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Reconstitution of Pure Acetylcholine Receptor in Phospholipid Vesicles and Comparison with Receptor-Rich Membranes by the Use of a Potentiometric Dye

Hans Lüdi*†, Hans Oetliker**, Urs Brodbeck*, Peter Ott*, Bernhard Schwendimann*** and Bernhard W. Fulpius***

* Medizinisch-chemisches Institut der Universität Bern, CH-3000 Bern 9, ** Physiologisches Institut der Universität Bern, CH-3012 Bern 9, *** Département de Biochimie, Université de Genève, Sciences II, CH-1211 Geneva 4, Switzerland

Summary. Acetylcholine receptor, isolated in Triton X-100 on a cobra α-neurotoxin affinity column was incorporated into unilamellar phospholipid vesicles by a detergent depletion method using Amberlite XAD-2. Vesicles of an average diameter of 25 nm were formed, as verified by freeze-fracture electron microscopy and gel filtration. 85 to 95% of the α-bungarotoxin binding sites of the reconstituted acetylcholine receptor were oriented towards the outside of the vesicles. In the reconstituted receptor one molecule of residual Triton X-100 per 2.5 α-bungarotoxin binding sites on the receptor molecule could be assessed. The reconstituted protein was not accessible to papain digestion, whereas the pure acetylcholine receptor, solubilized by Triton X-100 was split into smaller polypeptides under the same condition. Reconstituted acetylcholine receptor and receptor-rich membranes did not exhibit the same behavior as measured by use of a potentiometric dye. This is interpreted as an irreversible alteration of at least 95% of the receptors purified in the presence of Triton X-100. Furthermore, it could be shown that the fluorescence intensity changes induced by carbamylcholine in receptor-rich membranes did not reflect ion fluxes, but conformational changes of the protein or a displacement of the dye from the protein.

Key Words acetylcholine receptor · membrane reconstitution · phospholipid vesicles · indodicarbocyanine

Introduction

Reconstitution of the acetylcholine receptor (AcChR) from the electric organ of electric fishes has been carried out starting from receptor-rich membranes [6, 8, 11, 15, 23, 32, 33]. Sonication [8, 11] or addition of phospholipids dispersed in cholate were used as reconstitution procedures. In the latter case the detergent was subsequently removed by dialysis [6, 8, 11, 32, 33] and/or gel filtration [6, 23]. To our knowledge only Lindstrom et al. [15] and Boheim et al. [4] reported reconstitution of AcChR, which was purified on a cobra α-neurotoxin affinity column. Lindstrom et al. [15] have shown that, in addition to cholate, an excess of soybean phospholipids had to be present during all steps of the purification and reconstitution. It is common to most reconstitution procedures that the protein entity may not be completely separated from the lipid constituents of the original membrane. Therefore the receptor protein may be inserted into the artificial lipid environment together with a layer of strongly attached natural lipids.

To test if natural lipids are essential during purification and reconstitution to preserve the function we solubilized and purified AcChR in the presence of Triton X-100 on an affinity column according to Klett et al. [13] with modifications. The delipidated receptor was subsequently reconstituted into artificial phospholipid vesicles using a modified procedure of Gerritsen et al. [9] in which Triton X-100 was adsorbed to Amberlite XAD-2 beads.

The reconstituted system was characterized by freeze-fracture electron microscopy, density gradient centrifugation, gel filtration, antibody binding, papain digestion and by the use of a fluorescent probe. The latter studies were carried out, in addition, with receptor-rich membranes. The rationale for using a fluorescent potentiometric probe was the fact that, *in vivo* AcChR is contained in a membrane influenced by an electrical field (100,000 V/cm, derived from a membrane potential of 90 mV and a membrane thickness of 9 nm). Upon binding of an agonist, one ion channel mediates fluxes of up to 50,000 Na^+ ions/msec [12]. However, with conventional tracer flux measurements only the final state of agonist action can be detected, due to limited time resolution. Therefore, only a method capable of monitoring actual membrane potential, like the method described by Schindler and Quast [25] using planar lipid bilayers or the potentiometric dye technique allows to fol-

† *Present address:* Max-Planck-Institut für medizinische Forschung, Jahnstr. 29, D-6900 Heidelberg 1, West Germany

low continuously time-dependent agonist action under conditions close to the physiological situation. In the present investigation an attempt was made to detect agonist-induced ion fluxes by monitoring changes in fluorescence intensity of the potentiometric dye indodicarbocyanine [27, 31].

Abbreviations: AcChR: acetylcholine receptor protein; α-BuTx: α-bungarotoxin; AcCh: acetylcholine.

Materials and Methods

Materials

The assay components for the experiments were purchased from the following: Egg phosphatidyl-choline, Koch-Light Laboratories Ltd., Great Britain; valinomycin and papain (Papainase E.C. No. 3.4.22.2) from Papaya Latex, Sigma Chemical Co., St. Louis, Mo.; catalase, Böhringer Mannheim, West Germany; Triton X-100, Rohm and Haas, Philadelphia, Pa.; venom of *Bungarus multicinctus* (Lot BM 31-1), Miami Serpentarium, Fla.; Amberlite XAD-2, acrylamide, N,N'-methylene-bisacrylamide and Coomassie Brilliant Blue G-250, Serva Heidelberg, West Germany; 1,3,3,1',3',3'-hexamethylindodicarbocyanine NK 529, Nippon Kankoh Shikiso Kenkyusho, Okayama, Japan. ^{3}H-Triton X-100 (specific activity of 0.246 mCi/g) was a gift from Rohm and Haas. All other reagents were analytical grade products either from Fluka AG, Buchs, Switzerland or Merck, Darmstadt, West Germany.

Methods

Assay Procedures, Lipid Phosphorus and Protein Determination. The number of ^{125}I-α-BuTx binding sites on lipid-bound AcChR was determined with or without 1% Triton X-100 in the assay by the DEAE-cellulose filter disc method described by Klett et al. [13]. Catalase was assayed by the method of Aebi [1]. Lipid vesicles were quantified by measuring the lipid phosphorus according to Rouser et al. [21]. Protein concentration was measured by a modified method of Lowry et al. [16], using 0.5% sodium dodecylsulfate and 0.5% Triton X-100 in the assay mixtures of the protein samples and for the determination of the bovine serum albumin standards.

Isolation Procedures. The acetylcholine receptor from *Torpedo marmorata* was purified essentially as described by Klett et al. [13] for *Electrophorus electricus*. Three modifications were introduced: a) the buffer used for solubilization contained 1% (wt/vol) Triton X-100, 1 mM ethylene-diaminetetra-acetate and 1 mM phenylmethane-sulfonyl-fluoride; b) the affinity chromatography was performed with cobra α-neurotoxin linked to Sepharose CL-6B; c) the DEAE-cellulose chromatography was omitted. One mg of the purified receptor bound 10.5 nmol ^{125}I-labeled α-BuTx, purified and iodinated as described by Monnier and Fulpius [18]. The labeled α-toxin was further purified by ion-exchange chromatography as described by Vogel et al. [29].

AcChR-rich membrane vesicles were prepared according to Sobel and Changeux [28] with modifications (B. Schwendimann, *in preparation*). The specific activity was 3 to 8 nmol α-BuTx binding sites per mg protein.

Reconstitution Procedure. Unilamellar phospholipid vesicles were prepared as follows: 10 mg egg lecithin in 100 µl ethanol were dried in an evaporator and dispersed in 5 ml ice-cold buffer solution containing 200 mM KCl, 10 mM Tris-HCl, pH 7.4, and 0.5% (wt/vol) Triton X-100. Amberlite XAD-2 (1.5 g wet weight, washed with methanol according to Holloway [10]) was added and the solution was gently agitated with a magnetic stirrer at 4 °C. After 30 min 0.1 mg of pure acetylcholine receptor protein equilibrated in the above buffer was added (lipid/protein ratio 100:1 (wt/wt)). The resulting solution was stirred at 4 °C for 23.5 hr. Amberlite beads were removed by filtration through glass wool. For the electron-microscopy studies and for fluorescent dye experiments the lipid-to-protein ratio was decreased to 10:1 (wt/wt) and 20:1 (wt/wt), respectively. Vesicles prepared by the same procedure without adding the receptor protein are called protein-free phospholipid vesicles.

Freeze-Fracture Electron Microscopy. Samples of reconstituted AcChR were frozen as described by Moor et al. [19]. Fracturing was performed in a Balzers BAF 300 freeze-fracture apparatus. The replicas were examined in a Philips EM 206 electron microscope operated at 80 kV.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis. Electrophoresis in the presence of 0.1% sodium dodecylsulfate was performed by the method of Laemmli [14] with 10% acrylamide gels. Samples were reduced with 2% (vol/vol) β-mercaptoethanol and denatured for 1 hr at 40 °C in 2% sodium dodecylsulfate. Fixation, staining and destaining were carried out similarly to the method described by Downer et al. [7]. The gels were fixed in a solution of methanol/water/acetic acid, 5:5:1, (vol/vol/vol), for 15 min at 60 °C, stained in methanol/water/acetic acid, 5:4:1 (vol/vol/vol), with 0.2% Coomassie Brilliant Blue G-250 for 15 min at 60 °C and destained in 7% acetic acid.

Papain Digestion Experiments. Papain was activated as described by Arnon [2]. Either solubilized or reconstituted AcChR was incubated with activated papain at a ratio of 100:1 (wt/wt) in a buffer containing 200 mM KCl, 10 mM Tris-HCl, pH 7.4. If pure acetylcholine receptor was used the buffer contained 0.05% Triton X-100.

Fluorescence Studies. Fluorescence measurements were carried out in an SLM 4800/A spectrofluorometer (SLM Instruments Inc., Urbana, Illinois). Usually 100 µl of either protein-free vesicles or vesicles containing the reconstituted AcChR (200 µg phosphatidylcholine, 10 µg receptor protein) prepared in 200 mM KCl, 10 mM Tris-HCl, pH 7.4, were incubated in 2.5 ml of continuously stirred solution containing 200 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$, and 0.5 µg of indodicarbocyanine. The influence of the following components on the fluorescence intensity were studied: a) valinomycin, final concentration 5 pM; b) carbamylcholine, final concentration 1 to 30 µM; c) KCl 8 to 200 mM; d) NaCl 200 to 240 mM. Analogous experiments were carried out with 20 µl of AcChR-rich membranes (0.02 µg ml^{-1}). Spectral parameters were 680 ± 8 nm and 620 ± 8 nm for emission and excitation, respectively.

Results and Discussions

Reconstitution of Acetylcholine Receptor

Pure acetylcholine receptor in Triton X-100 was reconstituted as described in Materials and Methods. The Table summarizes constituents' recoveries

Table. Recovery of toxin binding sites, phospholipids and ^{3}H-Triton X-100 in the reconstituted AcChR

Time (hr)	α-Bungarotoxin binding sites (nmoles)	Total inorganic phosphorus (mg/ml)	^{3}H-Triton X-100 (mM)
0	–	2.00 (100%)	7.81 (100%)
0.13	–	1.96 (98%)	3.90 (50%)
0.5	1.05 (100%)	1.90 (95%)	1.95 (25%)
24	>0.84 (>80%)	1.60 (80%)	<0.07 (<1%)

Reconstitution was carried out as described in Materials and Methods. At zime zero Amberlite XAD-2 was added to a mixture of phosphatidylcholine, ^{3}H-Triton X-100 and unlabeled Triton X-100 (100% values). After 30 min purified AcChR was added and recovery of the components was monitored.

in the reconstitution mixture. After 24 hr more than 99% Triton X-100 was removed, whereas protein and phospholipids were recovered by more than 80%. Protein recovery was based on the total number of α-BuTx binding sites measured in the presence of 1% Triton X-100. Protein recovery was above 80% as long as the lipid/protein ratio was larger than 10:1 (wt/wt). Freeze-fracture electron microscopy revealed the presence of vesicles with an average diameter of 25 nm (Fig. 1).

Characterization of Reconstituted Acetylcholine Receptor

Density-Gradient Centrifugation. The amount of AcChR associated with lipid vesicles was assessed by density-gradient centrifugation. As shown in Fig. 2 more than 90% of the AcChR were recovered on top of the gradient together with lipid and residual Triton X-100. The fact that the sedimentation patterns of the protein and the lipid are not superimposed is explained by the high lipid-to-protein ratio (100:1, wt/wt) used in the reconstitution yielding a considerable amount of protein-free vesicles. Analogous results were obtained with a lipid-to-protein ratio of 10:1 (wt/wt), whereas with equal amounts of lipid and protein (wt/wt) a considerable fraction of AcChR aggregated during the reconstitution and appeared at the bottom of the density gradient (Fig. 2, arrow *A*).

When reconstituted AcChR was centrifuged through a density gradient containing 0.5% Triton X-100 the vesicles became disrupted and the monomeric AcChR was found at the position indicated by arrow *C* on Fig. 2 ($S_{4,w}=9$ S). The same result was obtained with pure AcChR.

To exclude electrostatic association between AcChR and the lipid vesicles, the reconstitution procedure and density-gradient centrifugation were also carried out under high ionic strength condition, namely 1 M NaCl in 10 mM Tris-HCl, pH 7.4. The yield of reconstituted receptor was comparable to that shown in the Table and all the protein floated together with the lipid on the top of the gradient.

These results provide evidence that the reconstitution procedure successfully incorporates the AcChR-protein complex into the lipid vesicles.

Gel Filtration of the Reconstituted AcChR. Gel filtration provides a means to independently determine the size of the vesicles and to remove radioactive impurities possibly present in the ^{3}H-Triton X-100 stock solution. As shown in Fig. 3 the vesicle diameter averaged 25 nm which is in agreement with the data from electron microscopy. In the peak fractions the amount of Triton X-100 (220 cpm/ml or 2.6×10^{-9} g/ml) present in the lipid vesicles was calculated and related to the number of toxin binding sites. Assuming a number of 5800 lecithin molecules per vesicle [30] and 2 toxin binding sites per receptor protein, about one molecule of Triton X-100 per AcChR (molecular weight 250,000) and one molecule of Triton X-100 per 10 vesicles were found. This means that on the average only every 10th vesicle contained a receptor protein when the lipid-to-protein ratio was 100:1 (wt/wt). In addition the amount of radioactivity per vesicle in the reconstitution mixture is larger before gel filtration than thereafter. This is most probably due to water-soluble radioactive impurities contained in the ^{3}H-Triton X-100, which are removed by the gel filtration.

The orientation in the vesicles of the reconstituted AcChR was determined by toxin binding site titration in the absence and in the presence of 1% Triton X-100. In Triton-free solutions the number of binding sites was 85 to 95% of that determined in presence of Triton X-100. This indicates that most of the α-BuTx binding sites faced the outside of the vesicles. A probable reason for the asymmetric distribution is the small size of the vesicles (small inner volume, high curvature of lipid bilayer). This small size might also explain our finding that at most one AcChR was found per vesicle when lipid-to-protein ratios were decreased.

Antibody Binding. Antibodies were raised against Triton X-100 solubilized AcChR. It was shown that in the reconstituted AcChR only 45% of the antigenic determinants, revealed by these antibodies, were found when compared with AcChR in a solution containing 1% Triton X-100.

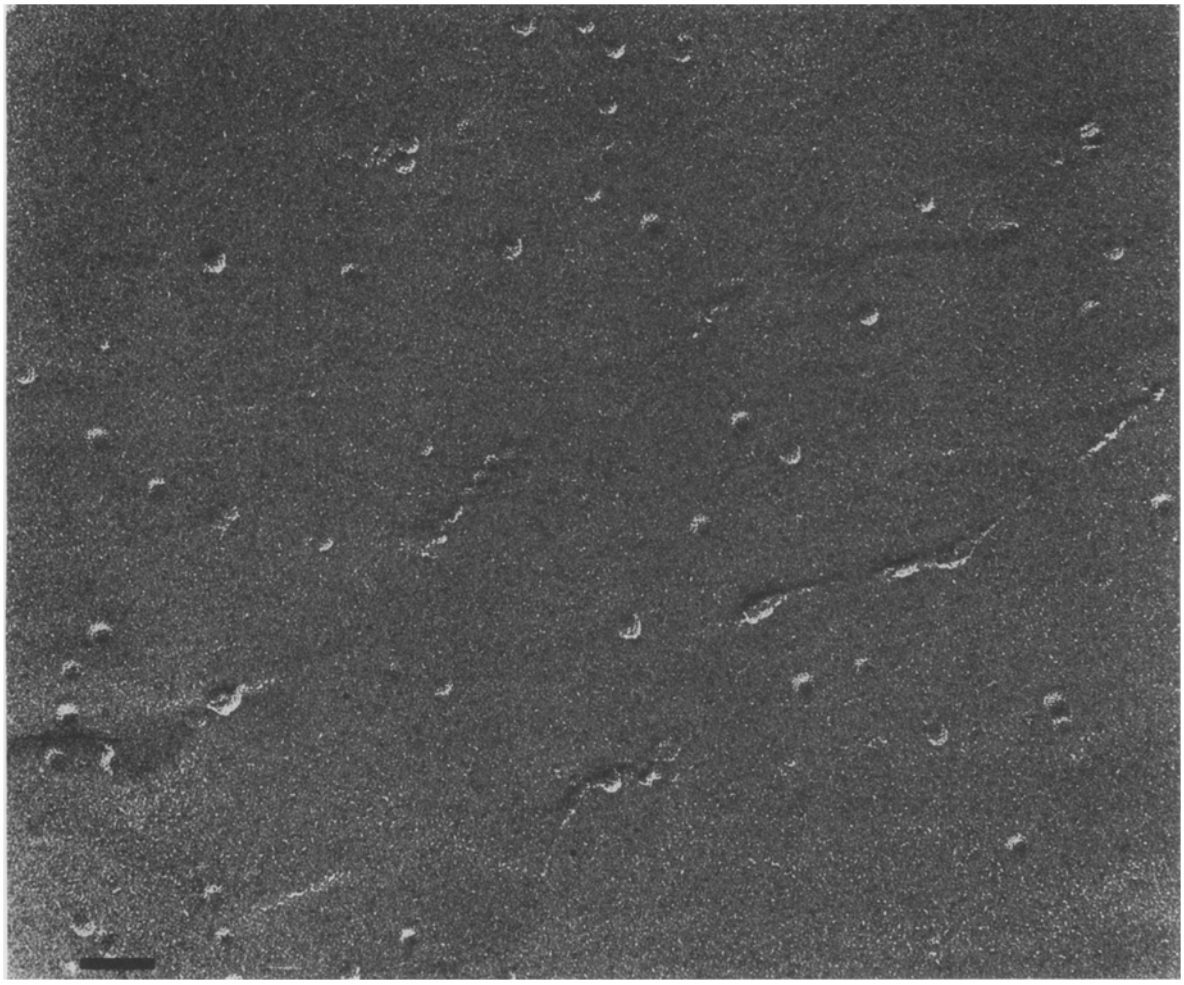

Fig. 1. Freeze-fracture electron microscopy of reconstituted vesicles. Electron micrograph of freeze-fractured lipid vesicles reconstituted with a lipid-to-protein ratio of 1:10 (wt/wt). The bar (−) represents 100 nm

Papain Digestion. Pure AcChR in a solution of Triton X-100 (1% vol/vol) was digested by papain. Figure 4*A* reveals that all 4 subunits originally present were split into polypeptides of smaller molecular weights. The reconstituted AcChR, on the other hand, was virtually insensitive to papain digestion (Fig. 4*B*).

The results described so far strongly suggest that the AcChR complex is indeed incorporated into the lipid bilayer structure with most of the α-bungarotoxin binding sites facing the outside. The results furthermore give indirect evidence for the membrane-spanning property of the protein complex.

Test for AcChR-Mediated Membrane Permeability. The main feature of the AcChR complex is to mediate ion fluxes across biological membranes upon binding of cholinergic agonists. As shown by Katz and Miledi at the motor endplate of skeletal muscle [12] binding of AcCh to one receptor molecule opens one channel which mediates a Na^+ influx in the order of 50,000 ions per msec. On the other hand "fluxes" of only 10 to 30 nmol Na^+/mg protein × sec were measured in vesicular systems [6, 8, 11, 15, 23, 32, 33]. This corresponds to 2 to 10 Na^+ ions lost or gained per receptor molecule in one second, which is about 7 orders of magnitude lower than the ion fluxes observed by Katz and Miledi [12] and can hardly be differentiated from Na^+ binding to the protein. In addition Schindler and Quast [25] using planar bilayers formed with native receptor-rich membranes from the electric organ of *Torpedo californica* obtained single-channel conductances of the same order of magnitude as described for the acetylcholine-activated channel in motor endplates of skeletal muscle.

The reasons for the large difference in the ion fluxes might be (i) the absence of an electrical

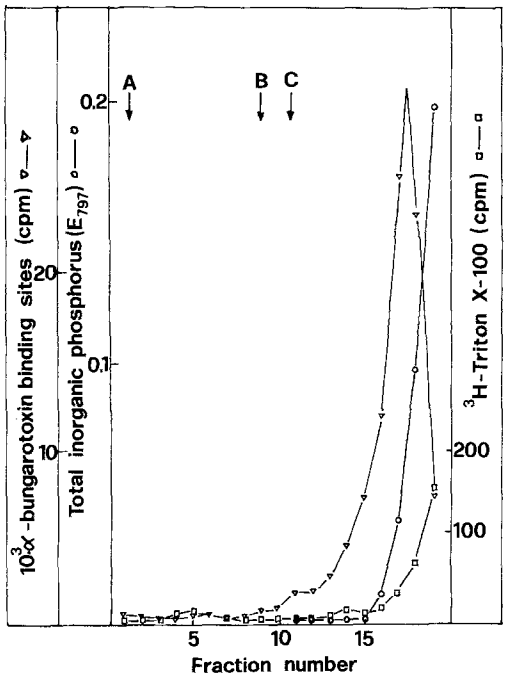

Fig. 2. Density gradient and gel filtration of reconstituted AcChR. Characterization by sucrose density gradient centrifugation of lipid vesicles with incorporated AcChR. 500 µl of vesicle solution were layered on top of a 5 to 30% continuous sucrose density gradient, prepared in the same buffer as used for the reconstitution but without Triton X-100. Gradients were centrifuged at 200,000 × *g* for 15 hr at 4 °C. Fractions of 0.6 ml were collected from the bottom and analyzed for toxin binding activity, ^{3}H-Triton X-100 and total lipid phosphorus. Arrow *A* indicates aggregated AcChR, arrow *B* peak fraction of catalase activity ($S_{4,w} = 11.4$ S). Arrow *C* indicates the peak fraction of monomeric AcChR (9 S)

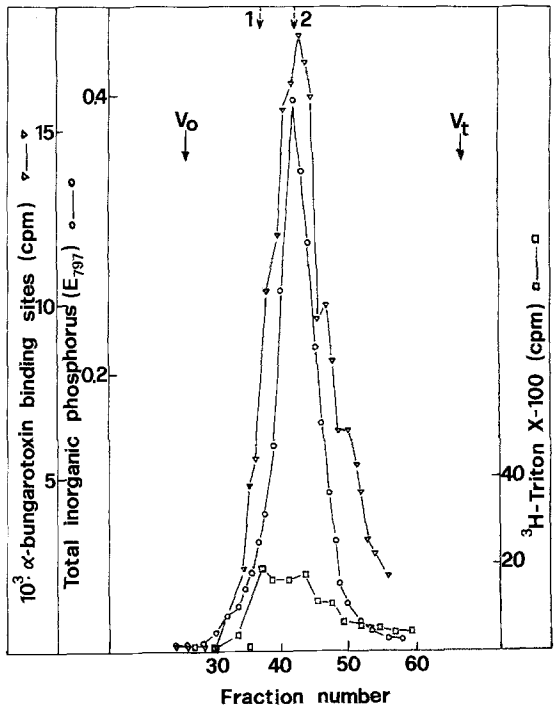

Fig. 3. Gel filtration of reconstituted AcChR. Characterization by gel filtration of lipid vesicles with incorporated AcChR. A 33 × 2.5 cm column of Sepharose 4 B was equilibrated with 20 mg of egg lecithin. 4 ml of vesicle solution were then applied to the column (flow rate 20 ml/hr). Fractions of 1.95 ml were collected and assayed for toxin binding sites, ^{3}H-Triton X-100 and lipid phosphorus. V_0 and V_t indicate void and total column volume. To determine the stokes-radii the column was calibrated according to Siegel and Monty [26]. Arrow 1: 30 to 32 nm; arrow 2: 20 to 26 nm

driving force for sodium in vesicular systems (*see also* Lindstrom et al. [15]) and (ii) an incomplete functional integrity due to a transient lack of minimum side pressure by the surrounding membrane lipids on the AcChR during solubilization. The importance of maintained side pressure for structure and function of the AcChR was emphasized by Schindler [24].

Recently, the time resolution of cation flux measurements has been improved using a rapid-mix quenched-flow technique [5, 20]. Ion fluxes of about 4 orders of magnitude lower than physiological values were obtained. To circumvent these difficulties with tracer flux measurements and because of the very small ratio of the vesicular inner space to suspension volume we tried to monitor changes in membrane potential due to carbamylcholine-induced ion fluxes in the reconstituted system by using a potentiometric dye as indicator for vesicular membrane potential which is expected to change upon sodium influx. To provide the electrical driving force for the Na^+ influx the vesicles were made inside negative by reconstituting them in 200 mM KCl buffer and diluting them into 200 mM NaCl buffer (1:25, vol/vol) in presence of the K^+-selective ionophore valinomycin (22 ng/ml). The potassium gradient established by this procedure (25:1, inside/outside) gives rise to a membrane potential of ~ -84 mV (between intravesicular space and outside solution) provided that the vesicles are permeable to a significant degree only to potassium. The amount of valinomycin added corresponds to 1 to 2 molecules per vesicle. To reach the potassium equilibrium potential of -84 mV, 3% of the potassium contained in the vesicles has to diffuse out; this raises the outside potassium concentration by a factor of 1.000003. Such an increase is clearly below the limit of detection of tracer flux measurements. To calibrate the fluorescence response with membrane potential protein-free vesicles were prepared in 200 mM KCl buffer and diluted into a 200-mM NaCl buffer con-

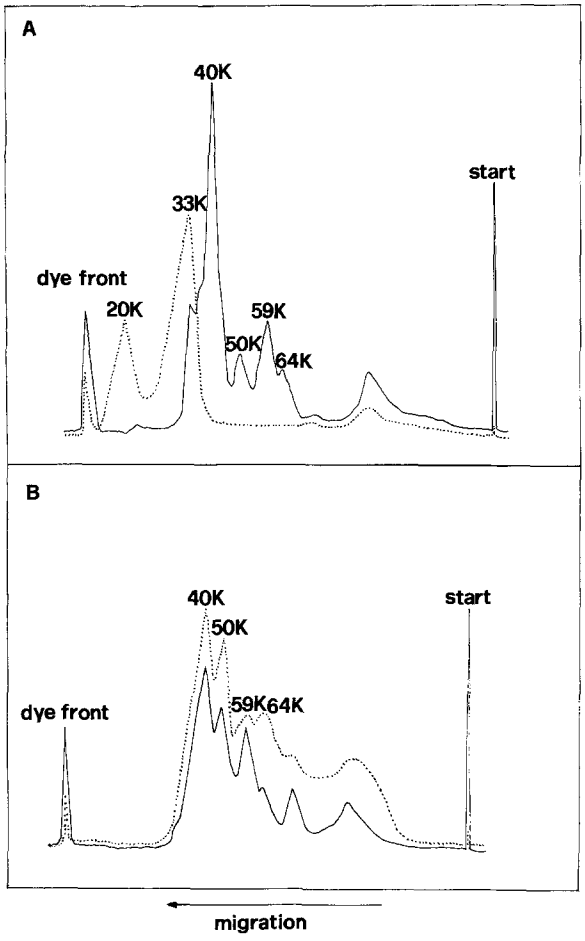

Fig. 4. Scans of sodium dodecylsulfate gels of pure and reconstituted AcChR before and after papain digestion. Samples for the electrophoresis were prepared as described in Materials and Methods. Proteolytic degradation was carried out for 1 hr. Gels were stained with Coomassie Brilliant Blue G-250 and scanned at 605 nm with a Gelman ACD-15 gel scanner. *A*) Pure AcChR (solid line) and pure acetylcholine receptor degraded with papain as described in Materials and Methods (dashed line). *B*) Reconstituted AcChR (solid line) and same sample treated with papain as in (*A*) (dashed line). The optical densities of the peaks represent relative values

taining the potentiometric dye (Fig. 5). An increase in fluorescence intensity was observed. The addition of valinomycin gave rise to a further increase in fluorescence intensity, which can be attributed to the development of a membrane potential. The stepwise addition of μl quantities of 3 M KCl to the suspension increases the external K^+ concentration and decreases the transmembrane potential. Consequently, a decrease of the fluorescence intensity is observed, reaching the level observed before the addition of valinomycin. This effect is not observed if NaCl is added instead of KCl. As changes in membrane potential of less than 10 mV are easily detected by the optical method (Fig. 5, inset) an electrogenic loss of 1 to 2 K^+ ions per vesicle can be monitored (for a detailed calculation *see* [17]).

An analogous experiment was carried out using vesicles containing the reconstituted receptor protein (Fig. 6). The results are similar to those shown in Fig. 5 for protein-free vesicles. The dependence of the fluorescence change on the logarithm of the external potassium concentration deviates from linearity at high outside potassium concentrations (inset, Fig. 6). This is most probably due to a dye-protein-ion interaction which is independent of membrane potential.

Figure 6 furthermore shows that the addition of 30 μM carbamylcholine did not reduce the transmembrane potential as the observed decrease in fluorescence (arrow 4, Fig. 6) is the result of dilution (arrow 5, Fig. 6). It could be excluded that residual Triton X-100 interferes with the potential dependence of the fluorescence intensity. Re-addition of more than 25 nM Triton X-100 to protein-free vesicles induced a smaller and overshooting decrease of the fluorescence intensity after the addition of KCl, an effect which is not observed with reconstituted AcChR.

The failure to measure agonist-induced changes in membrane potential led us to compare the reconstituted system with receptor-rich membrane vesicles, for which agonist-induced tracer fluxes had been reported [6, 8, 11, 15, 23, 32, 33]. The vesicles were dialyzed against 200 mM KCl 10 mM Tris-HCl, pH 7.4, overnight and diluted 125-fold into a solution of 200 mM NaCl in 10 mM Tris-HCl, pH 7.4, containing 0.5 μg fluorescent dye per 2.5 ml as shown in Fig. 7*A*. The fluorescence increase upon addition of valinomycin is much smaller than that observed previously and the size of the signal furthermore depends on the time of addition of the ionophore. If valinomycin is added prior to the AcChR-rich membranes, allowing to induce a high K^+ permeability without losing intravesicular K^+ or gaining Na^+ a more pronounced fluorescence increase is observed than when it is added after dilution of the membranes. This indicates that the receptor-rich membranes are much leakier than the reconstituted vesicles. In the latter the valinomycin-induced fluorescence increase remains constant for at least 15 min after dilution. Addition of KCl to AcChR-rich membranes results in a decrease of fluorescence intensity as expected.

Figure 7*B* shows the influence of carbamylcholine on the fluorescence intensity at a ten times lower concentration of the fluorescent probe (0.05 μg/2.5 ml). Instead of the expected decrease in fluorescence intensity, a dose-dependent in-

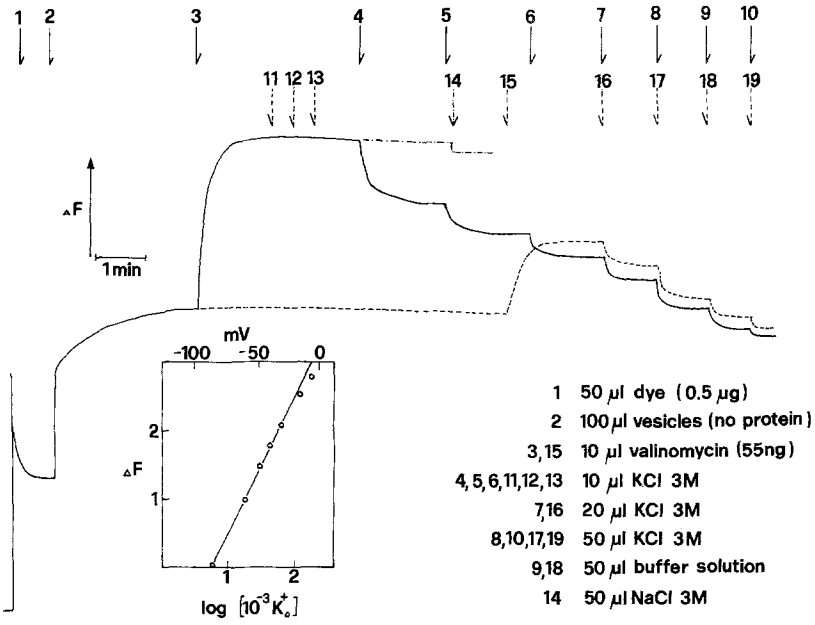

Fig. 5. Fluorescence intensity changes of indodicarbocyanine dye in the presence of protein-free vesicles. Fluorescence measurements were carried out as described in Materials and Methods. The incubation medium contained 0.2 M NaCl, 10 mM Tris-HCl, pH 7.4, and 2 mM $CaCl_2$. The membrane potential was changed stepwise by the addition of 3 M KCl (solid line). (—·—·): NaCl was added instead of KCl. (-----): Outside potassium concentration (K_0) was increased prior to the addition of valinomycin. Inset: Change of fluorescence (ΔF) versus logarithm of outside potassium concentration ($\log(K_0^+)$) and calculated membrane potential. Numbered arrows indicate addition of compounds

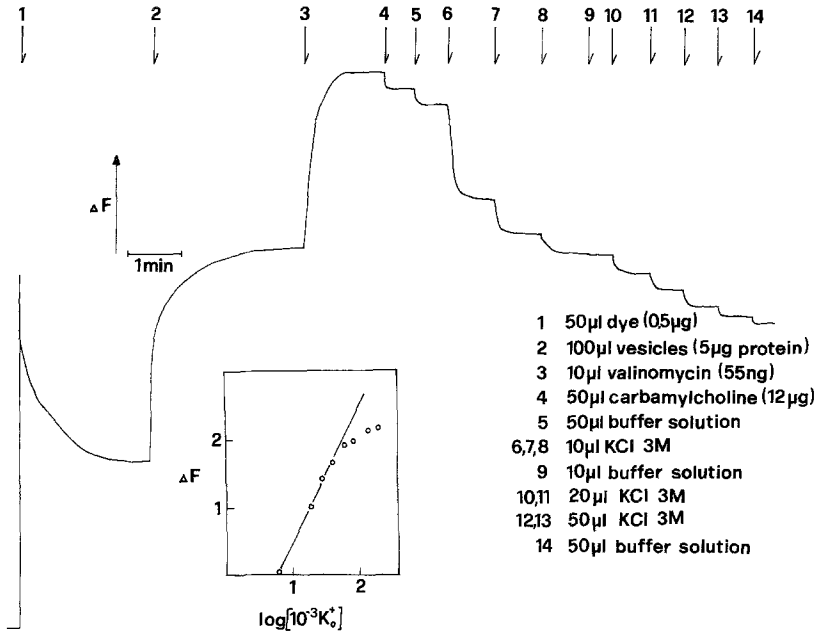

Fig. 6. Fluorescence intensity changes of indodicarbocyanine in presence of reconstituted AcChR. Same experiment as described in the legend of Fig. 5, but 100 µl of reconstituted AcChR was added instead of protein-free vesicles. Inset: Changes of fluorescence (ΔF) versus $\log(K_0^+)$

crease was observed, which is abolished by α-bungarotoxin and by the pre-incubation of the receptor-rich membranes with saturating concentrations of carbamylcholine. Furthermore, the increase in fluorescence is observed under conditions where no membrane potential is present, i.e. when the receptor-rich membranes are diluted into a solution of 200 mM KCl instead of 200 mM NaCl. These observations have to be attributed to a potential-independent carbamylcholine-sensitive dye-protein interaction. Thus the dye monitors a conformational change of the AcChR occurring after addition of the agonist, or the dye might be displaced from the protein into the lipid or aqueous phase. This potential-independent response could only be resolved at low dye concentrations.

The results shown in Figs. 5, 6 and 7*A* were independent of dye concentration, in the range used in these experiments; e.g. no carbamylcholine-induced dye response was observed in the reconstituted system even at 10 times lower dye and 2 times higher vesicle concentration. This situation was considered to be analogous to that used for the measurements with receptor-rich membranes

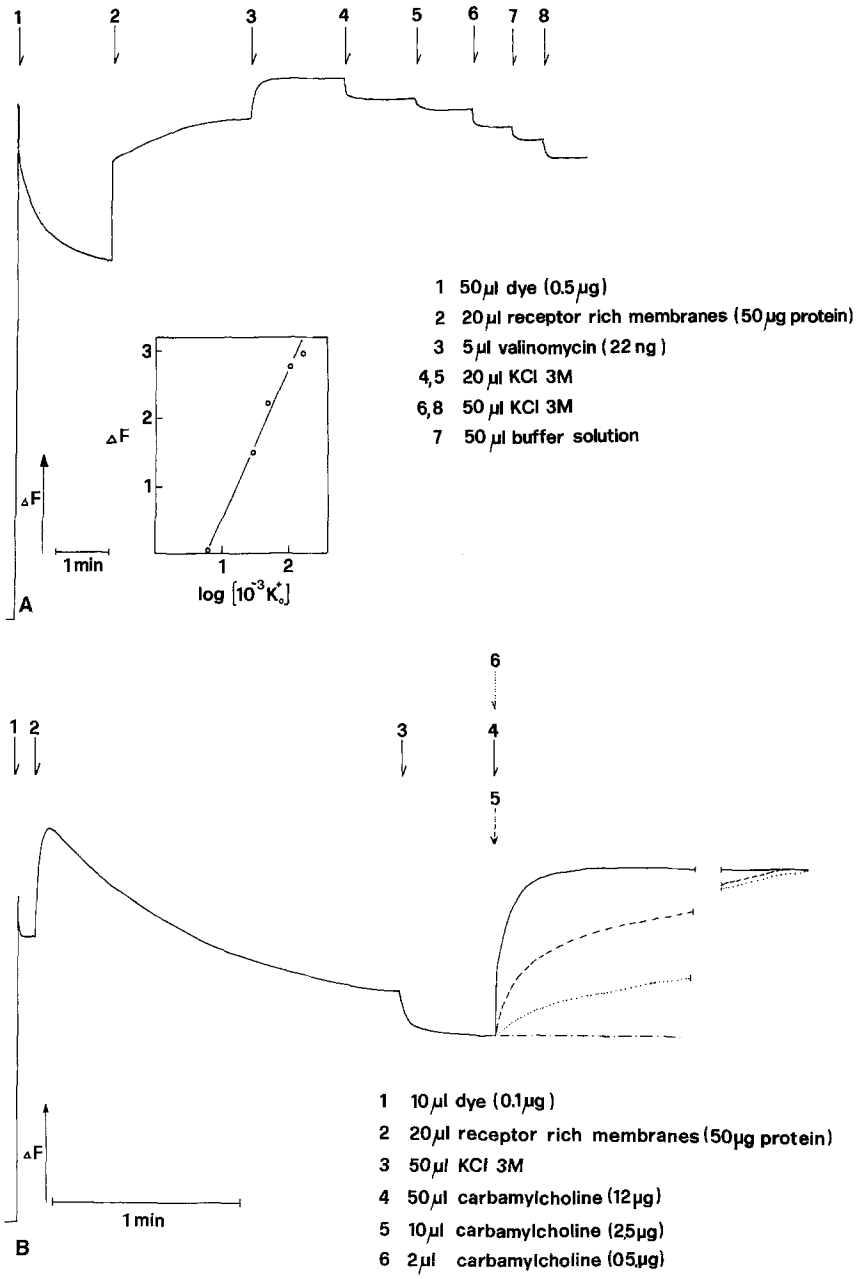

Fig. 7. Fluorescence intensity changes of indodicarbocyanine in presence of receptor-rich membranes. *A*) Same experiment as described in the legend of Fig. 5. 20 µl of receptor-rich membranes were used. Inset: Changes of fluorescence (ΔF) versus $\log(K_0^+)$. *B*) Same as in *A*, but valinomycin (55 ng) was added prior to the membranes. Different amounts of carbamylcholine were added at the arrows: 12 µg (2.7×10^{-5} M final concentration) (solid line); 2.5 µg (5.5×10^{-6} M final concentration (-----); 0.5 µg (1.1×10^{-6} M final concentration) (····). α-bungarotoxin (5×10^{-6} M) or carbamylcholine 2.7×10^{-5} M) were added prior to addition of the membranes (—·—·)

(Fig. 7*B*) as far as protein/lipid/dye ratio is concerned. Further reduction in dye/reconstituted receptor ratio was limited by optical noise. In addition, the same results as those shown in Figs. 5, 6 and 7 were obtained with Nile Blue A.

To sum up, these results suggest that the reconstitution procedure used in this study yields phospholipid vesicles containing the AcChR protein with 85 to 95% of its α-BuTx binding sites facing the outside. The potentiometric dye technique used to monitor changes in membrane potential is capable of detecting an electrogenic translocation across the lipid bilayer of a small number of ions per vesicle provided that the ion translocation occurs in a substantial fraction of the vesicular population.

In our reconstituted system we could not demonstrate an agonist-induced sodium influx. This, to us, means that at most 5% (our estimated limit of detection) of the reconstituted receptors were functioning as agonist-sensitive ion translocators.

The reasons for this might be, among others: 1) The use of Triton X-100 as detergent. 2) The removal of essential lipids during the purification procedure. 3) Low levels of Ca^{++} and Mg^{++} during isolation and purification to prevent proteolysis. 4) Unsuited environmental conditions for the AcChR.

Triton X-100 is thought to affect the AcChR in absence of lipids in an irreversible manner (*see*, for example, Barrantes [3]). In contrast to that Rüchel et al. [22] reported successful reconstitution of AcChR which was solubilized with and purified in Triton X-100 on an α-cobratoxin column. According to Boheim et al. [4] this preparation retains ligand recognition and ion-channel gating properties. Their conductance measurements were performed on lipid vesicles containing AcChR which were fused with black lipid membranes. In contrast to the measurements in our system, where an average vesicular membrane potential is monitored, their conductance changes could be demonstrated even if merely a small fraction of the reconstituted receptors has remained functional. It is therefore conceivable that the same receptor preparation would have been considered as "nonfunctional" if tested with the fluorescent dye technique.

In addition it should be noted that the experiments of Boheim et al. [4] were performed with an unnatural phospholipid (1-stearoyl-3-myristoyl-glycero-2-phosphocholine) "in the frozen state of membrane matrix." From our study we can not conclude whether or not missing essential lipids are responsible for the missing agonist sensitivity in the bulk part of our reconstituted system. It is uncertain to what extent our experiments confirm the requirement of mM levels of Ca^{++} and Mg^{++} in all steps of the isolation and reconstitution procedure as mentioned in Rüchel et al. [22]. Recently, Schindler [24] reported that a particular physical state (cohesive pressure) in the membrane as well as the presence of cholesterol is a prerequisite for the preservation of the AcChR function.

At present it is unclear what is the underlying process for the slow fluorescence change upon addition of an agonist to receptor-rich membranes stained with indodicarbocyanine. It seems interesting but rather speculative to note that the time course of this fluorescence change approximately parallels the time course of desensitization as shown in Schindler [24]. The fact that the indodicarbocyanine dye does not monitor changes in membrane potential could even indicate that these membranes are no more functional (i.e. do not exhibit a potassium conductance exceeding substantially the conductance for sodium), since the dye can be used in most membranes as an indicator of membrane potentials (e.g. pure phospholipid vesicles). The fact that the reconstituted system did not exhibit this fluorescence response must be taken as additional evidence for functional impairment of at least 95% of the AcChR entities during purification and reconstitution.

To our knowledge this communication is the first to report that a potentiometric dye monitors carbamylcholine binding to AcChR. The fact that the change in fluorescence intensity caused by depolarization and the fluorescence change associated with ligand receptor interaction have opposite signs prevented us from interpreting the results obtained with receptor-rich membranes as changes in membrane potential.

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