex (Sigma Chem), potassium chloride (analytical grade) (Fluka AG) and *myo*[2-$^3$H]inositol (Amersham). Solutions of drugs were prepared in 0.9% saline for organ bath experiments and in Krebs–bicarbonate buffer for biochemical assay of inositol phosphates.

## 3. Results

### 3.1. Responsiveness of airway tissue to spasmogens

In guinea-pig isolated trachea from animals of all ages, histamine, 5-HT and $K^+$ all produced concentration-dependent contraction such that the potency order was: 5-HT > histamine > $K^+$. In rat isolated tracheal tissue, histamine however failed to cause contraction, but concentration-dependent contraction was observed in response to both 5-HT and $K^+$, with the former being more potent.

Both 5-HT and $K^+$ were more potent in guinea-pig than rat isolated trachea.

### 3.2. Influence of age on tracheal responsiveness to spasmogens

#### 3.2.1. Potassium

During the maturation phase of the guinea-pig (birth to 12 weeks), the potency of $K^+$ declined significantly ($P < 0.0001$) from $1.90 \pm 0.03$ to $1.71 \pm 0.02$. No other evidence of age-related changes in $K^+$ potency was observed over the remainder of the age range examined (Fig. 1a). In contrast, the maximum contractile response to $K^+$ increased significantly ($P < 0.001$) between birth ($187.0 \pm 4.1$ mg weight; $n = 6$) and 20 weeks of age ($671.5 \pm 137.1$ mg weight; $n = 3$) (Fig. 1b) but remained stable thereafter.

In rat isolated tracheal ring preparations, the potency of $K^+$ demonstrated a progressive and small but significant ($P < 0.001$) increase with increasing animal age (Fig. 1c). Maximum contractile response to $K^+$ also changed significantly ($P < 0.01$, ANOVA) with increasing animal age (Fig. 1d). These changes were mainly due to a significant ($P < 0.01$, ANOVA) increase in $K^+$ $E_{max}$ during the maturation phase (4–12 weeks of age) with only an apparent fluctuation in data with no specific trend over the ages 20–136 weeks ($P < 0.05$, ANOVA).

#### 3.2.2. Histamine

The potency of histamine in guinea-pig isolated tracheal ring preparations increased 2 fold ($P < 0.05$) between birth ($5.11 \pm 0.09$) and the weaning age of 2 weeks ($5.40 \pm 0.07$). After weaning, histamine potency fell ($P < 0.05$) by 2 fold up to 20 weeks of age ($5.05 \pm 0.07$). With further increases in age up to 3 years, no further change in histamine potency was observed (Fig. 2). Maximum contractile responses to histamine did not change ($P > 0.05$)

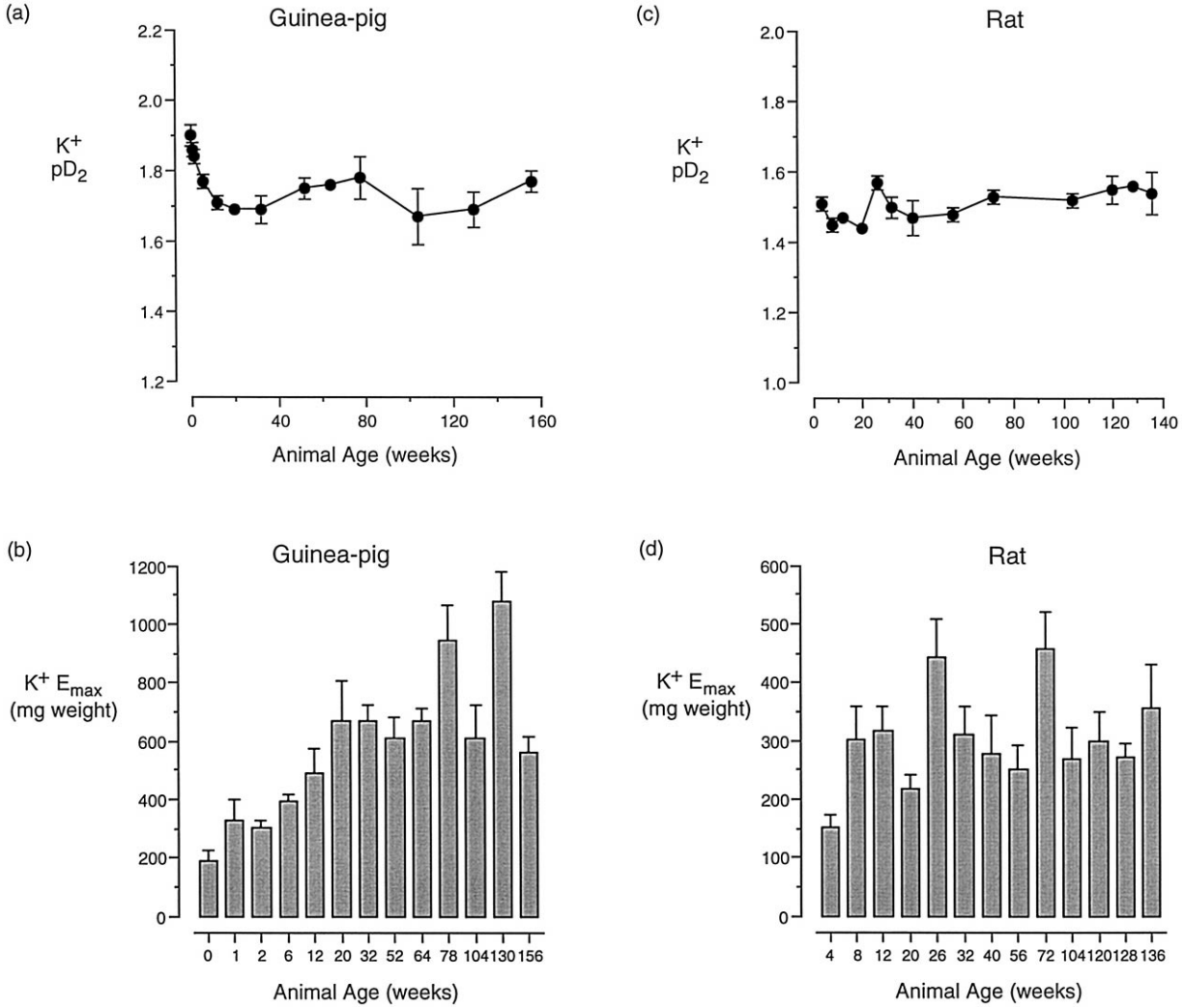


Fig. 1. Influence of age on potassium ($K^+$) (a) potency ($pD_2$) and (b) maximal contractile response (mg $E_{max}$) in guinea-pig isolated trachea and on potassium ($K^+$) (c) potency ($pD_2$) and (d) maximal contractile response (mg $E_{max}$) in rat isolated trachea. Data represented as mean ($n = 3$–9 animals, with up to 12 tracheal ring preparations obtained from each animal) with vertical lines representing the S.E.M.

with respect to animal age and remained within the range $69.2 \pm 18.5$ to $85.7 \pm 6.6\%$ $K^+$ $E_{max}$ ($n = 3–8$) over the entire age range examined (data not shown).

### 3.2.3. 5-HT

The contractile potency of 5-HT was greater in the guinea-pig than in the rat for almost all ages examined. In guinea-pig isolated tracheal ring preparations, the potency of 5-HT decreased significantly ($P < 0.0001$) between the ages of 1 and 156 weeks, representing a 25.1 fold loss of potency (Fig. 3a). Similarly, the maximum contractile responses of 5-HT, measured as a percent of maximum $K^+$-induced contraction, remained constant with respect to age during the maturation phase (newborn–12 weeks), but declined significantly ($P < 0.001$) with senescence (Fig. 3b). In rat tracheal tissue, neither the potency (Fig. 4) nor the maximal contractile effect of 5-HT changed significantly ($P > 0.05$) with respect to animal age over the age range examined with the latter remaining in the range $20.2 \pm 3.8$ to $40.5 \pm 4.8\%$ $K^+$ $E_{max}$ ($n = 3–8$; data not shown).

### 3.3. Inositol phosphate generation

In rat isolated tracheal tissue, no significant increases in inositol phosphate accumulation above basal levels were detected in tissue stimulated with histamine (1 mM), 5-HT (1 mM) or $K^+$ (120 mM) (data not shown). In guinea-pig isolated tracheal smooth muscle, however, significant levels of inositol phosphate accumulation above basal levels were observed in response to histamine (1 mM), but not 5-HT (1 mM) or $K^+$ (120 mM) (Fig. 5a). The proportion of total inositol phosphates accumulated by airway smooth muscle and cartilage components following histamine stimulation remained constant with respect to animal age with the ratio of smooth muscle to cartilage-derived inositol phosphate accumulation being in the range $3.1 \pm 0.2$ to $4.8 \pm 1.3$ ($n = 3–4$) over the age range 6 to 104 weeks. Basal levels of intracellular inositol phosphates (in the absence of agonist) were significantly ($P < 0.001$) decreased with increasing animal age (Fig. 5b), such that the level in tracheal smooth muscle from 2.5 year old (130 week) animals was only 26% of that observed in smooth muscle from 2 week old animals. Histamine-induced inositol phosphate accumulation above basal levels decreased significantly ($P < 0.001$) with increasing animal age (Fig. 5c) such that the levels of inositol phosphates induced by histamine in mature animals (26 weeks or older) was less than that of immature animals (up to 12 weeks of age) and

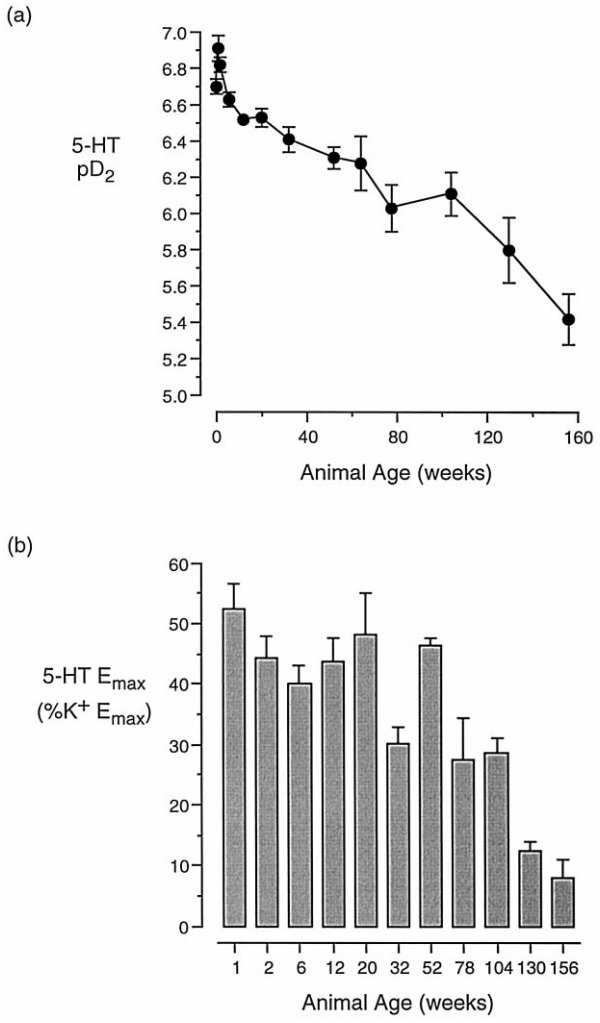


Fig. 3. Influence of age on (a) potency ($pD_2$) and (b) maximal contractile response ($E_{max}$, as % of maximum $K^+$-induced contraction) to 5-HT in guinea-pig isolated trachea. Data represented as mean ($n = 3–13$ animals, with up to 12 tracheal ring preparations obtained from each animal) with vertical lines representing the S.E.M.

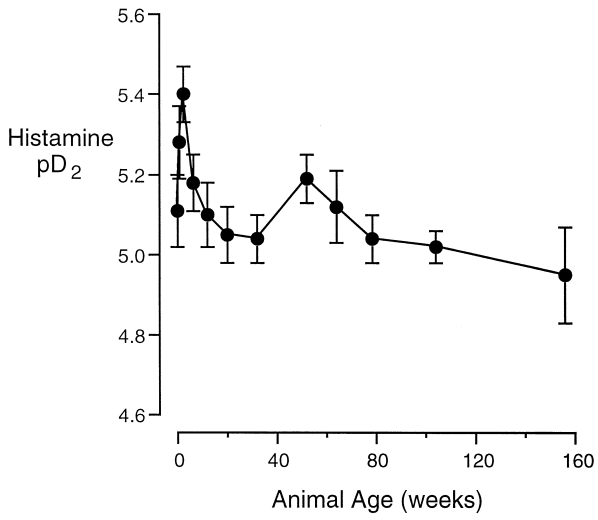


Fig. 2. Influence of age on the potency ($pD_2$) of histamine in guinea-pig isolated trachea. Data represented as mean ($n = 4–12$ animals, with up to 12 tracheal ring preparations obtained from each animal) with vertical lines representing the S.E.M.

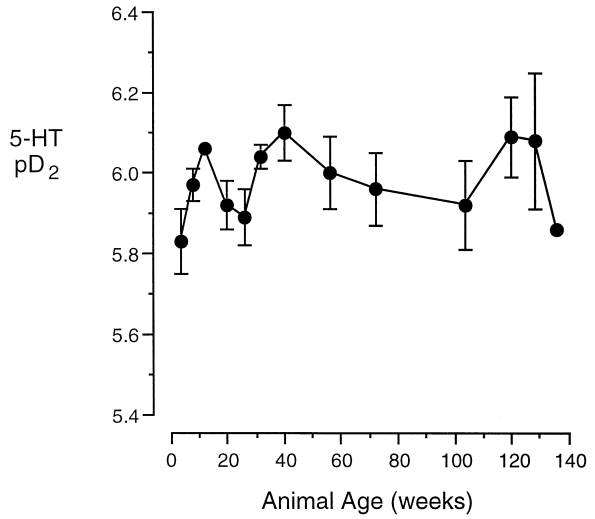


Fig. 4. Influence of age on the potency ($pD_2$) of 5-HT in rat isolated trachea. Data represented as mean ($n = 3–9$ animals, with up to 12 tracheal ring preparations obtained from each animal) with vertical lines representing the S.E.M.

that observed in animals 2.5 years (130 weeks) of age was only 12% that of 2 week old animals.

## 4. Discussion

In this study, we have demonstrated significant age-related changes in the responsiveness of guinea-pig and rat isolated tracheal tissue to histamine, 5-HT and $K^+$. In guinea-pig trachea, the potency of histamine was seen to change dramatically during the maturation phase of the animal (birth to 12 weeks), but did not alter with senescence. In the period between birth and weaning (2 weeks of age), the potency of histamine increased significantly, only to fall again following weaning to values at 20 weeks of age which were comparable to those at birth. The decline in histamine potency observed between 2 and 12 weeks of age is similar to the decline in histamine potency observed by Brink et al. (1980) over similar ages, and in other studies of young and old guinea-pigs (Duncan et al., 1983). It is interesting to note that the age at which histamine potency begins to fall is also the age at which a young guinea-pig is weaned. The reason for this is unclear, but similar trends have been observed in the pig around that species' weaning age of 4 weeks (Sparrow and Mitchell, 1990).

The most substantial change in potency of any of the spasmogens examined was that of 5-HT in the guinea-pig trachea which decreased 25.1 fold between 1 and 156 weeks of age. Bayol et al. (1985) demonstrated significant differences in tracheal and bronchial sensitivity to 5-HT between young and old guinea-pigs. In the present study, where many more age groups were examined over a larger and more accurately defined age range, it can be seen that the decline in tracheal sensitivity to 5-HT with age is a consistent trend. The changes in guinea-pig sensitivity to 5-HT with respect to age may reflect changes in the size and/or affinity of the 5-HT receptor population or changes

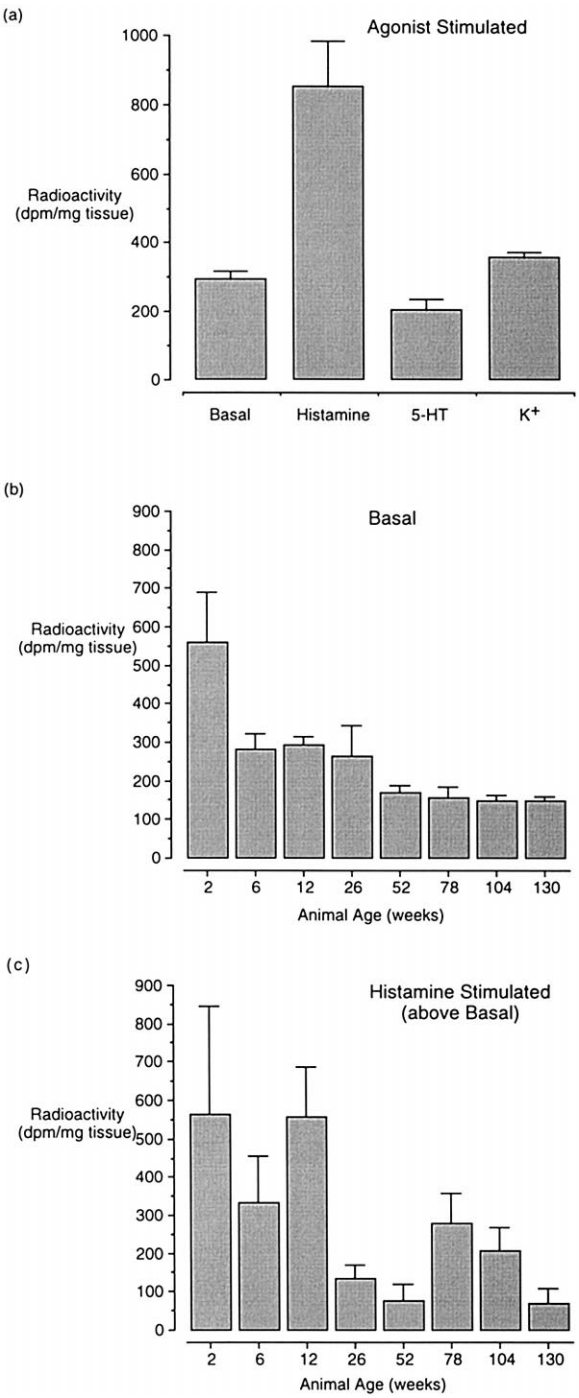


Fig. 5. (a) Inositol phosphate accumulation in isolated tracheal smooth muscle from 12 week old guinea-pigs in response to histamine (1 mM), 5-HT (1 mM) and $K^+$ (120 mM). (b) Basal inositol phosphate accumulation in guinea-pig isolated tracheal smooth muscle tissue and (c) histamine (1 mM)-induced inositol phosphate stimulation above basal with respect to animal age. Data expressed in terms of radioactivity (dpm) per mg tissue and represented as the mean of 3 animals with S.E.M. indicated by vertical lines.

in the efficiency of coupling to second messenger systems, or in uptake and metabolism of this amine. In sharp contrast, over a similar age range in the rat (4–136 weeks), no significant change in 5-HT potency was observed. These results clearly demonstrate significant species differences in the effects of age on responsiveness of tracheal tissue to 5-HT stimulation and suggests that either the changes with respect to animal age that occur in the guinea-pig leading to a decline in 5-HT potency do not occur in the rat, or that there are differences in the receptor subtypes present on tracheal smooth muscle between the two species.

Although small changes in $K^+$ potency were noted in the guinea-pig during maturation and in the rat with ageing, the results indicate that the contractile machinery of the airway smooth muscle in both species was not substantially altered with aging. Thus, any age-related changes in the potency or efficacy of other contractile agonists which stimulate specific cell surface receptors are more likely to be a result of changes at the receptor level, second messenger systems, or degradative enzymes within the tissue. When examining responses of isolated tracheal tissue from animals of different ages and therefore different sizes, maximal contractile responses to agonists including $K^+$, would be expected to increase with age during the maturation phase in proportion to the increase in muscle mass of the tissue. Indeed, in both guinea-pig and rat isolated tracheal tissue, an increase in $K^+$ $E_{max}$ was observed with respect to increasing animal age, particularly during the maturation phase of growth. While the weight of tracheal tissue can be measured at each age, this is not an accurate method for assessing age-related changes in muscle mass as the proportion of smooth muscle within the trachea may change at a rate dissimilar to that of other structures such as the cartilage, as is the case in human bronchi (Hislop and Haworth, 1989). Thus, normalization of contractile responses to agonists against the contractile responses induced by $K^+$ is a more appropriate means of controlling for muscle mass changes (Kong and Stephens, 1984; Dunn et al., 1989).

Following standardization of maximal contractile tension of histamine and 5-HT against that of $K^+$, it can be seen that despite changes in potency of some of these agonists, maximal contractile tension remained unchanged with respect to animal age. The exception was again 5-HT in the guinea-pig, in which a decreased maximal contractile effect was observed with increasing age, further suggesting an age-related decrease in the ability of guinea-pig isolated tracheal smooth muscle to contract in response to 5-HT stimulation.

To further examine the influence of animal age on tracheal responsiveness to spasmogenic stimulation, the second messenger system to which histamine $H_1$ receptors and 5-hydroxytryptamine 5-$HT_2$ receptors are reputedly linked, i.e. the inositol phosphate generation pathway, was studied in relation to animal age.

Consistent with previous studies (Henry et al., 1992), agonist stimulation of the non-smooth muscle, cartilaginous regions of both guinea-pig and rat isolated trachea resulted in increases in inositol phosphate accumulation above basal levels. It has previously been demonstrated that the majority of this cartilaginous component originated from the intercartilaginous region (Henry et al., 1992) which is known to contain a dense, highly ordered vascular network (McDonald, 1988). Thus, it is suggested that inositol phosphate generation from this region of the trachea could most likely be due to stimulation of vascular tissue. Whilst the majority of the tracheal tissue inositol phosphate generation is derived from airway smooth muscle, the present study has demonstrated that the ratio of smooth muscle to cartilage-derived inositol phosphate accumulation following histamine stimulation remains unchanged with respect to animal age.

As a function of animal age, basal levels of total intracellular inositol phosphate accumulation declined progressively and significantly in both guinea-pig and rat isolated tracheal tissue. This data perhaps suggests an age-related decline in the activity of this pathway which might be expected to be reflected in changes in agonist-induced inositol phosphate accumulation. Indeed, histamine-induced increases in inositol phosphate accumulation were shown to be significantly lower in tissue from older (26–130 weeks old) animals compared with that of younger (2–12 week old) animals. These results reflect similar changes in histamine potency observed functionally in isolated tracheal tissue with respect to animal age. However, whilst full functional contractile responses were maintained in tracheal tissue from aged animals (e.g. 130 weeks old), the levels of inositol phosphate accumulation in response to histamine in these tissues was not significantly greater than that of basal levels. This raises the question of whether only very small increases in inositol phosphate generation are required to induce contractile responses in these tissues, or whether the primary mechanism of action of histamine in mediating smooth muscle contraction in tracheal tissue from aged animals is no longer inositol phosphate dependent in these aged animals. In either case, a clear age-dependent reduction in inositol phosphate generating capacity has been detected in the guinea-pig.

Studies have shown that the 5-HT receptor subtype responsible for mediating contraction of guinea-pig isolated trachea is probably the 5-hydroxytryptamine 5-$HT_2$-subtype (Selig et al., 1988; Baumgartner et al., 1990; Ben-Harari et al., 1991). The 5-hydroxytryptamine 5-$HT_2$ receptor subtype mediates responses to 5-HT via the generation of inositol phosphates in brain and other non-airway tissues (Zifa and Fillion, 1992). In the present study however, 5-HT failed to significantly increase levels of inositol phosphate accumulation above basal in either guinea-pig or rat isolated tracheal tissue. This study was not designed to identify the 5-HT receptor subtype mediating contrac-

tion in guinea-pig trachea. However, these data suggest either that 5-hydroxytryptamine 5-$HT_2$ receptors were not linked to the inositol phosphate cascade or that a non-5-hydroxytryptamine 5-$HT_2$ receptor was activated.

In conclusion, this study has demonstrated significant age-related changes in airway responsiveness to histamine, 5-HT and $K^+$ in guinea-pig and rat isolated tracheal smooth muscle. In the guinea-pig, histamine and $K^+$ potencies were significantly altered during the maturation phase of growth but not with senescence, whereas 5-HT potency decreased significantly over the entire age range examined. No maturation phase changes were observed in the potencies of either 5-HT or $K^+$ in the rat, and senescence only brought about a small change in $K^+$ potency. Inositol phosphate generation in response to histamine also changed with respect to animal age and reflected changes in the functional responsiveness of the tissue seen with this drug. Thus, significant age-related changes and species differences in tracheal responsiveness to spasmogens have been described.

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