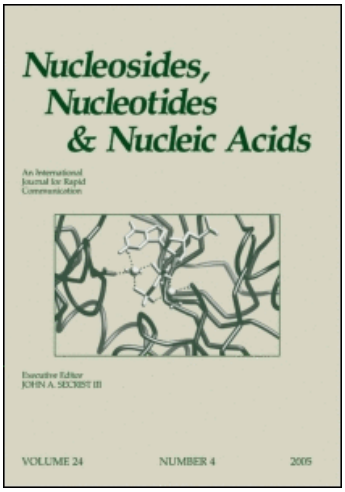

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EFFECT OF ENDOGENOUS PHOSPHOLIPIDS ON THE [^{3}H]R-PIA BINDING TO A_1 ADENOSINE RECEPTORS FROM PIG CEREBRAL CORTEX

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ABSTRACT

The effect of phospholipases on the membrane-bound and solubilized A_1 adenosine receptor has been studied. The results indicate that phospholipids are essential for the functionality of the A_1 adenosine receptor and that there is a co-solubilization of receptor and phospholipids.

In current models of membrane structure (1-2) phospholipids, in addition to maintain the structual identity of the membrane, play a key role in the interaction with protein components and may even modulate the activity of such proteins. Thus it has been proved that phospholipases directly modify the binding of some hormones to their specific receptors (3-5). There are no similar studies concerning adenosine receptors, although indirect evidence obtained by studying the solubilized A_1 adenosine receptors has suggested that lipids may prevent soluble receptor inactivation (6-7). In this paper we have analyzed the effects of phospholipases on A_1 adenosine receptor activity in intact brain membranes and soluble preparations.

Membranes and soluble receptor from pig brain cortex were obtained as previously described (8). As a general method [^{3}H]R-PIA binding to pig brain cortex adenosine receptors was measured after the incubation of the membranes or soluble extracts (0.7 to 0.8 mg protein/ml) with adenosine deaminase (0.2 Units/ml) and 2 mM $CaCl_2$ for 15 minutes at 25°C in 50 mM Tris-HCl buffer pH 7.4. Addition of one of the following enzymes, at the concentration (Units/ml) given in parentheses, was then made: phospholipase A_2 (0.10), phospholipase C (0.30) and phospholipase D (0.50). After standing at 25°C, 15 or 60 minutes, 6.5 nM [^{3}H]R-PIA was added and the incubation prolonged for 30 minutes. In this latter incubation the enzyme concentration, in all cases, was 8 % lesser due to the volume of reactives added. In incubations containing membrane suspensions free and bound ligand were separated by rapid filtration of

TABLE I. Effect of phospholipases on membrane-bound and soluble A_1 adenosine receptors. Phospholipases were incubated with membrane-bound or solubilized receptors (1 mg protein/ml) prior to the addition of [^{3}H]R-PIA (6.5 nM) as described elsewehre (8). Values are mean±SEM of four different experiments. Statistical significance versus controls were calculated according to Student's t-test: * P<0.1, ** P<0.05, *** P<0.005, **** P<0.0001

Treatment	Time	[^{3}H]R-PIA binding (pmol/min mg protein)			
	min	Membranes	Solubilized receptors	Membranes	Solubilized receptors
		(treatment before incubation with *cold* [^{3}H]R-PIA)		(treatment after incubation for 30 *minutes* with [^{3}H]R-PIA)	
Control	-	0.63±0.03	0.88±0.07	0.61±0.04	0.87±0.05
Phospholipase A_2	15	0.63±0.03	0.41±0.03***	-	-
(0.10 Units/ml)	60	0.62±0.02	0.33±0.04****	ND	0.41±0.02**
Phospholipase C	15	0.47±0.03***	0.75±0.05*	-	-
(0.30 Units/ml)	60	0.46±0.04***	0.74±0.04*	0.61±0.02	0.92±0.05****
Phospholipase D	15	0.36±0.01****	0.26±0.01****	-	-
(0.50 Units/ml)	60	0.37±0.02***	0.22±0.02****	0.62±0.03	0.73±0.03**

aliquots of 500 µl through Whatman GF/C filters, which were subsequently washed with 10 ml of ice-cold Tris buffer; the washing time was less than 8 seconds. The filters were presoaked in 0.3 % polyethylenimine (2-4 hours, pH 10) to improve the performance of the filtration (29). In all cases, after filtration, the filters were allowed to stand overnight with 10 ml Aquasol2 scintillation cocktail (New England Nuclear) at room temperature; radioactivity of vials was counted using a Packard 1500 TRI-CARB liquid scintillation counter with an efficiency of 50 %. When soluble extracts were used, polyethylene glycol (8000) and bovine gamma-globulins were added to the incubation mixture to a final concentration of 10 % and 1.3 mg/ml respectively. After further incubation for 10 minutes at 0°C the solution was filtered through Whatman GF/C filters (presoaked in polyethylenimine as indicated above), which were subsequently washed with 10 ml of 50 mM Tris-HCl buffer pH 7.4 containing 8 % polyethylene glycol. The final concentration of detergent prior to polyethylene glycol and gamma-globulins addition was 0.2 %; greater concentrations led to a marked decrease of specific binding.

Agonist protective effect against phospholipases was tested by radioligand binding experiments performed as described above except that 20 nM [^{3}H]R-PIA was incubated (30 minutes) with either membrane suspensions or soluble extracts before treatment for 90 minutes with phospholipases.In the final incubation period the radioligand concentration was 50 % lesser (10 nM).

Results appear in table I.

The phospholipases action upon the soluble receptor indicates that there exists co-solubilization of receptor and phospholipids. The lack of influence of phospholipase A_2 upon membranes is explained by the impossibility of cleaving bonds in the surrounding of the receptor when the membrane is intact. In the soluble extract the phospholipids accompanying the receptor molecular might be more exposed to the enzyme, the cleavage thus being possible. In contrast, phospholipase C and D affect the binding of the radiolabeled compound in both fractions. However, the effect of phospholipase D was stronger than the effect of phospholipase C in soluble extracts and in membranes. In consequence it should be pointed out that some parts of the phospholipid molecule are important for the interaction of R-PIA with the binding site of A_1 adenosine receptors. The binding is more altered by the negative net charge produced by phospholipase D than by the release of the whole polar head produced by phospholipase C. The interaction time between phospholipases and either membranes or soluble extracts did not produce any quantitative changes in the results obtained, 15 minutes being enough time to achieve the maximum effect. In membranes the ligand R-PIA is able to protect the receptor completely from attack by phospholipases. In soluble extracts the protective effect is only important in the case of phospholipases C and D. Thus it is suggested that the binding of the agonist on the receptor located in the membrane hides some polar heads of phospholipids which are essential for the functionality of the receptor. These phospholipids in the solubilized receptor molecule ar also covered by the ligand when it interacts with the "active site"; it seems however that the bond cleaved by phospholipase A_2, i.e. the ester bond between glycerol and the fatty acid in position two remains exposed.

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