Removal of Extracellular Mg\(^{2+}\) Suppresses Sulfation of Glycoconjugates Secreted from Rabbit Trachea in Culture

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ABSTRACT—The influences of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) concentrations on the basal secretion of glycoconjugates from rabbit trachea in organ culture were examined. Over 80% of the \(^{35}\)S-labeled and \(^{3}H\)glucosamine-labeled glycoconjugates secreted by the trachea were digested upon incubation with chondroitinase ABC. The basal secretion did not occur in the medium at 4°C, indicating an energy-dependent process. The basal secretion at 37°C of \(^{35}\)S-labeled glycoconjugates was prominently suppressed in Mg\(^{2+}\)-free Tyrode solution but not in Ca\(^{2+}\)-free Tyrode solution containing ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA). In contrast, the basal secretion of \(^{3}H\)glucosamine-labeled glycoconjugates was not affected by the Mg\(^{2+}\) concentration in the medium. The results suggest that extracellular Mg\(^{2+}\) largely contributes to sulfation of glycoconjugates basally secreted from rabbit trachea.

Keywords: Trachea (rabbit), Magnesium dependency, Sulfated glycoconjugates secretion, Chondroitin sulfate

Sputum consists of a complex mixture of macromolecules including proteins, serum-type glycoproteins, mucins, lipids, and nucleic acids (1), proteoglycans, and glycosaminoglycans (2, 3).

Bhaskar et al. (2) have reported material with features of proteoglycans in bronchial mucus aspirates from healthy volunteers, and Le Treut et al. (3) have presented some evidence for the presence of proteoglycans in the spuata of patients suffering from severe chronic bronchial hypersecretion. Hyaluronic acid has also been characterized in the bronchoalveolar lavage fluid of asthmatic patients (4). Furthermore, Rahmoune et al. (5) suggested a relationship between the presence of chondroitin sulfate proteoglycans in spuata and severe tracheobronchial infection in cystic fibrosis. Although the pathophysiological significance of the glycoconjugates remains unclear, it has been reported that sulfated glycoconjugates such as chondroitin sulfate, heparan sulfate and dextran sulfate may be potent inhibitors of human leukocyte elastase-mediated lung injury (6).

There are reports indicating the secretion of glycosaminoglycans by organ cultures of airway mucosa (7, 8) or from tracheal cells grown in culture (9, 10). There are also reports of an increased sulfation of glycoconjugates including glycosaminoglycans by cultured nasal epithelial cells from patients with cystic fibrosis (11). However, little is known about the regulation of the sulfated glycoconjugates secretion.

The aim of our study is to examine the role of extracellular Ca\(^{2+}\) and Mg\(^{2+}\) in the sulfated glycoconjugates secretion from rabbit trachea in organ culture by means of removal of each ion from culture medium.

MATERIALS AND METHODS

Materials

Medium 199, containing Earle's salts, was obtained from Nissui Pharmaceutical Co., Ltd. Penicillin and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Na\(_2^{35}\)SO\(_4\) (specific activity, 788.0 mCi/mmol), L-\(^{3}H\)serine (specific activity, 14.4 Ci/mmol) and D-[1,6-\(^{3}H\)]glucosamine hydrochloride (specific activity, 52.7 Ci/mmol) were purchased from New England Nuclear. Chondroitinase ABC (EC 4.2.2.4) from Proteus vulgaris, heparitinase (EC 4.2.2.8) and heparinase (EC 4.2.2.7) from Flavobacterium heparinum, hyaluronidase (EC 4.2.2.1) from
**Streptomyces hyalurolyticus**, keratanase (EC 3.2.1.103) from *Pseudomonas* sp., and endo-β-galactosidase (EC 3.2.1.103) from *Escherichia freundii* were obtained from Seikagaku Kogyo Co., Ltd.; and pronase was purchased from Calbiochem Co.

**Organ culture of rabbit trachea**

Organ culture was performed according to the method described by Gallagher and Kent (7). Twenty male New Zealand White rabbits (2.0–2.2 kg) were killed by intravenous injection of sodium pentobarbitone (50 mg/kg). The trachea of each rabbit was exposed by dissection, hemostats were clamped in position at both ends of it, and then the trachea was cut away from the bronchi and larynx. The trachea was washed in two changes of cold (4°C) Tyrode solution (120 mM NaCl, 4.75 mM KCl, 1.25 mM CaCl₂, 1.2 mM MgSO₄, 1.15 mM KH₂PO₄, 10 mM NaHCO₃ and 10 mM glucose, pH 7.4) before the segments of tissue damaged by the hemostats were cut away. After two further washes in the cold Tyrode solution, the trachea was cut into small segments (approx. 0.5 cm², 70–80 mg in wet weight), which were placed in a Falcon petri dish (100 mm in diameter). Medium 199 (30 ml) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and radioisotopes, such as Na²³S₀₄, 2 μCi/ml and [³H]glucosamine, 2 μCi/ml, were added to the dish, followed by incubation at 37°C in a 5% CO₂/95% air atmosphere for 20 hr.

After incubation, each segmented trachea was washed twice with 1 ml of cold Tyrode solution, and then 2 ml of Tyrode solution or the test solutions were added to the cultures in Falcon culture dishes (35 mm in diameter). Medium 199 (30 ml) containing penicillin (100 units/ml), streptomycin (100 μg/ml) and radioisotopes, such as Na²³S₀₄, 2 μCi/ml and [³H]glucosamine, 2 μCi/ml, were added to the dish, followed by incubation at 37°C in a 5% CO₂/95% air atmosphere for 20 hr.

After incubation, each segmented trachea was washed twice with 1 ml of cold Tyrode solution, and then 2 ml of Tyrode solution or the test solutions were added to the cultures in Falcon culture dishes (35 mm in diameter) for each chasing time. After incubation, the media were harvested, and 0.5-ml aliquots were removed and then filtration through 0.45-μm membrane filters (Millipore HAWP, Japan Millipore, Tokyo). The filters were wetted with 1 ml of 95% ethanol just before the addition of the culture medium-ethanol mixture, and then filtered with a 150-μm nylon mesh and centrifuged at 300 X g at 18°C for 10 min. Cell viability was assessed by the trypan blue exclusion test.

**Analysis of data**

Data are summarized as means ± S.E. Student’s unpaired t-test was used to analyze the data on the influence of temperature, and the other data was analyzed by Duncan’s multiple-range test.

**RESULTS**

**Tracheocytes viability**

To determine the integrity of tracheocytes in the tracheal tissues incubated for 25 hr in medium 199 and for 5 hr in normal Tyrode solution and Mg²⁺-free Tyrode solution, the viability of the pronase-dispersed tracheocytes was examined. More than 95% of the tracheocytes were viable, and ciliated cells had beating cilia.

**Effects of glycosidases on radiolabeled glycoconjugates secreted from the trachea in organ culture**

Figure 1 shows that the incubations with heparitinase, heparinase, hyaluronidase, keratanase or endo-β-galactosidase did not affect the count for ³⁵S-labeled and [³H]glucosamine-labeled macromolecules obtained on incubation of the rabbit trachea in Tyrode solution were dialyzed for 3 days against distilled water containing 0.1 mM toluenesulfonyl fluoride (TSF), lyophilized, resuspended in an appropriate buffer and then digested with enzymes, as follows: chondroitinase ABC (2.5 units/ml) for 4 hr in Tris-acetate buffer [0.1 M Tris-acetate, 0.15 M NaCl, 0.05% BSA (pH 8.0)]; heparitinase (2.5 units/ml) for 4 hr in Tris-acetate buffer, as above, adjusted to pH 6.8 and containing 5 mM CaCl₂ (pH 6.8); heparinase (2.5 units/ml) for 4 hr in Tris-acetate buffer and 5 mM CaCl₂ (pH 6.8); hyaluronidase (30 units/ml) for 10 hr in 0.1 M Tris-acetate, 0.15 M NaCl, 0.05% BSA and 5 mM CaCl₂ (pH 7.0); keratanase (1 units/ml) for 3 hr in 0.05 M Tris (pH 7.4); and endo-β-galactosidase (0.01 units/ml) for 4 hr in 0.05 M sodium acetate (pH 5.6). All incubations were performed at 37°C. Enzymatic digests were analyzed by the ethanol-precipitation method described above.
for the $^{35}$S- and $[^3H]$glucosamine-labeled glycoconjugates by about 85%.

**Influence of incubation temperature on the basal secretion of $^{35}$S-labeled and $[^3H]$glucosamine-labeled glycoconjugates in the Tyrode solution**

To determine whether the basal secretion of glycoconjugates is through an energy-dependent process, the influence of a cold medium on the secretion was examined. The amounts of $^{35}$S-labeled and $[^3H]$glucosamine-labeled glycoconjugates secreted into the medium after 1-hr incubation at 4°C were 19 ± 3% and 20 ± 3%, respectively, of that secreted after incubation at 37°C for the same time period (P < 0.01) (Fig. 2).

**Time course of the basal secretion of $^{35}$S-labeled and $[^3H]$glucosamine-labeled glycoconjugates**

$^{35}$S-Labeled and $[^3H]$glucosamine-labeled glycoconjugates were detectable within 15 min after the addition of Tyrode solution to the tracheal tissues (Fig. 3). The basal secretions of $^{35}$S-labeled and $[^3H]$glucosamine-labeled glycoconjugates reached a plateau at 1 hr (32.9 ± 3.5 and 65.3 ± 9.3 dpm/mg wet tissue weight, respectively).
Effects of Ca\(^{2+}\) and/or Mg\(^{2+}\) solution on the basal secretion of \(^{35}\)S-labeled and \(^{3}\)H]glucosamine-labeled glycoconjugates

As shown in Fig. 4, removal of Mg\(^{2+}\) suppressed by 80% the basal secretion of \(^{35}\)S-labeled glycoconjugates for 1 hr, while the basal secretion was not influenced in Ca\(^{2+}\)-free Tyrode solution containing EGTA (0.2 mM). A Mg\(^{2+}\)-free solution as well as a Ca\(^{2+}\) and Mg\(^{2+}\)-free solution had no effect on the basal secretion of the \(^{3}\)H]glucosamine-labeled glycoconjugates.

The suppression of the basal secretion of \(^{35}\)S-labeled glycoconjugates was dose-dependently recovered by the addition of Mg\(^{2+}\) but not by addition of Ca\(^{2+}\) (Fig. 5).

DISCUSSION

In the present study, \(^{35}\)S]-labeled and \(^{3}\)H]glucosamine-labeled glycoconjugates secreted from organ cultures of rabbit trachea were only digested with chondroitinase ABC, suggesting that a chondroitin sulfate proteoglycan is one of the main components in the basal secretion. The results seem to confirm the presumption by Gallagher and Kent (7). The trachea, however, is a complex structure in which the epithelial mucosa is born on the layer of submucosal connective tissue supported by rings of hyaline cartilage embedded in a network of collagen and elastic fibers. Under organ-culture conditions, nonepithelial regions can be expected to contribute to the total secretory activity of the system. In this regard, Bhasker et al. (8) also indicated that most of the macromolecular glycoconjugates basally secreted from airway mucosal explants were proteoglycans. Radioactive sulfate and glucosamine are incorporated not only by epithelial cells that contain secretory storage granules, but also by cells that do not contain them (12). Thus, at present, it is difficult to refer to which cell types secrete these sulfated glycoconjugates.

Radioactive glycoconjugates from rabbit trachea in organ culture were rapidly and continuously secreted, and the basal secretion of the glycoconjugates was abolished at 4°C, indicating that the basal secretion is functionally regulated through an energy-dependent process in the normal medium. The findings agree with those of Iwamoto et al. (13) who studied the metabolism of sulfated cell-surface macromolecules in dog tracheal epithelial cells in primary culture, although it has been suggested that the metabolism in cultured tracheal epithelial cells might not be representative of the entire spectrum of metabolism in intact tracheal segments (14).

Bogart et al. (15) reported that A23187 enhancing Ca\(^{2+}\) transport into tissues promoted mucus secretion from rabbit tracheal explants in a Ca\(^{2+}\)-rich solution and that Ca\(^{2+}\) chelation with EGTA abolished the ionophore-induced response. Conover and Conod (16) also showed A23187-inducing release of mucus-like in-

![Fig. 4](image)

Fig. 4. Effects of the Ca\(^{2+}\)-free and/or Mg\(^{2+}\)-free Tyrode solution on the basal secretion of \(^{35}\)S-labeled and \(^{3}\)H]glucosamine-labeled glycoconjugates in organ cultures of rabbit trachea. The Ca\(^{2+}\) concentration is 1.25 mM, Mg\(^{2+}\) 1.2 mM and EGTA 0.2 mM. The plus signs indicate incubation in the presence of each material and the minus that in the absence. Solid and shaded columns express data for \(^{35}\)S-labeled and \(^{3}\)H]glucosamine-labeled glycoconjugates, respectively. The value (dpm/mg wet tissue) for basal secretion in Tyrode solution is taken as 100%. Data are means ± S.E. for six experiments. *: Significantly different from the mean after incubation of the tissue in Tyrode solution. P < 0.01.

![Fig. 5](image)

Fig. 5. Effects of the Ca\(^{2+}\) (triangles) and Mg\(^{2+}\) (circles) concentrations in a Ca\(^{2+}\), Mg\(^{2+}\)-free Tyrode solution containing EGTA (0.2 mM) on the basal secretion of \(^{35}\)S-labeled glycoconjugates in an organ culture of rabbit trachea. The glycoconjugates were sampled after 1-hr incubation. The value (dpm/mg wet tissue) for basal secretion in the normal Tyrode solution at 1 hr is taken as 100%. Data are means ± S.E. for six experiments.
soluble material from rabbit trachea. Our results, however, demonstrated that extracellular Ca\(^{2+}\) is not important for the basal secretion of the glycoconjugates. Similarly, Barbieri et al. (17) showed that alteration of the Ca\(^{2+}\) concentration did not have a significant effect on the basal secretion of glycoconjugates by canine tracheal explants. On the contrary, Coles et al. (18) indicated in their early experiment that incubation of canine tracheal explants in Ca\(^{2+}\), Mg\(^{2+}\)-free medium induced an increase in the basal release of radiolabeled glycoproteins. Perhaps the reasons for these discrepancies are methodological.

The present results indicate that sulfation of glycoconjugates basally secreted depends on extracellular Mg\(^{2+}\). Changes in Mg\(^{2+}\) do have profound effects on cellular metabolism, structure and bioenergetics (19–23). Key enzymes or metabolic pathways (22–25), mitochondrial ion transport (22, 26–28), Ca\(^{2+}\) channel activities in the plasma membrane and intracellular organelles (19, 29, 30), ATP-requiring reactions, and structural properties of cells (20, 22, 31) and nucleic acids (19, 23, 24) are modified by changes in Mg\(^{2+}\) concentration. Although the defined mechanism of the reduced sulfation in Mg\(^{2+}\)-free medium was not investigated, it is probable that extracellular Mg\(^{2+}\) has a significant effect on sulfotransferases activity which requires Mg\(^{2+}\) and catalyzes the transfer of the sulfate ester group from 3'-phosphoadenosine-5'-phosphosulfate to glycoconjugates.

At present, it is rather difficult to draw a relationship between changes in extracellular Mg\(^{2+}\) concentration and the sulfated glycoconjugates contents in the pathological states of the airway. It is clear, however, that extracellular Mg\(^{2+}\) is capable of modifying sulfation of tracheal sulfated glycoconjugates in vitro.

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