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PYRENESULFONYL AZIDE: A COVALENT PROBE PERMITTING IN VITRO DESENSITIZATION OF LABELED ACETYLCHOLINE-RICH MEMBRANE FRAGMENTS FROM TORPEDO CALIFORNICA

J.M. Gonzalez-Ros, V. Šator, P. Calvo-Fernandez and M. Martinez-Carrion

Department of Biochemistry, Medical College of Virginia,
Virginia Commonwealth University, Richmond, Virginia 23298

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SUMMARY. Acetylcholine receptor-rich membrane fragments from Torpedo californica electroplax after covalent labelling at the protein-lipid boundary by nitrenes generated in situ from pyrenesulfonyl azide can bind [^{125}I]-α-bungarotoxin. The covalent attachment of 6-8 molecules of the fluorescent probe/receptor molecule also does not perturb the marked effect on the rate of α-bungarotoxin binding to electroplax membranes exerted by their preincubation with carbamylcholine. This phenomenon, which is analogous to pharmacological desensitization of receptors in synaptic junctions, is fully reversible upon removal of carbamylcholine (Quast, V., Schmerlik, M., Lee, T., Witzemann, V., Blanchard, V. and Raftery, M.A. (1978) Biochemistry 17, 2405-2414). Torpedo electroplax membranes, whether tagged with the covalent probe or freshly isolated, regain the original fast rate of α-bungarotoxin binding upon dilution of carbamylcholine.

INTRODUCTION

Exposures of AcChR[a]-enriched membrane fragments to cholinergic agonists slows the rate of α-Bgt binding to AcChR-rich membrane fragments. This effect has been observed in membrane preparations from Torpedo electric organ (1,2), and skeletal muscle (3) and it resembles pharmocological desensitization of the post synaptic membrane induced by cholinergic agonists (1,2,3,4). The prolonged exposure of AcChR membrane vesicles to agonists also causes a decrease in the sodium fluxes promoted by agonists (5).

Membrane environment appears to play an important role in maintaining the AcChR functional state, and minor perturbations of the membrane constituents affect, permanently, these state transitions (6,7,8) usually converting the receptor into a desensitized-like high affinity state. Every manipulation of the lipid environment so far attempted, including a recent report on the use of general anesthetic gases (9), induces perturbations on the "de-

(a) Abbreviations used are: AcChR, acetylcholine receptor; PySA, Pyrenesulphonyl azide; α-Bgt, α-Bungarotoxin; carb, carbamylcholine.

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sensitization" kinetics of the AcChR-rich membranes. This lack of manipulative tools has hampered attempts to identify possible structural changes in the receptor assembly which might be implicated in the functional state transitions. Recently, we have described PySA as a covalent photolabel for the AcChR subunits at the lipid-receptor interface in membrane fragment preparations of _Torpedo californica_ (10). In this communication we report that _in situ_ modification of AcChR does not perturb the _in vitro_ desensitization behavior of membrane-bound AcChR induced by cholinergic ligands.

MATERIALS AND METHODS

α-Bgt was obtained from _Bungarus multicinctus_ venom (Sigma Chemical Co.) following published purification procedures (11). The preparation of [^{125}I]α-Bgt and the synthesis and [^{3}H]-labeling of PySA were performed as described previously (10).

Carbamylcholine was purchased from Aldrich Chemical Co. Solubilized AcChR and AcChR-enriched membrane fragments were prepared from _Torpedo californica_ electroplax (Pacific Biomarine) as previously described (10).

Protein concentrations were determined by the method of Lowry _et al._ (12) and the specific activities of the preparations were determined by the DEAE-cellulose filter disc procedure using [^{125}I]α-Bgt (13). The AcChR preparations had a specific activity of 9 nmoles of α-Bgt binding sites/mg protein, and the AcChR-enriched membrane fragments commonly showed 1 to 2 nmoles of α-Bgt binding sites/mg protein. For the PySA labeling procedures (10) membrane fragments showing low affinity for Carb (1 μM) were used. The time courses of [^{125}I]α-Bgt binding to the AcChR-enriched membrane fragments were followed according to Quast _et al._ (1978) (14); the experimental details are given in the legends to the figures.

RESULTS

AcChR labeling with PySA. PySA partitions, almost quantitatively, into the lipid core of the AcChR-enriched membrane fragments (>96%). Upon irradiation about 10% of the photogenerated nitrene covalently binds to the AcChR protein (about 27 molecules per molecule of the receptor), predominantely to the 48,000 and 55,000 molecular weight subunits, presumably in contact with the lipid core of the AcChR-enriched membrane fragments (10). PySA can also label Triton X-100 solubilized AcChR. The effect of the presence of the covalent label on the toxin binding capacity of AcChR is shown in Table I. In both, the solubilized and membrane-associated states of the AcChR, the amount of the [^{125}I]α-Bgt bound is unaffected by the attachment of the probe to the receptor.

Table I

SPECIFIC ACTIVITIES OF ISOLATED AcChR AND AcChR-ENRICHED MEMBRANE FRAGMENTS PREPARATIONS BEFORE AND AFTER LABELING WITH PySA PHOTOPRODUCTS

	Specific Activity of AcChR (nmole of -Bgt/mg protein)		moles PySA-bound / mole AcChR
	Native	PySA-labeled	
Isolated AcChR	9.38	9.84 ± 0.54 (a)	28 ± 5
AcChR-enriched membrane fragments	1.61	1.61 ± 0.09 (a)	27 ± 7 (b)

[a] Values corrected for fluorescence quenching induced by PySA photoproducts, since the yellow-brownish color of the photoproducts in the system under study causes an apparent decrease of 12-20% (depending on the concentration of PySA) in the radioactivity caused by the quenching of fluorescence of the scintillation fluid.

[b] Measured after solubilization with detergent and isolation of AcChR as indicated in Ref. 10.

Effect of covalent attachment of PySA on low-high ligand affinity state transitions of AcChR. AcChR-enriched membrane fragments can undergo changes in affinity for cholinergic ligands as a response to prolonged exposure to these ligands (1,2,14). These changes resemble the receptor desensitization phenomenon observed in electrophysiological experiments with intact tissue (1-4). The well-known α-Bgt time dependent binding process of AcChR-enriched membrane fragments (1,2,9,14) is not abolished by the covalent labeling of AcChR with photogenerated pyrenesulfonyl nitrene. Figures 1A and 1B show identical α-Bgt binding behavior in both freshly prepared and in PySA labeled AcChR-enriched membrane fragments from the electric organ of *Torpedo californica*. In the absence of Carb, the rate of the interaction of α-Bgt with the receptor in both non labeled and labeled membranes is higher than after exposure of the membranes to the agonist. Also, as expected (14), when the membranes are incubated with Carb for 30 minutes prior to addition of α-Bgt, the initial amount of the α-neurotoxin bound is significantly lower than when Carb and $[^{125}I]$α-Bgt are added simultaneously to the membrane preparations.

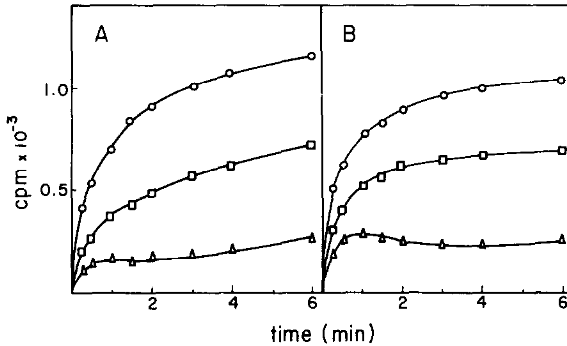

FIGURE 1. Time dependence of $[^{125}I]\alpha$-Bgt binding to AcChR-enriched membrane fragments. In all assays the concentration of AcChR was 0.25×10^{-7} M in α-Bgt binding sites. $[^{125}I]\alpha$-Bgt concentration was 2.5×10^{-7} M. Ca^{++} free-Ringer's solution used as solvent. (A) Native AcChR-enriched membrane fragments. (B) AcChR-enriched membrane fragments labelled with PySA (approximately 0.2 μmole PySA per mg protein) after irradiation with long wavelength ultraviolet light for 15 minutes at 20° (see Methods). o—o, $[^{125}I]\alpha$-Bgt binding in the absence of Carb; □—□ , Carb, (final concentration 10^{-6} M) and $[^{125}I]\alpha$-Bgt added simultaneously to membrane fragments suspension at zero time; Δ—Δ membrane fragments suspension incubated 30 minutes in the presence of 10^{-6} M Carb prior to the addition of $[^{125}I]\alpha$-Bgt.

Upon a 40 fold dilution of the carb containing sample the rate of α-Bgt binding reverts, within a 15 minute period, to that observed with the same membranes in the absence of carb pretreatment. This reversal of "desensitization" is observed in both freshly prepared membranes and after the photolabel has been attached.

DISCUSSION

The property of the AcChR to undergo transitions from low to high ligand affinity states in response to the presence of agonists is very sensitive to variations into the membrane integrity. Indeed, these transition properties of the receptor in its membrane environment are lost upon solubilization with detergents (2,15) although solubilized receptor is able to bind cholinergic ligands (2,16,17,18,19,20). Even small concentrations of Triton (~1 μM), insufficient to solubilize membranes, cause a loss of this characteristic feature of native AcChR (6). Specific membrane perturbants, such as local

anesthetics and phospholipase A (isolated from snake venom), have been shown to induce similar loss of sensitization-desensitization shifts in Torpedo membranes (7,8). Therefore, it appears that there is a strong dependence of the sensitivity to desensitization of the AcChR on any perturbation of the lipid. Even when the perturbing agent is a gas it affects the carb induced desensitization as a potentiation of the agonists effects (9). In the latter example, however, it is not clear whether the general anesthetic effects are reversible.

On the other hand, ligand binding induced conformational transition(s) of AcChR has been proposed. Bonner _et al_. (22) and Barrantes (4) monitored slight changes in intrinsic fluorescence of the receptor during its association with various agonists and Witzemann and Raftery (21) have observed an increase of the AcChR labelling with a water soluble probe, ethidium azide, in the presence of cholinergic ligands. Postulated conformational transitions may not only involve water exposed regions of the receptor molecule but may also encompass the subunits in contact with the hydrocarbon core of the membranes which appear to be exquisitly sensitive to the state of lipids in their proximity. Therefore, it is of importance to develop non perturbing probes for these subunits. Ideally, such probes should have the following properties: 1) do not perturb the ability of the AcChR to undergo the conformational change itself, 2) allow for the AcChR to undergo reversible sensitization-desensitization shifts and 3) provide a spectroscopic handle to monitor events in the strategic area. PySA appears advantageous for this purpose. It can be bound to the subunits situated in the lipid-protein interface (10) and does not cause alterations in the ligand affinity states characteristic of the native AcChR-enriched membrane fragments. In addition, pyrene derivatives, due to their fluorescence properties such as long fluorescence lifetimes and extreme solvent sensitivity of vibrational fine structure of their fluorescence emission spectra, can be useful in providing information about possible variations in their environment (23,24,25,26,27,28).

From this work, it is apparent that not all perturbations of membrane components necessarily lead to loss of cholinergic ligand induced, low to high, affinity state transitions of the receptor. We have previously shown that introduction of pyrene alone into the lipid phase of the membrane did not affect those affinity state transitions (28). Now it is evident that, in situ, covalent labeling of the AcChR subunits in contact with the lipid phase does not affect the transition either. The fact that pyrene and PySA are bulky compounds renders the approach of covalent modification more encouraging for the design of smaller probes with different spectroscopic characteristics. Finally, the question of why pyrene compounds do not affect the sensitization-desensitization affinity state transitions and other perturbations of the membrane do, must be addressed. The answer may rest on the difference between perturbing agents. Pyrene compounds, and general anesthetics, are hydrophobic and presumably remain, and perturb, mostly the hydrophobic part of the lipid layer. On the other hand, other reagents such as detergents, phospholipase A or local anesthetics can cause drastic changes in the distribution of properties of the polar heads of the lipid. The latter changes may be significant in "freezing" the AcChR conformation in only one affinity state.

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