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Immobilization and Characterization of a Liposome-Mediated Reconstituted Nicotinic Acetylcholine Receptor

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Abstract: A nicotinic acetylcholine receptor (nAchR) isolated from the electric tissues of *Torpedo californica* has been reconstituted into a vesicle comprising a bifunctional azo-ligand (Bae 1) compound, and a liposome containing phospholipids and cholesterol (1 : 1, w/w). The liposome-mediated reconstituted receptor showed a concentration-dependent response to cholinergic drugs in a lithium ion flux assay. This liposome-mediated reconstituted nAchR was immobilized onto an electrode using various synthetic polymers which were tested for their response to the cholinergic ligands. The immobilized nAchR not only exhibited a linear response to a wide range of cholinergic ligand concentrations but also retained an operational stability which lasted for longer than 6 days. Thus, this result provides a basis for application of the immobilized nAchR-based biosensor in detecting cholinergic ligands *in vitro*.
Key words: biosensor, cholinergic drug, nicotinic acetylcholine receptor, protein immobilization, receptor reconstitution.

The nicotinic actylcholine receptor (nAchR) is a pentameric glycoprotein (α_2, β, γ, δ) at the cell surface which acts as a chemically gated cation channel (Stroud and Finer-Moore, 1985). Binding of acetylcholine to the receptor triggers a conformational change of the subunits and causes the opening of an ion channel across the plasma membrane (Witzemann and Raftery 1978; Kristler *et al.*, 1982). nAchR is found in high concentrations in the electric organs of electric fishes, and it can be purified in fairly large quantities (Raftery *et al.*, 1980; Conti-Tranconi and Raftery, 1982). nAchR of skeletal muscle is the molecular target for neurotoxin therapeutic agents, and drug abuse treatments (O'Brien *et al.*, 1972; Eldefrawi *et al.*, 1973). Neurotoxins, such as α-Naja toxin and α-bungarotoxin have been used extensively to study nAchR because they bind to nAchR with a high affinity (Franklin and Potter, 1972; Colquhoun and Rang, 1976). Due to high affinity and specificity of the receptors for particular ligands, nAchR has a number of distinct advantages as a biological element used in biosensors.

A receptor-based biosensor is a device that couples a receptor protein to a transduction system, sensing changes in the receptor when it binds to its substrates. Several transduction modes have already been used, including optical signal generation-based fluorescence and bioluminescence (Rogers *et al.*, 1989), an electric field (Gotoh *et al.*, 1987) and a chemomechanical signal resulting from alteration of the mechanical properties of proteins (Taylor *et al.*, 1988). However, the development of a receptor-based biosensor has been limited by the inherent instability of the receptors once they are extracted from their natural membrane environments. Although many attempts have been made to stabilize the receptors in lipid vesicle bilayers, there have been many problems in the maintenance of stability (Hugamir *et al.*, 1979; Nelson *et al.*, 1980; Rogers *et al.*, 1991). Recently, it has been reported that a pH electrode was developed by incorporation of an enzyme into a membrane on the surface of a sensor (Tor and Freeman, 1986). The present studies were performed to design a novel system in which nAchR is reconstituted in a mixture of an azoligand compound (*e.g.*, Bae 1) and immobilized onto an electrode with a stable polymeric matrix. Results demonstrate that the resulting biosensor prototype is able to detect particular classes of substances within 10 to 15 min at submicromolar concentrations and is stable in use for 100 h or more. Herein for the first time a new kind of prototype for

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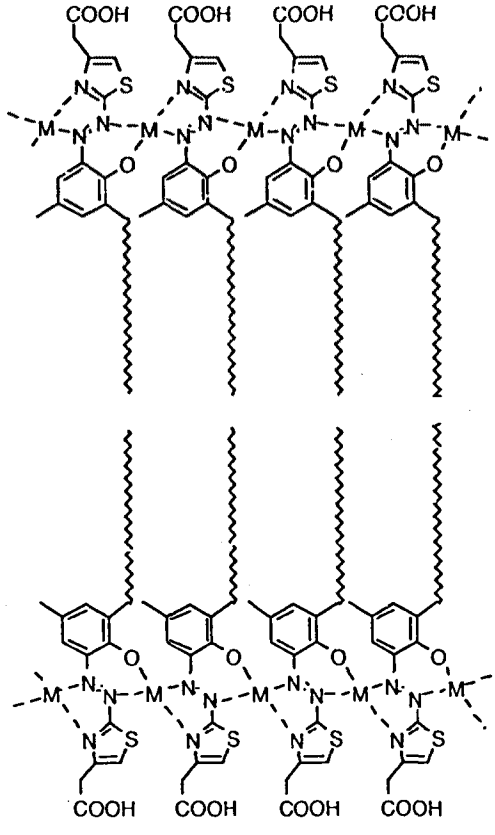
a nAchR-based biosensor is reported.

Experimental Procedures

Materials

All reagents and solvents used were of analytical grade or better. Acetylcholine, glyoxal, acrylamide, methacrylamide, hydrazine hydrate, Triton X-100, polyethylene glycol (M.W=2,000), polypropylene glycol (M.W=2,000), asolectin (phosphatidyl choline 50%, from soy bean), bean and 2,4-toluene diisocyanate were obratined from Sigma Chemical Co (St. Louis, USA). The azoligand compound(potassium-3-[[3[*n*-dodecyl-2carbamoylethyl]-2-hydroxyl-5-methyl-phenyl]azo]-4-hydroxybenzene sulfonate; Bae 1, Scheme 1) was synthesized as described by Suh and Shim (1994). nAchR was isolated from the electric organ of *T. californica* employing α-bungarotoxin affinity chromatographic procedures following solubilization of proteins with Triton X-100 as described by Rogers *et al.* (1989). Polyacrylamide-methacylamide prepolymer was prepared as described by Tor and Freeman (1986) with minor modification. Polyurethane prepolymer was synthesized according to the method of Fukui *et al.* (1987).

Reconstitution of nAchR

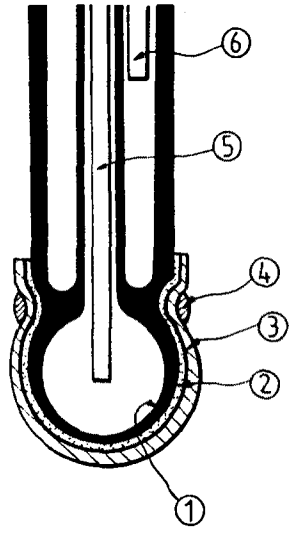


Scheme. 1. Bilayer structure of azoligand compound (Bae 1) formed by coordination with transition metal (Suh and Shim, 1994).

Twenty five mg of a mixture of asolectin-cholesterol (6 : 1) was dissolved in 5 ml of cholate (2% w/v) and sonicated at 25℃ to clarify the solution. Approximately 1.2 mg of an azoligand compound was dissolved in 250 μl of distilled water and mixed with an asolectin solution. The optimum ratios between the components (*e.g.*, asolectin:cholesterol and/or lipid : azoligand) were empirically determined. The mixture was heated to 90° C and sonicated for 5 min to clarify. For reconstitution of nAchR in lipid vesicles, the detergent-solubilized receptor was suspended in a lipid/cholate mixture to yield a final concentration of 0.5 mg/ml protein, 1.5% (w/v) cholate, and 5 mg/ml lipid. Forty μl of ferric chloride (10 mM) per 1 mg of azoligand compound in lipid was added to the receptor-lipid mixture. The receptor protein-lipid cholate mixture (designated as reconstituted nAchR) was dialyzed for 48 h, with three changes of buffer, against 500 volumes of buffer A (100 mM Hepes buffer containing 10 mM sodium cholate, 100 mM NaCl, 3 mM NaN_3, and 0.1 mM $CaCl_2$; pH 7.4) at 4℃.

Immobilization of nAchR on electrode

Polyacrylamide-methacrylamide copolymer : A 20



Scheme 2. Schematic representation of nAchR-based pH electrode. nAchR gel layer is formed as follows: (i) reconstitution of nAchR with a vesicle containing azo ligand compound and lipids- phospholipid plus cholesterol (1 : 1); (ii) immobilization of the reconstituted nAchR obtained as above procedures with a synthetic polymers such as polyacrylamide-methacrylamide copolymer, polyurethane homopolymer, or polyurethane copolymer; (iii) coating the immobilized nAchR solution on the surface of pH electrode; and crosslinking the nAchR coated on the surface of electrode with glyoxal. In the case of using polyurethane polymer as immobilization matrix, the dialysis film layer on the surface of glass membrane can be omitted in electrode assembly. The numbers marked are; ①, electrode glass membrane; ②, dialysis membrane; ③, O-ring; ④, liposome-mediated nAchR gel layer; ⑤, electrode; ⑥, reference electrode.

% (w/v) prepolymer solution was made by addition of dry polyacrylamide-methacrylamide prepolymer to distilled water while stirring gently at room temperature. Approximately 0.2 ml of reconstituted nAchR solution was added in drops to 1.8 ml of a 20% prepolymer solution. A pH electrode was cleaned, dried, and fixed in a vertical position. Nitrocellulose dialysis membrane (molecular cut off=12,000 daltons) was fixed on the surface of a pH electrode glass with an O-ring. The nAchR-prepolymer mixture (1~2 ml) was coated on the dialysis membrane layer formed on the pH electrode glass, and cross-linked using 1% glyoxal, as described by Tor and Freeman (1986). The representative ststematic structure is shown in Scheme 2.

Preparation of polyurethane prepolymer: Three solutions were prepared and then reacted with 2,4-toluene diisocyanate to produce polyurethane prepolymer. These solutions were 1) polyethylene glycol (PG), 2) 80% (v/v) polyethylene glycol plus 20% (v/v) polypropylene glycol (PGP1), and 3) 50% (v/v) polyethylene glycol plus 50% (v/v) polypropylene glycol (PGP2). Approximately 0.2 g of polyurethane polymer was added 0.6 ml of the reconstituted nAchR solution, stirred vigorously to form a viscous solution, then poured onto a glass plate followed by covering with another glass for 1~2 h at room temperature. After the cover plate was removed a polymeric film with immobilized nAchR was obtained. The polymer film was capped on a pH electrode membrane with an O-ring (Scheme 2).

Lithium flux assay

The reconstituted nAchR vesicle was dialyzed twice against 500 volumes of buffer A, and against buffer B (145 mM sucrose in 10 mM Hepes, pH 7.4) at 4℃ for 60 h. The 115 μl sample was then mixed with 5 μl of 2 M LiCl and 2.5 μl of a cholinergic drug solution. The entrapped Li^+ was separated from external Li^+ by filtering through a 1.5 ml Dowex 50 WX-8-100 cation-exchange column which had been equilibrated with 3.0 ml of a sucrose solution (170 mM, in 1.3 mg/ml of B.S.A). The column was immediately eluted with 1.6 ml of the 175 mM sucrose solution and an aliquout of each fraction (~200 μl) was analyzed by atomic absorption spectrometer (A.A.S) at 670 nm (Schurholz *et al.*, 1992). All assays were performed in duplicate. Quantitation of Li^+ uptake was performed using the following equation (Schurholz *et al.*, 1992):

$$\text{Uptake (\%)} = \frac{\text{Concentration of } Li^+ \text{ ions in the eluted fraction}}{\text{Concentration of } Li^+ \text{ ions in the assay solution}} \times 100$$

Characterization of nAchR immobilized electrodes

The nAchR electrode was incubated in 4.5 ml of buffer C (10 mM Tris-HCl containing 15 mM KCl and 1 mM NaCl, pH 7.0). The potential base line of the electrode was then recorded with a pH meter in mV mode. Following establishment of a steady base line, 0.5 ml of the cholinergic drug solution in the same buffer was added and the potential change was observed until a steady value was obtained.

Other assays

The protein concentration was determined by the Lowry assay method (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Results and Discussion

Reconstitution of nAchR

To facilitate a functional reconstitution of nAchR, a lipid vesicle was prepared in which the ratio of phospholipid (asolectin, PC) to cholesterol was 6 : 1 (w/w) according to the procedure described by Eldefrawi, *et al.* (1988). Addition of the azoligand compound to a lipid vesicle was examined for facilitation of effective reconstitution of nAchR. Four types of liposomes were

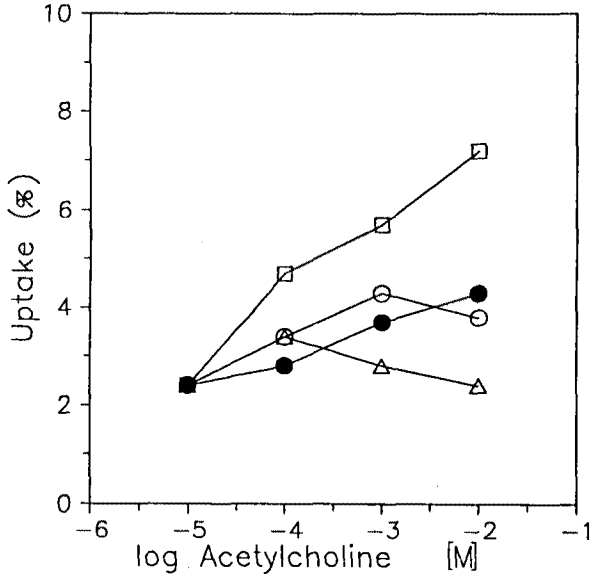


Fig. 1. Activity of the reconstituted nAchR measured by agonist-induced uptake of Li^+ into the reconstituted vesicles. For the ion flux assay, samples (115 μl) were mixed with 5 μl of 2 M LiCl and 2.5 μl acetycholine chloride solution. Entrapped Li^+ in the vesicle was separated from external cations by filtration through a 1.5 ml Dowex 50WX-8-100 cation exchange column. The column was eluted with 1.6 ml of sucrose solution (175 mM), and combined fractions of 200 μl were analyzed by atomic absorption (AAS) at 670 nm. The contents of the azoligand compound in vesicles are as follows; ○–○ : 0% (w/w), △–△ : 10% (w/w), □–□ : 50% (w/w), and ●–● : 90% (w/w).

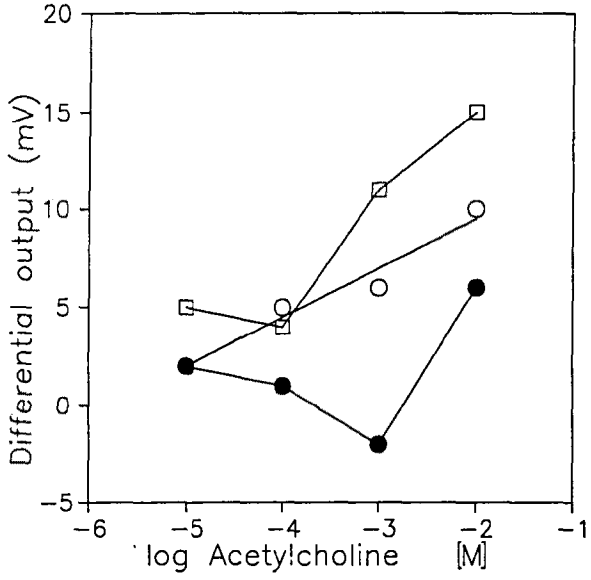


Fig. 2. Influence of buffer system on the response of nAchR electrode to acetylcholine concentration. nAchR electrode was made by coating the nAchR, reconstituted in a vesicle containing azo ligand compound (Bae 1) and asolectin, and immobilized in polyacrylamide- methacrylamide copolymer matrix on the surface of pH electrode following crosslink polymer matrix with glyoxal. The differential output is a value substrated from the potential response of nAchR electrode to that of pH electrode coated by polyacrylamide-methacylamide poymer layer containing no nAchR (●–●: buffer D (15 mM sodium phosphate, contain 150 mM KCl and 10 mM NaCl, pH 7.0); □–□: buffer E (10 mM Tris-HCl, containing 150 mM KCl and 10 mM NaCl, pH 7.0); and ○–○ buffer C (10 mM Tris-HCl, containing 15 mM KCl and 1 mM NaCl, pH 7.0). The values represent means of duplicate samples of two independent experiments.

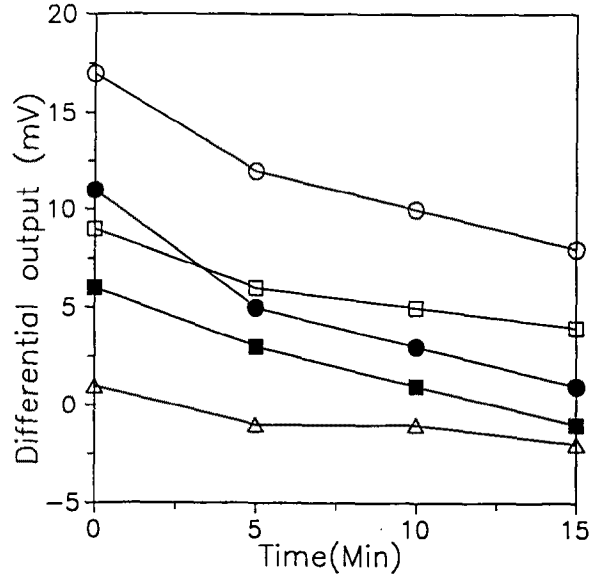


Fig. 3. Response curve for acetylcholine exhibited by nAchR electrode containing polyacylamide-methacrylamide copolymer as a matrix for nAchR immobilization. The assay was carried out in buffer C at room temperature (○–○: 10^{-2} M, ●–●: 10^{-3} M, □–□: 10^{-4} M, ■–■: 10^{-5} M, and △–△: control). The values represent means of duplicate samples.

prepared containing different amounts of azoligand in lipid vesicles (PC : cholesterol = 6 : 1), and each type was tested for activity by a lithium flux assay. Fig. 1 shows that the optimum ratio of azoligand compound to lipids in vesicle is 1 : 1, in which dose response of the reconstituted nAchR to acetylcholine appears to be a linear. These results indicate that the functional activity of reconstituted nAchR is greatly influenced by the ratio of the azoligand compound to lipids. It also suggests that the azoligand compound contains an important matrix component in stabilizing the reconstituted system. This unique feature of the azoligand compound has been identified as a structural molecule forming a stable bilayer membrane by coordinative polymerization with transition metals (Suh and Shim, 1994). Scheme 1 shows the bilayer structure of the azoligand compound (Bae 1) formed by metal ions (M).

Characteristics of the AchR immobilized electrode

Immobilization of the liposome-mediated reconstituted nAchR was carried out using a pH electrode and various prepolymers. To test the function of each nAchR immobilized electrode the optimum buffer system for immobilized nAchR was first determined by examining the effect of the buffer system on the response of the receptor to a cholinergic agonist (acetylcholine), following calibration of the electrode at room temperature in the different buffer systems containing no ligand. Fig. 2 shows the influence of the buffer systems cn the response of the nAchR immobilized electrode to aectylcholine. The response of the electrode using the nAchR immobilized in the polyacrylamide-methacrylamide copolymer as a sensing probe to cholinergic drugs was also affected by the composition and ionic strength of the applied buffer. This result indicates that the best medium for reconstituted nAchR is buffer C (10 mM Tris, containing 15 mM KCl and 1 mM NaCl, pH 7.0), in which a linear response was observed with a concentration range between 1 μM and 10 mM acetylcholine. To test the effect of a synthetic matrix used for immobilization of nAchR on the electrode, changes in the signal output with time were measured using buffer C. Fig. 3 shows the acetylcholine concentration response curve by the nAchR immobilized electrode using the polyacrylamide-methacrylamide copolymer as a matrix for nAchR immobilization in buffer C at room temperature. This system appears to offer high sensitivity and a linear response to the substrate concentration at submicromolar concentrations. The response time of the electrode was less than 20 min under steady-state conditions. To test electrode stability the nAchR immobil-

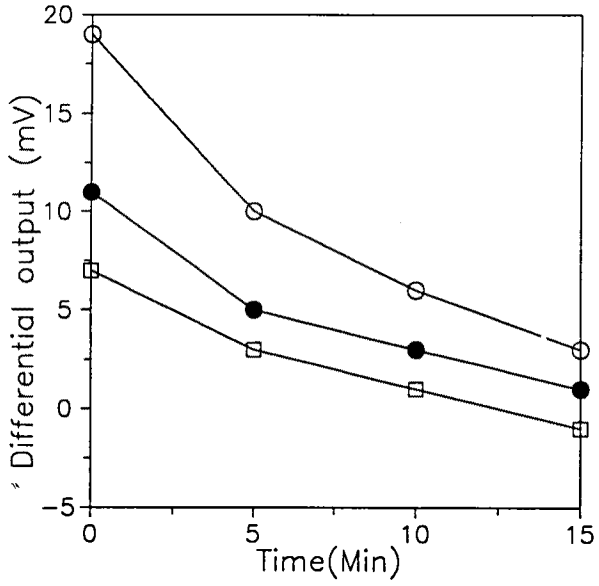


Fig. 4. Response curve for acetylcholine exhibited by nAchR electrode containing polyacylamide-methacrylamide copolymer as a matrix for nAchR immobilization after 100 h storage in buffer C at 25℃. The assay was carried out in buffer C at room temperature (○–○ : 10^{-2} M, ●–∧ : 10^{-3} M, □–□ : 10^{-4} M). All values represent the mean of duplicate samples.

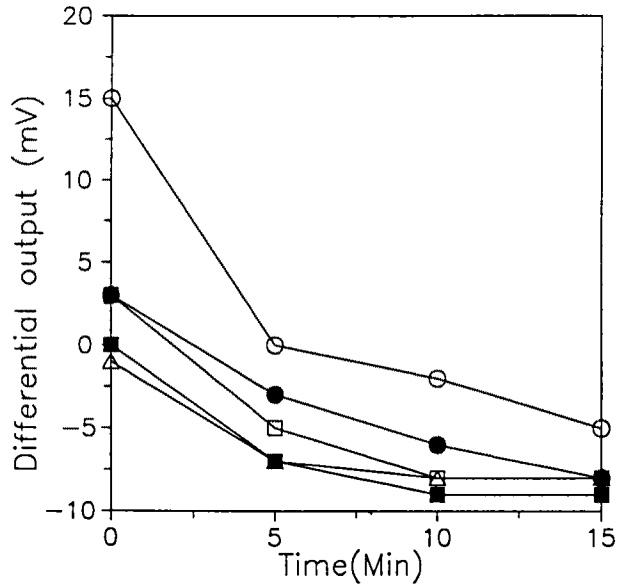


Fig. 5. Response curve for acetylcholine exhibited by nAchR electrode using polyacylamide-methacrylamide copolymer as a matrix for nAchR immobilization after 150 h in buffer C at 25℃. The assay was carried out in buffer C at room temperature (○–○ : 10^{-2} M, ●–● : 10^{-3} M, □–□ : 10^{-4} M, ■–■ : 10^{-5} M , and △–△ : control). All values represent the mean of duplicate samples.

ized electrode was stored for 100 h and 150 h, then the response to acetylcholine was examined. Figs. 4 and Fig. 5 show the acetylcholine concentration response curves produced by nAchR electrodes which had been stored for 100 h and 150 h, respectively, at room temperature using the polyacrylamide-methacrylamide copolymer as a matrix for nAchR immobilization that As shown in Fig. 4, the response of the electrode stored for 100 h to aceylcholine was conserved, while the response of the electrode stored for 150 h was not. This result indicates that immobilized nAchR using the polyacrylamide-methacrylamide copolymer as a matrix is stable longer than 100 h. To examine the influence of the nAchR source in immobilization conditions, three different types of nAchR were prepared: crude protein, Triton X-100 solubilized protein, and protein purified using an α-bugarotoxin affinity column which had been reconstituted with the same liposome vesicle the containing an azo-ligand compound and a lipid(phospholipid : cholesterol = 6 : 1). When nAchR was reconstituted in a vesicle containing an azo-ligand compound (Bea-1) and liposomes containing phospholipids and cholesterol (1 : 1, W/W), there was a good linear response to a wide range of acetylcholine concentrations from 1 μM to 1 mM. With nAchR solubilized by Triton X-100, there was no response to the acetylcholine concentration. With crude nAchR there was a linear response to the acetylcholine only at higher concentrations (100 μM to 10 mM). This result is consistent with the data presented in Fig. 1, and suggests that the azo-ligand compound contains a functional

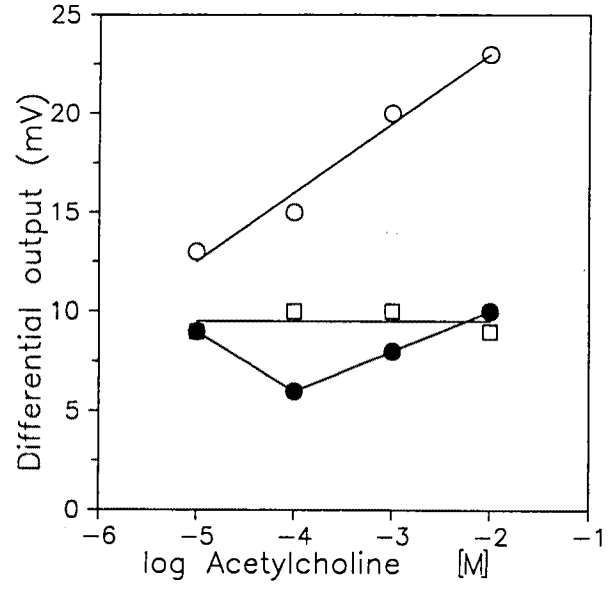


Fig. 6. Influence of the source of acetylcholine receptor on response curve to acetylcholine exhibited by nAchR electrode containing polyacylamide-methacrylamide copolymer as a matrix for nAchR immobilization. The assay was carried out in buffer C at room temperature (○–○ : nAchR reconstituted in a vesicle comprising azo ligand compound (Bae 1) and lipids-phospholipid plus cholesterol = 6 : 1, ●–● : crude nAchR, and □–□ : nAchR solubilized with Triton X-100). All values represent the mean of duplicate samples.

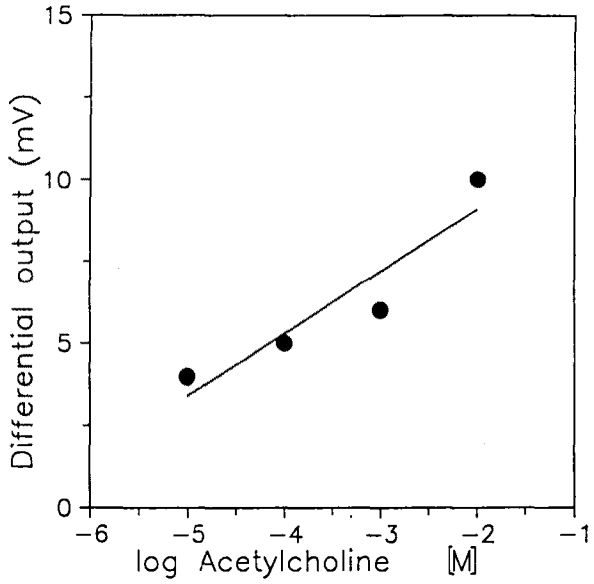


Fig. 7. *Response curve for acetylcholine exhibited by nAchR electrode containing polyurethane hetropolymer as a matrix for nAchR immobilization. Polyurethane heteropolymer has a diol component consists of 80% polyethylene glycol and 20% polypropylene glycol. The assay was carried out in buffer C at room temperature. Differential output was explained as a value substrated from response of nAchR electrode to that of pH electrode coated with polyurethane film containing no nAchR. All values represent the mean of duplicate samples.*

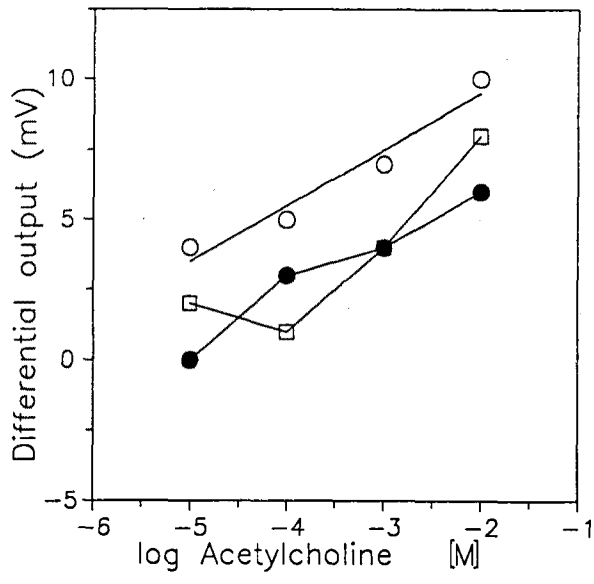


Fig. 8. *Influence of the compositions of diol component of polyurethane polymer on response curve for acetylcholine concentration by nAchR electrode using polyurethane homopolymer or hetropolymer as a matrix for nAchR immobilization. The determination was done in 10 mM Tris-HCl , containing 15 mM KCl and 1 mM NaCl, pH 7.0 buffer, at room temperature. Differential output is determined by a value substrated from response of nAchR electrode to that of pH electrode coated with polyurethane film containing no nAchR (○–○: 80% polyethylene glycol and 20% polypropylene glycol, ●–●: 100% polyethylene glycol, and □–□: 50% polyethylene glycol and 50% polypropylene glycol.) All values represent the mean of duplicate samples.*

ingredient for the immobilization matrix and enhances stability of the liposome vesicle by formation of a stable coordinated bilayer complex with the phospholipid liposome.

In a storage stability test of an nAchR immobilized electrode using the polyacrylamide-methacrylamide copolymer as the polymer matrix for immobilization of nAchR, a linear electrode response to acetylcholine concentration, which continued up to 150 h, was observed. This remarkable stability is an improvement over previously reported nAchR-based biosensors (Rogers *et al.*, 1989; Gotoh *et al.*, 1987; Tayer *et al.*, 1988). It is assumed that diminution of the response of the electrode to the acetylcholine concentration after storage for 100 h may result from swelling of the polyacrylamide-methacrylamide copolymer matrix in an aqueous environment.

To solve this problem, a polymer was used which was more hydrophobic than the polyacrylamide-methacrylamide copolymer. A polyurethane polymer was chosen to substitute for the hydrophilic polyacrylamide-methacrylamide copolymer as a polymer matrix for nAchR immobilization. The prepolymer form of the polyurethane polymer is soluble in water and is compatible with the nAchR solution, and it could easily be formed into a stable, thin film suitable for immobilizing nAchR. Fig. 7 shows the response curve for the acetylcholine concentration by the nAchR electrode using the polyurethane polymer as a matrix for nAchR immobilization. In this case a linear response of the electrode to the acetylcholine concentration was observed, although the response was weaker than the polyacrylamide-methacrylamide polymer as a matrix. Polyurethane has various properties, probably due to its diol component. In general, polyethylene glycol as the diol component results in the hydrophilic property of polyurethane, while polypropylene glycol exhibits a hydrophobic property to polymers (Fukui *et al.*, 1987). Therefore, the composition ratio of polyethylene glycol to polypropylene glycol in the diol components for providing an optimum environment for the electrode using the polyurethane polymer as a matrix for nAchR immobilization is important. Fig. 8 shows the influence of the diol component compositions of polyurethane polymer on the response curve to the acetylcholine concentration. With a diol compositions of 80% polyethylene glycol and 20% polypropylene glycol, a linear response was observed in a wide range of acetycholine concentraion (mM to μM), similar to the case of an electrode with the polyacrylamide-methacrylamide polymer as a matrix for nAchR immobilization. However,

with 50% polyethylene glycol and 50% polypropylene glycol, or with 100% polyethylene glycol, the sensitivity for acetylcholine was low.

In conclusion, these results provide a basis for application of azoligand as an effective component in immobilization of nAchR. Furthermore, these data indicate that synthetic polymers, such as polyacrylamide-methacrylamide and polyurethane, could be an useful matrix for designing nAchR-based biosensors.

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