Mechanical stretch-induced serotonin release from pulmonary neuroendocrine cells: implications for lung development

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Submitted 14 April 2005; accepted in final form 9 August 2005

Pan, Jie, Ian Copland, Martin Post, Herman Yeger, and Ernest Cutz. Mechanical stretch-induced serotonin release from pulmonary neuroendocrine cells: implications for lung development. Am J Physiol Lung Cell Mol Physiol 290: L185–L193, 2006. Pulmonary neuroendocrine cells (PNEC) produce amine (serotonin, 5-HT) and peptides (e.g., bombesin, calcitonin) with growth factor-like properties and are thought to play an important role in lung development. Because physical forces are essential for lung growth and development, we investigated the effects of mechanical strain on 5-HT release in PNEC freshly isolated from rabbit fetal lung and in the PNEC-related tumor H727 cell line. Cultures exposed to sinusoidal cyclic stretch showed a significant 5-HT release inhibitible with gadolinium chloride (10 nM), a blocker of mechanosensitive channels. In contrast to hypoxia (PO2 ~ 20 mmHg), stretch-induced 5-HT release was not affected by Ca2+-free medium or nifedipine (50 μM), excluding the exocytic pathway. In H727 cells, stretch failed to release calcitonin, a peptide stored within dense core vesicles (DCV), whereas hypoxia caused massive calcitonin release. 5-HT released by mechanical stretch is derived predominantly from the cytoplasmic pool, because it is rapid (~5 min) and is releasable from early (20 days of gestation) fetal PNEC containing few DCV. Both mechanical stretch and hypoxia upregulated expression of tryptophan hydroxylase, the rate-limiting enzyme of 5-HT synthesis. We conclude that mechanical strain is an important physiological stimulus for the release of 5-HT from PNEC via mechanosensitive channels with potential effects on lung development and resorption of lung fluid at the time of birth.

mechanosensitive ion channels; amine storage pools; neuroendocrine cell secretion; hypoxia-induced secretion; fetal lung fluid

THE SYSTEM OF PULMONARY NEUROENDOCRINE CELLS (PNEC) is composed of amine (serotonin, 5-HT)- and peptide (e.g., bombesin, calcitonin)-producing cells widely distributed within the airway mucosa of human and animal lungs (13, 34, 40). PNEC occur as single cells and as distinctive innervated clusters, neuroepithelial bodies (NEB). Whereas solitary PNEC populate the mucosa of the trachea and bronchi up to the terminal bronchioles, NEB are found only within intrapulmonary airways, where they appear concentrated at airway branch points (7). Pulmonary NEB function as airway O2 sensors, because they express a membrane-bound O2-sensing molecular complex composed of an O2-sensitive K+ channel coupled to an O2-sensing protein, NADPH oxidase (9, 10, 45). Hypoxia activates the O2 sensor, leading to Ca2+-dependent exocytosis of dense core vesicles (DCV), the storage site of amine and peptides, and the release of these mediators acting locally or via vagal afferents (9, 10). Hypoxia induced 5-HT release from NEB cells is dose dependent and occurs within the physiological range expected in the airway (PO2 ~ 95 mmHg) (17).

The precise role of solitary PNEC and whether they are functionally distinct from NEB is not known, although they share identical neuroendocrine and molecular markers as well as innervation (13, 34, 40). PNEC/NEB appear prominent in fetal and neonatal lungs but are less conspicuous in the lungs of adults because of a “dilutional” effect of postnatal lung growth (20).

PNEC are thought to play an important role in lung development, because they are the first cell type to differentiate in early fetal lung and produce amine and peptides with growth factor-like properties (11, 12, 39). In addition, two morphological types of PNEC have been recognized in human fetal lungs, one having a flask shape with apical cytoplasmic processes reaching the airway lumen, or the so-called “open type,” and PNEC with elongated dendritic-like cytoplasmic processes extending along the basement membrane without a luminal contact (“closed type”) (13, 34, 40). The PNEC type with dendritic cytoplasmic processes is found only in fetal or neonatal lungs (39). Furthermore, this anatomic arrangement could facilitate local/paracrine effects, because these dendritic cytoplasmic processes make direct contact with several adjacent epithelial cells as well as being in close proximity to the subbasement membrane mesenchyme to affect epithelial/mesenchymal interactions. The basal location of PNEC cytoplasmic processes and their anchoring to the basement membrane via various specialized cell-cell adhesion molecules (cadherins, selectins, cell adhesion molecules) likely play a significant role in the transmission of mechanical stimuli (23). Therefore, we postulated that this strategic anatomic location of PNEC in the airway also could be ideally suited to receive stimuli generated by the mechanical forces known to be important for lung growth and differentiation, particularly the fetal breathing movements and expansion of the airways by lung fluid secretion (24). Experimental studies have shown that mechanical stretch stimulates proliferation of fetal lung cells and affects lung maturation by inducing production of extracellular matrix and differentiation of alveolar cells (27). This mechanical stretch-induced cell proliferation is mediated through intracellular signal transduction pathways, particularly the protein tyrosine kinase-phospholipase Cy-protein kinase C axis (28).
Mechanical forces induce the synthesis and secretion of several extracellular matrix molecules, including fibronectin, proteoglycans, and glycosaminoglycans (43), as well as downregulating the expression of surfactant proteins B and C as a reflection of conversion of alveolar type II cells to type I cells (26). In the present study we examined the effects of mechanical stretch on 5-HT release by using cultures of PNEC isolated from rabbit fetal lungs from early and late gestation and the release of 5-HT and calcitonin in a carcinoid tumor cell line (H727) as a convenient model representative of PNEC in human lung (6). We used a combination of morphological, biochemical, and molecular methods to define the mechanism and the release pathway for mechanical stretch-induced 5-HT secretion. Our findings indicate that during lung development, the release of 5-HT from PNEC occurs via mechanosensitive channels independently of Ca\(^{2+}\)-mediated exocytosis, the predominant pathway of hypoxia-induced secretion. Because 5-HT is an amine with pleiotrophic biological activities, its local effects could be modulated by mechanical stretch coordinate with other physical force- and hormone-dependent mechanisms involved in lung development and neonatal adaptation.

**MATERIALS AND METHODS**

**Animals and cell culture.** For animal experiments, New Zealand White rabbits at 20 (E20) and 26 days of gestation (E26) were used. The dams were killed with an overdose of pentobarbital sodium, and the fetuses were removed. All animal experiments were approved by the local Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines. The lungs with trachea attached were removed en bloc. Portions of lung tissue were used for morphological studies on NEB (see below). Primary cultures of PNEC/NEB were established using previously published methods with some modifications (45). To obtain intact bronchial trees, we teased the lung parenchyma and vasculature away from the airways under a dissecting microscope. The bronchial trees were minced with a razor blade, and airway epithelial cells were isolated by 20-min digestion with purified dispase (1,000 U/ml; Goto Shusei, Tokyo, Japan). To enrich the mixed airway cell preparation in PNEC/NEB, we subjected the cell preparation to three courses of differential plating in serum-free medium. The carcinoid tumor cell line H727 (related to classic small cell lung cancer) was obtained from American Type Culture Collection and cultured as previously reported (44). Both cell preparations (enriched rabbit fetal PNEC/NEB and H727 cells) were seeded onto collagen I BioFlex plates (Flexcell International, Hillsborough, NC) and maintained in RPMI 1640 plus 10% FBS and α-MEM medium with 10% FBS, respectively, at 37°C in a 5% CO\(_2\) incubator. Cyclical mechanical strain. Cells grown until 85–90% confluent on LabTech chamber slides, using the same media and culture conditions.

**Cyclical mechanical strain.** Cells grown until 85–90% confluent on the flexible surface of the BioFlex plates were subjected to cycles of stretch and relaxation with the use of a computer-driven, vacuum-operated Flexcell strain unit FX-4000 (Flexcell International). Before stretching, cells were rinsed in culture medium and then switched to CO\(_2\)-independent serum-free medium (GIBCO BRL, Burlington, ON, Canada) containing 50 μM pargyline (to prevent 5-HT reuptake) for 10 min. Sinusoidal cyclic stretch frequency of 0.5 Hz producing a 9% elongation of cells was applied in the presence and absence of gadolinium chloride (GC; 10 nM) (Sigma, St. Louis, MO), a widely used blocker of stretch-activated channels. The specificity, potency, and mode of action of GC has been a subject of previous reviews (22). The media were collected after 5, 10, 15, 30, and 90 min of stretch. As a control, media were collected from nonstretched cultures maintained in parallel. All collected supernatants were centrifuged at 1,500 g for 5 min to remove cell debris and nonadherent cells and then stored at −80°C until assay.

**Immunohistochemistry and electron microscopy.** Samples of human fetal and neonatal lung used in our previous studies were utilized for the demonstration of 5-HT-immunoreactive PNEC with dendritic-like processes (11, 12). The lung specimens from human lungs as well as fetal rabbit lungs (E20, E26) were fixed in 10% neutral buffered formalin and embedded in paraffin according to routine histological procedures. Paraffin sections were cut at 5 μm and dewaxed. For antigen retrieval, the sections were microwaved in 10 mM citrate buffer at pH 6.0. Endogenous peroxidase was quenched with 3% H\(_2\)O\(_2\), and nonspecific binding was blocked with 20% normal goat serum for 30 min at room temperature. The following primary antibodies were used: monoclonal antibody against serotonin (5-HT) (7) and calcitonin (11, 12, 39). The immunohistochemistry procedures using the avidin-biotin complex method (Vectorstain ABC kit; Vector Laboratories, Burlingame, CA) or the indirect immunofluorescence method were carried out as previously reported (7, 11, 12, 32). As negative control, primary antibody was omitted.

**Effects of hypoxia and Ca\(^{2+}\) on 5-HT and calcitonin release.** We assessed the effects of hypoxia and extracellular Ca\(^{2+}\) on 5-HT release from rabbit fetal PNEC/NEB cells (used as positive control) for comparison with those induced by mechanical strain. Supernatants were collected after exposure of cultures to hypoxia (5% O\(_2\)-5% CO\(_2\)-90% N\(_2\)) for 90 min. To test the effects of extracellular Ca\(^{2+}\)-stimulated 5-HT secretion, we replaced bicarbonate buffer salt solution (24 mM CaCl\(_2\)) with an equimolar Ca\(^{2+}\)-free bicarbonate buffer salt solution. We used nifedipine (50 μM; Sigma) to block the voltage-dependent L-type Ca\(^{2+}\) channel known to be involved in hypoxia-induced 5-HT release from NEB cells (17). Normoxic control conditions (5% CO\(_2\), 20% O\(_2\)) included 90-min incubation at 37°C in 1 ml of bicarbonate buffer solution at pH 7.4 containing 116 mM NaCl, 5 mM KCl, 24 mM NaHCO\(_3\), 2 mM CaCl\(_2\), 1.1 mM MgCl\(_2\), 10 mM HEPES, and 5.5 mM glucose, and 50 μM pargyline was added to prevent 5-HT reuptake. All collected samples were lyophilized under vacuum and stored at −80°C until assay.

**Measurement of 5-HT release.** To quantify 5-HT release into the media, we reconstituted the lyophilized samples in 200 μl of distilled water and assayed using a commercially available ELISA (IBL) kit as previously reported (20). Briefly, the assay was performed as follows: samples and 5-HT standard dilutions were applied to 96-well microtiter plates previously coated with goat anti-rabbit antibody, followed by 50 μl of biotin-labeled 5-HT and 50 μl of rabbit antibody against 5-HT. After incubation overnight at 4°C and washing with PBS, 150 μl of goat anti-biotin antibody alkaline phosphatase conjugate in Tris buffer was incubated for 2 h at room temperature with gentle mixing and was washed again; p-nitrophenylphosphate substrate was added, and the enzyme reaction was terminated after 60 min by addition of 50 μl of 1 M sodium hydroxide. Absorption was measured at 405 nm with the use of a microtiter plate reader (Versamax; Molecular Devices, Sunnyvale, CA), and the concentration of 5-HT was calculated from the reference curve by using the SOFTmax PRO version 3.0 software program (Molecular Devices). For 5-HT, the data were finally normalized as nanograms per 10,000 PNEC, and the number of PNEC was determined by counting 5-HT-positive-stained cells in each cultured well before and after treatment in triplicates. As negative control, rabbit lung fibroblasts were run in parallel. The calcitonin in culture supernatants released from the same number of H727 cells was asayed in triplicate with an ELISA kit (BioSource-Europe, Nivelles, Belgium) according to the manufacturer’s specifications. Values of triplicate cultures differed from the mean by no more than 5%. The nonparametric, unpaired t-test was used for comparison between the two groups with Prism GraphPad version 5.01 software (GraphPad Software, San Diego, CA). P values of <0.05 were considered statistically significant.
containing protease inhibitor cocktail (Sigma) (3). As positive control, tissue from rabbit midbrain was homogenized in 200 µl of lysis buffer. Samples were homogenized with a sonicator in 1.5-ml polystyrene microcentrifuge tubes and allowed to sit for 15 min on ice before spinning in a microcentrifuge (Eppendorf 5415) at 10,000 rpm for 10 min at 4°C. The supernatants were collected, and protein concentrations (Bio-Rad RC/DC protein assay standardized against BSA standard) were adjusted to 2 mg/ml with 0.1 M Tris-buffered saline (TBS). Samples were heated after Laemmli sample buffer was added with 2-mercaptoethanol and then subjected to 10% SDS-polyacrylamide gel electrophoresis and supported polyvinylidene difluoride membranes. The blots were incubated at 4°C overnight in blocking buffer containing TBS (150 mM NaCl, 50 mM Tris·HCl, pH 7.4) with 0.05% Tween 20 and 5% nonfat dry milk. The blots were incubated with sheep anti-rabbit tryptophan hydroxylase (TPH) antibody (Chemicon, Temecula, CA) and monoclonal antibody against β-actin (Sigma) in TBS with 0.05% Tween 20 containing 3% milk. After washings, rabbit anti-sheep secondary antibody (Pierce, Rockford, IL) diluted 1:10,000 and goat anti-mouse IgG secondary antibody (Chemicon, Temecula, CA) and monoclonal antibody against β-actin (Sigma) were added for 1 h. The membranes were washed and incubated using the Attoglow Western blot system with Millennium Enhancer (BioChain, Hayward, CA) and exposed to autoradiography film. The band densities within the linear range were analyzed with NIH Scion Image software. Values were obtained for individual samples (4 separate experiments performed in duplicate) and normalized to the mean β-actin used as housekeeping protein on the same blot with same exposure time. Means ± SE were calculated for each lane (n = 4). The nonparametric, unpaired t-test was used for comparison between the two groups with Prism GraphPad version 5.01 software (GraphPad Software).

RESULTS

Immunolocalization of 5-HT. We used immunostaining for 5-HT to visualize PNEC with dendritic-like cytoplasmic processes (11, 12). In human fetal/neonatal lung, these cells appeared most prominent within the epithelium of small and medium size bronchi, particularly in a tangential plane of section (Fig. 1A). The dendritic-like processes varied between 50 and 250 µm in length and ran in a longitudinal orientation (Fig. 1B). Such an arrangement would favor transmission of physical forces generated by the breathing movements. Similar

![Image](https://example.com/image1.png)

**Fig. 1.** Serotonin (5-HT)-immunoreactive pulmonary neuroendocrine cells (PNEC) in airway mucosa of developing human lung. A: PNEC within mucosa of small peripheral airway of human fetal lung at 22 wk of gestation. PNEC formed lateral dendritic-like cytoplasmic processes (arrows) extending along the basement membrane. AL, airway lumen. Immunoperoxidase staining for 5-HT; magnification, ×400. B: two closely placed PNEC within the mucosa of a human neonatal lung. Both cells formed unilateral dendritic-like cytoplasmic processes (arrows) extending along the basement membrane (arrowheads). One PNEC also shows an apical cytoplasmic process extending toward the airway lumen. Immunoperoxidase staining for 5-HT; magnification, ×400.

Western blot analysis for tryptophan hydroxylase expression. Cells from individual 35-mm culture wells and dishes (stretched, nonstretched, and exposed to hypoxia conditions) were lysed and homogenized separately in 100 µl of lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 10 mM EDTA, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol, 400 mM sodium orthovanadate, 50 mM NaF, and 2% SDS) for 5-HT; magnification, ×400.

![Image](https://example.com/image2.png)

**Fig. 2.** Cultures of PNEC from rabbit fetal lung immunostained for 5-HT. A: control nonstretched culture from early (E20) gestation rabbit lung with small cell clusters and single cells strongly immunoreactive for 5-HT representing PNEC. Magnification, ×400. B: the same culture (E20) as in A, after 90 min of cyclical stretch, showing well-preserved 5-HT-immunoreactive PNEC. Magnification, ×400. C: control (nonstretched) culture from E26 rabbit lung with larger PNEC clusters immunoreactive for 5-HT and a few isolated PNEC with short cytoplasmic processes. Magnification, ×400. D: the same culture (E26) as in C, after 90 min of cyclical stretch, showing well-preserved 5-HT-immunoreactive PNEC. Magnification, ×400.
cells also are found in the epithelium of larger airways in rabbit fetal/neonatal lungs with most of them innervated by a complex network of interconnecting fine varicose nerve fibers originating in the submucosa (data not shown) (32). PNEC/NEB isolated from early (E20) or late (E26) rabbit fetal lungs and grown on collagen I BioFlex membranes appeared well preserved and were similar to other in vitro preparations used in our previous studies (45). In cultures immunostained with an antibody against 5-HT, the cytoplasm of PNEC/NEB was strongly positive in samples from both early (E20) and late (E26) gestational age (Fig. 2, A and C). These 5-HT-immunoreactive cells either formed small cell aggregates or appeared as isolated cells with short cytoplasmic processes, and their viability and morphological appearance did not change after 90 min of cyclical stretch (Fig. 2, B and D). Ultrastructural analysis of PNEC/NEB in rabbit fetal lungs in situ and in vitro is shown in Fig. 3. The electron microscopy of PNEC/neuroepithelial bodies (NEB) in rabbit fetal lungs in situ and in vitro, A: parts of 3 NEB cells in E20 fetal rabbit lung with nucleus (Nu) and cytoplasm containing prominent ribosomes, occasional strands of rough endoplasmic reticulum (rer), a few mitochondria (mi), and sparse dense core vesicles (DCV; arrow). Magnification, ×12,000. B: culture of PNEC from E20 fetal rabbit lung after 3 days in vitro with a well-preserved nucleus and larger as well as more numerous DCV compared with those in A. Magnification, ×12,000. C: NEB cells in E26 fetal rabbit lung with numerous cytoplasmic DCV and scattered mitochondria. Magnification, ×12,000. D: numerous pleomorphic electron dense DCV in cytoplasm of PNEC from E26 rabbit fetal lung after 3 days in culture. Magnification, ×12,000.

Fig. 4. Morphological characteristics of H727 tumor cell line. A: diffuse positive cytoplasmic staining for 5-HT in practically all tumor cells, whereas nuclei are negative. Magnification, ×1,000. B: positive immunostaining for calcitonin with fine granular cytoplasmic pattern corresponding to localization in DCV. Magnification, ×1,000. C: ultrastructural appearance of representative H727 tumor cell with well-preserved nucleus and cytoplasm containing abundant DCV (arrows), a few lysosomes (ly), and microfilaments (mfi). Magnification, ×11,000.
culture from E20 gestation lungs revealed abundant ribosomes, few mitochondria, and strands of rough endoplasmic reticulum, but only very occasional DCV (Fig. 3, A and B). Hence, in these early fetal samples, the presence of strong cytoplasmic staining for 5-HT in the face of a paucity of DCV is consistent with the distribution of 5-HT in a nongranular cytoplasmic pool. As expected, NEB cells from later in gestation (E26) contained more numerous DCV that appeared concentrated near the basement membrane (Fig. 3, C and D). After 3 days in culture, PNEC in a sample from E20 fetal lungs showed more advanced cytoplasmic differentiation with a somewhat increased number of DCV (Fig. 3B), but still fewer compared with the cultures from E26 lungs (Fig. 3D). Immunostaining of H727 cells revealed strong positive staining of the cytoplasm for 5-HT in a diffuse pattern (Fig. 4A) and a granular immunostaining for calcitonin (Fig. 4B). Ultrastructural analysis of H727 cells showed cells with clear cytoplasm containing variable numbers of classic DCV, pleiomorphic lysosomes, and other organelles characteristic of a neuroendocrine neoplasm (Fig. 4C).

Effect of mechanical stimulation on 5-HT release. To assess the effect of mechanical stimulation on 5-HT release from monolayer cultures of airway epithelium from fetal rabbit lungs enriched in PNEC and cultures of H727 tumor cell line, we designed stretch patterns to evaluate the influence of frequency and amplitude under normal fetal breathing conditions. We measured the release of 5-HT into extracellular medium using a highly sensitive ELISA competitive for 5-HT. In our initial experiments, we found that significant amounts of 5-HT were released from E26 cultures after 90 min of cyclic stretching (60/min) compared with the nonstretched baseline level (94.47 ± 18.50 vs. 22.06 ± 6.67 ng 5-HT/10^5 PNEC, P < 0.001, Student’s t-test) (Fig. 5A). Preincubation of the cells with the blocker of stretch-activated channels, GC (22), significantly inhibited the stretch-induced 5-HT release, suggesting that it occurred via mechanosensitive channels. However, GC did not completely inhibit 5-HT release to the level of nonstretched control baseline (36.65 ± 11.68 vs. 20.43 ± 7.15 ng 5-HT/10^5 PNEC, P < 0.05, Student’s t-test) (Fig. 5A). Interestingly, cultures from E20 rabbit lungs under identical stretch conditions showed almost triple the amount of released 5-HT compared with the cultures from E26 (272.05 ± 76.11 vs. 94.47 ± 18.50 ng 5-HT/10^5 PNEC, P < 0.001, Student’s t-test) (Fig. 5B). In addition, the inhibitory effects of GC on 5-HT release in E20 cultures were more pronounced and were closer to the nonstretched basal level (51.32 ± 9.50 vs. 49.59 ± 7.33 ng 5-HT/10^5 PNEC, P > 0.05, Student’s t-test) (Fig. 5, A and B). The differences in the rate of release and the effects of GC between cultures at E20 and E26 suggest that the 5-HT release may occur from different storage pools that could be developmentally regulated.

Cultures of H727 cells used as a model for PNEC in human lung showed similar 5-HT release characteristics under the same mechanical strain conditions as those used for rabbit fetal PNEC. After 90 min of cyclic stretch, there was significant release of 5-HT compared with nonstretched control cultures, and 5-HT release was inhibited by preincubation with GC (10 nM) to the level of nonstretched controls (Fig. 5C). On the other hand, there was no effect on calcitonin release under identical mechanical stretch conditions (Fig. 6A), although exposure to hypoxia caused a massive release of calcitonin that

Fig. 5. Mechanical stretch-induced 5-HT release from cultures of PNEC from fetal rabbit lungs and H727 tumor cell line. A: PNEC cultures from E26 rabbit fetal lung subjected to 90 min of cyclical stretch, showing significant 5-HT release into medium (expressed as ng 5-HT/10^5 PNEC) compared with nonstretched control cultures (*P < 0.001, by Student’s t-test). This stretch-induced 5-HT release was blocked by incubation with 10 nM gadolinium chloride (GC) (**P < 0.05, by Student’s t-test), a blocker of mechanosensitive channels. The degree of GC-related inhibition was slightly above baseline (nonstretched cultures). No release was detected in cultures of rabbit fibroblasts (RbF) used as negative control. B: mechanical stretch-induced 5-HT release from PNEC in E20 fetal rabbit lung cell cultures subjected to the same conditions as in A, showing that the concentration of 5-HT released into the medium is nearly 3-fold higher compared with E26 cultures (A). Blockade with GC (10 nM) showed near total inhibition of 5-HT release with levels near baseline (**P < 0.05, by Student’s t-test). C: mechanical stretch-induced 5-HT release from H727 tumor cell line under the same experimental conditions as PNEC primary cultures (A and B). The mean concentration of 5-HT is significantly higher in medium from stretched cultures compared with nonstretched control. (**P < 0.05, by Student’s t-test). Incubation with GC (10 nM) caused near complete inhibition of stretch-induced 5-HT release (**P < 0.001, by Student’s t-test). Human lung fibroblasts (HLF) were used as negative control.
was inhibited by nifedipine, a blocker of L-type Ca\(^{2+}\) channels (Fig. 6B). These findings are consistent with calcitonin being stored mainly in DCV and its release dependent on a Ca\(^{2+}\)-mediated exocytic mechanism.

To examine the time course of stretch-induced 5-HT release, we exposed the cultures for various time intervals (0, 5, 10, 15, and 30 min) to cyclic stretch. Significant release of 5-HT was observed within the first 5 min in both rabbit E26 PNEC cultures and H727 cells, with a plateau reached in E26 cultures within 30 min (Fig. 7A). A similar response was observed with H727 cells, even though the amount of 5-HT released was almost 15 times lower than in fetal PNEC cultures (Fig. 7B).

This pattern of rapid rate of release by mechanical stretch is in keeping with the idea that this source of 5-HT is derived from the cytoplasmic pool, by analogy with other small molecules, such as ATP known to be released by mechanical strain (30).

To further define the mechanism of stretch-induced 5-HT release, we compared mechanical stimulus-evoked 5-HT release with hypoxia-induced 5-HT release (17). As expected, cultures exposed to acute hypoxia (5% O\(_2\)) for 90 min showed significant release of 5-HT, inhibited by removal of extracellular Ca\(^{2+}\) or incubation with nifedipine (50 µM), a blocker of voltage-activated Ca\(^{2+}\) channels, essential for the exocytosis of DCV and hypoxia-induced 5-HT release (13, 17) (Fig. 8A). In contrast, incubation with GC (10 nM), a blocker of mechanosensitive channels, had no effect on hypoxia-induced 5-HT release (Fig. 8A). In cultures subjected to 90 min of cyclic stretch and incubated in either a Ca\(^{2+}\)-free medium or a combination of Ca\(^{2+}\)-free medium plus nifedipine, there was no significant reduction in 5-HT release compared with nonstretched control cultures (P = 0.05) (Fig. 8B). These results suggest that the 5-HT release pathway in PNEC/NEB that is evoked by mechanical strain is independent of hypoxia stimulus-evoked neurotransmitter secretion.

Expression of TPH modulated by mechanical stretch. To determine the effects of mechanical stretch on 5-HT synthesis in PNEC/NEB, we assessed the expression of TPH, the rate-limiting enzyme in 5-HT synthesis. The expression of TPH mRNA has been previously documented in both rabbit lung PNEC and PNEC-related tumor cell lines (13, 31, 44). We assessed expression of TPH at a protein level by performing Western blot analysis of cultures of PNEC from E26 rabbit fetal lung subjected to either cyclic mechanical stretch or hypoxia (Fig. 9). A specific band corresponding to TPH with an electrophoretic mobility of ~55 kDa was detected in extracts of rabbit PNEC culture lysates from both sets of experiments. Densitometric analysis of the TPH-specific bands showed a near threefold increase in cell homogenates from both mechanically stretched and hypoxia-stimulated PNEC cultures compared with the nonstretched/normoxic control cultures. The homogenates from stretched cells treated with GC or hypoxia-challenged cells treated with nifedipine showed a similar TPH-immunoreactive band and the same electrophoretic mobility. There was no apparent change in TPH expression level, indicating that this enzyme is not affected by these blockers.

Fig. 6. A: cultures of H727 tumor cell line exposed to 90 min of mechanical stretch, showing levels of calcitonin in the culture medium similar to those in nonstretched controls. Incubation with GC (10 nM) had no effect on calcitonin release in either stretched or nonstretched samples. B: effects of hypoxia on calcitonin release from H727 tumor cells. Exposure of H727 cell cultures for 90 min of hypoxia (HOX-5% O\(_2\)) resulted in massive release of calcitonin into the medium compared with basal release observed under normoxia (NOX). Hypoxia-induced calcitonin release was inhibited by nifedipine (50 µM), a blocker of L-type Ca\(^{2+}\) channels.

Fig. 7. Time course of mechanical stretch-induced 5-HT release from E26 rabbit (Rb) fetal lung PNEC culture (A) and H727 tumor cells (B). In both cell preparations, significant 5-HT release occurred within the first 5 min of stretching and reached plateau after 30 min.
could promote differentiation of adjacent epithelial cells (11, 12, 15, 38, 39, 42). A similar role has been postulated for 5-HT, a pleiotropic amine highly expressed in PNEC, because its actions include that of a mitogen and a morphogen, particularly in the developing central nervous system (25, 42). In addition, various 5-HT receptors have been identified in alveolar, bronchial, and vascular smooth muscle components of the lung (35, 41).

The rabbit fetal lung culture model used in this study is particularly well suited for studies on PNEC/NEB, because these cells are relatively numerous in this species, and their morphology and development, as well as expression of neuroendocrine markers including 5-HT, have been well characterized (7, 34, 40). Furthermore, the function of NEB as hypoxia-sensitive chemoreceptors has been confirmed in this species with cellular and molecular biology approaches (9, 10, 17, 45). The rabbit model also approximates PNEC/NEB in developing human lung, including expression of 5-HT during early stages of lung development as well as the expression of gastrin-releasing peptide and 5-HT receptors (41, 42). The advantages of human lung carcinoma tumor line H727 include its derivation from a carcinoid tumor expressing both 5-HT and calcitonin and that it can be grown as a monolayer suitable for mechanical stretch experiments.

The principal finding of the present study is the demonstration of the release of 5-HT by mechanical strain from PNEC/NEB isolated from rabbit fetal lungs and from the H727 tumor cell line, representative of human PNEC. The relative amount of stretch-releasable 5-HT was significantly higher in PNEC cultures from early (E20) gestation compared with E26 or H727 cells. At E20, PNEC are in early stages of differentiation and contain only sparse DCV, whereas in PNEC cultures from both E26 and H727 cells, more numerous DCV are present in the cytoplasm. This finding is consistent with the notion that the released 5-HT is derived mostly from the nongranular cytoplasmic pool, because PNEC at E20 show strong cytoplasmic immunoreactivity for 5-HT but only a few DCV. The evidence for the existence of cytoplasmic and granular pools

DISCUSSION

In human fetal lungs, the first PNEC immunoreactive for 5-HT are identified at ~8 wk of gestation, when the lung consists of a few primitive epithelial tubes embedded within abundant, poorly differentiated mesenchyma (31). At the ultrastructural level, the primitive airway epithelial cells, the precursors of ciliated, mucus, and serous cells, appear undifferentiated and contain large amounts of cytoplasmic glycolgen with only occasional cell organelles (15). In contrast, early PNEC are characterized by sparse cytoplasmic glycolgen and the appearance of more numerous cell organelles, including mitochondria, the Golgi complex, rough endoplasmic reticulum, and a very occasional DCV, the first definitive sign of neuroendocrine differentiation (11, 12, 15). The finding that PNEC are the first cell type to differentiate in early stages of fetal lung development led to the suggestion that these cells could promote differentiation of adjacent epithelial cells (11,
for the storage and release of catecholamines has been well documented in early studies on the metabolism of biogenic amines, including 5-HT (29). For example, studies using isolated synaptosomes have demonstrated both Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent 5-HT release mechanisms corresponding to the granular and cytoplasmic compartments, respectively (19). The mechanical stretch-induced 5-HT release was inhibited by GC, a blocker of mechanosensitive channels, but there was no effect on the stretch-induced 5-HT release when PNEC cultures were exposed to Ca\textsuperscript{2+}-free medium or to nifedipine, a selective blocker of L-type voltage-activated Ca\textsuperscript{2+} channels. These findings indicate that the mechanical strain-induced 5-HT release occurs via mechanosensitive channels; however, the precise nature of such channels in PNEC is at present unknown. A large number of mechanosensitive channels have been identified in eukaryocytes involved in the cell physiology and mechanotransduction, including the perception of touch, hearing, osmotic gradients, cell swelling, and other activities (21). Furthermore, mechanical stimuli are known to cause changes in the plasma membrane and subsequently affect exocytic traffic, including secretion of extracellular matrix proteins, surfactant, various growth factors, and hormones such as atrial natriuretic hormone, angiotensin II, and endothelin I (2). The mechanical strain-induced 5-HT release could be analogous to well-characterized mechanosensitive release of ATP, a small molecule that acts as a neurotransmitter of mechanosensation in various tissues (4). Recent studies have indicated that purinergic mechanisms, including ATP release, may be involved in PNEC/NEB function, particularly the chemotransmission and modulation of hypoxia signaling via P2X\textsubscript{2-3} receptors expressed on NEB cells and on the nerves that innervate them (5, 16). Reported candidates for ATP release pathways include ABC transporter proteins (e.g., CFTR, P-glycoprotein), hemi gap-junction channel, and exocytosis of ATP-containing vesicles (22). Interestingly, PNEC/NEB express both CFTR (20) and the hemi gap-junction protein connexin43 (unpublished observation). The present study indicates that the exocytosis of DCV is apparently not involved in the stretch-induced 5-HT release from PNEC/NEB, although it appears to be the major pathway for hypoxia-stimulated release (13, 17). The massive release of calcitonin by hypoxia but the lack of stretch-induced release of calcitonin (stored in cytoplasmic DCV) in H727 cells also supports this conclusion.

The existence of distinct pools of 5-HT that may be released by different mechanisms is in keeping with the multimodal function postulated for PNEC/NEB in the lung. Such functions may be developmentally regulated, because in PNEC from early stages of lung development, the cytoplasmic pool of 5-HT predominates. The role of locally released 5-HT in early stages of lung development may involve effects on cell proliferation and differentiation as well as that of a neurotransmitter signaling via emerging PNEC innervation (32). During the later stages of lung development, the stretch-induced 5-HT release could be complemented with hypoxia-stimulated exocytosis mechanism once the necessary cell machinery develops for the synthesis and storage of amine and peptides within DCV. It has been postulated that 5-HT released from PNEC/NEB in fetal lung may be involved in the regulation of lung fluid production and clearance, and hence may play a role during the transition from fetal to extraterine life. In experiments using isolated guinea pig fetal lungs, it has been observed that 5-HT reduced lung fluid production and increased its absorption, particularly in fetal lungs close to term (8). This effect was inhibited by both the 5-HT receptor blocker and amiloride, suggesting that it may act through the classic amiloride-sensitive Na\textsuperscript{+}-based reabsorptive system.

Our finding of mechanical strain-induced upregulation of TPH, the rate-limiting enzyme in 5-HT synthesis, is in keeping with a physiological response to the depletion of biogenic amine involved in a feedback homeostatic mechanism. The lack of inhibitory effects of GC (blocker of mechanosensitive channels) on TPH expression suggests that the regulation of TPH synthesis may involve other cellular components of the mechanosensitive apparatus that sense cellular deformation, including integrins, microfilaments, and microtubules (33). This interpretation is supported by a similar TPH response observed in PNEC cultures exposed to hypoxia that was not affected by incubation with nifedipine, suggesting involvement of a Ca\textsuperscript{2+}-independent pathway (36).

Postnatally, stretch-mediated 5-HT release could be involved in airway signaling via vagal mucosal stretch receptors. Recent studies by Adriaensen et al. (1) on the innervation of NEB in rat lung revealed a complex neural network, suggesting multimodal receptor function including that of mechanosensitive receptors. The mechanical strain-induced 5-HT release also could play a role in the pathogenesis of ventilator-induced chronic lung disease in preterm infants with broncho-pulmonary dysplasia in which hyperplasia of PNEC has been documented (18). Hence, our finding of a potential novel mechanism for 5-HT release should stimulate further studies related to pulmonary physiology and pathophysiology, particularly during the perinatal period. In addition, our findings on this novel 5-HT release mechanism may be relevant to other organ systems such as the gastrointestinal tract, known to contain numerous 5-HT-containing enterochromaffin cells and the gut wall being subjected to mechanical stretch.

Acknowledgments

We thank V. Wong for excellent technical assistance with immunohistochemistry and electron microscopy.

Grants

This work was supported by Canadian Institutes of Health Research Operating Grant MOP15270 (to E. Cutz and H. Yeger).

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