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TOLUENE DIISOCYANATE CAUSED ELECTROPHYSIOLOGICAL DISTURBANCES IN THE UPPER AIRWAYS WALL

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Abstract
Objectives: Toluene diisocyanate (TDI) due to its widespread use in industry is one of the most common and well-known causes of occupational asthma and Reactive Airways Dysfunction Syndrome (RADS). In this study the impact of TDI on the electrophysiological properties of the airways wall, particularly on the mechanisms of absorption of sodium ions and chloride ions secretion was evaluated. **Materials and Methods:** Isolated rabbit tracheal wall (from outbred stock animals) was mounted in an apparatus for electrophysiological experiments by means of Ussing method and was mechanically stimulated by the jet flux of specified fluid directed onto the mucosal surface of the tissue from a peristaltic pump. The measured parameters were: transepithelial potential difference under control conditions (PD, mV), after mechanical stimulation (dPD or physiological reaction of hyperpolarization, mV) and electric resistance (R, Ω*cm^2). When TDI (0.035 mM) was added to stimulation fluid, only the immediate reaction was identified and when it was added to incubation fluid and other experimental fluids, the late (post-incubation) reaction was determined. The experiments involving the inhibition of Na^+ by amiloride and Cl^- by bumetanide were also performed. **Results:** A series of functional tests for 72 pieces of tracheal wall from 36 animals were performed. It has been shown that short-term exposure to TDI significantly changed the course of reactions to mechanical stimulation. Also after incubation in the presence of TDI, the reactions to mechanical stimulation were changed in relation to control conditions. **Conclusions:** The immediate reaction of the isolated rabbit tracheal wall after exposure to TDI depends on the duration of exposure and on the physiological condition of the tissue in respect of sodium and chloride ion transport.

Key words:
Airways, Transepithelial Ion Transport, Toluene Diisocyanate, TDI

INTRODUCTION

Diisocyanates are one of the most common and well known causes of occupational asthma [1–7]. They are highly active low molecular weight compounds [2–5,6,8,9].
Clinical and experimental studies of functional disturbances of respiratory tract observed after TDI exposure on humans or on animal models have been reported [1–3,10–14]. In particular, it has been proved that high concentrations of TDI are irritant to airways, while low concentration may produce allergy. However, the limits of the sensitising (allergic) or irritating concentrations are overlapping [5,7,9,12].
As a result of TDI exposure, instead of IgE antibodies characteristic for asthma, often IgG antibodies are produced which indicate allergy without signs of asthma [4–10,15,16].

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Reversible airway obturation after TDI exposure is referred to as IIA — Irritant Induced Asthma, formerly RADS — Reactive Airways Dysfunction Syndrome, or simply occupational asthma [4,7,8,17] and is explained by contraction of airways smooth muscles, hypersecretion of mucus, swelling of airways mucosal tissue (until total closure of airway lumen) [4,6–9,18,19], while some authors stress the excessive production of airway surface liquid [17,20].

Abnormal production of interleukins, inflammatory and allergic mediators [3,4,6,9,16,18–22], disturbed relation between parasympathetic and sympathetic neurotransmitters [13,18,19,22], and disturbed production of tachykinins (sensory neuropeptides) [8,9,13,17–19,21,23,24] are supposed to be involved in pathogenesis of TDI related airway spasms.

In studies published so far, relatively little attention has been given to disturbances of transepithelial ion transport which is substantial for all processes related to airway surface liquid [13,17,20,22,25].

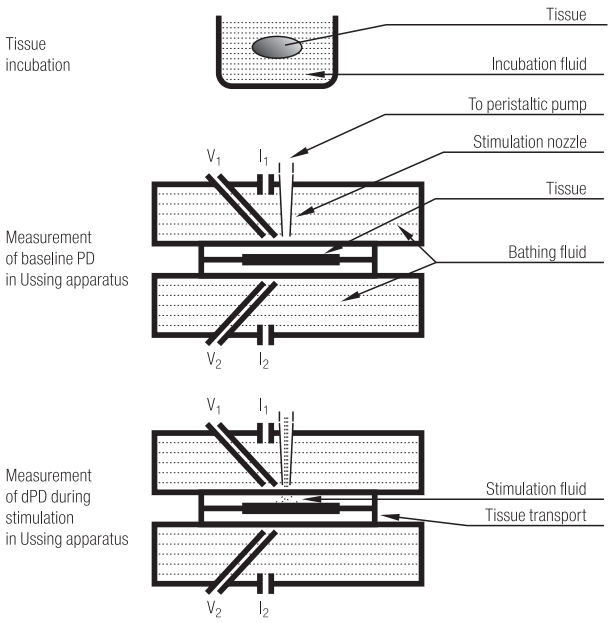
It is generally recognised that the processes of sodium ion reabsorption decrease, whereas those of chloride ion secretion increase the production of airway surface liquid, thus making the airway lumen diameter wider or narrower, respectively [20,25–28]. Processes of transepithelial ions transport generate and maintain electric field on epithelial surface [13,20,26,27].

The question whether TDI exposure affects airway transepithelial electrogenic ion transport has been studied by few authors [13,17], and the aim of this study was observation of electrophysiological variables of isolated airway wall mounted in the Ussing apparatus in the presence of TDI. Particular attention was paid to the question whether sodium reabsorption inhibitable by amiloride and chloride secretion inhibitable by bumetanide may be influenced by TDI.

MATERIALS AND METHODS

The experiments were performed on isolated tracheal wall mounted in the Ussing apparatus for electrophysiological measurement of epithelial tissue. The tracheae were excised from adult New Zealand rabbits of both sexes, outbread stock, weighing between 3.5 and 4.0 kg, obtained from a commercial supplier of experimental animals.

Animals were asphyxiated with high concentration of CO_2 (about 60% in the inhaled air). Then superficial tissues of the neck and the chest were removed and the tracheae were gently excised, immediately washed with Ringer solution, trimmed of fat and connective tissue, cut along the membranous part and divided into pieces of about 2.5 cm^2. Then the tracheae were incubated for about 60 minutes according to experimental plan and mounted in the Ussing apparatus adapter. The area of the studied part of the tissue was about 1 cm^2. The experiments consisted of continuous measuring of the changes in transepithelial potential (dPD, mV) and electric resistance (R, Ω*cm^2) under control conditions, and after mechanical stimulation of epithelial sensory receptors by gentle rinsing of the mucosal surface with bathing fluid [13,17,20,22,29–31]. The modification of the Ussing apparatus (Fig. 1) with the stimulation device has been described and published elsewhere [13,17,20,22,29–31]. Gentle mechanical stimulation was done by jet flux from ca. 1.2 mm dia. nozzle located 12 mm away from the mucosal surface of the tissue. The jet flux was produced by a peristaltic pump and was directed onto the tissue at some angle. For most



I_1, V_1 — current and voltage electrodes, respectively, from mucosal surface of tissue.
I_2, V_2 — current and voltage electrodes, respectively, from interstitial surface of tissue.
In stationary (control) conditions pump was off, during the stimulation the pump was on and mucosal surface of the trachea was rinsed by stimulation fluid specified in experiment descriptions.

Fig. 1. The scheme of Ussing chamber experiments to study electrophysiological reactions of epithelial tissue to mechanical stimulation [32].

experimental groups a 15 s stimulation was repeated three times with lag time of 45 s, but a single 30 s stimulation was applied when inhibitors of transepithelial ion pathways were used. Between consecutive stimulations, about 10 minutes were allowed for restoration of the control conditions (stimulations with tested substances without TDI addition).

Mechanical stimulus was modified in some experiments by addition of test substances (amiloride, bumetanide, DMSO and TDI) only to stimulation fluid and resultant changes were described as immediate reaction to test substance. When test substances were added to all experimental fluids, including incubation fluid, the experiments showed late reactions to test substances.

An EVC 4000 apparatus (WPI, USA) for electrical measurements and experimental data acquisition computer system MP 100 (BioPac, USA) were used. The preamplifiers of EVC 4000 were connected to Ag/AgCl electrodes. The electrodes were connected with the Ussing apparatus by means of elctrolytic bridges made of polyethylene tubing filled with electrolyte solution with agar.

The solutions used in the experiments were (concentrations given in mM): Ringer solution without additions (RH) — Na^+ 147.2; K^+ 4.0; Ca^{2+} 2.2; Cl^- 155.6 and Hepes (N-[2-Hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid]) 10.0 and RH with the additions of amiloride 0.01; bumetanide 0.01; TDI 0.035 or DMSO 1%.

Ethics

The experiments were approved by Local Committee for Ethical Animal Experiments of the Universities of Bydgoszcz.

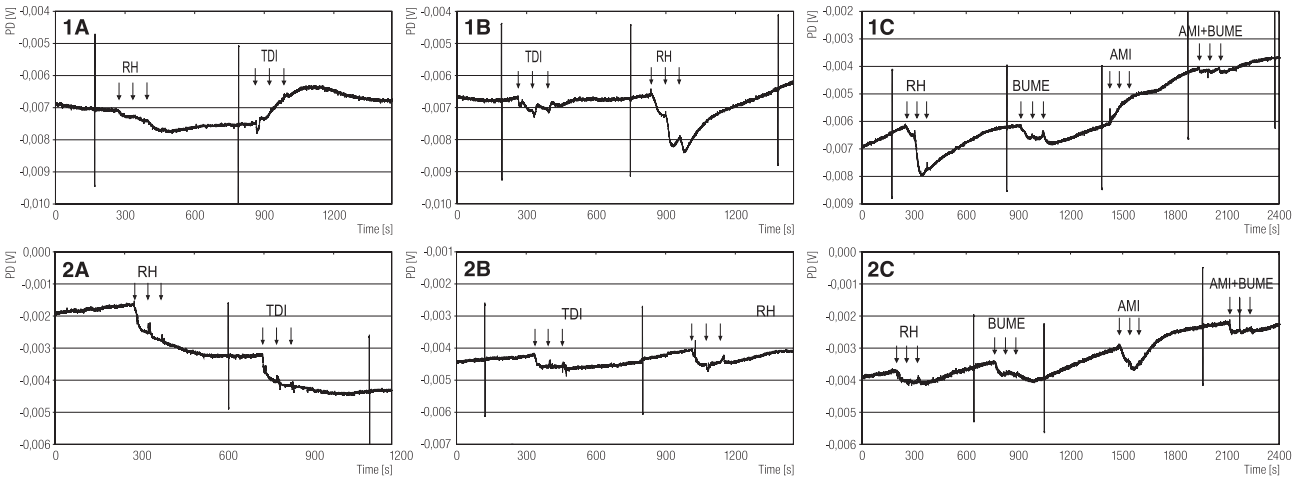
Statistics

The results have been presented in terms of time courses of the transepithelial potential differences of individual experiments as an example for the whole experimental group. The results of whole group were presented in tables as the mean ±SD. Statistical significance was verified with Mann Whitney test. Significance limits were set at $p \leq 0.05$.

RESULTS

The experiments were performed on 72 specimens of isolated tracheal wall from 36 rabbits.

Thirty specimens were incubated in Ringer solution with addition of amiloride during 30 minutes, and then during next 30 minutes in Ringer solution without additions



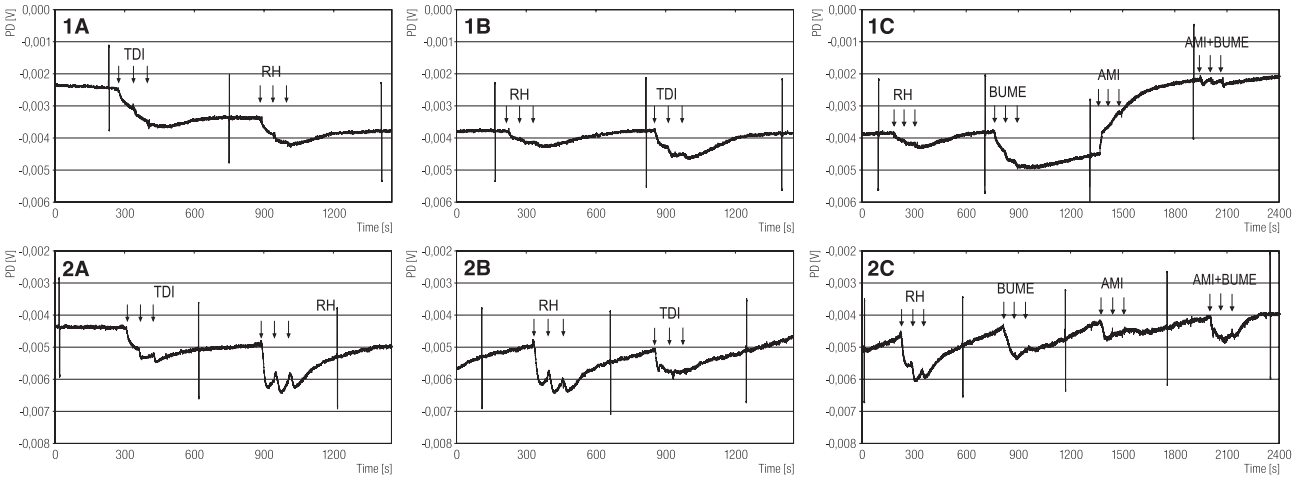
The upper panel shows repeated reactions to TDI (1A and 1B) for tissue specimens sensitive to amiloride inhibition and the lower panel (2A and 2B) for tissue specimens insensitive to amiloride inhibition, while 1C and 2C show test reactions to transport inhibitors for both types of tissues.
Single experiments are shown.
Tissues were stimulated (↓↓↓) three times for 15 seconds with 45 seconds intervals. RH — stimulation without TDI; TDI — stimulation with addition of TDI to stimulation fluid.
BUME, AMI, AMI+BUME — stimulation after addition of bumetanide, amiloride or both, respectively, to stimulation fluid.
PD — transepithelial electrical potential difference.

Fig. 2. Reactions of isolated tracheal wall to the mechanical stimulation after addition of TDI to stimulation fluid only.

Table 1. Reactions of isolated tracheal wall to the mechanical stimulation without and with TDI addition to stimulation fluid only

Type of experiments	Experimental variables	Influence of TDI				Functional pharmacological test			
		RH-1	TDI-1	TDI-2	RH-2	RH	BUME	AMI	AMI + BUME
Amiloride sensitive tissues (AS) (n = 10)	PDp (mV)	–5.18±2.03	–5.31±2.07	–4.82±1.89	–4.68±1.9	–4.61±2.03	–4.7±2.11	–4.33±1.9	–3.35±1.68
	dPD (mV)	–0.77±0.72[a]	0.56±0.71[*a]	–0.04±0.46[*b]	–0.66±0.61[b]	–0.68±0.5[c]	–1.02±0.39	0.21±0.6[d*c]	–0.03±0.27[*c]
	PDk (mV)	–5.56±2.04	–4.73±1.92	–4.7±1.94	–4.71±2.18	–4.81±2.11	–4.41±1.91	–3.54±1.76	–3.18±1.67
	R (Ω*cm2)	188±55	192±58	204±82	203±89	184±57	209±105	191±60	202±62
Amiloride insensitive tissues (AI) (n = 5)	PDp (mV)	–5.56±3.46	–6.57±3.66	–7.11±3.55	–7.38±3.77	–7.53±4.07	–7.43±4.41	–6.78±3.76	–6.02±4.45
	dPD (mV)	–0.98±0.85[a]	–0.45±0.95[*a]	–0.65±0.72	–0.76±0.85	–0.4±0.28	–0.16±0.42	–0.27±0.32[*d]	–0.54±0.42
	PDk (mV)	–6.54±3.77	–7.11±3.34	–7.54±3.64	–7.74±4.05	–7.69±4.35	–7.09±3.35	–6.31±4.49	-5.69±4.17
	R (Ω*cm2)	207±51	219±65	217±73	236±79	235±94	235±103	249±106	291±67

AS — amiloride-sensitive tissues.
AI — amiloride-insensitive tissues; consecutive reactions to mechanical stimulations.
RH-1, RH-2 — first and second reaction with Ringer solution as stimulation fluid (without addition of TDI to stimulation solution).
TDI-1, TDI-2 — first and second reaction in the presence of TDI in stimulation solution.
BUME, AMI, AMI+BUME — reactions with addition of bumetanide or amiloride or both to Ringer solution, respectively.
PDp — transepithelial potential difference (mV) before stimulation.
dPD — the difference between maximum value after stimulation by gentle washing and control value before stimulation (mV).
PDk — transepithelial potential difference (mV), after stimulation.
R — transepithelial resistance (Ω*cm^2).
The indicated values represent mean ±SD.
n — number of experiments.
*[abcd] — statistically significant difference ($p \leq 0.05$) compared with reaction RH-1 marked as [a], or with reaction RH-2 marked as [b], or with reaction RH marked as [c] and comparison between reactions with amiloride from AS and AI tissues marked as [d].



Explanations and abbreviations as in Figure 2. Single experiments are shown.

Fig. 3. Reactions of isolated tracheal wall to the mechanical stimulation after incubation of the tissues in the presence of TDI for 0.5 h.

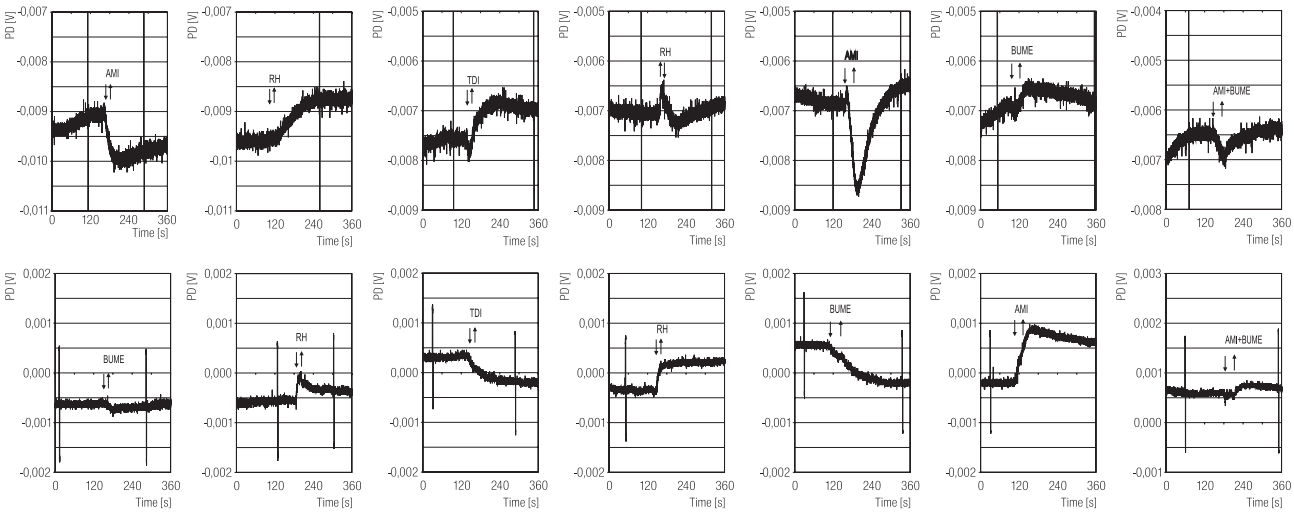
Table 2. Reactions of isolated tracheal wall to mechanical stimulation after incubation of tissues in the presence of TDI for 30 minutes

Type of experiments	Experimental variables	Influence of TDI				Functional pharmacological test			
		TDI-1	RH-1	RH-2	TDI-2	RH	BUME	AMI	AMI + BUME
Amiloride sensitive tissues (AS) (n = 10)	PDp (mV)	–5.39±4.19	–5.35±4.14	–5.23±4.14	–4.98±3.70	–5.13±4.31	–4.68±4.27	–4.48±3.66	–3.18±3.40
	dPD (mV)	–0.66±0.41	–0.45±0.32	–0.52±0.31[b]	–0.99±1.12[*b]	–0.45±0.23[c]	–0.46±0.52	0.23±0.77[d*c]	–0.18±0.39[*c]
	PDk (mV)	–5.61±4.27	–5.39±4.28	–5.25±4.06	–5.32±4.30	–4.83±4.28	–4.64±3.45	–3.44±3.71	–3.02±3.43
	R (Ω*cm^2)	167±68	173±73	190±81	176±70	178±71	184±82	194±82	218±129
Amiloride insensitive tissues (AI) (n = 4)	PDp (mV)	–7.86±4.05	–8.45±3.42	–7.86±3.25	–6.68±3.30	–6.66±2.58	–6.37±2.10	–6.04±1.70	–4.89±1.61
	dPD (mV)	–1.45±0.71	–1.27±0.74	–1.56±1.04	–1.36±1.17	–1.22±1.28[c]	–0.54±0.28[*c]	–0.33±0.25[*c,d]	–0.32±0.16[*c]
	PDk (mV)	–8.77±3.31	–8.51±3.04	–7.46±3.11	–7.1±2.59	–6.99±2.25	–6.35±1.44	–5.65±2.46	–4.48±1.33
	R (Ω*cm^2)	230±70	230±68	228±64	228±58	243±79	233±65	239±53	262±66

Explanations and abbreviations as in Table 1.

(n = 15) and the results are shown in Figure 2 and Table 1, or with addition of TDI (n = 15) (Fig. 3, Table 2).

The reactions of tissues to the first exposure after addition of TDI to stimulation fluid only was described as immediate and showed qualitative differences according to tissue sensitivity to amiloride inhibition of dPD. As shown in Figure 2 — 1C-AMI, when amiloride depolarized the tissue, TDI also caused depolarization (Fig. 2 — 1A-TDI),



Tissues were stimulated for 30 seconds — arrows denote start (↓) and termination (↑) of stimulation.
Abbreviations as in Figure 2.

Fig. 4. Influence of toluene diisocyanate (TDI) on hyperpolarization reaction after mechanical stimulation in conditions of inhibition of sodium ion transport by amiloride (upper panel) and inhibition of chloride ion transport by bumetanide (lower panel) in isolated tracheal wall.

Table 3. Influence of toluene diisocyanate (TDI) on hyperpolarization after mechanical stimulation of isolated tracheal wall during inhibition of sodium ion transport by amiloride (upper panel) or inhibition of chloride ion transport by bumetanide (lower panel)

Type of experiments	Experimental variables	AMI	RH	TDI	RH	BUME	AMI	AMI+BUME
In the presence of amiloride (n = 14)	PDp (mV)	–4.78±2.09	–5.91±3.45	–5.00±2.48	–3.91±1.67	–3.53±1.05	–3.38±1.98	–2.35±1.11
	dPD (mV)	–0.39±0.32[a]	0.01±0.12[*a]	–0.12±0.09[*a]	–0.04±0.09[c]	0.18±0.04[*c]	–0.51±0.30[d *c]	–0.25±0.14[*c]
	PDk (mV)	–5.36±3.34	–5.50±2.65	–4.62±2.15	–3.88±1.70	–3.19±1.03	–3.38±1.98	–2.27±1.04
	R (Ω *cm^2)	236±68	245±74	227±72	225±64	200±40	217±67	179±61
		BUME	RH	TDI	RH	BUME	AMI	AMI+BUME
In the presence of bumetanide (n = 12)	PDp (mV)	–2.22±1.59	–2.73±1.71	–2.75±1.89	–2.54±1.83	–1.98±1.42	–1.85±1.84	–2.06±1.19
	dPD (mV)	–0.19±0.09[b]	0.20±0.10	–0.39±0.17[*b]	0.24±0.11[c]	–0.19±0.05[*c]	0.55±0.70[*c,d]	–0.17±0.08[*c]
	PDk (mV)	–2.15±1.59	–2.50±1.63	–3.03±1.64	–2.07±1.50	–2.29±1.55	–0.91±1.55	–1.84±1.26
	R (Ω *cm^2)	209±73	228±89	226±85	191±63	202±64	207±70	193±69

Explanations and abbreviations as in Table 1.
[*abcd] — statistically significant difference ($p \leq 0.05$) compared with reaction AMI marked as [a], or with reaction BUME marked as [b], or with reaction RH marked as c and comparison between reactions with amiloride from upper and lower panels marked as [d].

and the next stimulation without this irritant (Fig. 2 — 1B-RH) caused augmented reaction. These results were fully confirmed for the whole AI (amiloride inhibitable, n = 10) group, see Table 1. For the AU(amiloride uninhabitable, n = 5) group presented also in Table 1 and in Figure 2 — 2A, 2B and 2C there was no depolarization in the presence of amiloride and the hyperpolarization reactions to mechanical stimulation did not differ either with or without TDI in the stimulation fluid.

Two different types of late reactions were also observed when mechanical stimulation was applied to tissues incubated in the presence of TDI. In that group, when the tissue responded to stimulation in the presence of amiloride by depolarization, the dPD were smaller if stimulation fluid was without TDI (Fig. 3 — 1A, 1B and 1C, Table 2, group AI). In the other tissue group, the tissue responded to stimulation in the presence of amiloride by hyperpolarization and dPD were augmented if stimulation fluid was without TDI (Fig. 3 — 2A, 2B and 2C, Table 2, group AU).

In the 14 of 24 tissue samples which were incubated in Ringer solution with amiloride, the pattern of reactions after the stimulation with TDI was changed in comparison to stimulation before TDI application (shown in Figure 4, upper panel and Table 3).

After incubation in Ringer solution with bumetanide, most of analysed tracheal specimens (13 of 18 tested) showed a different pattern of reactions to stimulation than in the former group incubated with amiloride, and the pattern was not changed after stimulation with TDI (Fig. 4, lower panel and Table 3).

DISCUSSION

Despite many years of studies and development of suitable animal models, the pathogenesis of isocyanate induced asthma has not been completely explained [5].

In particular, local epithelial changes in airways after isocyanate challenge may have important influence on airway lumen obturation and airway clearance [13,17,20,22, 29–31], and this question for the first time was addressed in this study.

It has been demonstrated that gentle mechanical stimulation by rinsing of mucosal side of isolated rabbit tracheal wall produces changes in electrogenic ion transport, which are affected by mucokinetic drug and physiological ligand [17,29–31]. Therefore, in this study, electrophysiological variables of isolated tracheal wall were recorded to see if isocyanates were able to change ion transport in airways.

Characteristics of experimental model

The front part of isolated rabbit trachea mounted in Ussing apparatus for electrophysiological measurements was the experimental model in this study. Tissue specimens were excised from CO_2-asphyxiated animals of outbred stock. Such animals have different alleles in their gene loci and that may be an explanation for the diversity of reaction types after TDI challenge.

The front part of tracheal wall, suitable for mounting as barrier between chambers of Ussing apparatus, was prepared by cutting across the membranous part of the trachea in such a way that sensory fibres, nervous cells and important intrawall nerves were well preserved [13,17,20,22,29–31].

The physiological reaction of hyperpolarization (also denoted dPD) to gentle mechanical stimulation of mucosal surface of isolated tracheal wall was achieved by providing the Ussing apparatus with the nozzle connected to peristaltic pump and directed onto mucosal surface of the specimen. The jet flux from this nozzle rinsing the tracheal epithelial surface was mechanical stimulus. Reactions obtained by addition of chemicals to the stimulation fluid only were considered to be immediate, while those noted after addition of test substances to all experimental fluids, including incubation fluid, were regarded to represent late reactions.

The transepithelial electrical potential difference (PD), changes of this parameter after stimulation (dPD) and the electrical resistance of tissue (R) were recorded continuously. The PD is directly related to transepithelial bidirectional electrogenic ion flux, and the transepithelial resistance (R) indicates functional stability of tight junctions (epithelial cell extracellular connections) [22,29,30–32].

Sodium reabsorption and/or chloride secretion are both important ionic fluxes responsible for production and maintaining of electrical potential of epithelial surface [20,22] so the incubation of the tissue in the presence of amiloride, a sodium ion transport inhibitor, or in the presence of bumetanide, a chloride transport inhibitor may be useful in determining which transport pathway is influenced by the test substance. It was experimentally demonstrated that the highest values of PD were observed after incubation of the tissue in the presence of amiloride and then additionally only in Ringer fluid — such procedure evidently stimulated transepithelial fluxes not only for sodium ions.

In preliminary experiments it was shown that TDI concentrations between 0.35 and 140 μmol/l influenced ion currents in isolated tracheal wall (not described in this publication). Exposure of tracheae to 35 μmol/l of TDI was used in these experiments because the electrophysiological reactions were characteristic and repeatable.

Immediate reaction to TDI exposure

The *in vitro* experiments show that tracheal epithelium responds almost immediately to isocyanate exposure by deep depolarization most probably related to inhibition of sodium ion flux, as it occurred only in tissues which were sensitive to amiloride inhibition of dPD. In our opinion, the disturbances of airway ion transport evoked by TDI were related to intrawall regulatory processes rather than to direct inhibition of sodium channel, as the consecutive stimulation without TDI caused greater hyperpolarization than that recorded before application of TDI (Fig. 2, Table 1).

Late reactions to TDI exposure

After the incubation of isolated tracheal wall with TDI, the tissues responded to mechanical stimulation in a way which differed from the immediate reactions.

The tracheae sensitive to amiloride inhibition showed greater reaction to stimulation in the presence of TDI in stimulation fluid than without the drug, while opposite

changes were typical for tissue insensitive to amiloride (Fig. 3, Table 2).

Reaction to TDI exposure after inhibition of Na^+ or Cl^- transport

When sodium ion transport in tracheal epithelium was inhibited by amiloride, the expected pharmacological model of tissues insensitive to amiloride, the immediate action of TDI changed the reaction to mechanical stimulation even more than in the first series of this study. Besides, the conclusion that TDI modified the ion transport of trachea by changing the local regulatory processes in airway wall seems additionally supported by the fact that after TDI exposure the course of hyperpolarizations in the presence of different test substances was variable (upper panels of Fig. 4 and Table 3).

Inhibition of transepithelial Cl^- pathway with bumetanide, although in some way it represents an experimental model for tissues sensitive to amiloride inhibition, caused that TDI influenced the hyperpolarization reactions only slightly (lower panels of Fig. 4 and Table 3).

The marked modifications of hyperpolarization reactions by TDI were noted in experimental groups where sodium ion transport was clear-cut (upper panel of Fig. 2 and Table 1) and also in other groups where it was inhibited by amiloride. These findings were to some extent in contrast with the current opinion about the dominant role of chloride transport in airways [31]. The role of sodium ion transport for function of airway epithelium has been eventually recognised [30] but there are still many unresolved questions.

The thorough comparison of experimental group of amiloride sensitive tissues with tissues incubated in presence of bumetanide (which showed the same sensitivity) and also the amiloride insensitive tissues with those incubated in the presence of amiloride (in both groups in presence of amiloride after stimulation hyperpolarization was observed) supports the idea about some other transport processes being involved in the transepithelial potential difference in addition to the Na^+ and Cl^- only.

The diversity of reactions observed in this study is explainable by genetic and regulatory variability of experimental material. The only way to eliminate the effects of genetic variability would involve the use of inbred stock experimental animals. The stabilization of local regulatory mechanisms would be possible by blockade of receptors of important transmitters and mediators. In particular, it is advisable to check receptors and transmitters of -C-fibres which secrete inhibitory tachykinins and stimulatory CGRP [8,13,20–24,33].

Besides, in the light of other studies, also on other animal models [6,10,11,13,14,21], and because it was shown that TDI decreased the tissue concentration of SP (substance P), NKA (neurokinin A) and CGRP (calcitonin gene related peptide) [21,23,24,33], it seems reasonable to infer that disturbances of transepithelial transport may be at least in part related to TDI interfering with interepithelial C-fibre endings.

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