The hydrophobic, photoreactive probe 3-(trifluoromethyl)-3-(m-[125I]iodophenyl)diazirine ([125I]TID) was used to characterize the effects of lipids and detergents on acetylcholine receptor (AChR) conformation. Affinity purified AChR reconstituted into dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidyl acid (DOPA), and cholesterol showed the same pattern of [125I]TID-labeling and demonstrated the same reduction in labeling of all four subunits upon desensitization by the agonist carbamoylcholine, as partially purified AChR in native lipids. On the basis of the patterns of [125I]TID incorporation, reconstitution into DOPC/DOPA also appeared to stabilize the resting (functional) conformation of the AChR, while reconstitution in DOPC/cholesterol or DOPC alone largely desensitized the AChR. The effects of lipids on the functional state of the AChR was determined independently by measuring the ability of AChR reconstituted into different lipid combinations to undergo the change in affinity for agonist diagnostic of desensitization. The dramatic reduction in the apparent levels of [125I]TID associated with the subunits of the AChR observed upon agonist-induced desensitization was shown not to be due to a change in affinity for tightly bound lipid. Solubilization of affinity purified AChR reconstituted into DOPC/DOPA/cholesterol by the non-ionic detergents octyl glucoside, Triton X-100, and Tween 20 (final detergent concentration = 1%) was shown to produce the same pattern of [125I]TID-labeling as desensitization by agonist, while solubilization in 1% sodium cholate appeared to stabilize a conformation of the AChR more similar to the resting state.

Prolonged exposure to agonist induces the AChR to isomerize to a non-conductive, desensitized conformation (recent reviews of the AChR include Stroud et al., 1990; Guy and Hucho, 1987; and McCarthy et al., 1986). Earlier studies have shown that the function of the AChR is sensitive to its lipid environment. Full function appears to require the presence of cholesterol or cholesterol analogues (Daiziel et al., 1980; Criado et al., 1982; Jones and McNamee, 1988), and phospholipids with negatively charged head groups also appear important (Ochoa et al., 1983; Fong and McNamee, 1986). In Fourier transform infrared spectroscopy studies, cholesterol and negatively charged phospholipids have been shown to alter the secondary structure of the AChR (Fong and McNamee, 1985). The AChR is also sensitive to the degree of saturation of phospholipid fatty acid side chains (Fong and McNamee, 1986), and free fatty acids inhibit the activity of the AChR, presumably acting through the lipid phase (Andreasen and McNamee, 1980). Solubilization of the AChR in non-ionic detergent also stabilizes a non-functional conformation of the AChR (Gonzalez-Ros et al., 1980). These studies indicate that a fairly specific combination of lipids, acting at the hydrophobic, membrane-spanning region of the AChR, are required to maintain the AChR in a functional conformation, and that inappropriate lipids or detergents disrupt this highly sensitive lipid-protein interface.

It is not surprising that the AChR should have these narrow lipid requirements. Other membrane proteins, such as the rat brain sodium channel (Feller et al., 1985) and various ATPases (Vemuri and Philipson, 1989) also appear to require negatively charged phospholipids for maximal function. In the case of ion channels, the net surface charge of lipid membranes has been shown to affect ion conductance, with both sarcoplasmic reticulum K⁺ channels (Bell and Miller, 1984) and Ca²⁺-activated K⁺ channels (Moczlydowski et al., 1985) showing higher conductances in negatively charged phospholipid bilayers, compared to neutral phospholipids. Neutral lipids are also important, and membrane proteins such as the Na⁺,K⁺-ATPases (Vemuri and Philipson, 1989) and the GABA transporter (Shoufani and Kanner, 1990) also appear to have a requirement for cholesterol. In addition, the length and degree of saturation of the fatty acid side chains affects the properties of membrane proteins such as the insulin receptor (Gould et al., 1982) and rhodopsin (Baldwin and Hubbell, 1985), probably as a function of membrane fluidity. The lipid requirements of some membrane proteins can be quite complex. The μ opioid receptor requires both negatively charged phospholipids and fatty acid side chains containing two or more double bonds for full binding activity (Hasegawa et al., 1987). Finally, most membrane proteins require a minimal number of lipids to function or to remain stable in detergent solution, although a few membrane proteins, such as bacteriorhodopsin (Huang et al., 1981), can be

The abbreviations used are: AChR, acetylcholine receptor; BgTx, bungarotoxin; [lWI]TID, 3-[N-morpholino]propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DOPC, dioleoylphosphatidylcholine; DOPA, dioleoylphosphatidyl acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
completely delipidated with no irreversible effects.

The hydrophobic, photoreactive probe 3-(trifluoromethyl)-3-\((\text{m-}[\text{125I}]\text{iodophenyl})\)diazirine (\(\text{[125I]}\)TID) was designed to label the amino acids at the protein-lipid interface of intrinsic membrane proteins. Brunner and Semenza (1981) and has been used to identify the membrane-spanning regions of several proteins (Frielle et al., 1982; Hoppe et al., 1984; Meister et al., 1985; Brunner et al., 1985; Lenard and Vanderoef, 1990). Earlier studies demonstrated that all four subunits of the AChR were labeled with \(\text{[125I]}\)TID (White and Cohen, 1988; McCarthy and Stroud, 1989a). This strongly suggests, in agreement with earlier work (summarized by Popot and Chang, 1984; McCarthy et al., 1986), that each AChR subunit spans the lipid bilayer. Surprisingly, in the earlier \(\text{[125I]}\)TID-labeling studies, the extent of \(\text{[125I]}\)TID incorporation into the subunits of the AChR was found to be sensitive to the conformational state of the protein. Agonist-induced desensitization of the AChR significantly reduced the level of \(\text{[125I]}\)TID incorporation into all four subunits, most dramatically the \(\gamma\) subunit, while binding of the agonist \(\alpha\)-bungarotoxin (BgTx) had no effect (White and Cohen, 1988; McCarthy and Stroud, 1989a).

We report here the effects of the lipid environment and detergent solubilization on the patterns of \(\text{[125I]}\)TID incorporation into the AChR. Reconstitution of the AChR into lipids previously shown to desensitize the AChR, or solubilization in some non-ionic detergents, caused the same changes in the levels of \(\text{[125I]}\)TID labeling of the AChR subunits as desensitization due to prolonged agonist. These studies show that conditions as disparate as long term agonist exposure, reconstitution into certain lipids, or detergent solubilization, procedures which were earlier shown to desensitize the AChR on the basis of functional assays, all induce the same conformation of the AChR, on the basis of a structural assay.

**EXPERIMENTAL PROCEDURES**

**Materials**—\(\text{[125I]}\)TID (8–10 Ci/mmol) and \(\text{[125I]}\)-BgTx (2000 Ci/mmol) were purchased from Amersham Corp. Egg lecithin (phosphatidylcholine, predominantly dioleoyl) and dioleoyl phosphatidic acid were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Cholesterol and cholate were purchased from Sigma. Octyl glycoside (1–0–N-Octyl-\(\beta\)-D-glucopyranoside) was purchased from Boehringer Mannheim. Triton X-100 and Tween 20 were purchased from Bio-Rad. Live Torpedo californica were purchased from Marinus (Long Beach, CA). All other chemicals were reagent grade.

**Preparation of Torpedo Muscle**—Electric organs were excised in a cold room from pithed Torpedo californica. The electric organs were washed in dialysis buffer (10 mM MOPS, 100 mM NaCl, 0.1 mM EDTA, 0.25% NaN\(_3\), pH 7.5) containing 0.2 mM phenylmethylsulfonyl fluoride, and then cut into small, approximately 1-inch cubes prior to freezing in liquid nitrogen. The excised electric organ fragments were stored at -70 °C. AChR-enriched membranes were prepared essentially following the protocol of Ochoa et al. (1983) with some changes. In this protocol, 600 g of frozen electric organ fragments was partially thawed and homogenized for 3 × 30 s in a Waring blender in 570 ml of homogenization buffer (10 mM NaH\(_2\)PO\(_4\), 5 mM EGTA, 0.02% NaN\(_3\), pH 7.4) made 0.1 mM in phenylmethylsulfonyl fluoride and 10 mM in iodoacetamide just prior to use. The homogenate was centrifuged for 10 min at 4,000 g, and the supernatant was collected through eight layers of cheesecloth. The pellets were rehomogenized in 240 ml of homogenization buffer for 2 × 30 s in a Virtis model 23 homogenizer at 70% of maximum and centrifuged as above. The supernatant of the last centrifugation was then centrifuged for 4.5 h at 27,000 × g, and the resulting supernatant was discarded. The AChR-enriched membrane pellets were resuspended in 100 ml of dialysis buffer, further dispersed using a 50-ml Potter-Elvejem homogenizer, and frozen at -70 °C.

After resuspension and reconstitution of the AChR into defined lipids was performed largely according to the method of Eilena et al. (1983) with modifications. AChR-enriched membranes were diluted to 250 ml in dialysis buffer (about 4 mg/ml) and solubilized by the addition of 250 ml of 2% cholate in dialysis buffer. This solution was stirred at 4 °C for 30 min and then centrifuged for 3 h at 27,000 × g to sediment-insoluble material. For the affinity purification of the AChR, bromoacetylcholine bromide, prepared according to Damle et al. (1978), was used to derivatize 50 ml of Bio-Rad Affi-Gel 10 (Blusans and McNamara, 1990) and poured into a 2.5 × 20-cm Bio-Rad 20/20 chromatography column. The cholate-solubilized AChR was applied to the affinity column overnight at 4 °C, at a flow rate of 1–2 ml/min. To reconstitute the AChR into different lipid mixtures, the affinity column was washed with the lipids of choice. Basically, the column was first washed with ~200 ml of dialysis buffer containing 1% cholate and lipids (A) until no more eluting protein absorbance at 280 nm was detected in a spectrophotometer. This wash also replaced native lipids with defined lipids. Three more 50-ml washes at higher lipid concentrations (2.24 mg/ml) were performed with dialysis buffer containing 1% cholate (lipid wash B), to exchange defined lipids for native lipids completely. Subsequently, the column was washed at lower lipid concentrations (0.09 mg/ml) with 150 ml of dialysis buffer containing 0.5% cholate (lipid wash C), to lower the final lipid/protein ratio. AChR was eluted from the column in 10 mM MOPS, 10 mM carbamoylcholine, 250 mM NaCl, 0.1 mM EDTA, 0.5% cholate, 0.02% NaN\(_3\)), again containing low concentrations of lipids (0.09 mg/ml, lipid wash D).

In these experiments, affinity purified AChR was reconstituted into four different lipid mixtures. For reconstitution into dioleoylphosphatidylcholine (DOPC, actually egg lecithin), lipid wash A contained 1.14 mM DOPC, lipid wash B contained 2.86 mM DOPC, and lipid wash C and D contained 0.11 mM DOPC and 0.32 mM lipid wash B. For reconstitution into DOPC and cholesterol (molar ratio 3:1), lipid wash A contained 0.86 mM DOPC and 0.29 mM DOPC, lipid wash B contained 2.14 mM DOPC and 0.74 mM DOPC, and lipid washes C and D contained 0.086 mM DOPC and 0.029 mM cholesterol. For reconstitution into DOPC, DOPA, and cholesterol (molar ratio 3:1:1), lipid wash A contained 0.78 mM DOPC, 0.26 mM DOPA, and 0.26 mM cholesterol, lipid wash B contained 1.91 mM DOPC, 0.64 mM DOPA, and 0.64 mM cholesterol, and lipid washes C and D contained 0.19 mM DOPC, 0.64 mM DOPA, and 0.64 μM cholesterol.

AChR was eluted from the affinity column with lipid wash D. Fractions containing AChR (determined by measuring their absorbance at 280 nm) were pooled and dialyzed against 4 × 4 liters of dialysis buffer, to remove the carbamoylcholine and cholate, and reconstitute the AChR into the defined lipids. Yields averaged 15–25 mg, and the dialyzed, reconstituted AChR was typically stored at -70 °C. Concentrations of detergent and lipid in dialysis buffer for reconstituted AChR appeared 100% pure on the basis of SDS-PAGE analysis and \(\text{[125I]}\)-BgTx binding (Schmidt and Raftery, 1973) of the various preparations of AChR averaged 7.67 ± 0.77 nmol of BgTx bound/mg of protein. The amount of phospholipid present was determined by phosphate assay (Ames, 1966). The molar phospholipid/protein ratio was 387 ± 28.1 for AChR reconstituted into DOPC, 224 ± 51 for AChR reconstituted into DOPC and DOPA, 262 ± 10:1 for AChR reconstituted into DOPC and cholesterol, and averaged 220 ± 66:1 for three different preparations of AChR reconstituted into DOPC, DOPA, and cholesterol. These phospholipid/protein ratios have been shown to be sufficient to support full AChR function (Jones et al., 1988).

**\(\text{[125I]}\)TID Labeling**—Labeling of the AChR with \(\text{[125I]}\)TID was performed largely as described previously (McCarthy and Stroud, 1989a). All photolabeling experiments were performed at 22 °C. In those cases where the effects of factors on AChR were tested, reaction mixtures contained 44.8 pmol of AChR/μl of \(\text{[125I]}\)TID/100-μl aliquot. Experiments on the effects of detergents were carried out at 297 pmol of AChR/μl of \(\text{[125I]}\)TID/45-μl aliquot, and the AChR was incubated with detergent (at concentrations mentioned in text) for 20 min at 22 °C prior to application of Photolabeling was performed by exposing samples to long wavelength UV lamp (\(\lambda_{\text{max}} = 366 \text{ nm}) for 12 min at a distance of 3 cm. \(\text{[125I]}\)TID incorporation into the AChR peaked
within 8–10 min of exposure to UV light. Photolabeling was terminated by removing the reaction mixtures from the UV light. The reaction mixtures were then frozen and stored for subsequent analysis, or used immediately.

**SDS-PAGE—**[125]ITID-labeled AChR were reduced with 2 mM dithiothreitol for 30 min at 22 “C prior to gel separation. In studies of the effects of lipid composition on [125]ITID incorporation, the AChR 100-μl aliquots of the reduced reaction mixtures were centrifuged in a microfuge for 15 min, the supernatant removed, and the concentrated AChR pellet was resuspended in 15 μl of 2 X sample buffer (Laemmli, 1970). In studies of the effects of detergents, 45-μl aliquots of the reduced reaction mixtures were mixed directly with 15 μl of isoelectric focusing sample buffer (Dunbar, 1988) prior to gel analysis. SDS-PAGE separation of the [125]ITID-labeled AChR subunits was performed as described previously (McCarthy and Stroud, 1989a) with minor modifications. 9% polyacrylamide separating mini-gels were used, for better separation of the AChR subunits, and occasionally the gels were dried on a Hoeffer SE 1150 slab gel dryer. Autoradiograms were prepared using Kodak X-Omat RP film at ~70 “C, typically in the presence of a Lightning-Plus intensifying screen (Du Pont-New England Nuclear. [125]ITID incorporation into the subunits of the AChR was quantitated by cutting out the bands of interest and the amount of [125]ITID present in each band was determined in a Packard Counter.

**Delipidation—**[125]ITID-labeled AChR preparations were delipidated by either cold ethanol or chloroform/methanol extraction. For ethanol extraction, 224.4 μl of [125]ITID-labeled AChR membranes (66 μg of protein total) were reduced with 2 mM dithiothreitol and then stored overnight at -20 “C in 1 ml of absolute ethanol. The AChR then pelleted in a microfuge, the supernatant (which contained extracted lipids and free [125]ITID) was removed, and the pellet was dried under argon. Chloroform/methanol extraction was performed using a scaled-down version of the Bligh and Dyer protocol (Bligh and Dyer, 1959). 100 μl of [125]ITID-labeled AChR membranes were reduced with 2 mM dithiothreitol and then mixed sequentially with 200 μl of methanol, 200 μl of CHCl3, and 300 μl of water. After phase separation, the mixture was centrifuged in a microfuge for 4 min. The upper aqueous phase was carefully removed and the white protein precipitate collected. In some cases a second chloroform/methanol extraction was performed. The precipitated protein was dried under argon. Both cold ethanol and chloroform/methanol-extracted preparations were reduced with 2 mM dithiothreitol, resuspended in 2 X sample buffer (Laemmli, 1970), and subjected to SDS-polyacrylamide gel analysis as described above.

**Toxin Binding Assay—**To measure the state of the AChR reconstituted into different lipids, we used the toxin binding assay of Walker et al. (1981), with modifications, to determine the initial rate of toxin binding. AChR (50 nm in toxin-binding sites) was mixed with [125]I-BgTx (30 nM) alone, with carbamylcholine (2 μM) and [125]I-BgTx added after pre-equilibration with 2 μM carbamylcholine, to a final volume of 700 μl in toxin-binding buffer (10 mM Na2HPO4, 100 mM NaCl, 0.5 mg/ml bovine serum albumin, pH 7.0). To determine the amount of [125]I-BgTx specifically bound by the AChR, 100-μl aliquots of the reaction mixture were removed at 15, 75, 135, 195, 245, and 300 s after mixing, and applied to DE81 filter discs. Free [125]I-BgTx was removed by washing the filters in wash buffer (10 mM NaH2PO4, 100 mM NaCl, 0.1% Triton X-100, pH 7.5). The filters were then dried under a heat lamp, and the amount of [125]I-BgTx remaining was determined by γ counting. The data were plotted as the counts/min corresponding to the amount of BgTx bound at any one time divided by the counts/min corresponding to the maximal amount of BgTx binding, and pseudo-first order rate constants were calculated by non-linear least squares analysis.

**RESULTS**

Our earlier studies of [125]ITID incorporation were performed upon AChR which was only 70% pure (McCarthy and Stroud, 1989a). To work with more pure protein, and to test the effects of the lipid environment on the incorporation of [125]ITID into the AChR, we have affinity purified the AChR and reconstituted it into different combinations of lipids. Initially, we characterized affinity purified AChR reconstituted into DOPC/DOPA/cholesterol, as this combination of lipids has been shown in the past to support fully functional AChR (Ochoa et al., 1983; Fong and McNamee, 1986). As shown in Fig. 1, AChR reconstituted into this combination of lipids demonstrates the same pattern of [125]ITID incorporation as partially purified AChR in native lipid membranes (compare lanes 2 and 4). Enhanced labeling of the γ subunit is observed in both cases, although the effect is less dramatic for reconstituted AChR. The ratio of [125]ITID incorporated into the γ subunit relative to the α subunit (γ/α) for partially purified AChR in native lipids was 4.46 ± 33 in our earlier study (McCarty and Stroud, 1989a) and has averaged about 3 in more recent experiments (see below), while the γ/α ratio for affinity purified AChR reconstituted into DOPC/DOPA/cholesterol averages 2.3 ± 0.19 (see Table I). The calculated molar levels of [125]ITID incorporation into the subunits of AChR reconstituted into DOPC/DOPA/cholesterol are indicated in Table I, column 1. The levels of [125]ITID incorporation are much lower than we reported earlier for partially purified AChR in native lipids (McCarty and Stroud, 1989a). This is probably due in part to changes in the experimental set-up, as we now typically observe lower levels of [125]ITID incorporation into partially purified AChR as well. However, approximately twice as much [125]ITID is incorporated/subunit of partially purified AChR compared to affinity purified AChR reconstituted into DOPC/DOPA/cholesterol.

In earlier studies, pre-equilibration with the agonist carbachol dramatically reduced the level of [125]ITID incorporation into all four subunits of partially purified AChR (White and Cohen, 1988; McCarthy and Stroud, 1989a). As shown in Fig. 2, pre-equilibration with 25 μM carbamylcholine had a similar effect on affinity purified AChR reconstituted into DOPC/DOPA/cholesterol. The amount of label incorporated into all four subunits was diminished 0.5–3-fold and the enhanced labeling of the γ subunit was eliminated (γ/α ratio = 0.75). Carbachol reduced the maximal level of label incorporation, but the rate of label incorporation remained the same (data not shown). This suggests that [125]ITID labeling sites which are present in resting state AChR are sterically blocked or removed upon desensitization by carbachol.

The combination of DOPC/DOPA/cholesterol had been shown in earlier work to stabilize the resting conformation of the protein, while reconstitution into DOPC or DOPC/cholesterol desensitized the AChR (Fong and McNamee, 1986). To determine if the changes in AChR conformation induced by different lipid environments resulted in altered patterns of

**Fig. 1.** [125]ITID-labeling patterns are the same for both partially purified AChR in native lipids and affinity purified AChR in defined lipids. [125]ITID photolabeling was performed for 72 min at 22 “C as described under “Experimental Procedures.” Lane 1, SDS-PAGE of [125]ITID-labeled, partially purified AChR in native lipids, stained with Coomassie Blue. Lane 2, autoradiogram of lane 1. Lane 3, SDS-PAGE of [125]ITID-labeled, affinity purified AChR reconstituted into DOPC/DOPA/cholesterol, stained with Coomassie Blue. Lane 4, autoradiogram of lane 3. The positions of the AChR subunits are indicated.
Conformations of the Acetylcholine Receptor

Levels of [\(^{125}\)I]TID incorporation into the subunits of AChR, reconstituted into different combinations of lipids, in the presence or absence of 25 μM carbamylcholine (carb). Data reported as moles of [\(^{125}\)I]TID/mole of AChR subunit. γ/α is the ratio of label incorporated into the γ subunit relative to the α subunit. The values in parentheses are the standard error. n = 4 for DOPC/DOPA/cholesterol and DOPC/cholesterol and n = 3 for DOPC and DOPC/DOPA.

| Subunits | DOPC/DOPA/chol | DOPC/DOPA | DOPC/cholesterol | DOPC |
|----------|----------------|-----------|------------------|------|
|          | -carb          | +carb     | -carb            | +carb|
| α        | 0.18 (0.07)    | 0.12 (0.02)| 0.10 (0.02)      | 0.05 (0.01)|
| β        | 0.24 (0.08)    | 0.08 (0.03)| 0.14 (0.03)      | 0.06 (0.01)|
| γ        | 0.42 (0.17)    | 0.09 (0.03)| 0.27 (0.04)      | 0.08 (0.01)|
| δ        | 0.15 (0.05)    | 0.08 (0.02)| 0.13 (0.03)      | 0.06 (0.01)|
| γ/α      | 2.3            | 0.75      | 2.7              | 1.6   |
|          | −-carb          | +carb     | −-carb            | +carb |
| α        | 0.08 (0.01)    | 0.05 (0.01)| 0.07 (0.03)      | 0.06 (0.03)|
| β        | 0.08 (0.02)    | 0.08 (0.02)| 0.08 (0.02)      | 0.08 (0.02)|
| γ        | 0.12 (0.02)    | 0.07 (0.01)| 0.04 (0.01)      | 0.06 (0.03)|
| δ        | 0.08 (0.03)    | 0.07 (0.01)| 0.05 (0.01)      | 0.04 (0.02)|
| γ/α      | 1.7            | 1.2       | 1.0              | 0.8   |

[\(^{125}\)I]TID labeling, we reconstituted affinity purified AChR into DOPC, DOPC/DOPA, and DOPC/cholesterol. As shown in lanes 1 and 2 of Fig. 3, the [\(^{125}\)I]TID-labeling pattern of AChR reconstituted into DOPC/DOPA is similar to AChR in both native lipids and DOPC/DOPA/cholesterol. In the absence of carbamylcholine, AChR reconstituted into DOPC/DOPA demonstrates the enhanced labeling of the γ subunit typical of resting state AChR (γ/α ratio = 2.7; Table I). The labeling of all four subunits is reduced upon equilibration with carbamylcholine. The levels of [\(^{125}\)I]TID incorporation into AChR reconstituted into DOPC/DOPA appear lower than for AChR reconstituted into DOPC/DOPA/cholesterol (Table I). However, this may not reflect a difference in the conformation of the AChR induced by different lipid environments. While the ratio of [\(^{125}\)I]TID/protein was kept constant in all labeling experiments, the lipid/protein ratio varied between different reconstitutions. As [\(^{125}\)I]TID also nonspecifically labels the fatty acid side chains of lipids, strict comparisons of the levels of [\(^{125}\)I]TID incorporation should only be made between protein reconstituted at the same lipid/protein ratios. Therefore, the values for the levels of [\(^{125}\)I]TID incorporation into the subunits of AChR reconstituted into different lipid mixtures listed in Table I are relevant only at the lipid/protein ratio at which they were determined.

[\(^{125}\)I]TID labeling of AChR reconstituted into DOPC/cholesterol in the absence of carbamylcholine differed from the pattern observed for AChR in DOPC/DOPA/cholesterol (Fig. 3, lanes 3 and 4). Labeling of the γ subunit was less enhanced (γ/α ratio = 1.7), and the reduction in [\(^{125}\)I]TID labeling of the α, β, and δ subunits upon equilibration with carbamylcholine was slight or nonexistent (Table I). The [\(^{125}\)I]TID-labeling pattern of AChR reconstituted into DOPC was the same in absence or presence of carbamylcholine (Fig. 3, compare lanes 5 and 6), and there was no enhanced labeling of the γ subunit in the absence of carbamylcholine (γ/α ratio = 1; Table I). The lack of an effect of carbamylcholine on the incorporation of [\(^{125}\)I]TID into AChR reconstituted into DOPC suggests that this lipid can not stabilize the resting state conformation of the AChR, in agreement with earlier work (Criado et al., 1982; Fong and McNamee, 1986). The [\(^{125}\)I]TID-labeling pattern of AChR reconstituted into DOPC/cholesterol is intermediate, suggesting that this combination of lipids can only partially stabilize AChR in the resting state.

As an independent assay of the functional conformation of the AChR reconstituted into different combinations of lipids, we used the toxin rate binding assay (Walker et al., 1981). In this assay, the initial rate of \(^{125}\)I-BgTx binding to the AChR is determined either in the absence of agonist, with agonist and toxin added simultaneously, or with agonist added 30 min prior to the addition of toxin to desensitize the AChR. If the AChR is initially in the resting state, the rate of toxin binding when added at the same time as agonist is intermediate to the rates observed in the absence of agonist and upon prior equilibration with agonist. This is due to the greater ability of agonist to compete with toxin binding to desensitized AChR, rather than resting state AChR, as a result of the \(\sim\)1000-fold greater affinity of desensitized AChR for agonist.
observed in the absence of carbamylcholine (circles) and upon pre-equilibration with carbamylcholine (triangles). This indicates that AChR reconstituted into DOPC/DOPA/cholesterol is in the functional, resting state, in agreement with earlier work (Criado et al., 1982; Fong and McNamee, 1986).

Conversely, the rates of toxin binding upon co-equilibration or pre-equilibration with carbamylcholine were approximately the same for AChR reconstituted into DOPC (Fig. 4b) or DOPC/cholesterol (Fig. 4c), indicating that the AChR in these instances was largely in the desensitized state even in the absence of agonist, again in agreement with previous studies (Criado et al., 1982; Fong and McNamee, 1986) and with the pattern of $^{[125]}$I(TID incorporation. On the basis of the toxin rate binding assay, AChR reconstituted into DOPC/DOPA also appeared largely desensitized (Fig. 4d), although the pattern of $^{[125]}$I(TID incorporation into AChR reconstituted into these lipids is similar to that of AChR in the resting state.

The dramatic reduction in $^{[125]}$I(TID incorporation into all four subunits of the AChR upon desensitization by agonists is unusual, given the nonspecific mode of $^{[125]}$I(TID labeling of membrane proteins. If TID is functioning essentially to "spray paint" the amino acid side chains at the protein/lipid interface as expected, it is difficult to visualize how desensitization could diminish the extent of label incorporation short of removing the AChR from the bilayer, which we know does not occur. A possible explanation is that in resting state AChRs, tightly bound, $^{[125]}$I(TID-labeled lipid remains associated with the AChR subunits even during SDS-gel electrophoresis and that this tight association between protein and lipid is lost upon desensitization. For example, tight, but non-covalent, association of $^{[125]}$I(TID-labeled lipid and colicin E1 on SDS gels has been observed. To test for this possibility, we extensively delipidated $^{[125]}$I(TID-labeled AChR by both cold ethanol and chloroform/methanol extraction, to determine if delipidation altered the effects of carbamylcholine-induced desensitization, or the calculated levels, of $^{[125]}$I(TID incorporation into the subunits of the AChR. As shown in Fig. 5, AChR reconstituted into DOPC/DOPA/cholesterol demonstrated the same levels of $^{[125]}$I(TID incorporation, and the same $\gamma/\alpha$ ratio, both before and after extensive delipidation by cold ethanol (compare lanes 1 and 3). The relative extent of delipidation is indicated by the reduction in label running at the dye front, where free $^{[125]}$I(TID and $^{[125]}$I(TID-labeled lipids migrate on SDS gels. In addition, the same reduction in label incorporation upon desensitization with 25 $\mu$m carbamylcholine was observed both prior to and following delipidation (Fig. 5, lanes 2 and 4). Similar effects were seen upon extensive delipidation with chloroform/methanol (data not shown). Therefore, the reduction in apparent $^{[125]}$I(TID incorporation observed upon desensitization of the AChR is not due to a change in affinity for some lipid but must involve another mechanism.

Finally, we looked at the effects of detergent solubilization on the pattern of $^{[125]}$I(TID labeling of the AChR. The $^{[125]}$I(TID-labeling pattern of AChR solubilized in the detergents octyl glucoside, Triton X-100, and Tween 20 appears the same as agonist-desensitized AChR (compare lanes 4–6 of Fig. 6 with lane 2). Intriguingly, solubilization in the non-ionic detergent sodium cholate did not appear to desensitize the AChR (Fig. 6, compare lanes 1 and 3). As the partition coefficient of $^{[125]}$I(TID into membrane vesicles and mixed lipid-detergent-protein micelles is most likely different, direct comparisons of the overall amounts of $^{[125]}$I(TID incorporation in the presence or absence of detergents are not possible. In
fact, it appears that $[^{125}]$TID partitions less efficiently into the mixed lipid-detergent-protein micelles, which is why the overall levels of $[^{125}]$TID incorporation appear lower in the presence of detergents. However, the ratio of label incorporation into AChR subunits is largely determined by the local conformation and can, therefore, be used as a measure of the state of the AChR. The ratio of label incorporated in the $\gamma$ and $\alpha$ subunits after solubilization in octyl glucoside or Triton X-100 is similar to that observed for membrane-bound AChR after desensitization by agonist, although AChR solubilized in 1% Tween 20 does not appear to be completely desensitized (Table II). However, the $\gamma/\alpha$ ratios of resting state AChR and AChR solubilized in 1% sodium cholate are both characteristic of resting state AChR. To investigate the effects of sodium cholate on the $[^{125}]$TID-labeling patterns of the AChR further, we photolabeled the AChR over a range of sodium cholate concentrations (0.1–3.0%). Only at higher concentrations of sodium cholate (2.5–3.0%) did we observe the $[^{125}]$TID labeling pattern characteristic of agonist-desensitized AChR (data not shown). For all four detergents, when AChR was solubilized with concentrations of detergent sufficient to produce the $[^{125}]$TID-labeling pattern characteristic of desensitized AChR, addition of 25 $\mu$m carbamylcholine did not further reduce the levels of $[^{125}]$TID incorporation (data not shown). It therefore appears that solubilization in non-denaturing detergents may induce or stabilize the desensitized conformation of the AChR, although in the case of sodium cholate, concentrations of detergent well beyond the critical micelle concentration are necessary.

**DISCUSSION**

We describe here the effects of alterations in the lipid environment on the conformation of the AChR, as detected by the variable incorporation of the hydrophobic, photoactive probe $[^{125}]$TID. In earlier studies, performed upon partially purified AChR in native lipids, $[^{125}]$TID was found to label all four subunits of resting state AChR, in agreement with other evidence indicating that each subunit spanned the lipid bilayer at least once. The subunits were labeled non-stoichiometrically, however, as the $\gamma$ subunit was labeled two to four times greater than any of the other subunits (White and Cohen, 1988; McCarthy and Stroud, 1989a). $[^{125}]$TID-labeling of the AChR was not affected by the binding of the antagonist BgTx or the noncompetitive blocker phencyclidine, but the extent of label incorporation into all four subunits was reduced 2–8-fold upon desensitization by agonist. This effect was shown to be specific, as agonist-induced reduction of $[^{125}]$TID incorporation was blocked by prior binding of BgTx (White and Cohen, 1988; McCarthy and Stroud, 1989a), and $[^{125}]$TID labeling of the intrinsic membrane protein bacteriorhodopsin was shown not to be affected by the presence of carbamylcholine (McCarthy and Stroud, 1989a). A large peptide containing the first three, putative membrane-spanning regions of the $\alpha$ subunit was determined by White and Cohen (1988) to contain the carbamylcholine-sensitive $[^{125}]$TID-labeling site(s).

In the work described here, we have used the pattern of $[^{125}]$TID incorporation into the subunits of the AChR as a measure of the conformation of the AChR reconstituted into different lipids and after detergent solubilization. These studies were performed upon affinity purified AChR, reconstituted into defined lipids, to minimize errors associated with impure and uncharacterized material. First, we examined AChR reconstituted into DOPC/DOPA/cholesterol, as this combination of lipids was previously shown to support the functional, resting state conformation of the AChR (Criado et al., 1984; Fong and McNamee, 1986). Similar to observations with AChR in native lipids, $[^{125}]$TID labeled all four subunits of AChR reconstituted into DOPC/DOPA/cholesterol but was preferentially incorporated into the $\gamma$ subunit. This suggests that the non-stoichiometric levels of $[^{125}]$TID incorporated into the $\gamma$ subunit is a function of the resting state conformation of the AChR, which is stabilized by both native lipids and DOPC/DOPA/cholesterol, and not due to specific interactions between native lipids and the $\gamma$ subunit.

As was true for partially purified AChR in native lipids, addition of carbamylcholine reduced the maximal level of $[^{125}]$TID incorporation into all four subunits of AChR reconstituted into DOPC/DOPA/cholesterol. The most likely cause for this reduction in label incorporation is that equilibration with carbamylcholine induced the desensitized conformation of the AChR. As shown by the toxin rate binding assay, AChR reconstituted in DOPC/DOPA/cholesterol is largely in the resting state, but upon pre-equilibration with agonist is capable of undergoing the change in affinity for agonist characteristic of desensitization. The molar levels of $[^{125}]$TID incorporated into the subunits of AChR reconstituted into DOPC/DOPA/cholesterol, both in the presence and absence of carbamylcholine, is lower than observed for partially puri-

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**Table II**

| Memb - carb | Memb + carb | Cholate | OG | TTX-100 | TWEEN |
|-------------|-------------|---------|----|---------|-------|
| $\gamma/\alpha$ | 2.58 (0.09) | 1.02 (0.07) | 2.04 (0.12) | 0.95 (0.01) | 1.08 (0.03) | 1.47 (0.16) |
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fied AChR in native lipids. This may be due to some extent to differences in the lipid/protein ratio of the various preparations, but reduced levels of $^{[35]S}$TID labeling appears to be a characteristic of affinity purified, reconstituted AChR. In addition, the magnitude of the reduction in labeling observed upon desensitization with carbamylcholine is smaller with AChR reconstituted into DOPC/DOPA/cholesterol than with AChR in native lipids. In particular, desensitization reduces labeling of the α subunit by only about 50% for AChR in DOPC/DOPA/cholesterol, compared to the 2-fold reduction observed for AChR in native lipids. A possible explanation is that the proportion of the AChR in the resting state compared to the desensitized state is higher for AChR in native lipids than for AChR reconstituted into DOPC/DOPA/cholesterol, although at the qualitative level the toxin rate binding assays of both forms of AChR are indistinguishable (data not shown). It is also possible that $^{[35]S}$TID partitions differently into the two lipid mixtures.

Conversely, on the basis of the pattern of $^{[35]S}$TID labeling, AChR reconstituted solely into DOPC appears desensitized even without prior equilibration with carbamylcholine. There is no significant change in the level of $^{[35]S}$TID incorporation, and labeling of the γ subunit is not enhanced relative to the α subunit ($γ/α$ ratio = 1). This conclusion is supported by the toxin rate binding assay and also by earlier work (Criado et al., 1984; Fong and McNamee, 1986). The $^{[35]S}$TID-labeling pattern of AChR reconstituted into DOPC/cholesterol is similar. AChR in DOPC/cholesterol appears largely desensitized even without exposure to agonist, although there is some enhanced labeling of γ subunit. Again, both the toxin rate binding assay and work by other groups support the same conclusion. However, interpretation of the $^{[35]S}$TID-labeling pattern of AChR reconstituted into DOPC/DOPA is not as straightforward. On the basis of $^{[35]S}$TID labeling, AChR in DOPC/DOPA appear largely in the resting state in the absence of agonist, as equilibration with carbamylcholine reduces the labeling of all four subunits 2–3-fold, and labeling of the γ subunit is significantly enhanced relative to the other subunits ($γ/α$ ratio = 2.7), both characteristics of AChR in the resting state. However, on the basis of the toxin rate binding assay, AChR reconstituted into DOPC/DOPA appears desensitized even in the absence of agonist. Given earlier studies which suggest that AChR reconstituted in DOPC/DOPA is capable of fluxing ions in response to agonist binding (i.e. is in the resting state) (Ochoa et al., 1983), and the technical difficulties inherent in the toxin rate binding assay, AChR reconstituted in DOPC/DOPA is most likely stabilized largely in the resting state, in agreement with the $^{[35]S}$TID-labeling pattern.

Intrinsic membrane proteins such as the AChR function by definition in the presence of lipids. The lipid composition of the electric organs of different species of electric fish, from both the Pacific and Atlantic oceans, are quite similar, suggesting that the native lipid environment of Torpedo AChR is functionally conserved (Barrantes, 1989). Electrophysiological experiments are approximately equinolar in phospholipids and cholesterol. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidyserine account for 80–90% of the total phospholipids (Gonzalez-Ros et al., 1982; Rotstein et al., 1987). The composition of the fatty acid side chains is remarkable, demonstrating a fairly typical complement of saturated, monounsaturated, and polyunsaturated long chain fatty acids (Rotstein et al., 1987).

The specific lipid requirements of the AChR have been studied by reconstituting the AChR in different combinations of lipids (reviewed by Barrantes, 1989). AChR reconstituted solely into phospholipids appeared largely desensitized, on the basis of the toxin rate binding assay (Criado et al., 1982; Fong and McNamee, 1986), and usually did not flux ions in response to agonist binding (Ochoa et al., 1983; Fong and McNamee, 1986), although some flux response was observed for AChR reconstituted in a mixture of DOPC/DOPA (Ochoa et al., 1983). Cholesterol is clearly necessary to support fully functional AChR. Cholesterol, or the cholesterol analog cholesteryl hemiacetate, were shown both to stabilize the resting state conformation of the AChR (Criado et al., 1982; Fong and McNamee, 1986) and to support the ability of the AChR to flux ions (Dalziel et al., 1980; Killian et al., 1980; Ochoa et al., 1983; Criado et al., 1984; Fong and McNamee, 1986). There is evidence for nonannular cholesterol-binding sites on the AChR (Jones and McNamee, 1988), and cholesterol was observed to increase the α-helical content of AChR by Fourier transform-infrared spectroscopy analysis (Fong and McNamee, 1987). Cholesterol may also be important for the formation of sealed vesicles, a requirement for ion fluxing assays (Earnest et al., 1987). There is some controversy as to which mixture of phospholipids and neutral lipids satisfies the minimal lipid requirements of the AChR. Barrantes and co-workers found a mixture of the neutral phospholipid phosphatidylethanolamine and cholesterol hemiacetate worked best to support the functional state of the AChR. Cholesterol may also be important for the functional conformation of the AChR in native lipids. The ability of agonist to desensitize AChR in this lipid mixture, shown by the toxin rate binding assay, was paralleled by the reduction in the ratio of $^{[35]S}$TID incorporation into all four subunits for AChR in native lipids. Conversely, AChR reconstituted into DOPC/cholesterol, AChR reconstituted into lipids shown to stabilize the functional conformation of the AChR in earlier work, also demonstrated the $^{[35]S}$TID-labeling pattern of resting state AChR in native lipids. The ability of agonist to desensitize AChR is less well characterized in this combination of lipids to support intermediate levels of AChR ion-fluxing ability. However, on the basis of the toxin rate binding assay, AChR in DOPC/DOPA appeared largely desensitized even without prior equilibration with carbamylcholine, as equilibration with carbamylcholine reduces the labeling of all four subunits 2–3-fold, and labeling of the γ subunit is significantly enhanced relative to the other subunits ($γ/α$ ratio = 2.7), both characteristics of AChR in the resting state. However, on the basis of the toxin rate binding assay, AChR reconstituted into DOPC/DOPA appears desensitized even in the absence of agonist. Given earlier studies which suggest that AChR reconstituted in DOPC/DOPA is capable of fluxing ions in response to agonist binding (i.e. is in the resting state) (Ochoa et al., 1983), and the technical difficulties inherent in the toxin rate binding assay, AChR reconstituted in DOPC/DOPA is most likely stabilized largely in the resting state, in agreement with the $^{[35]S}$TID-labeling pattern.

Our studies of the effects of lipids on the structure of the AChR, as measured by the patterns of $^{[35]S}$TID incorporation, are largely in agreement with the earlier work summarized above. AChR reconstituted into DOPC/DOPA/cholesterol, lipids shown to stabilize the functional conformation of the AChR in earlier work, also demonstrated the $^{[35]S}$TID-labeling pattern of resting state AChR in native lipids. The ability of agonist to desensitize AChR is less well characterized in this combination of lipids to support intermediate levels of AChR ion-fluxing ability. However, on the basis of the toxin rate binding assay, AChR in DOPC/DOPA appeared largely desensitized. It is possible that this combination of lipids is supporting partially desensitized AChR, characterized by the $^{[35]S}$TID-labeling pattern of resting state AChR and still able to show some ion fluxing ability, but sufficiently inactivated to appear desensitized in the toxin rate binding assay. We have also studied the effects of detergent solubilization on AChR $^{[35]S}$TID labeling. The effects of detergents on the functional conformation of the AChR are less well characterized. At concentrations below the critical micelle concentration, the non-ionic detergents Triton X-100 and sodium cholate were shown to act like desensitizing local anaesthetics (Changeux et al., 1976), while at concentrations above its...
critical micelle concentration, the effects of solubilization in sodium cholate on agonist binding (Briley and Changeux, 1978) and the fluorescence of a fluorescent agonist analog (Heidmann et al., 1978) were more characteristic of resting state AChR. Solubilization in octyl glucoside also appeared to desensitize the AChR, on the basis of the toxin rate binding assay (Gonzalez-Ros et al., 1980). The difference in the conformational state stabilized by sodium cholate, relative to that induced by octyl glucoside or Triton X-100, appears to extend to the ability of these detergents to support the functional reconstitution of the AChR. After removal of potassium cholate (Epstein and Racker, 1978) or sodium cholate (Briley and Changeux, 1978), the AChR can be reconstituted into the appropriate lipids to regenerate the resting state. However, reconstitution of functional AChR after solubilization in octyl glucoside is less straightforward. Functional AChR can be reconstituted into native Torpedo lipids (Gonzalez-Ros et al., 1980), but not asolectin (Anholt et al., 1981), following octyl glucoside solubilization. In circular dichroism studies, spectra of both membrane-bound and sodium cholate-solubilized AChR appear essentially identical (Mielke and Wallace, 1988), suggesting that the change in conformation induced upon cholate solubilization is small at best. However, it should be noted that, both in the circular dichroism measurements (Mielke and Wallace, 1988) and in tritium-hydrogen studies (McCarthy and Stroud, 1989b), the global change in conformation between the resting and desensitized states of the AChR appears quite small.

The effects of detergents we have observed on the patterns of $[^{125}]$TID incorporation into the AChR are interesting. We expected that all non-ionic detergents would desensitize the AChR as this seems the usual response to perturbations of the hydrophobic environment of the AChR. However, as shown in Fig. 6, solubilization in 1% sodium cholate appears not to desensitize the AChR, at least on the basis of $[^{125}]$TID labeling. This is supported to some extent by the earlier work cited above and may explain why AChR solubilized in sodium cholate can be easily reconstituted in a functional state, whereas AChR solubilized in other detergents is in some cases permanently desensitized. It may be that cholate helps to stabilize cholesterol-binding sites on the AChR (Jones and McNamee, 1988) and that stabilization of these sites might be crucial for function. However, at higher concentrations (>2.5%), sodium cholate did appear to desensitize the AChR. This effect may be permanent, as in earlier studies of sodium cholate solubilization of affinity purified AChR reconstituted at approximately the same lipid/protein ratio, high concentrations of sodium cholate permanently eliminated ion-fluxing ability of the AChR (Jones and McNamee, 1988). The observation that solubilization in low concentrations of sodium cholate appears to preserve the resting state of the AChR suggests that for x-ray crystallographic studies, crystals of AChR solubilized in sodium cholate might be best, as the protein should be present in a more functional conformation than if solubilized in other detergents.

We report here that different combinations of lipids, or certain detergents, appear to induce the same, final desensitized conformation of the AChR as prolonged exposure to agonist, on the basis of the patterns of incorporation of the hydrophobic probe $[^{125}]$TID. We are currently extending these studies to determine if different classes of local anesthetics also have the same effects on the patterns of $[^{125}]$TID labeling. These studies may allow us to determine whether these disparate agents and conditions induce the same desensitized state, or multiple, nonfunctional conformations and will perhaps yield insights into the molecular mechanism of desensitization.

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