



Phosphodiesterase IV and neutral endopeptidase in airways from developing and allergen sensitized rabbits

Gary L. Larsen^{a,*}, Joan E. Loader^a, Cori Fratelli^a, June-ku Brian Kang^a,
Azzeddine Dakhama^a, Giuseppe N. Colasurdo^b

^a*Department of Pediatrics, Division of Pediatric Pulmonary Medicine, National Jewish Medical and Research Center, 1400 Jackson Street, Denver, CO 80206, USA*

^b*Department of Pediatrics, Division of Pulmonary Medicine, University of Texas, Houston Medical School, Houston, TX, USA*

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Abstract

Phosphodiesterase IV (PDE IV) and neutral endopeptidase (NEP) may modulate the neurally mediated nonadrenergic noncholinergic inhibitory (NANCI) response. This response is not present in normal rabbits until 2 weeks of age. Allergen sensitization and challenge of fully grown 13-week old rabbits decreases the NANCI response. Our goal was to assess NANCI as a function of age and allergen sensitization. Tracheal smooth muscle (TSM) rings from normal 1-, 2-, and 13-week old rabbits plus ragweed immune as well as ragweed immune/challenged (I/C) 13-week old rabbits were assessed. Colorimetric assays of PDE IV and NEP activity were conducted on TSM from each group. NANCI responses were obtained in the presence and absence of Ro 20-1724 (PDE IV inhibitor) and/or phosphoramidon (Phos; NEP inhibitor) after contraction of TSM with neurokinin A. In normal TSM, there was no difference in PDE IV as a function of age. Conversely, NEP decreased significantly from 1 to 13 weeks of age. The immune and I/C groups had decreases in NEP and increases in PDE IV that were significant. Neither Ro 20-1724 nor Phos alone or together increased NANCI responses in TSM from 1- or 2-week old rabbits. However, both enhanced relaxation in TSM from normal, immune, and I/C 13-week old rabbits with an additive effect when drugs were combined. This work demonstrates (1) normal maturational changes in NEP but not PDE IV within TSM of this species; (2) modulation of the NANCI response by inhibitors of PDE IV and NEP in 13- but not 2-week old rabbits; (3) increased PDE IV and decreased NEP levels in the immune and I/C groups with reconstitution of NANCI responses by the combination of inhibitors. We conclude that mediation of the NANCI response is different in normal 2- and 13-week old rabbits. Both PDE IV and NEP modulated relaxation in fully grown rabbits, but had no effect at the younger age. Furthermore, both ragweed sensitization alone and ragweed challenge of immune rabbits altered NANCI via increases in PDE IV and decreases in NEP.

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1. Introduction

An important part of the balance determining airway patency is provided by neural control mechanisms [1,2]. The nonadrenergic noncholinergic inhibitory (NANCI) response is a neural pathway that mediates relaxation of airways in several mammalian species including humans. We have reported that NANCI responses are functionally absent early

in life in 2 species: rabbits [3] and ferrets [4]. In both species, NANCI responses normally develop between birth and when the animal is fully grown. We also reported that parenteral sensitization of newborn rabbits to an allergen beginning in the first week of life led to a functional deficiency in this pathway when the animals were fully grown [3]. This occurred as a consequence of the process of active sensitization via a parenteral route without direct introduction of allergen into the airways. Challenge of airways with relevant allergen in sensitized rabbits led to even greater loss of the ability to relax the airway via stimulation of nerves [5].

*Corresponding author. Tel.: +1 303 398 1201; fax: +1 303 2760 2189.
E-mail address: larseng@njc.org (G.L. Larsen).

The purpose of this study was to delineate the biochemical mechanisms responsible for absence of the NANCi response in newborn rabbits, and dysfunction of this pathway following allergen sensitization alone and following allergen sensitization coupled with airway challenge with relevant allergen. Because of the clinical observation that inhibition of neutral endopeptidase (NEP) protects against exercise-induced airway obstruction [6], one focus of the study was on the role of this membrane bound metalloproteinase in modulating NANCi responses. In addition, we also elected to address the potential importance of phosphodiesterase IV (PDE IV) in relaxant responses. This latter avenue of investigation was based in part on the observation that an inhibitor of this enzyme facilitated the NANCi response to nerve stimulation in human bronchi [7].

2. Methods

2.1. Animals

New Zealand White rabbits were bred locally in Denver (altitude 5280 feet) to produce litters for study. All procedures employed in this work were approved by the Animal Care and Use Committee at National Jewish Medical and Research Center and conformed to NIH guidelines (Guide for the Care and Use of Laboratory Animals, NIH Publication, Revised 1996, National Academy Press, Washington, DC).

2.2. Sensitization of rabbits to allergen

Rabbit kits were actively immunized with mixed ragweed extract (Center Labs, Port Washington, NY), 0.25 ml of a 1:20 dilution (w/v) given with 0.25 ml adjuvant (aluminum hydroxide, 10 mg/ml normal saline) intraperitoneally beginning at less than 24 h of life. This was followed by weekly injections of ragweed extract for the next three weeks. Beginning at the fifth week of life, the rabbits were injected intraperitoneally with extract every other week ending on the eleventh week of life with a final subcutaneous dose of 1.0 ml ragweed extract and 1.0 ml adjuvant. This protocol has previously been shown to produce an allergen-specific IgE response in rabbits [8,9].

2.3. Aerosol exposure

Aerosol exposure was accomplished by ultrasonic nebulization of 1% ragweed diluted in sterile phosphate-buffered saline for 45 min. For this process, rabbits were placed into a Plexiglas box with a removable top (dimensions 41 × 21 × 25 cm). The ragweed solution was aerosolized into one end of the box by using an ultrasonic nebulizer (Aerosonic model 5000D, DeVilbiss, Somerset, PA). At the other end of the box were two small air holes (0.5 cm diameter) to ensure a continuous cross-current flow of air. As defined by laser nephelometry, more than 98% of

the particles were in the range of 1–5 µm in mass median diameter.

2.4. NANCi relaxant responses in airways from rabbits

Tracheal smooth muscle (TSM) segments in the form of tracheal rings of approximately 1 cm in length were obtained from normal control rabbits at 1, 2, and 13 weeks of age and also from 13-week old rabbits that were ragweed immunized as outlined above (immune group) or ragweed immunized plus exposed to an aerosol of ragweed solution (immune challenged group: I/C). After anesthesia with xylazine (5 mg/kg) and ketamine (35 mg/kg), animals were sacrificed by exsanguination. The trachea was then removed and placed in modified Krebs-Henseleit (K-H) solution. The TSM segments were cleaned of loose connective tissue, placed in 3 ml organ baths (Harvard Apparatus Co. Inc, Millis, MA), and supported longitudinally by triangular stainless steel wire supports. The lower support was attached to a hook at the base of the organ bath, and the upper support was attached with a gold chain to an isometric force transducer (model FT.03C, Grass Instrument Co., Quincy, MA). The latter was mounted on a rack and pinion clamp permitting adjustments of the resting length of each TSM segment. The tissues were bathed in modified K-H solution (in mM): 118NaCl, 25NaHCO₃, 2.8CaCl₂·2H₂O, 1.17MgSO₄, 4.7KCl, 1.2KH₂PO₄, and 2 gm/l dextrose. The baths were aerated with a 95% O₂–5% CO₂ gas mixture, and a pH of 7.43 ± 0.03 was established for the duration of each experiment. The temperature of the baths was maintained at 37 °C. Each TSM segment was equilibrated in the bath for 60 min at an optimal resting tension of 1.5 g. This optimal resting tension was established by assessing its maximal contractile response to the following standard electrical field stimulation (EFS): 4 V, 1 ms pulse duration, 30 Hz stimulus frequency. During this equilibration time, the tissues were challenged 2 times with KCl at a final concentration in the bath of 120 mM. Segments of TSM that would not develop a contractile response were excluded from further studies. Tissues were rinsed with fresh K-H solution periodically and allowed to relax to their initial tension after reaching maximal contraction.

Recordings of resting tensions and TSM contractile responses were made on a SensorMedics Dynagraph Recorder (R612) equipped with type 9853A couplers, 461D preamplifiers and 412 amplifiers (SensorMedics Corp., Anaheim, CA). The EFS was delivered by a Grass S44 stimulator connected to a stimulus isolation unit (Grass SIU5). The stimulus (1–30 Hz, 1-ms duration) was applied transmurally across the tissues by means of parallel platinum electrodes (each 0.3 cm²) and tension was recorded. To evaluate NANCi responses, experiments were performed in the presence of atropine (Aldrich, Milwaukee, WI) at a final concentration of 1 × 10^{−6}, propranolol (Sigma, St. Louis, MO) at a final concentration of

5×10^{-6} M and indomethacin (Sigma) at a final concentration of 1×10^{-5} M in the bath. After obtaining a plateau response with neurokinin A (NKA, Sigma) at a final concentration of 5×10^{-6} M, EFS was applied at stimulation frequencies ranging from 2.5 to 30 Hz to determine the frequency of stimulation that produced maximal relaxation. Changes in tension from baseline NKA contractile response were recorded and expressed as percent relaxation (mean $\pm$ SEM). Results of NANCi responses were expressed as percent relaxation at 20 Hz because this stimulation frequency gave the maximal NANC relaxation in rabbits at each age studied. NKA was used to induce TSM tone due to its lack of influence on cholinergic neurotransmission (G.L. Larsen and G.N. Colasurdo, unpublished observations). The degree of contraction to NKA was carefully controlled in that a concentration of NKA producing approximately 50% of the maximal contractile response to methacholine was used (5×10^{-6} M). Preliminary dose-response curves to NKA in this and prior studies [3,5] found that this degree of contraction allowed us to subsequently produce relaxant response with nerve stimulation that were reproducible and easy to quantitate. At the end of each experiment, the TSM segments were blotted on a gauze pad and weighed.

In selected experiments, the PDE IV inhibitor Ro 20-1724 (Calbiochem, La Jolla, CA) and/or the NEP inhibitor phosphoramidon (Phos; Sigma, St. Louis, MO) was added to the bath before nerve stimulation to produce NANCi responses. The concentration of Ro 20-1724 employed (10^{-4} M) was based on dose-response curves that demonstrated this concentration of drug completely reversed NKA-induced contractions in TSM segments from normal 2- and 13-week old rabbits (data not shown). For Phos, the dose employed (10^{-5} M) was based on published dose-response curves using TSM segments from 2-week old rabbits [10] and fully grown rabbits [11]. In those studies, the isometric tension generated in response to substance P was plotted versus the concentration of Phos with curves at both ages demonstrating a plateau in contractile responses at approximately 10^{-6} M Phos. For the studies reported here, Ro 20-1724 was added to the tissue bath before NKA while Phos was always added to the bath once a stable NKA-induced contraction was established. Adding Phos after instead of before producing the contraction allowed use of the same concentration of NKA in TSM segments from both groups of rabbits. The preparations were stable in terms of NKA-induced tension during the subsequent 30 min when measurements of NANCi responses were obtained.

2.5. Neutral endopeptidase (NEP) activity assay

The membrane fraction of TSM was isolated according to the method of Carvalho et al. [12]. Tracheal tissues were homogenized in 8 volumes (w/v) of 50 mM Tris-HCl buffer, pH 7.5, using a Virtishear homogenizer (Virtis Co., Inc., Gardiner, New York). The homogenate was centri-

fuged at 10,000g for 15 min and the pellet discarded. The supernatant was then centrifuged at 60,000g for 1 h in a Beckman Optimax centrifuge (Beckman Coulter, Inc, Fullerton, CA) to isolate the membrane protein. The pellet was washed 3 times and centrifuged as above and then resuspended in 5 volumes (w/v) of the 50 mM Tris buffer with sonication. An aliquot was removed for protein determination using the BioRad standard protein assay (BioRad Laboratories, Hercules, CA). The enzymatic assay for NEP was based on a modification of the method of Orłowski and Wilk [13] using Z-gly-gly-leu-pNA as the substrate. Duplicate samples (50 μ l) were preincubated at 37 °C for 20 min with and without 10^{-4} M thiorphan in a final volume of 450 μ l Tris-HCl buffer. Subsequently, 12.5 μ g of aminopeptidase was added to each tube and the reaction started by adding substrate at a final concentration of 0.3 mM. The final volume of 500 μ l mixture was incubated at 37 °C for 120 min. A time course performed in our laboratory of 30–240 min proved this time to be optimal (data not shown). The reaction was stopped with 250 μ l trichloroacetic acid (40% w/v). The mixture was then centrifuged at 10,000g for 5 min and the supernatant removed for determination of *p*-nitroaniline. The colorimetric determination of *p*-nitroaniline modified from Goldberg and Rutenburg [14] consisted of adding 250 μ l of the above mixture to 250 μ l of a 0.1% solution of sodium nitrite. After 3 min, excess sodium nitrite was degraded by adding 250 μ l of a 0.05% solution of ammonium sulfamate and allowing the reaction to take place for 2 min. A volume of 500 μ l of a 0.5 mg/ml solution of N-(1-naphthyl) ethylene diamine dihydrochloride was then added and incubated for 10 min. The resultant pinkish-violet color was read at 545 nm. The nmoles of product liberated/tube of reaction mixture was calculated from a standard curve of *p*-nitroaniline (0–36 nmol). The values calculated from the tubes with thiorphan were subtracted from the duplicate tubes without the inhibitor to account for specific NEP activity. These corrected values were then divided by the time and amount of protein per tube to give a final value of nmoles of *p*-nitroaniline liberated/minute/mg protein.

2.6. Phosphodiesterase IV (PDE IV) activity assay

This method was based on the work of Hosono [15]. Approximately 0.5 cm of airway smooth muscle (tracheal or bronchial) was homogenized in 1 ml of 80 mM Tris-HCl buffer (pH 7.5) using a Virtishear homogenizer. The homogenate was centrifuged at 10,000g for 15 min at 4 °C and the supernatant removed for protein measurement (BioRad) and for PDE IV activity determination. A total of 100 μ l of sample supernatant was added to tubes containing 250 μ l Tris-HCl with 2 mM MgSO₄. A duplicate tube for each sample contained 10^{-4} M Ro 20-1724, a specific PDE IV inhibitor. This dose of Ro 20-1724 was chosen based on dose-response curves for both Ro 20-1724 and IBMX (3-isopropyl-1-methylxanthine; Sigma, St. Louis, MO). The latter is a non-specific inhibitor of both

cAMP and cGMP phosphodiesterases. A plateau in inhibition of phosphodiesterase activity was seen at a 10^{-4} M concentration of Ro 20-1724 while total phosphodiesterase activity continued to decrease with 10^{-3} M IBMX (data not shown). The tubes were incubated for 20 min at 37°C before adding 50 μl of a 140 U/mg solution of alkaline phosphatase and 50 μl of a 2 mM solution of cAMP. The total mixture was incubated at 37°C for 90 min and the reaction stopped with 50 μl of 55% trichloroacetic acid with centrifugation for 5 min at 4°C , 10,000g. A volume of 250 μl of the supernatant was then removed and added to 250 μl of coloring reagent consisting of 1 volume 6 N H_2SO_4 , 2 volumes H_2O , 1 volume 2.5% ammonium molybdate and 1 volume 10% ascorbic acid. This solution was incubated for 90 min at 37°C and the blue color read at 820 nm. The amount of phosphate liberated in each tube was calculated using a standard curve ranging from 0 to 5 nmol. The values from the tubes with Ro 20-1724 were subtracted from their duplicate tubes without the inhibitor to account for specific PDE IV activity and then divided by the time and amount of protein per tube to give a final value of pmoles phosphate liberated/min/mg protein.

2.7. Statistical analysis

Data are expressed as means $\pm$ SEM. A one-way analysis of variance (ANOVA) was used in data analysis with the Tukey-Kramer Honestly Significant Differences test of multiple comparisons applied to the analysis. Significance was determined at the $P = 0.05$ level.

3. Results

3.1. NANCi relaxant responses in airways from rabbits

Neurally mediated relaxant responses are shown as a function of age in both Figs. 1 and 2. Full frequency responses are shown in Fig. 1 for TSM from normal 2- and 13-week old rabbits while Fig. 2 depicts the effects of neural stimulation at 20 Hz only and includes 1-week old rabbits. This frequency of stimulation was chosen for Fig. 2 based on the plateau in response shown in Fig. 1 at 20 and 30 Hz as well as the observation that this lower frequency is closer to normal vagal function observed in vivo [16]. In this species, no NANCi response was present at 1 week of age, but was present by the second week of life. As shown in Fig. 1, the magnitude of the response was not significantly different in normal 2- vs. 13-week old rabbits at any frequency of stimulation ($27.1 \pm 4.7\%$ vs. $31.8 \pm 1.6\%$ at 20 Hz for TSM from 2- and 13-week old rabbits, respectively).

The effect of ragweed sensitization is also shown in terms of the full frequency response curve in the first figure. Relaxation was significantly less in TSM from immune 13-week old rabbits compared to 13-week old normal animals at stimulation frequencies of 5 Hz and above (Fig. 1). The responses in the ragweed immune and ragweed I/C groups

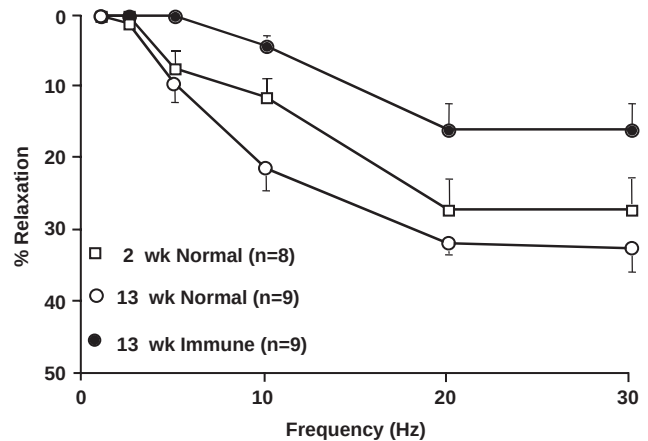


Fig. 1. The NANCi relaxant response is shown for TSM from normal 2- and 13-week old rabbits plus 13-week old immune rabbits. The response in normal 2- and 13-week olds was not significantly different at any frequency of stimulation. Conversely, relaxation was significantly less in TSM from immune 13-week old rabbits compared to 13-week old non-immune (normal) animals at stimulation frequencies of 5 Hz and above. At both 10 and 20 Hz, responses were also significantly different between tissues from 2-week old normal versus 13-week old immune rabbits.

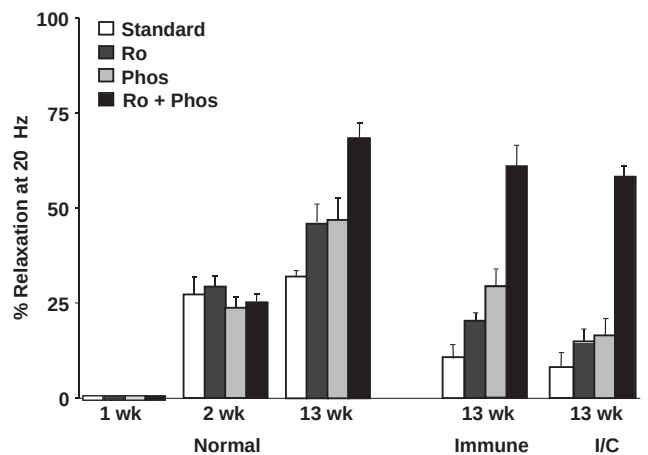


Fig. 2. Relaxant responses of TSM to neural stimulation at 20 Hz are shown under standard bath conditions (see text) and with 10^{-4} M Ro 20-1724 (Ro) and/or 10^{-5} M phosphoramidon (Phos) added to the tissue bath as both a function of age and immune status. In terms of age, responses were assessed in tissues from normal rabbits at 1, 2, and 13 weeks of age. In terms of immune status, responses were assessed in both ragweed immune and ragweed I/C 13-week old animals. Normally, TSM from 1-week old rabbits had no relaxant response under any of the conditions of study. The NANCi response became apparent at 2 weeks of age, and was of the same magnitude as that seen at 13 weeks of age. However, the NANCi response was not modulated by Ro and/or Phos at 2 weeks of age, but was significantly increased by this combination of drugs at 13 weeks of age. The NANCi response was significantly decreased in both the immune and I/C groups when compared to normal 13-week old animals, but was significantly upregulated by the combination of an inhibitor of neutral endopeptidase and PDE IV.

were similar. This is shown at 20 Hz in Fig. 2 where the responses for both the immune and I/C groups were significantly decreased compared to the normal group ($31.8 \pm 1.6\%$ vs. $12.0 \pm 4.6\%$ and $10.5 \pm 3.8\%$ at 20 Hz for

TSM from 13-week old normal, immune and I/C groups, respectively). At 20 Hz, responses were also significantly different between tissues from 2-week old normal versus 13-week old immune and I/C rabbits.

3.2. Modulation of NANCi responses

Relaxant responses of TSM to neural stimulation at 20 Hz is shown under standard bath conditions (see Section 2) and with 10^{-4} M Ro 20-1724 (Ro) and/or 10^{-5} M phosphoramidon (Phos) added to the tissue bath as both a function of age and immune status in Fig. 2. In terms of age, TSM from 1-week old rabbits had no relaxant response under any of the conditions of study. The NANCi response became apparent at 2 weeks of age, and was noted to be of the same magnitude at 13 weeks of age (values given above). However, the NANCi response was not modulated by Ro and/or Phos at 2 weeks of age, but was significantly increased by the combination of drugs at 13 weeks of age (relaxation at 20 Hz = $68.2 \pm 4.4\%$, Fig. 2). In terms of immune status, responses were also assessed in TSM from both ragweed-immune and I/C 13-week old animals. The NANCi response was significantly decreased in both immune and I/C groups, but was significantly upregulated by the combination of an inhibitor of NEP and PDE IV (relaxation at 20 Hz = $61.0 \pm 10.3\%$ and $58.3 \pm 3.2\%$ in ragweed immune and I/C groups, Fig. 2).

3.3. Neutral endopeptidase (NEP) activity assay

Shown in Fig. 3 are levels of NEP in TSM from normal rabbits of three different ages, and in tissues from 13-week old immune and I/C rabbits. As displayed, NEP decreased significantly as a function of age in TSM from normal rabbits (15.6 ± 0.4 vs. 2.0 ± 0.2 nmol/min/mg protein in

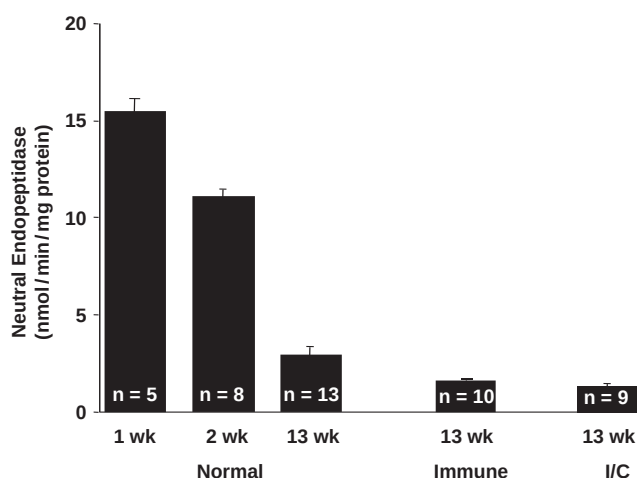


Fig. 3. Neutral endopeptidase (nmol/min/mg protein) is shown in TSM from normal rabbits of three different ages, and in tissues from 13-week old immune and I/C rabbits. Normally, NEP decreased significantly as a function of age. In addition, both ragweed immunization alone and immunization followed by ragweed challenge led to further significant decreases in NEP levels in TSM from 13-week old rabbits.

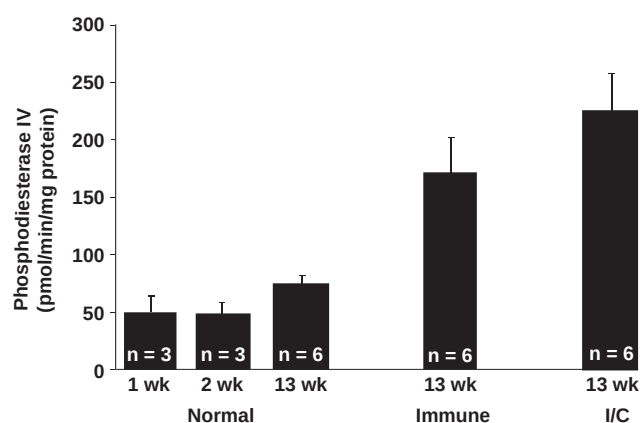


Fig. 4. Phosphodiesterase IV activity is shown in TSM from normal rabbits of three different ages, and in tissues from 13-week old immune and I/C rabbits. Normally, PDE IV did not change as a function of age. However, both ragweed immunization alone and immunization followed by ragweed challenge led to significant increases in PDE IV levels in TSM from 13-week old rabbits.

TSM from rabbits 1 and 13 weeks of age, respectively). In addition, ragweed immunization alone and immunization followed by ragweed challenge both led to significant decreases in NEP levels in TSM from 13-week old rabbits (1.4 ± 0.1 and 1.5 ± 0.1 nmol/min/mg protein in TSM from immune and I/C rabbits, respectively).

3.4. Phosphodiesterase IV (PDE IV) activity assay

Shown in Fig. 4 are levels of PDE IV activity for TSM from normal rabbits of three different ages, and in tissues from 13-week old immune and I/C rabbits. In normal TSM, PDE IV did not change significantly as a function of age. However, both ragweed immunization alone and immunization followed by ragweed challenge led to significant increases in PDE IV levels in TSM from 13-week old rabbits (73.2 ± 8.3 vs. 168.7 ± 34.4 and 223.8 ± 35.6 pmol/min/mg protein in TSM from normal, immune, and I/C rabbits, respectively).

4. Discussion

Both PDE IV and NEP may modulate the neurally mediated NANCi response in humans [6,7] as well as other species including rabbits. This response is not present in normal rabbits until 2 weeks of age, and may be altered (diminished) in magnitude by allergen sensitization alone [3] and allergen sensitization plus airway challenge with appropriate allergen [5]. Thus, our goal in conducting this work was to assess NANCi and factors that modulate NANCi as a function of both age and allergen sensitization. As part of this process, assays of PDE IV and NEP activity were performed on TSM from various groups of rabbits. In addition, NANCi responses were obtained in the presence and absence of Ro 20-1724 and/or phosphoramidon (Phos). In TSM from normal rabbits, there was no

difference in PDE IV as a function of age. Conversely, NEP decreased significantly from 1 to 13 weeks of age. The immune and I/C groups had additional decreases in NEP compared to tissue from normal rabbits and also developed increases in PDE IV that were significant. Neither Ro 20-1724 nor Phos alone or together brought out relaxant responses in TSM from 1-week old rabbits, and neither compound alone or together increased the NANCi response in TSM from 2-week old rabbits. However, both enhanced relaxation in TSM from normal, immune, and I/C 13-week old rabbits with an additive effect when drugs were combined.

Bioactive peptides within the lung can be degraded by peptidases including NEP (EC 3.4.24.11) [17]. In the lungs of different animal species and humans, NEP has been localized in the basal cells of the epithelium, alveolar type II cells, submucosal glands, airway smooth muscle, post-capillary venules, and nerves [18]. This membrane bound metalloproteinase is a degrading enzyme for bronchoconstrictor (SP, NKA, etc.) as well as bronchodilator peptides (VIP, atrial natriuretic peptide) within airways. In humans, NEP inhibition by thiorphan reduced exercise induced airway obstruction during the recovery period following exercise [6]. These results suggest that an NEP substrate with activity as a bronchodilator (such as VIP) moderates airway obstruction due to exercise in patients with asthma. Given this ability to effect bronchodilator responses, changes in NEP within an airway could alter NANCi responses. For example, an increase in NEP within airways (as shown in Fig. 3) could be responsible for the lack of a NANCi response early in development such as demonstrated within rabbit airways in Fig. 2. However, this did not appear to be the reason for the absent NANCi response at 1 week of age in that inhibition of NEP did not bring out the response. In addition, despite the increase in NEP still seen at 2 weeks of age, inhibition of NEP failed to enhance the response. Thus, it was only in TSM from fully grown 13-week old normal, immune, and I/C rabbits that inhibition of NEP augmented EFS-induced relaxation. This occurred at a time when NEP activity was lowest in normal rabbit TSM, and even lower after immunization with or without allergen challenge. The reason for this paradox is currently unexplained, but might be related to changes in the levels of other bioactive amines that normally occur in this species. For example, levels of substance P within airways and bronchoalveolar fluid are normally high shortly after birth, and fall with maturation in this species [19]. Thus, the balance of bronchoconstrictive to bronchoprotective peptides may be a contributing factor in inhibiting neurally mediated relaxation at the younger ages. This issue requires additional study.

The mediator(s) of the NANCi system is not as well characterized in this species as in humans. However, this study presents evidence that mediation of this relaxant response in mature rabbits is in part via the cyclic 3',5'-adenosine monophosphate (cAMP) pathway. Part of this evidence is based on our observations showing that the

maximal NANCi response in normal fully grown rabbits can be significantly increased with use of the PDE IV inhibitor, Ro 20-1724. As shown in Section 3, we also have evidence that Ro 20-1724 will partially restore neurally mediated relaxation in rabbits sensitized and challenged with ragweed. This occurs in these two immunized groups at a time when PDE IV is elevated from normal (Fig. 4), and thus is easier to understand than the augmentation produced by the NEP inhibitor phosphoramidon. Conversely, zaprinast, a PDE V inhibitor, does not alter the normal relaxant response in this species [3]. PDE IV is predominantly responsible for hydrolyzing cAMP in ASM while PDE V is the major enzyme involved in metabolizing cyclic 3',5'-guanosine monophosphate (cGMP) [20]. Given our prior published observations [3] plus other unpublished observations from our laboratory (Colasurdo and Larsen), nitric oxide does not appear to contribute in a significant manner to the NANCi response in this species. Thus, the mediator of the fully grown rabbit's NANCi response appears to be one or more neuropeptides producing relaxation by generation of cAMP. Fernandes et al. [7] reported that Ro 20-1724 facilitated NANCi responses to EFS in human bronchi while zaprinast did not. Thus, airways of mature rabbits and humans appear to be similar in this respect.

One unexpected finding from this series of investigations was that the normal neurally mediated relaxant response appeared to differ as a function of age in this species of animals. The response was absent at 1 week of age and could not be unmasked by either Phos and/or Ro 20-1724. While a response was found in TSM from 2-week old rabbits, it was not modulated by either drug or their combination. Conversely, the response in fully grown normal rabbits (13 weeks of age) was altered by both drugs alone or in combination. These observations unveil a previously unrecognized complexity in the NANCi response in this species. It is functionally absent at birth, and the nature of the response (and thus possibly mediation of the response) may change during growth of this species. To more fully understand this pathway, additional studies are required to identify the mediators that lead to relaxation as a function of age.

The NANCi response seen in the 13-week old immune and I/C groups under standard conditions (when neither inhibitor was in the bath) was similar (Fig. 2). Thus, the question can be raised about the need to study both groups. In earlier work involving 13-week old immune and I/C groups, significant differences in the NANCi response were found with the response in the I/C group significantly more compromised compared to the response in the immune group [5]. This was not the case in the current work where NANCi responses were not significantly different in these groups. We believe this likely reflects a difference in the manner in which allergen challenges were conducted in the two studies. In the work of Fame et al. [5], rabbits were intubated for allergen challenge while in the current study, the rabbits were not intubated and inhaled

allergen in a normal manner while within a chamber into which allergen was nebulized (detailed in Section 2). The concentration of allergen in the nebulized solution in each set of experiments was similar. However, we expect that rabbits intubated for allergen delivery had more allergen deposition within their airways than the I/C group utilized in this study. Thus, a difference in total allergen deposition within airways (and therefore the magnitude of the challenge) likely explains the divergent results in these two studies. In this work, the responses under normal conditions as well as when the inhibitors alone or in combination were in the tissue baths were similar.

To put these findings into context, study of neural mechanisms that may be important in human diseases involving the airways has been challenging. As reviewed by Widdicombe [21], the evidence for involvement of neural mechanisms in animal models of allergic asthma has been widely studied and documented. However, similar studies in humans are seldom practical. Thus, conclusions drawn from use of models are inferential in terms of the human process. Yet, observations from this and similar studies show the potential for mechanisms of neural control to change as both a function of the age of development and as a result of pathologic processes early in life. As such, they give clues as to pathways that may be important in human disease. In terms of phosphodiesterase inhibitors, clinical intervention with this class of drugs is of interest for several reasons as reviewed by Grootendorst and Rabe [22]. While the inhibitor used in this study is not a candidate for clinical use, other inhibitors in this class that have less side effects in humans are being studied. The potential of inhibitors of NEP to modulate human asthma was noted above [6]. This current work suggests that at least in this mammalian model, the combination of the two classes of medication may be more beneficial than either alone in terms of preserving the bronchoprotective NANCi response in fully grown animals. Thus, these observations may help clinical investigators more precisely develop studies that address similar processes in humans. The potential of such mechanisms to be important in diseases such as childhood asthma merits additional investigations in both models of disease and through clinical investigations.

This current work demonstrates that the NANCi component of neural control undergoes significant post-natal changes within airways of rabbits. Within this species, other components of neural control also undergo dramatic post-natal changes. In this respect, maturation of the cholinergic component of neural control has recently been addressed [19]. As with the relaxant response, the contractile response undergoes significant alterations during early development in rabbits. Furthermore, it is now apparent that insults to an airway early in life may alter normal post-natal maturation of both pathways.

To summarize, this work demonstrates normal maturational changes in the activity of NEP but not PDE IV within TSM in this species. Modulation of the NANCi

response by inhibitors of PDE IV and NEP was seen in airways from normal 13- but not 2-week old rabbits. Furthermore, increased PDE IV and decreased NEP levels were seen in the immune and I/C groups with reconstitution of NANCi responses by a combination of inhibitors for NEP and PDE IV. We conclude that modulation of the NANCi response is normally different in 2- and 13-week old rabbits. Both PDE IV and NEP modulated relaxation in fully grown rabbits, but had no effect at the younger age. Furthermore, ragweed sensitization alone as well as sensitization and specific allergen challenge altered NANCi via increases in PDE IV and decreases in NEP. In these latter two groups, inhibitors of PDE IV and NEP helped restore relaxant responses. Thus, the stage of airway development as well as the immune (allergen-sensitized) status of the host may be important factors in determining the magnitude of relaxation and the biochemical pathways that modulate this bronchoprotective response.

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