fractionated by reverse-phase HPLC[15,16]. Kelly et al. have identified four fractions which cross-react with digoxin-specific antibodies from normal human plasma. Three fractions were associated with Na^+,K^+-ATPase inhibitory activity, but one had no detectable effect on Na^+,K^+-ATPase activity[16]. Furthermore, it should be recognized that steroids may interfere in the digoxin radioimmunoassay systems. Butler et al.[17] already pointed to this possibility in 1967. Dehydroepiandrosterone sulfate, one of the major steroids in human plasma, has actually been claimed to represent the major digoxin-like immunoreactivity in normal adult plasma[18]. In the present radioimmunoassay system, the cross-reactivities of digoxin antibody with the known steroids were less than 0.001%. However, it is still possible that the combinations of the known and unknown steroids considerably contribute to the digoxin-like immunoreactivity in rat plasma.

Our findings clearly demonstrate that major substances detected by digoxin-like immunoreactivity and direct Na^+,K^+-ATPase inhibitory activity are completely different, at least in rat plasma. The fact that dioxin-like immunoreactivity was reduced during acute expansion strongly supports the contention that digoxin-like immunoreactivity may not reflect the EDF level specifically. More specific methods suitable for accurate measurement of EDF need to be established.

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Azelastine inhibits bronchial hyperreactivity to acetylcholine in guinea pigs

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Summary. Contractile responses to acetylcholine were measured using isolated tracheae obtained from actively sensitized guinea pigs 0.5, 1, 5, 20, 24, 48 and 72 h after antigen challenge. Tracheal contractions to acetylcholine and to histamine were significantly increased 20 h but not 0.5, 1, 5, 24, 48 and 72 h after antigen challenge indicating bronchial hyperreactivity. When animals were pretreated with azelastine and then exposed to antigen challenge, concentration-response curve to acetylcholine did not differ from that obtained in control (non-challenged) tracheae. It is likely that azelastine is able to inhibit bronchial hyperresponsiveness to chemical mediators of bronchial asthma.
Key words. Guinea pig; active immunisation; in vitro; acetylcholine; azelastine; hyperreactivity; trachea; contraction.

Several definitions of bronchial asthma have been proposed. Bronchial asthma has been defined by the American Thoracic Society Committee on Diagnostic Standards[1] as a 'disease characterized by an increased responsiveness of the trachea and bronchi to a variety of stimuli'. The bronchial hyperreactivity is a central feature of nearly all asthmatics, regardless of etiology. In clinical trials, it has been demonstrated that allergen exposure increases bronchial responsiveness to putative chemical mediators of asthma[2-5]. The mechanisms underlying bronchial hyperreactivity are not well understood but it is likely that allergen-induced inflammation is related to the increase in nonallergic bronchial responsiveness. In the therapy of chronic reversible obstructive airways disease in which allergic inflammation and bronchial hyperresponsiveness are significant pathophysiological factors there are useful drugs that can prevent the late asthmatic reaction and reduce bronchial hyperreactivity. Mazzoni et al.[6] showed that asthmaprophylactic drugs such as disodium cromoglycate, ketotifen or hydrocortisone reduce platelet-activating factor (PAF)-induced bronchial hyperreactivity to bombesin or histamine.

Azzelastine (4-(p-chlorobenzyl)-2-(hexahydro-1-methyl-1H-azepine-4-yl)-1-(2H)-phtalazinone), a new antiallergic/asthmaprophylasctic agent, showed a potent and long-acting inhibitory effect in several animal models of allergic reactions, such as bronchospasm[7], passive cutaneous anaphylaxis[8,9] and passive Arthus reactions[8,10]. It is well established that azelastine has potent receptor-blocking properties against histamine, LTC_4 and LTD_4 in vitro and in vivo[7,11,12]. Azelastine has also been shown to inhibit histamine release from rat peritoneal mast cells[13] and immunologically induced LTC_4 release from chopped lung of guinea pigs[14]. Moreover, it has been demonstrated that azelastine possesses anti-PAF effects both in vitro and in vivo[15]. Based on these results it was of interest to investigate whether azelastine is capable of inhibiting increased airway hyperresponsiveness to a cholinergic stimulus in guinea pigs.

Materials and methods. Male Hartley guinea pigs (300–350 g) (Fa. Savo, Kisslegg, FRG) were actively immunized with ovalbumin (70 mg i.m.)[16]. Allergic bronchospasm was induced by aerosolized ovalbumin (5% w/v in saline) 21 days later. The challenge was chosen in such a way that it caused

a profound bronchospasm without lethality of animals. Only responder animals were used in further studies. 0.5, 1, 5, 20, 24, 48 and 72 h after antigen challenge the animals were sacrificed and tracheal spirals were suspended in an organ bath under 1 g preload in oxygenated Tyrode's solution at 37 °C. After an equilibration period of 30 min, contractions to 10^{-4} mol/l acetylcholine were recorded isotonically[17]. Contractions induced by 10^{-4} mol/l acetylcholine of tracheae obtained from control unchallenged animals were taken as 100%. In an additional experiment, changes in contractile responses to histamine were also investigated. Responses of tracheae obtained from challenged animals were calculated as a percentage of the control response. In a separate study, 2 h prior to antigen challenged animals were orally treated either with azelastine (3 mg/kg) or with placebo (1% carboxymethylcellulose). 20 h after antigen challenge, tracheal spirals were prepared and cumulative concentration response curves to acetylcholine were performed. Maximal response of control (unchallenged, non-treated) tracheal spirals was obtained in our system at 10^{-3} mol/l acetylcholine (intrinsic activity). The height of this response was defined as 100%. All other contractions were expressed as a percentage and related to the control response. Drug- and placebo-treated groups were compared by Student's t-test for unpaired samples.

Results. In tracheae obtained from control unchallenged animals, 100% contraction induced by acetylcholine at 10^{-4} mol/ml corresponded to 64% of maximum response evoked by 10^{-3} mol/ml of acetylcholine. When tracheae were obtained from challenged animals 0.5, 1 and 5 h after antigen challenge, the responsiveness to acetylcholine (10^{-4} mol/l) was not increased. A maximum in contractile response was observed 20 h after challenge: the acetylcholine-induced contraction was enhanced by 40%. At 24, 48 and 72 h after challenge, the responsiveness to acetylcholine declined again (fig. 1).

Tracheae obtained from animals exposed only to ovalbumin aerosol or from animals actively sensitized but not challenged showed steadily increased contractions to cumulatively enhanced concentrations of acetylcholine. The estimated pD_2-values were 4.43 and 4.23, respectively. Cumulative concentration response curve to acetylcholine was shifted to the left when tracheae of sensitized guinea pigs were removed and investigated 20 h after antigen challenge. The pD_2-value slightly increased up to 4.78 indicating a higher sensitivity to acetylcholine. The maximal response to acetylcholine observed at 10^{-3} mol/l acetylcholine was increased by 38% in comparison to the control response. In other words, the intrinsic activity of acetylcholine increased up to 1.38 in sensitized and challenged animals. By contrast, tracheae obtained from guinea pigs pretreated with azelastine 2 h prior to antigen challenge showed no signs of hyperreactivity: cumulative concentration response curve to acetylcholine did not differ from control curves. The calculated pD_2-value with 4.02 was slightly lower than those observed in control tracheae. The intrinsic activity of acetylcholine remained unchanged at 0.97 (fig. 2).

Contractile response of tracheae to histamine was investigated at 10^{-5} mol/l. At this concentration, the contraction of control tracheae amounted to 42% of the maximum response. The same concentration of histamine enhanced the contractility of tracheae obtained from sensitized and challenged animals up to 79%.

Discussion. Isolated tracheae of actively sensitized and challenged guinea pigs proved to be more sensitive to acetylcholine and histamine than control tracheae indicating the development of a non-specific hyperreactivity. The maximum of this hyperreactivity was observed 20 h after antigen exposition, as recently also demonstrated by Fügner[18].

The mechanism of bronchial hyperreactivity is not yet known in detail. In our present study, we found that the increased responsiveness of tracheae obtained from sensi-

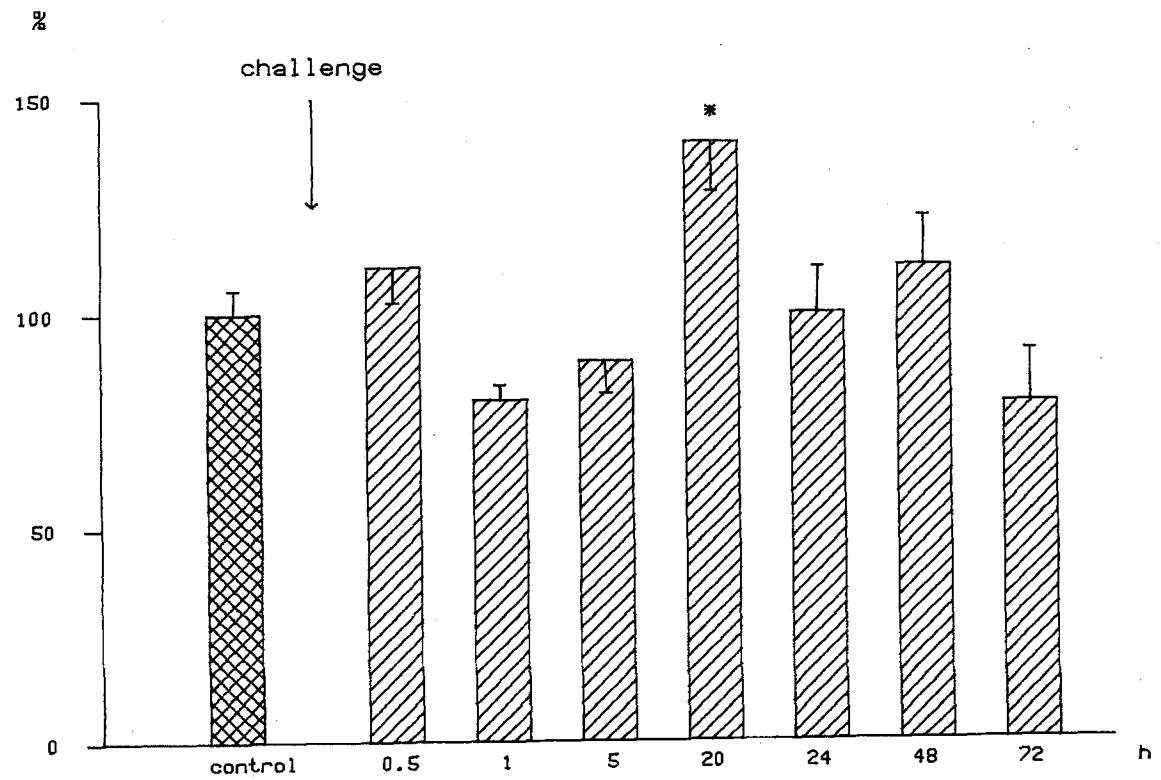

Figure 1. Contraction response to acetylcholine (10^{-4} mol/l) of tracheae obtained from actively immunized guinea pigs 0.5, 1, 5, 20, 24, 48 and 72 h after antigen challenge (n = 6; *p > 0.05 in comparison to non-challenged control group (the tracheal spasm induced by 10^{-4} mol/l acetylcholine was setted as 100%).

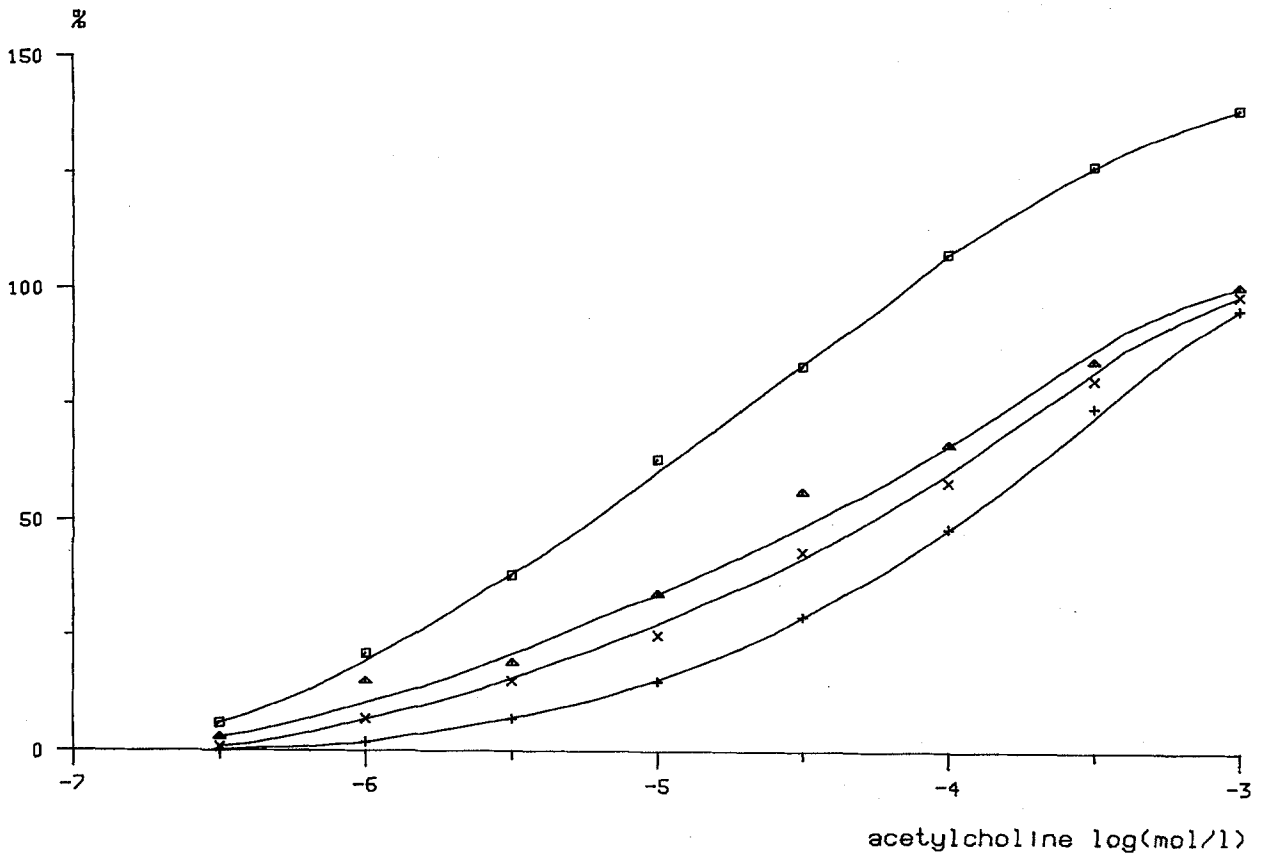

Figure 2. Concentration-response curves to acetylcholine using isolated tracheal spirals. Tracheae were obtained from non-immunized but ovalbumin-aerosol-treated (X); sensitized but non-challenged (△); sensitized, challenged, placebo- (□) and azelastine-treated (+) guinea pigs. The extent of tracheal contraction is given in percentage of maximally induced contraction observed at 10^{-3} mol/l acetylcholine using tracheae obtained from control animals (X). Values (△, +, X) observed between 3×10^{-6} and 10^{-3} were significantly different from values marked by (□). Each point represents the mean result from 6–10 individual values. SE means have been omitted for clarity, but for any single point did not exceed 15%.

Effect of azelastine on acetylcholine-induced contractions (mm) of tracheae obtained from sensitized guinea pigs 20 h after antigen challenge (mean ±SD, N = 6)

Concentration of acetylcholine (mol/l)	Contraction response to acetylcholine (mm)		
	Sensitized but not challenged animals	20 h after antigen challenge	
		Animals receiving vehicle	Animals receiving azelastine (3 mg/kg, p.o.)
3×10^{-7}	0.5 ± 0.4	0.7 ± 0.5	0.0
10^{-6}	1.9 ± 1.1	2.6 ± 1.4	0.2 ± 0.2
3×10^{-6}	3.9 ± 2.2	4.7 ± 2.6	0.8 ± 0.5
10^{-5}	5.7 ± 3.0	7.8 ± 3.3	1.9 ± 0.8*
3×10^{-5}	6.9 ± 3.6	10.2 ± 3.5*	3.6 ± 0.7
10^{-4}	8.3 ± 4.1	13.2 ± 4.3*	5.9 ± 0.7
3×10^{-4}	9.5 ± 4.6	15.2 ± 4.6*	8.2 ± 0.9
10^{-3}	12.3 ± 5.7	20.5 ± 5.6*	12.2 ± 2.1

* significantly different from control value obtained in sensitized but not challenged animals.

tized and challenged guinea pigs was accompanied by an increased intrinsic activity and an obviously increased affinity of acetylcholine to the muscarinic receptors. The observed higher intrinsic activity of acetylcholine may be explained by spare receptors which have been activated by events involved in the late phase reactions of bronchial asthma. There is also growing evidence that inflammation is important in the development of a non-specific bronchial hyperreactivity [19]. It is possible that the continuous secretion of inflammatory mediators (histamine, leukotrienes, prostaglandins, PAF) both from mast cells and other effector cells (particularly eosinophils and monocytes) plays a central role in this process [20]. Darius et al. [21] found that histamine is responsible for the early component, PAF for the middle component, and leukotrienes for the later component of pulmonary anaphylaxis in animals. It can also be assumed that inflammatory events, mediators released in these phases lead to a higher overall sensitivity of bronchial smooth muscle resulting in enhanced contractions to various mediators. Pretreatment of animals with azelastine led to a total abolition of tracheal hyperreactivity to acetylcholine, although, azelastine has no cholinolytic activity [7]. The effect of azelastine on histamine-induced hyperreactivity was not investigated due to the histamine H_1-antagonistic property of the com-

pound. Azelastine has been demonstrated to show anti-PAF activities both in vitro and in vivo [15]. Moreover, it inhibits the release and synthesis of leukotrienes [14, 22]. It is likely that azelastine, due to its complex pharmacodynamic profile, is capable of inhibiting bronchial hyperreactivity in animals.

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Stimulation of sodium current by cyclic AMP is mediated through protein phosphorylation in *Euhadra* neurons

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Summary. The protein kinase inhibitors, protein kinase inhibitor isolated from rabbit muscle and isoquinolinesulfonamide, abolished the inward Na current which was elicited by cAMP.
Key words. Na current; cAMP; protein kinase; protein kinase inhibitor; protein phosphorylation; H-8; snail neuron.

There exists a surfeit of evidence on the role of cyclic AMP (cAMP) as an intracellular messenger regulating neuronal activity. As an example, recent studies have shown that intracellular cAMP injection into molluscan neurons alters membrane properties which are coupled with activation of transmembrane Na^+ inward current [1–4]. In addition, Costa et al.[5] demonstrated the selective phosphorylation of the Na^+ channel's α subunit with cAMP-dependent protein kinase (PK) and suggested that this phosphorylation may possibly be an important step in activating this channel. Recently, we demonstrated that enhancement of intracellular cAMP causes Na^+ conductance in *Euhadra* neurons[6]. Based on Greengard's theory that all effects of cAMP are mediated by protein phosphorylation [7], it is highly probable that the increment in sodium conductance may be also generated through cAMP-dependent PK which in turn, phosphorylates membrane proteins forming ionic channels in *Euhadra* neurons. In this regard, we tested the possibility that protein phosphorylation may be involved in cAMP-induced opening or activation process of Na^+ channels in snail neurons, using several pharmacological agents, the pressure injection method and the voltage clamp technique.

Materials and methods. All experiments were carried out on neurons RC-2 and RC-3 identified in the right caudal cluster of an isolated subesophageal ganglion of the snail, *Euhadra peliomphala*[6]. Procedures for dissection and the basic formula for the normal snail saline were previously described [8, 13]. The arrangements for the voltage-clamp circuitry were described elsewhere[6]. In brief, both recording and current microelectrodes, with acceptable tip resistances ranging from 1–5 MΩ, were filled with 3 M KCl. A third electrode was used for pressure injection. The membrane potential of these neurons was usually held at −50 mV with voltage step commands of 5 s duration. Clamp current was measured by a virtual ground circuit through an I–V converter. Membrane potential was kept at the holding potential (Vh) for at least 10 s before stepping to different potentials. Constant pressure (2.0 kg/cm^2) injection lasting for 1 s into the neurons was performed through an electrode with an outside tip diameter of 1–1.5 μm in which cAMP (0.1 mM), catalytic subunit of cAMP-dependent protein kinase (PKC, 1.0 mg/ml), 5,5'-dethobis (2-nitrobenzoic acid) (Nb_{S2})-inactivated PKC (1.0 mg/ml) or cAMP-dependent PK inhibitor (PKI, 1 mg/ml) was contained, as previously described [9]. Both PKC and PKI were obtained from bovine heart and from rabbit muscle, respectively, and purified in the laboratory of Dr H. Komuro (Kanagawa Dental College, Yokosuka), who also kindly provided Nb_{S2}-inactivated PKC. These were backloaded into the tip of a microelectrode. The other agent used for inhibiting cAMP-dependent PK was isoquinolinesulfonamide (H-8, Seikagaku Kogyo Co., Ltd., Tokyo) [10].

Results and discussion. PKI is a heat-stable 10-kDa protein that possesses high affinity binding to PKC and is able to block its activity [11, 12]. In the present experiment, both PKI