

# Side Specific Effect of 5-Hydroxytryptamine on NaCl Transport in the Apical and Basolateral Membrane of Rat Tracheal Epithelia

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Received January 14, 2005

Accepted August 23, 2005

On-line available October 17, 2005

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## Summary

5-Hydroxytryptamine (5-HT) can be released from mast cells and platelets through an IgE-dependent mechanism and may play a role in the pathogenesis of allergic bronchoconstriction. However, the effect of 5-HT on ion transport by the airway epithelium is still controversial. The objective of this study was to determine whether 5-hydroxytryptamine (5-HT) regulates NaCl transport by different mechanisms in the apical and basolateral membrane of tracheal epithelia. We studied the rat tracheal epithelium under short-circuit conditions *in vitro*. Short-circuit current ( $I_{sc}$ ) was measured in rat tracheal epithelial monolayers cultured on porous filters. 5-HT inhibited  $Na^+$  absorption [measured *via*  $Na^+$  short-circuit current ( $I_{sc}^{Na}$ )] in the apical membrane and stimulated  $Cl^-$  secretion [measured *via*  $Cl^-$  short-circuit current ( $I_{sc}^{Cl}$ )] in the basolateral membrane. Functional localization using selective 5-HT agonists and antagonists suggest that  $I_{sc}^{Cl}$  is stimulated by the basolateral membrane-resident 5-HT receptors, whereas  $I_{sc}^{Na}$  is inhibited by the apical membrane-resident 5-HT<sub>2</sub> receptors. The basolateral addition of 5-HT increases intracellular cAMP content, but its apical addition does not. The addition of BAPTA/AM blocked the decrease of  $I_{sc}^{Na}$  which was induced by the apical addition of 5-HT, and 5-HT increased intracellular Ca concentrations. These results indicate that 5-HT differentially affects  $I_{sc}^{Na}$  and  $I_{sc}^{Cl}$  across rat tracheal monolayers through interactions with distinct receptors in the apical and the basolateral membrane. These effects may result in an increase of water movement towards the airway lumen.

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## Key words

5-hydroxytryptamine • Tracheal epithelia • NaCl transport

## Introduction

5-Hydroxytryptamine (5-HT) is found in high concentrations in platelets, enterochromaffin cells located throughout the gastrointestinal tract, and in certain regions of the brain (Garrison and Rall 1990). 5-HT in the airways is primarily synthesized by and released from neuroendocrine cells in the airways in response to

alterations in airway gas chemical composition (Johnson and Georgieff 1989). It has been demonstrated to be a potent bronchoconstrictor and to stimulate proliferation of smooth muscle cells (Seuwen *et al.* 1988) and fibroblasts (Nemecek *et al.* 1986, Meloche *et al.* 1992). It is well established that 5-HT-secreting tumors are associated with an altered secretory and absorptive balance resulting in diarrhea and bronchorrhea (Brown

1977; Donowitz *et al.* 1977). This association of 5-HT with increased salt and fluid secretion has generally been explained on the basis of 5-HT as a secretagogue (Thompson 1977). Studies for electrolyte transport in intact tissues support the presence of 5-HT receptors in the rat colonic mucosa (Bunce *et al.* 1991, Siriwardena *et al.* 1991, Engelmann *et al.* 2002), salivary gland (Fain and Berridge 1979), jejunum (Kellum *et al.* 1994) and conjunctival epithelia (Alvarez *et al.* 2001).

However, the exact mechanism by which 5-HT causes these actions is still debatable. Cooke (1984) showed that the action of 5-HT in the guinea pig ileal mucosa is in part mediated by the submucosal plexus of nerves through an atropine-sensitive pathway. Siriwardena *et al.* (1991) showed that changes in electrolyte transport induced by 5-HT may be mediated by non-neural pathways as well as neural pathways in rat colon. Jegris *et al.* (1982) reported that 5-HT inhibits Na<sup>+</sup> absorption in baboon broncheal mucosa. Studies using rabbit tracheal sheet and dog tracheal mucosal preparations also suggested that 5-HT affects NaCl transport (Jegris *et al.* 1982, Tamaoki *et al.* 1997, Greczko and Tyrakowski 2001).

However, studies using mucosal preparations cannot provide direct evidence about the presence of 5-HT receptors in epithelium itself, because they contain heterogeneous groups of cells and nervous elements. Jung *et al.* (1997) reported that 5-HT directly stimulates Cl transport in cultured rat tracheal epithelia. However, the sidedness of 5-HT effect on Na and Cl transport in airway epithelia is still not clear.

The aim of the present study was to evaluate the sidedness of 5-HT on Na and Cl transport in airway epithelia. Na and Cl current were separately measured to evaluate the effects of 5-HT on Na and Cl transport, after rat tracheal epithelial monolayers had been cultured on permeable filters and mounted into a Ussing chamber system.

## Material and Methods

### Primary cell culture

Sprague Dawley rats (200-300 g) were anesthetized with an intraperitoneal injection of pentobarbital sodium (120 mg/100 g b.w.). Primary cell cultures were treated by a minor modification of the procedures described previously (Hwang *et al.* 1996). Briefly, freshly excised tracheae were incubated at 4 °C for 18-24 h in a Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free, serum-free modified

Eagle's minimum essential medium (MEM) containing 1 mg/l protease XIV (Sigma, St. Louis, MO). The epithelial cells were removed from the airways by scraping the epithelial surface of the tracheae, and the cells were then washed with fresh MEM medium (see above) containing fetal bovine serum (FBS) to neutralize the protease. After the final wash, the cells (by 10<sup>6</sup>) were resuspended in DMEM/Ham's F-12 mixture containing insulin, transferrin, hydrocortisone, triiodothyronine, prostaglandin E, epidermal growth factor and 5 % FBS (K1-10 medium), and 0.5 ml of final cell suspension was added to each permeable filter supports that were coated with collagen type I (12 mm SNAP-WELL; Costar, Cambridge, MA). The cells were incubated in an atmosphere of 5 % CO<sub>2</sub>-95 % air at 37 °C in the K1-10 medium containing 5 % FBS and 500 U/l penicillin and 500 ng/l streptomycin. Typically, on the 4th or 5th day after seeding the cells, transepithelial resistance, measured by EVOM epithelial ohmmeter (World Precision Instruments, Sarasota, FL) or by Ohm's law after steady-state voltage and current were recorded for a particular monolayer, the values being above 1000 Ω x cm<sup>2</sup>.

### Electrophysiology

Transepithelial electrophysiological measurements were performed in a modified Ussing chamber constructed to accept SNAPWELL filter (World Precision Instrument). The short-circuit current ( $I_{sc}$ ) was measured with a DVC-1000 voltage-current clamp (World Precision Instrument) in the voltage clamp mode. The filters above 1000 Ω x cm<sup>2</sup> in transepithelial resistance measured by an EVOM epithelial ohmmeter were used for all experiments. The bath solution was a Krebs' bicarbonate Ringer's solution (KBR) that was composed of (in mM) 140 NaCl, 2.3 K<sub>2</sub>HPO<sub>4</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 1.2 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, and 10 HEPES, 10 glucose (pH 7.4). Both mucosal and serosal bath solutions were subjected at constant recirculation, maintained at 37 °C, and oxygenated gently with 100 % O<sub>2</sub>. In these measurements of secretory Cl<sup>-</sup> current, 10 μM amiloride was added to the mucosal side to block absorptive Na<sup>+</sup> current ( $I_{sc}^{Na}$ ) to measure  $I_{sc}^{Cl}$  specifically. To measure  $I_{sc}^{Na}$ , the bath solution on mucosal and serosal sides was replaced with Cl<sup>-</sup>-free, sodium gluconate solutions that was composed of (in mM) 136 sodium gluconate, 3 potassium gluconate, 2 MgSO<sub>4</sub>, 10 HEPES, 10 glucose, and 2 CaSO<sub>4</sub>, adjusted to pH 7.4. Under these conditions,  $I_{sc}$  is equivalent to  $I_{sc}^{Na}$ .

### Measurement of cAMP

Tracheal monolayers were grown on permeable supports, washed, and stimulated on the serosal side with 5-HT (50  $\mu$ M) and forskolin (5  $\mu$ M) and on the apical side with 5-HT (50  $\mu$ M) until maximal  $I_{sc}$  changes occurred (usually 3 min after addition of agents). At this time the monolayer was removed from the Ussing chamber and placed into ice cold trichloroacetic acid. A radioimmunoassay kit (Amersham, Arlington Heights, IL) was used to measure cAMP concentration.

### Measurement of changes in intracellular free $Ca^{2+}$

After adherence of cells to cover slips, the cover slips were incubated in a control bath solution containing 2  $\mu$ M Fura 2-AM for 60 min at 37 °C. Cells were then washed in a fresh buffer to remove the residual Fura loading solution. Fura-2-loaded cells were placed in a temperature controlled superfusion chamber positioned on the stage of an inverted microscope. Changes in intracellular  $Ca^{2+}$  were quantitated using a fluorescence imaging system (Universal Imaging, Downingtown, PA) or a spinning excitation filter wheel coupled to a photometer system employing a Hamematsu (Bridgewater, NJ) photomultiplier tube and signal conditioner (Texas A and M University, College Station, TX). Specimens were exposed to excitation wavelengths of 340, 360, or 380 nm as appropriate and fluorescence emission measured at 510 nm. Changes in intracellular  $Ca^{2+}$  levels were expressed in terms of the 340/380 nm ratio.

### Materials

All chemicals and drugs were purchased from Sigma, unless otherwise indicated. FBS was purchased from GIBCO BRL. Thapsigargin was purchased from RBI. 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) was purchased from Molecular Probes (Eugene, OR).

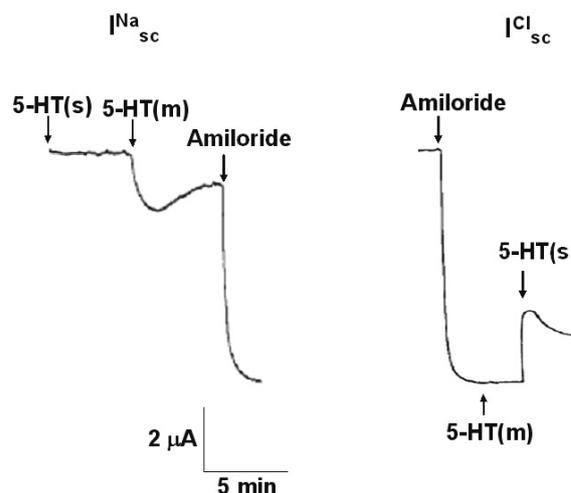
### Data presentation and statistical analysis

Data were presented as agonist-induced maximal changes of  $I_{sc}$  from the baseline (means  $\pm$  SEM). Comparisons between groups and matched pairs were made using Student's *t*-test (two-sided). Differences were assumed to be significant when  $P < 0.05$ .

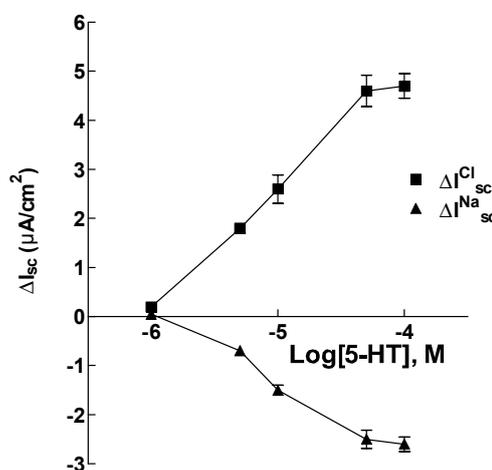
## Results

### Effect of 5-HT on $I_{sc}^{Na}$ and $I_{sc}^{Cl}$

In normal KBR solution, the rat tracheal

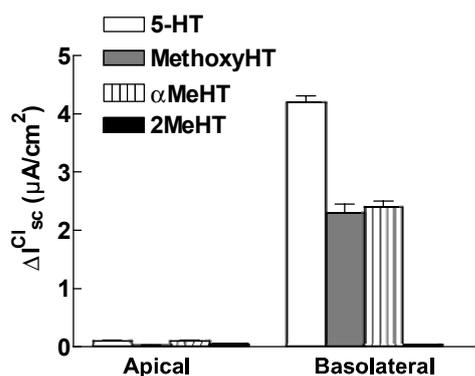


**Fig. 1A.** Representative current record illustrating the effects of 5-HT on  $I_{sc}^{Na}$  and  $I_{sc}^{Cl}$ . 5-HT (50  $\mu$ M) was added to the apical (m) and basolateral (s) bath solutions.



**Fig. 1B.** Dose-response curve for 5-HT-induced effect on  $I_{sc}$ . Values shown are means  $\pm$  SE for 4 experiments.

monolayers exhibited a transmembrane potential difference (PD) of  $-15.7 \pm 2.8$  mV (mucosal side) and a basal  $I_{sc}$  of  $13.2 \pm 2.1$   $\mu A/cm^2$ . To determine whether extracellular 5-HT regulate electrogenic  $Na^+$  and/or  $Cl^-$  transport,  $I_{sc}^{Na}$  and  $I_{sc}^{Cl}$  across monolayers of rat tracheal epithelial cells mounted in Ussing chambers were measured. In the absence of  $Cl^-$  in the bathing solution, the apical addition of 5-HT (10  $\mu$ M) induced inhibition of  $I_{sc}^{Na}$  and the addition of 10  $\mu$ M amiloride completely inhibited  $I_{sc}$ , but its basolateral addition did not (Fig. 1A). In contrast, the basolateral addition of 5-HT in the presence of 10  $\mu$ M amiloride significantly increased  $I_{sc}^{Cl}$ , but its apical addition did not (Fig. 1A). 5-HT-induced increase of  $I_{sc}^{Cl}$  was blocked by the addition of the basolateral bumetanide (20  $\mu$ M). In the dose-response



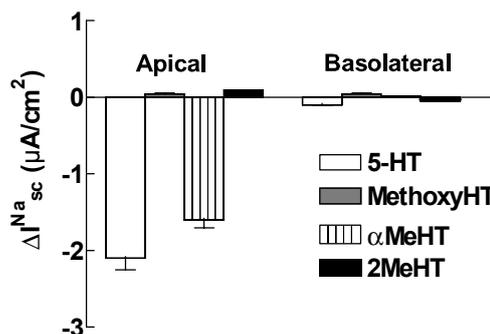
**Fig. 2.** Polarity of 5-HT receptor agonist effects on  $I_{sc}^{Cl}$ . Sidedness of the effects of 5-HT, 5-methoxytryptamine, 2-methyl-5-HT, and  $\alpha$ -methyl-5-HT on  $I_{sc}^{Cl}$  is shown. The agonists were added to the apical or basolateral solution. Values shown are means  $\pm$  SE for 5 experiments.

experiment 5-HT showed the maximal response at 50  $\mu$ M, and the  $EC_{50}$  was about 5  $\mu$ M (Fig 1B).

#### Pharmacological identification of 5-HT receptors

To identify the subtypes of 5-HT receptors that regulate  $I_{sc}^{Na}$  and  $I_{sc}^{Cl}$  in the apical and basolateral membrane in tracheal epithelia, effects of 5-HT receptor agonists on  $I_{sc}^{Na}$  or  $I_{sc}^{Cl}$  were examined. 5-methoxytryptamine, a 5-HT<sub>4</sub> receptor agonist, and  $\alpha$ -methyl-5-hydroxytryptamine, a 5-HT<sub>2</sub> agonist, induced  $I_{sc}^{Cl}$  increase, but 2-methyl-5-hydroxytryptamine, a 5-HT<sub>3</sub> receptor agonist, did not. A relative agonist potency was exerted by 5-HT > 5-methoxytryptamine =  $\alpha$ -methyl-5-hydroxytryptamine (Fig. 2).

In the case of  $I_{sc}^{Na}$ , 5-HT and  $\alpha$ -methyl-5-hydroxytryptamine induced inhibition (Fig. 3). In this experiment, we used 10  $\mu$ M 5-HT to maximize the effect of antagonists. The effects of 5-HT receptor antagonists on the responses to 5-HT receptor agonists were tested. ICS-205-930 (10  $\mu$ M), a 5-HT<sub>4</sub> receptor antagonist, or ketanserin, a 5-HT<sub>2</sub> receptor antagonist, (1  $\mu$ M) failed to inhibit the  $I_{sc}^{Cl}$  rise induced by 5-HT (10  $\mu$ M), although ICS-205-930 or ketanserin inhibited the 5-methoxytryptamine (10  $\mu$ M) or  $\alpha$ -methyl-5-HT (10  $\mu$ M) response, respectively (Fig. 4A). The combined treatment of ICS-205-930 and ketanserin still failed to inhibit 5-HT-induced rise of  $I_{sc}^{Cl}$ . To determine the possibility that the inhibitory effects of 5-HT antagonists depend on the concentration of 5-HT to be used, we also tested the effects of 5-HT antagonists on the responses induced by lower concentrations of 5  $\mu$ M 5-HT. However, ICS-205-930 or ketanserin did not affect the responses (data not shown). In the case of  $I_{sc}^{Na}$ , ketanserin inhibited the effect of 5-HT and  $\alpha$ -methyl-5-HT (Fig. 4B).



**Fig. 3.** Polarity of nucleotide receptor agonist effects on  $I_{sc}^{Na}$ . Sidedness of the effects of 5-HT, 5-methoxytryptamine, 2-methyl-5-HT, and  $\alpha$ -methyl-5-HT on  $I_{sc}^{Na}$  is shown. The agonists were added to the apical or basolateral solution. Values shown are means  $\pm$  SE for 5 experiments.

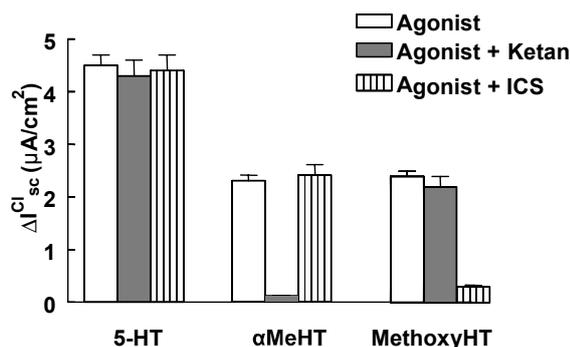
#### Signaling mechanism of the 5-HT action

To determine whether the observed effects of 5-HT were dependent on adenylate cyclase activity, the effect of pretreatment of forskolin on the 5-HT-induced response was examined. Forskolin (5  $\mu$ M) was initially added to the serosal side of a monolayer and, after an additional 5 min, 5-HT (50  $\mu$ M) was added to the apical or basolateral membrane. When 5-HT was added to cells that had been stimulated previously with the maximal concentration of forskolin (5  $\mu$ M), no significant increase of  $I_{sc}^{Cl}$  was observed, indicating that the action of 5-HT on  $I_{sc}^{Cl}$  is mostly mediated by an increase in the cAMP content. However, 5-HT-induced decrease in  $I_{sc}^{Na}$  was not affected by the pretreatment with forskolin (Fig. 5). To confirm this possibility, changes in cAMP concentration by 5-HT were measured. The basolateral addition of 5-HT (50  $\mu$ M) and forskolin (5  $\mu$ M) increased intracellular cAMP concentration by 18.4 and 37.3 fold, respectively, whereas the apical addition of 5-HT did not (Table 1).

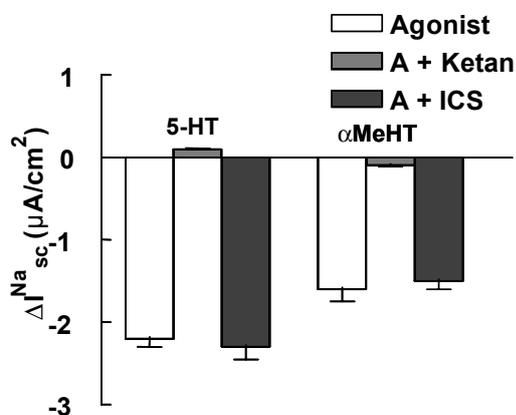
**Table 1.** Effects of 5-HT on cAMP content in tracheal epithelia.

	cAMP content
Control	17.1 $\pm$ 2.1
+ Forskolin	638.8 $\pm$ 34.2 *
+ 5-HT (Basolateral)	315.3 $\pm$ 24.5 *
+ 5-HT (Apical)	15.6 $\pm$ 1.2

cAMP is expressed in pmoles cAMP/filter. Values are mean  $\pm$  SE for 3 experiments. \*  $p < 0.01$ , significantly different from control data



**Fig. 4A.** Effects of 5-HT antagonists on 5-HT agonists-induced  $I_{sc}^{Cl}$ . To examine effects of antagonists, ketanserin (1  $\mu$ M) and ICS-205-930 (10  $\mu$ M) were applied to the basolateral side before 10 min of addition of 5-HT (10  $\mu$ M), 5-methoxytryptamine (10  $\mu$ M) or  $\alpha$ -methyl-5-HT (10  $\mu$ M). Values shown are means  $\pm$  SE for 5 experiments. \*  $p < 0.05$ , significantly different from the data of agonist alone.

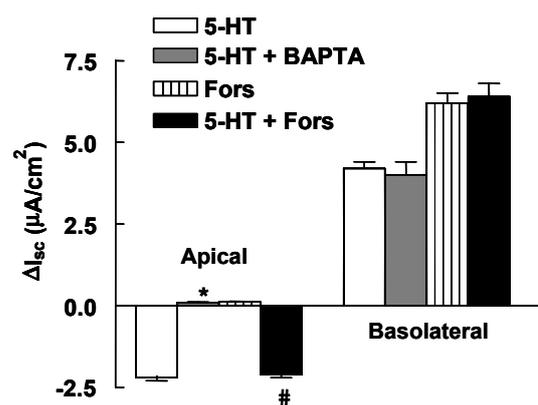


**Fig. 4B.** Effects of 5-HT antagonists on 5-HT agonists-induced  $I_{sc}^{Na}$ . To examine effects of antagonists, ketanserin (1  $\mu$ M) and ICS-205-930 (10  $\mu$ M) were applied to the apical side 10 min before addition of 5-HT (10  $\mu$ M) or  $\alpha$ -methyl-5-HT (10  $\mu$ M). Values shown are means  $\pm$  SE for 5 experiments. \*  $P < 0.05$ , significantly different from the data of agonist alone.

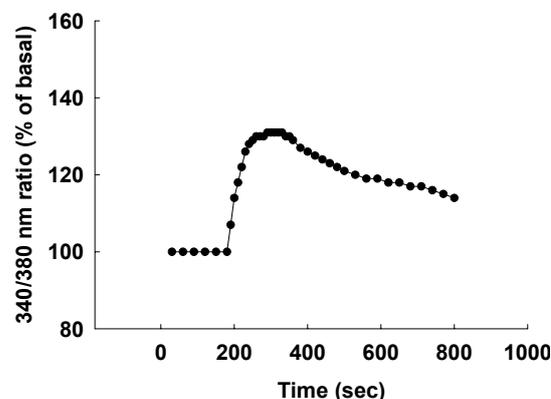
To determine the role of cytosolic  $Ca^{2+}$  in mediating the response to 5-HT, the effect of BAPTA/AM was examined. Pretreatment of BAPTA/AM (10  $\mu$ M, 1 h, basolateral) did not affect the 5-HT-induced  $I_{sc}^{Cl}$  increase, but completely inhibited the 5-HT-induced  $I_{sc}^{Na}$  decrease (Fig. 5A). To determine whether 5-HT affect intracellular  $Ca^{2+}$  concentrations, 50  $\mu$ M 5-HT was added into Fura-2/AM-loaded cells grown on coverslips. The addition of 5-HT increased intracellular  $Ca^{2+}$  concentrations in rat tracheal epithelial cells (Fig. 5B).

## Discussion

The present study has shown that 5-HT stimulated  $I_{sc}^{Cl}$  and inhibited  $I_{sc}^{Na}$  in tracheal epithelia in a dose-dependent manner. The responses were mediated by



**Fig. 5A.** Effect of BAPTA and forskolin on 5-HT-induced effects on  $I_{sc}^{Cl}$  and  $I_{sc}^{Na}$  in rat tracheal epithelia. After the treatment of forskolin (Fors, 5  $\mu$ M) or BAPTA/AM (10  $\mu$ M) to the basolateral side, 5-HT (50  $\mu$ M) was added to the apical or basolateral bath solution. Values shown are means  $\pm$  SE for 4 experiments. \*  $P < 0.05$  significantly different from the data of 5-HT alone.



**Fig. 5B.** Effect of 5-HT on intracellular  $Ca^{2+}$  concentrations of primary cultured rat tracheal epithelia. At the indicated time points, 5-HT (50  $\mu$ M) was added to coverslips containing Fura-2-loaded rat tracheal epithelial cells.

interaction with the different receptors in the apical and basolateral membrane, because the 5-HT response on  $I_{sc}^{Na}$  and  $I_{sc}^{Cl}$  was only observed by the apical or the basolateral addition, respectively.

Trans epithelial  $Cl^-$  secretion which occurs in the airway epithelium is dependent on two types of ion channels, namely  $Cl^-$  channels at the apical membrane and  $K^+$  channels at the basolateral membrane (Welsh 1987, Boucher 1994).  $Cl^-$  enters the cells across the basolateral membrane *via* an electrically neutral  $Na^+-K^+-2Cl^-$  cotransporter.  $Cl^-$  then exits across the apical membrane through  $Cl^-$  channels. The  $Cl^-$  channels in the mucosal membrane of tracheal epithelia are regulated by changes in cAMP and  $Ca^{2+}$  levels (Anderson and Welsh 1991).

In this study the pretreatment of forskolin

completely inhibited the 5-HT response on  $I_{sc}^{Cl}$ , the basolateral addition of 5-HT, not its apical addition, increased intracellular cAMP levels, and the pretreatment of BAPTA/AM did not affect the 5-HT response on  $I_{sc}^{Cl}$ , indicating that cAMP mediates the secretory response of chloride to 5-HT in the basolateral membrane of rat tracheal epithelia. The 5-HT-induced increase in intracellular  $Ca^{2+}$  concentrations in tracheal epithelial cells that were grown on coverslips may be the result of the activation of the apical 5-HT receptors not of the basolateral 5-HT receptors. It has been shown that the culture of epithelial cells on culture dishes or coverslips, disrupts the polarity of the distribution of membrane protein but this not the case of those cultivated on the permeable support. It has been known that 5-HT increases  $Cl^-$  secretion in human jejunal mucosa *via* the activation of 5-HT<sub>4</sub> receptor (Kellum *et al.* 1994) and porcine conjunctival epithelia (Turner *et al.* 2003), its action being mediated by cAMP.

Many agents have been reported to influence airway epithelial functions through the alteration of intracellular  $Ca^{2+}$  concentrations. In the airway smooth muscle 5-HT induces contraction *via* 5-HT<sub>2</sub> receptor the action of which is mediated by the activation of phospholipase C and by the subsequent increase in intracellular  $Ca^{2+}$  concentration (Cohen *et al.* 1985, Baumgartner *et al.* 1990). In this experiment, the pretreatment of BAPTA/AM and 5-HT<sub>2</sub> receptor antagonist did inhibit the responses of 5-HT on  $I_{sc}^{Na}$  and 5-HT increased intracellular  $Ca^{2+}$  concentrations. The data indicate that alterations of cytosolic  $Ca^{2+}$  level through the activation of 5-HT<sub>2</sub> receptor in the apical membrane of rat tracheal epithelia plays important role in the responses of  $I_{sc}^{Na}$  to 5-HT.

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According to some reports it seems that 5-HT induced  $Cl^-$  secretion in the intestine by a non-neural pathway and may be mediated through the 5-HT<sub>4</sub> receptor (Budhoo and Kellum, 1994, Kellum *et al.* 1994). Because these authors used mucosal preparations for their experiment, which have mixed populations of cells, the results did not provide direct information about the presence and identity of 5-HT receptors in the intestinal epithelium itself. In our experiments, the  $I_{sc}^{Cl}$  response elicited by 5-HT in tracheal epithelia was not inhibited by single or combined treatment of 5-HT<sub>2</sub> and 5-HT<sub>4</sub> receptor antagonists (Fig. 5), although 5-HT<sub>2</sub> or 5-HT<sub>4</sub> agonist itself induced an  $I_{sc}^{Cl}$  increase (Fig. 3). This is consistent with the previous study using nonpermeabilized preparation of primary cultured epithelia (Jung *et al.* 1997) in which only 10  $\mu$ M antagonists were used. In this experiment, we tried to use higher concentrations of antagonists, but we could not measure ion-specific  $I_{sc}$  due to disruption of the tight junction by nonspecific effects of antagonists in permeabilized tracheal epithelia. However, the antagonists failed to inhibit the stimulatory action of  $I_{sc}^{Cl}$  by lower concentrations of 5-HT (5  $\mu$ M). Therefore, these data suggest that the 5-HT response in rat tracheal epithelia may be mediated by different 5-HT receptors except 5-HT<sub>4</sub> and 5-HT<sub>2</sub>, although we still cannot exclude the possibility that antagonists failed to block the interaction between 5-HT and their receptors completely in the antagonist concentrations used in the experiment.

In conclusion, the present study indicates that 5-HT has differential effects on  $Na^+$  and  $Cl^-$  transport in the apical and basolateral membrane of primary cultured rat tracheal epithelia, which may be mediated by the interaction with different receptors.

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