The Effects of Phospholipase and 2-Chloroadenosine Treatments on α_2 -Adrenoceptors in Rat Vas Deferens

Yasuhiro WATANABE, Yoshiro KAMINOH*, Toshifumi KAGIYA**,
Hidehiko KAWABATA*** and Hiroshi YOSHIDA

Department of Pharmacology I, *Intensive Care Units, **Internal Medicine I and
***Orthopedics, Osaka University School of Medicine, Osaka 530, Japan

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The effects of phospholipases on various neurotransmitter and hormone binding sites have been reported (1–3). In some cases, the affinity of the ligand binding sites was changed by the products of phospholipolysis. In the other cases, the masked binding sites appeared due to digestion of the membrane.

It has been reported that the ^3H -clonidine binding sites, which are considered to be α_2 -adrenoceptors (4–6), are not detectable so far in rat vas deferens (4, 7–9). We reported that ^3H -clonidine binding sites increased with aging (9) and with sympathetic denervation by the treatments with reserpine (4) and adenosine receptor agonists (8). However, the mechanism of action of 2-chloroadenosine (2CA), an adenosine receptor agonist, on ^3H -clonidine binding sites is unknown. So we examined the effects of phospholipase C (PL-C) and phospholipase A_2 (PL- A_2) treatment on α_2 -adrenoceptor binding sites in rat vas deferens and compared the effects of PL-C treatment with that of 2CA.

Male Wistar rats, 7–8 weeks old, were used. The animals were sacrificed by decapitation. Vasa deferentia were removed rapidly and placed in ice-cold 0.32 M sucrose. After being freed from the serosa coat with blood vessels and connective tissues, the vas deferens was homogenized in 160 volumes of 50 mM Tris-HCl buffer (pH 7.4) in a polytron (Kinematica, Switzerland) at a setting of 5 for 30 sec. The suspension was filtered through nylon cloth (140 mesh). The homogenate was centrifuged for 15 min at 100,000 $\times g$, and then the pellet was resuspended in 160 volumes of buffer (50 mM Tris-HCl buffer, pH 7.4, 1 mM CaCl_2). This crude membrane fraction was used for the

binding assay. The concentration of protein in the suspension, determined by the method of Lowry et al. (10), was $447 \pm 15 \mu\text{g/ml}$ (mean $\pm$ S.E.M., $n=15$).

The treatments with PL-C, PL- A_2 , 1,2-diglyceride or 2CA were performed by preincubation of the tissue for 15 min at 37°C before the binding assay. When the effect of bovine serum albumin (BSA) was examined, the PL-C (0.2 U/ml)-treated and control homogenates were incubated in Tris-HCl (50 mM, pH 7.4) containing 1% BSA at 0°C for 30 min and then centrifuged at 100,000 $\times g$ for 15 min (11). The pellet was resuspended and used for the binding assay as mentioned above.

The reaction was started by adding ^3H -clonidine or ^3H -yohimbine to 1 ml of test tissue. When the inhibition experiments were performed, the reaction was started by adding ^3H -yohimbine concurrently with α -agonists. After incubation for 30 min at 25°C, the reaction was terminated by passing the reaction mixture through a Whatman GF/F glass filter. The filter was then washed 3 times with 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4). The filters were placed directly in a Triton/toluene-based scintillation cocktail, and the radioactivity was measured in a liquid scintillation counter. Specific binding was defined as the difference between the total counts on the filter in the absence and presence of 10 μM unlabeled 1-norepinephrine (NE).

Drugs used in the present studies included ^3H -clonidine (23.8 Ci/mmol), ^3H -yohimbine (89.7 Ci/mmol, New England Nuclear); clonidine-HCl (Boeringer Ingelheim); 1,2-diglycerides (porcine liver lecithin, Serdary

Research Laboratories, Inc.); yohimbine-HCl, 2CA, PL-A₂ and PL-C (Sigma). Other drugs and chemicals were obtained from commercial sources.

The data were expressed as the mean $\pm$ S.E.M. Tests of significance were evaluated using Student's *t*-test, *P* values less than 0.05 being considered significant.

³H-Clonidine binding sites in the rat vas deferens membrane fraction before and after the treatment with PL-C are shown in Table 1. The binding assay was done at the concentration of 14 nM ³H-clonidine. In the untreated tissue, ³H-clonidine binding sites were not detectable in the crude membrane fraction as reported previously (4, 7, 8, 9). In the crude membrane fraction after PL-C treatment at the concentrations of 0.2 and 0.4 U/ml, the ³H-clonidine binding sites were detected; but after PL-C treatment at the concentration of 0.1 U/ml, we could not detect ³H-clonidine binding sites in the membrane fraction. Preincubation with phospholipase C for 15 min was enough to evoke the maximal effect on ³H-clonidine binding because no further increase in the binding sites was observed when a longer preincubation period of 30 min or 45 min was carried out. Next, the effects of the treatment

with PL-A₂ (0.25–8.0 U/ml) were studied. The ³H-clonidine binding sites were not detected after the treatment with PL-A₂ at these doses.

As shown in Table 1, the treatment with 2CA (10 μ M) increased the ³H-clonidine binding sites. Addition of 1.25 mM theophylline completely abolished the effects of 2CA when it was added simultaneously with 2CA at the beginning of preincubation (Table 1). In addition, we examined the effect of 10 μ M 2CA in the crude membrane fractions treated with PL-C. The amount of ³H-clonidine binding sites in the examination was not significantly different from that obtained after PL-C treatment only. The preincubation with 1,2-diglycerides (0.1 mg/ml) had no effects on ³H-clonidine binding sites (Table 1). In addition, the incubation of PL-C treated homogenates with 1% BSA did not reverse the effects of PL-C treatment on ³H-clonidine binding (data not shown).

The results calculated by the Scatchard analysis of ³H-clonidine and ³H-yohimbine binding in the membrane fraction are shown in Table 2. In the untreated tissue, ³H-clonidine binding sites were not detectable. After treatment with PL-C (0.2 U/ml) or 10 μ M 2CA the *B*_{max} values were 9.1 ± 2.2

Table 1. The effects of PL-C, 2CA and 1,2-diglyceride on ³H-clonidine binding sites in rat vas deferens homogenate and crude membrane fraction

Tissue	Treatment	Binding sites	n
Homogenate	no	not detectable	
	PL-C (0.8 U/ml)	0.43 ± 0.15 pmol/g wet weight	4
	2CA (10 μ M)	0.52 ± 0.15 pmol/g wet weight	5
Membrane fraction	no	not detectable	
	PL-C (0.1 U/ml)	not detectable	
	(0.2 U/ml)	6.1 ± 1.0 fmol/mg protein	10
	(0.4 U/ml)	5.1 ± 0.9 fmol/mg protein	6
	2CA (10 μ M)	5.5 ± 1.3 fmol/mg protein	4
	2CA (10 μ M) and theo. (1 mM)	not detectable	3
	PL-C (0.2 U/ml) and 2CA (10 μ M)	6.2 ± 1.7 fmol/mg protein	4
	1,2-diglyceride (0.1 mg/ml)	not detectable	

The concentration of ³H-clonidine was 14 nM. Preincubation with PL-C or 1,2-diglyceride was performed at 37°C for 15 min, and then ³H-clonidine or/and 2CA was added into the incubation mixture. theo: theophylline

Table 2. Scatchard analysis of ^3H -clonidine and ^3H -yohimbine binding with rat vas deferens crude membrane fractions

Treatment	^3H -Clonidine			^3H -Yohimbine		
	K_d (nM)	$B_{\max}^*$	n	K_d (nM)	$B_{\max}^*$	n
No treatment	not detectable		(13)	4.9 ± 0.4	9.2 ± 0.5	(4)
PL-C (0.2 U/ml)	6.0 ± 2.1	9.1 ± 1.4	(3)	6.7 ± 1.4	8.7 ± 0.5	(3)
2CA (10 μM)	3.0 ± 1.4	6.7 ± 0.8	(3)	5.5 ± 0.7	9.8 ± 0.6	(3)

The treatment with PL-C or 2CA was performed as described in the text. *fmol/mg protein.

and 6.7 ± 1.2 fmol/mg protein, respectively, and the K_d values were 6.0 ± 3.0 and 3.0 ± 2.0 nM, respectively. The dose-response of the effect of preincubation with 2CA was studied. ^3H -clonidine binding sites were not detected with 10^{-8} M 2CA, though when the preincubation with 2CA was done at the concentrations of 10^{-7} M and 10^{-6} M, the $B_{\max}$ values of the ^3H -clonidine binding sites were 0.61 ± 0.21 (4) and 0.78 ± 0.33 (4) pmoles/g wet weight, respectively. On the other hand, ^3H -yohimbine binding sites were detectable even in the untreated tissue. The $B_{\max}$ and K_d values of ^3H -yohimbine binding obtained after the treatments with 0.2 U/ml PL-C or 10 μM 2CA were not significantly different from those without treatment with PL-C or 2CA as shown in Table 2. Next, the effects of the preincubation with 10^{-5} M 2CA or phospholipase C treatment on the inhibition by an α -agonist in ^3H -yohimbine (3 nM) binding were also studied. At the concentration of 10^{-5} M, both NE and clonidine inhibited the specific ^3H -yohimbine binding completely. The preincubation with 10^{-5} M 2CA reduced the IC_{50} values of clonidine significantly from $11 \pm 3 \times 10^{-8}$ M to $4 \pm 2 \times 10^{-8}$ M (4) and that of NE from $10.8 \pm 2.0 \times 10^{-9}$ M (4) to 4.0×10^{-9} M (4). The phospholipase C treatment also reduced the IC_{50} value of clonidine significantly from $10 \pm 3 \times 10^{-8}$ M (3) to $0.9 \pm 0.2 \times 10^{-9}$ M (3).

It has been reported that the PL-C treatments increased the insulin receptors in rat liver plasma membrane (1) and GABA (2) or benzodiazepine binding sites (3) in rat brain synaptic plasma membranes. In these reports, it was suggested that PL-C acts upon the polar head groups of phospholipids and causes the changes in the affinity or the

number of binding sites in those receptors. In the present study, it seems that the affinity of the α_2 -agonist (clonidine) to the receptors in rat vas deferens increased by the digestion of some phospholipids in the membranes with PL-C or the treatment with adenosine receptor agonists such as 2CA or adenosine (Table 2). The mechanism by which 2CA increases ^3H -clonidine binding is unknown, but since 2CA and theophylline are an agonist and an antagonist, respectively, of adenosine receptors, the activation of adenosine receptors by the agonists may cause the increase of α_2 -agonist affinity to the receptors.

In the present study, 2CA caused no further changes in the ^3H -clonidine binding sites in rat vas deferens membrane fraction after PL-C treatment (Tables 1 and 2). Therefore, the results suggest that the changes in the composition of phospholipids surrounding the α_2 -adrenoceptor and adenosine receptor in rat vas deferens membranes result in the loss of stimulation of adenosine receptors by its agonist, 2CA, or the inhibition on the interaction between α_2 -adrenoceptor and adenosine receptor. The treatments with adenosine receptor agonists and phospholipase C may also cause an increase of α_2 -agonist affinity to the receptor by the same mechanism.

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