# Dissociation of digoxin-like immunoreactivity and $Na^+,K^+$-ATPase inhibitory activity in rat plasma

K. Yamada, A. Goto, M. Ishii, M. Yoshioka[a] and T. Sugimoto

*Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1- Hongo, Bunkyo-ku, Tokyo 113 (Japan), and [a]Faculty of Pharmaceutical Science, Setsunan University, Hirakata (Japan)*
*Received 7 June 1988; accepted 26 July 1988*

*Summary.* We measured endogenous digitalis-like factor (EDF) in rat plasma during acute saline infusion by two different procedures. $Na^+,K^+$-ATPase inhibitory activity in the rat plasma significantly increased during saline loading (7.8 ± 2.2 vs 2.5 ± 0.9%, with and without acute saline loading, respectively, $p < 0.05$). On the other hand, the plasma digoxin-like immunoreactivity significantly decreased during acute saline loading (16.9 ± 1.6 vs 32.0 ± 2.8 pg digoxin equivalents/ml, with and without acute saline loading, respectively, $p < 0.01$). These results indicate that the major substances detected by digoxin-like immunoreactivity and direct $Na^+,K^+$-ATPase inhibitory activity are completely different, at least in rat plasma.
*Key words.* Endogenous digitalis-like factor; digoxin-like immunoreactivity; $Na^+,K^+$-ATPase inhibitor.

We have recently shown that endogenous digitalis-like factor (EDF), which is determined by its inhibitory effects on $^3H$-ouabain binding to intact human erythrocytes, definitely exists in human urine and dog plasma[1,2]. Although the chemical nature and the production site of EDF remain to be elucidated, EDF, supposed to be a circulating natural ligand for the cardiac glycoside receptor of $Na^+,K^+$-ATPase, could play an important role in the regulation of sodium excretion and may be related to the pathogenesis of hypertension in humans and animals[3-6]. Because of the lack of specific assay methods, a variety of different procedures have been employed to monitor digitalis-like activity. Some investigators have measured EDF by its cross-reactivity with anti-digoxin antibody[7-9] and others by the ouabain-displacing activity from $Na^+,K^+$-ATPase, or the $Na^+,K^+$-ATPase inhibitory activity[10-12]. However, the possibility that each different assay method detects completely different substances has not been ruled out.

In this study, we have measured plasma EDF of the rat during acute saline loading by two different procedures and found that the two assay methods may actually detect totally different substances.

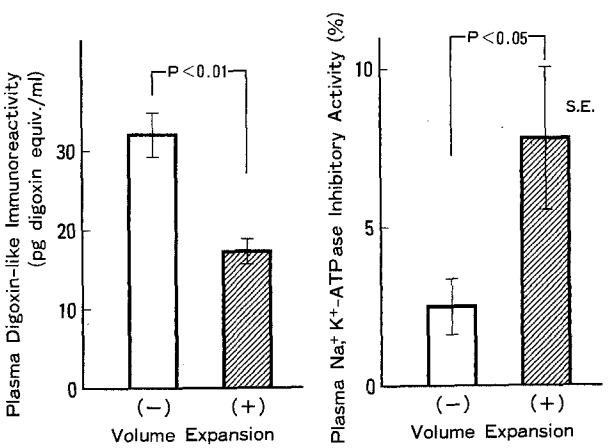
*Materials and methods.* Male SD rats (b. wt 406 ± 36 (SE) g, n = 10) were anesthetized with pentobarbital (40 mg/kg, i.p.) and PE-50 catheters were inserted into the right carotid artery and the right jugular vein. 30 min after the operation, a physiological saline solution, which was equal in volume to 10% of body weight, was infused i.v. for 40 min. Another 9 rats (b. wt 389 ± 35 g) without saline infusion served as controls. Blood samples were obtained from the right carotid artery at the end of the infusion into heparinized syringes. 5 ml of plasma was mixed with 10 ml of methanol and the mixture was kept at 4 °C for 16 h. After filtration through filter paper, the filtrate was evaporated and lyophylized. The resulting residue was dissolved in 8 ml of distilled water and the solution was applied to Amberlite XAD-2 (3 ml). After washing with 30 ml of distilled water, EDF was eluted with 8 ml of methanol. The eluent was evaporated and the residue was redissolved in 0.5 ml of distilled water.

EDF was determined by two methods. First, digoxin-like immunoreactivity was measured by radioimmunoassay according to the method of Gruber et al.[13] using an anti-digoxin antibody purchased from Miles Laboratories, Inc. (Elkhart, USA). Second, $Na^+,K^+$-ATPase inhibitory activity was measured using fluorescent high-pressure liquid chromatography (HPLC) which separates adenine compounds according to the method described previously[14]. In brief, 40 µl of 5 mM ATP was added to 60 µl of reaction mixture, which contained dog kidney $Na^+,K^+$-ATPase (Sigma, St. Louis, USA) and plasma extract. Incubation was performed at 37 °C for 20 min. The reaction was stopped by boiling and the mixture was applied to fluorescent HPLC after conversion into fluorescent derivatives. Percentage of inhibitory activity was calculated by the formula: $(X_0 - X_i) \times 100/(X_0 - X_{100})$, where X is the peak-height ratio of ADP/(ADP + ATP) in the chromatogram, $X_{100}$ is X in the presence of $10^{-3}$ M ouabain, $X_0$ is X without addition of ouabain, and $X_i$ is X in the presence of plasma extract. The data are expressed as mean ± SEM and were analyzed by the unpaired Student's t-test.

*Results.* The plasma digoxin-like immunoreactivities were 16.9 ± 1.6 and 32.0 ± 2.8 pg digoxin equivalents/ml in the rats with and without acute saline loading, respectively. The plasma digoxin-like immunoreactivity significantly decreased during acute saline loading ($p < 0.01$). On the other hand, $Na^+,K^+$-ATPase inhibitory activity in the plasma significantly increased during acute saline loading (7.8 ± 2.2 vs 2.5 ± 0.9%, with and without acute saline loading, respectively, $p < 0.05$). There was no correlation between the values of EDF assayed by the two methods used here.

*Discussion.* Endogenous digitalis-like factor (EDF) has been postulated to be implicated in the regulation of sodium excretion and the pathophysiology of hypertension. In this context, plasma volume expansion is supposed to increase the plasma levels of EDF. In the current study, the ability of plasma to inhibit dog kidney $Na^+,K^+$-ATPase activity significantly increased during acute saline infusion. In contrast, digoxin-like immunoreactivity in plasma fell off significantly during acute volume expansion.

Many investigators have measured digoxin-like immunoactivity to assess the level of the putative EDF. This approach was originally based on the premise that a substance which binds to a specific endogenous receptor may also bind to an antibody raised against that substance. However, this assumption may not be universally valid. Recent reports indicate the dissociation of digoxin-like immunoreactivity from $Na^+,K^+$-ATPase inhibitory activity, when plasma or urine is




The digoxin-like immunoreactivity and $Na^+,K^+$-ATPase inhibitory activity in the plasma of the rat with or without acute saline loading.

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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0014-4754/88/11-120992-02$1.50 + 0.20/0


## Azelastine inhibits bronchial hyperreactivity to acetylcholine in guinea pigs

U. Achterrath-Tuckermann and I. Szelenyi *

*Department of Pharmacology, ASTA Pharma AG, POB 100503, D-6000 Frankfurt/Main 1 (Federal Republic of Germany)*
*Received 30 June 1988; accepted 13 July 1988*

*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