

## Mucosal inhibition of cholinergic contractions in ferret trachea can be transferred between organ baths

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*Mucosal inhibition of cholinergic contractions in ferret trachea can be transferred between organ baths. A. Ullman, C.G. Löfdahl, N. Svedmyr, L. Bernsten, B.E. Skoogh.*

**ABSTRACT:** The influence of the mucosa on the contractile responses to cholinergic nerve stimulation in an *in vitro* nerve muscle preparation of ferret trachea was studied. Repeated contractions were induced by alternating direct vagal nerve stimulation (DNS) and electrical field stimulation (EFS). With intact mucosa there was a marked successive decrease of the contractile responses. During 60 minutes the responses decreased to  $46 \pm 8$  % of baseline (Mean  $\pm$  SEM,  $n=6$ ), compared to  $86 \pm 2$  % in preparations, in which the mucosa was initially removed. The mucosa dependent inhibition could be partly blocked by indomethacin ( $10 \mu\text{M}$ ). The inhibitory effect could be transferred via the bath fluid from a donor preparation with intact mucosa to a recipient preparation with removed mucosa. Fluid transferred from a donor preparation with removed mucosa or from indomethacin treated preparations did not affect the contractile responses in the recipient preparation. We conclude that ferret tracheal mucosa can release a factor which inhibits the contractile responses to cholinergic nerve stimulation. The release of this factor can be blocked to a major part by indomethacin and the factor can be transferred from a donor to a recipient preparation.

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After the discovery of an endothelium derived relaxant factor (EDRF) [1], which is obligate for the dilating effect of a number of drugs, the question of whether there is a similar mechanism in the airway mucosa affecting bronchial tone was raised. Lately several authors have reported that removal of the epithelium alters the response to contractile as well as relaxant stimuli in airway smooth muscle *in vitro*. It has been shown in a number of different species including man [2-10].

Involvement of cyclooxygenase products in the epithelial modulation of bronchial tone has been discussed [2-4, 9, 11, 12], although there are conflicting reports about the effect of indomethacin. Species differences as well as involvement of more than one factor, possibly both prostaglandins and other metabolites, could explain these differences.

While developing an *in vitro* nerve-muscle preparation of ferret trachea [13-15], originally designed to study parasympathetic ganglionic transmission, we observed that removal of the mucosa over the tracheal muscle prevented an otherwise profound fading of contractile responses to repeated, phasic cholinergic stimulations. We therefore undertook the present study with the following aims: firstly, to see if the observed successive decrease in contractile force is due to

accumulation of a factor released from the mucosa; secondly, to determine whether prostaglandins or other cyclooxygenase products are involved in this inhibitory mechanism. Thirdly, to examine if it is possible to transfer this factor (or factors) between isolated preparations, which would indicate an accumulation not only in the tissue but also in the surrounding fluid. Our preparation also made it possible to evaluate if ganglionic transmission was affected, besides postganglionic structures.

### Materials and methods

The study was performed *in vitro* using a nerve-muscle preparation of ferret trachea. Male ferrets were rendered unconscious by electric shocks and killed by exsanguination. The trachea, with intact right recurrent and vagus nerves, was rapidly removed and immersed in an organ bath filled with 200 ml Krebs Ringer solution (KR) of the following composition (in mM): NaCl 118, KCl 5.9,  $\text{CaCl}_2$  2.5,  $\text{MgSO}_4$  1.2,  $\text{NaH}_2\text{PO}_4$  25.5 and glucose 5.6, maintained at  $38^\circ\text{C}$  and gassed with 94%  $\text{O}_2$  to 6%  $\text{CO}_2$ . Ascorbic acid was added to the KR in a concentration of 0.3 mM as an antioxidant to prevent the formation of oxygen free radicals. The

bath was continuously flushed by prewarmed KR, 10 ml·min<sup>-1</sup>.

In the organ bath the trachea was split along the anterior long axis and flapped open. One side was attached by pins and the opposite side was connected to three strain gauges (Grass FT 03) for recording of isometric muscle tension at optimal resting tension (7–10 g). In some preparations the mucosa covering the tracheal muscle was removed by cutting along the borders between the membranous part and the cartilaginous part. Care was taken not to damage the mucosa in other parts of the trachea. Histological examination verified that the epithelium and a major part of the submucosal tissue was removed over the tracheal muscle by this method.

Phasic contractions were evoked by nerve stimulation, for 20 sec every 2 minutes. This was done by alternating activation of postganglionic fibres through electrical field stimulation (EFS) and activation of pre-ganglionic fibres through direct nerve stimulation (DNS). EFS was given *via* two platinum electrodes placed one above and one below the tracheal muscle. DNS was applied to the vagal nerve and to the laryngeal end of the recurrent nerve simultaneously *via* two suction electrodes. Electrical impulses were biphasic square waves with a duration of 0.25 msec (DNS) or 0.5 msec (EFS). The frequencies used were 2 and 12 Hz for DNS and 12 Hz for EFS. The current for DNS was supramaximal (22 mA). The current for EFS was supramaximal (1500 mA) at start of the experiment, which gave a somewhat stronger contraction than DNS. Therefore the two types of contractions were matched by reducing the current for EFS (600–1000 mA). The stimulations were given repeatedly in the following sequence: DNS,2Hz; EFS,12Hz; DNS,12Hz; EFS,12Hz.

#### *Effect of mucosal removal on the successive decrease of phasic contractions*

Experiments were carried out both in preparations where the mucosa was initially intact and then later removed (M[+]) and in preparations where the mucosa was removed initially (M[-]). In each type of preparation experiments were done with and without indomethacin (10 μM).

After an equilibration period of 60 min the electrical stimulations were started. The contractile responses at 30 minutes after the start of stimulation were used as baseline. The successive decrease in contractile force was observed during 60 min after baseline measurements. The mucosa was then removed by gentle dissection with the preparation still in the organ bath, connected to the force transducers. After this dissection the resting tension was readjusted. Separate experiments showed the optimal resting tension to be the same in preparations with intact and with removed mucosa. M[-] experiments had an identical design except that the mucosa in these preparations was removed initially.

#### *Transfer experiments*

In the transfer experiments we used the same nerve-muscle preparations as described above, with initially removed mucosa, as a recipient preparation. In this contractions were induced by alternating DNS, 2Hz and DNS, 12Hz, every two minutes, throughout the experiments. Only the 2 Hz data are presented here.

Fluid was transferred from a similar preparation with

Table 1. – Contractile responses in untreated preparations and in preparations treated with indomethacin (n=6)

		Baseline		60 min		120 min	
		g		% of baseline		% of baseline	
		M[+]	M[-]	M[+]	M[-]	M[+]	M[-]
Untreated							
2 Hz	DNS	28.0±4.4	28.4±2.5	46±8	*** 86±2	87±7	82±6
12 Hz	DNS	45.0±4.8	42.8±3.8	72±7	*** 94±2	95±4	89±1
12 Hz	EFS	46.5±5.0	44.3±3.4	69±5	*** 96±1	97±4	87±3
Indomethacin							
2 Hz	DNS	32.6±2.3	27.6±3.6	80±3	* 89±3	93±3	80±4
12 Hz	DNS	50.8±2.7	43.0±4.5	91±2	97±1	97±2	90±2
12 Hz	EFS	52.0±2.8	45.3±4.4	82±4	97±1	99±3	94±1

M[+]: The mucosa initially intact and then removed immediately after recording of the 60 min value; M[-]: The mucosa removed initially; \*: p<0.05; \*\*: p<0.01; \*\*\*: p<0.001.

intact mucosa in a parallel donor bath with identical volume, temperature and gassing conditions. After equilibration the flushing of Krebs-solution in the donor bath was stopped and the preparation was stimulated to phasic contractions by EFS, 12Hz (1000 mA), every two minutes, for 60 minutes. Thereafter the flushing of the recipient bath was stopped and the bath was emptied and refilled with the fluid from the donor preparation (T1). Thirty minutes later the recipient bath was washed out and the flushing resumed. After the transfer of its fluid the donor bath was refilled with fresh Krebs solution and flushed for 60 minutes with no stimulations. The flushing was then stopped again and the EFS was resumed for 60 minutes, which after the transfer procedure was repeated (T2). In a separate series of experiments the donor preparation was treated with indomethacin (10  $\mu$ M) between T1 and T2.

**Drugs:** Indomethacin (MSD) was dissolved in 95% ethanol giving a final bath concentration of ethanol less than 0.1%.

**Statistical analysis:** The results are expressed as means  $\pm$  SE. Statistical evaluation of the data, expressed as a percentage of baseline, was made by Student's *t*-test for paired or unpaired observations. Differences were considered to be significant when  $p < 0.05$ . In all experiments *n* equals the number of animals. All performed significance testings are shown in table 1.

## Results

### *Effect of mucosal removal on the successive decrease of phasic contractions*

Baseline responses were similar in preparations with intact mucosa M[+] and preparations with mucosa initially removed M[-] (table 1). In M[+] preparations there was a successive decrease in contractile responses. Sixty minutes after baseline measurements the mean contractile response to DNS, 2Hz had decreased to 46% compared to 86% ( $p < 0.01$ ) in M[-] preparations (table 1). For the stronger, DNS, 12Hz, contractions there were similar changes (table 1). Removal of the mucosa in M[+] preparations restored the contractions. At 120 minutes (60 minutes after mucosa removal) the contractile forces were similar in M[+] and M[-] preparations (table 1).

The decrease in contractile responses to EFS and DNS (12Hz) in M[+] preparations was similar (table 1). Thus, the inhibition of responses to pre and postganglionic activation was similar, which indicates a postganglionic effect.

Baseline responses were similar with and without indomethacin. The marked successive decrease in contractile responses in M[+] preparations was significantly attenuated ( $p < 0.01$ ) in the presence of indomethacin (table 1). However M[+] preparations with indomethacin present showed a slightly more pronounced decrease of contractile responses ( $p < 0.05$ ) compared to indomethacin treated M[-] preparations. On the other hand indomethacin did not affect contractile responses in M[-] preparations (table 1).

Table 2. - Transfer experiments. Contractile responses to DNS, 2Hz in recipient preparations with removed mucosa.

	Baseline 1 g	T1 % decrease	Baseline 2 g	T2 % decrease
Untreated n=4	34.8 $\pm$ 4.5	9 $\pm$ 2	33.4 $\pm$ 4.2	33 $\pm$ 4
Indomethacin treatment after T1 n=4	28.5 $\pm$ 7.0	12 $\pm$ 2	23.8 $\pm$ 5.5	4 $\pm$ 5

T1 and T2: effect of the first and second transfer of the bath fluid from a donor preparation with intact mucosa; DNS: direct nerve stimulation.

### *Transfer experiments*

Transfer of fluid from the donor preparation did inhibit the contractile responses in the recipient bath. This phenomenon was observed after both T1 and T2 in all four experiments (fig 1, table 2). However, the magnitude of the inhibition was greater after T2 (33 $\pm$ 4%) compared to T1 (9 $\pm$ 2%). The inhibitory effect of the transferred fluid could, to a major part, be rapidly reversed by washing the recipient bath.

In two experiments fluid was transferred from donor preparations with removed mucosa, with no effect on the contractile responses in the recipient preparation.

In four experiments the donor preparation, with intact mucosa, was treated with indomethacin immediately after T1. This means that T1 was performed without indomethacin as in the previous series, and the effect of T1 was as expected similar in these two series. However, addition of indomethacin blocked the inhibitory effect of T2 (table 2).

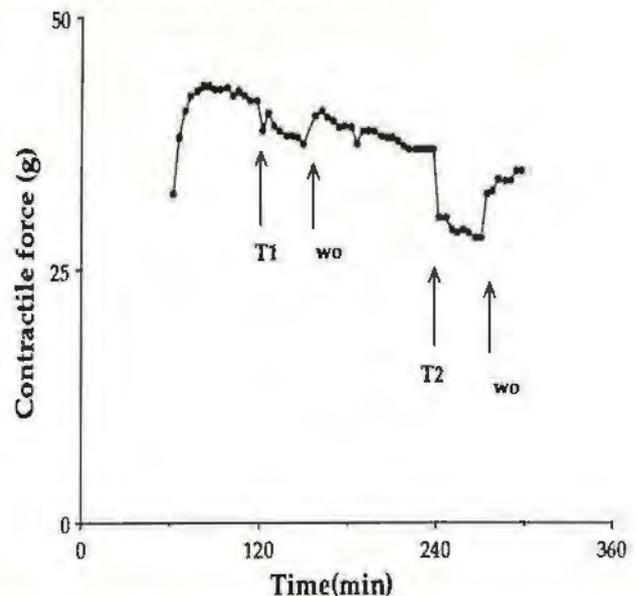


Fig. 1. - A typical transfer experiment. Contractile responses to 2 Hz direct nerve stimulation (DNS) (g) in the recipient preparation. T1: first transfer, T2: second transfer, wo: wash-out.

### Discussion

We have demonstrated in this study a marked successive decay of repeated contractions induced by nerve stimulation in tracheal preparations with intact mucosa. This decay can be reversed by selectively removing the mucosa over the tracheal muscle. The successive decay indicates accumulation of (an) inhibitory factor(s). The factor can be transferred *via* the surrounding fluid medium.

The inhibitory effect can be blocked to a major part, but not totally abolished, by indomethacin (10  $\mu$ M), indicating involvement of prostaglandins or other cyclooxygenase products. Release of PGE<sub>2</sub> has been demonstrated from the rabbit bronchial epithelium and from cultured canine epithelial cells as well as from the pig nasal mucosa [9, 16, 17]. It is well known that prostaglandins of the E-series have bronchodilator properties in animals and man [18, 19]. It has also been shown that exogenous arachidonic acid causes relaxation of guinea-pig trachea isolated with intact epithelium, whereas in epithelium denuded preparations or indomethacin treated preparations, this relaxation was converted to contraction [11, 12]. Thus, it is obvious that airway epithelium can release relaxant prostaglandins and our data show that prostaglandins participate in the mucosa dependent inhibition of smooth muscle contractions in ferret trachea. However, we found a small but significant mucosa dependent inhibition even in the presence of indomethacin. This might be due to an incomplete inhibition of cyclooxygenase activity. Alternatively, it could be explained by additional release of a cyclooxygenase independent factor. Partial blocking effects of epithelium derived relaxation by cyclooxygenase inhibition has also previously been reported [3, 4]. In bovine trachea no effect at all of indomethacin was found on epithelial modulation of smooth muscle contraction [2]. Thus, it seems likely that the mucosa derived inhibition in ferret trachea is mediated to a major part through release of prostaglandins and to a minor part through release of cyclooxygenase independent factors.

The transfer experiments show that inhibitory factors are released and accumulated not only within the tissue but in the surrounding fluid as well. An unexpected finding was that the second transfer of bath fluid (T2) caused a much more pronounced effect. This might be due to a time dependent increased sensitivity in the recipient preparation or to increasing release of inhibitory factor(s) from the donor preparation.

The fairly marked inhibitory effect of T2 was completely blocked by indomethacin treatment of the donor preparation. This indicates that the factor(s) transferred *via* the bath fluid is a cyclooxygenase product. We could not demonstrate transfer of any cyclooxygenase independent factor, possibly due to low bath concentration or to chemical instability.

Cholinergic contractions induced by postganglionic (EFS) and preganglionic (DNS) nerve stimulation were inhibited to a similar degree. Thus, the inhibition occurs at a postganglionic site, although we cannot

exclude a small ganglionic effect at low frequencies, as we compared pre- and postganglionic activation only at 12 Hz. We have not yet clarified whether the inhibition is a direct effect on the smooth muscle or if it affects the transmission at the neuro muscular junction. The latter possibility is supported by earlier studies showing the ability of prostaglandins to modulate transmission at the cholinergic neuro muscular junctions [20-22].

In our preparations we only removed the mucosa over the tracheal muscle. This technique was more effective and reproducible than rubbing off the epithelium from the whole circumference (own unpublished observations from an *in vitro* ring preparation of ferret trachea). The pronounced effect of this limited mucosal removal indicates that this part of the mucosa might be more important for modulating smooth muscle contractions than the mucosa covering the cartilage. A possible explanation is that the close relationship between the muscle and mucosa is necessary for the release or activity of the relaxant or inhibitory factors. Differences in biochemical activities or neural or vascular supply might also be important.

In this work we studied the effects of removal of the epithelium and a major part of the subepithelial tissue covering the tracheal muscle. This means that several possible cellular sources have to be considered as responsible for the release, such as epithelial cells, inflammatory cells or neural structures within the mucosa.

It has earlier been hypothesized that, in rat trachea, the epithelium dependent modulation of muscle tone is under neural, maybe peptidergic, control [23]. Our results may also indicate an interaction between the airway mucosa and the airway nervous system. Further studies are required to clarify the mechanisms of release and action of this inhibitory factor.

We conclude that ferret trachea *in vitro* can release (a) mucosa derived factor(s) which inhibits the contractile responses to cholinergic nerve stimulation. The release and accumulation of this factor can be activated by electrical field stimulation and the release can be blocked by indomethacin, indicating involvement of prostaglandins. Finally this factor can be transferred *via* the surrounding fluid medium.

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*L'inhibition des contractions cholinergiques par la muqueuse dans la trachée du furet peut être transférée par les bains d'organes.* A. Ullman, C.G. Löfdahl, N. Svedmyr, L. Bernsten, B.E. Skoogh.

RÉSUMÉ: Nous avons étudié l'influence de la muqueuse sur les réponses contractiles à la stimulation nerveuse cholinergique dans une préparation muscle-nerf de la trachée du furet in vitro. Des contractions répétées ont été induites en alternance par stimulation nerveuse vagale directe (DNS) et par stimulation par champ électrique (EFS). Quand la muqueuse est intacte, on note une diminution marquée successive des réponses contractiles: au cours de 60 minutes, les réponses laissent jusqu'à 46±8% des valeurs basales (moyenne: ±SEM, n=6), par comparaison avec une diminution jusqu'à 86±2% dans les préparations dont la muqueuse a été initialement enlevée. L'inhibition muqueuse-dépendante peut être partiellement bloquée par l'indométhacine (10 µM). L'effet inhibiteur peut être transféré par le liquide du bain d'une préparation donneuse à muqueuse intacte à une préparation receveuse dont la muqueuse est réséquée. Par contre, le liquide provenant d'une préparation donneuse avec muqueuse réséquée, ou les préparations traitées à l'indométhacine, n'affectent pas les réponses contractiles de la préparation receveuse. Nous concluons que la muqueuse trachéale du furet peut libérer un facteur qui inhibe les réponses contractiles à la stimulation nerveuse cholinergique. La libération de ce facteur peut être largement bloquée par l'indométhacine et le facteur peut être transféré d'une préparation donneuse à une préparation receveuse.

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