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Separate Sites of Low and High Affinity for Agonists on *Torpedo californica* Acetylcholine Receptor†

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ABSTRACT: We have studied alkylation of the membrane-bound acetylcholine receptor (AcChR) from *Torpedo californica* electric organ by the cholinergic agonist bromoacetylcholine (BrAcCh). Following reduction of the AcChR with dithiothreitol (DTT) under strictly controlled conditions, a single class of binding sites was covalently labeled by BrAcCh. The extent of alkylation was dependent on the concentration of both DTT and BrAcCh and reached a maximum when a number of sites equivalent to the number of α-bungarotoxin (α-BTx) binding sites were labeled. The reaction with BrAcCh was completely inhibited by saturating concentrations of α-BTx. On the contrary, complete alkylation of the AcChR with [^{3}H]BrAcCh consistently inhibited only ~50% of α-BTx binding. The effects of DTT reduction and subsequent BrAcCh alkylation on the cation-gating properties of the AcChR were investigated in rapid kinetic experiments. DTT reduction resulted in a slight decrease in the maximum cation flux and a small shift in the effective dissociation constant to higher acetylcholine (AcCh) concentration. The flux response was completely inhibited by maximal alkylation of the membrane vesicles by BrAcCh. A low-affinity binding site for AcCh, which is likely to be important in AcChR activation, has been revealed for *T. californica* AcChR by studying the effects of cholinergic ligands on the fluorescence of a probe, 4-[(iodoacetoxy)ethylmethylamino]-7-nitro-2,1,3-benzoxadiazole (IANBD), covalently bound to the AcChR protein. Maximal labeling by BrAcCh did not affect the binding of AcCh to the low-affinity binding site, as monitored by changes in the fluorescence of this probe. This low-affinity binding site must therefore be distinct from the site labeled by BrAcCh. The results strongly support the notion that the nicotinic AcChR contains multiple binding sites for cholinergic ligands.

The nicotinic acetylcholine receptor (AcChR)[1] is the first neurotransmitter receptor to be identified as a molecular entity and purified to homogeneity after solubilization both in nondenaturing detergents and in the native membrane-bound form [reviewed in Conti-Tronconi & Raftery (1982)]. The function of the AcChR in the postsynaptic membrane is to mediate, in response to agonist binding, a rapid ion flux through a cation-selective channel contained within the AcChR molecule [reviewed in Conti-Tronconi & Raftery (1982)]. Channel activation occurs rapidly following neurotransmitter release, and the channel remains open for a few milliseconds. After exposure to agonist for longer periods, desensitization leads to channel closing and a loss of the permeability response (Katz & Thesleff, 1957). Quantitative in vitro studies of the ligand-binding and cation-gating properties of the membrane-bound *Torpedo* AcChR can be made since isolation of postsynaptic membrane fragments containing the AcChR as the only protein component is possible (Neubig et al., 1979; Elliott et al., 1979). Upon exposure to cholinergic agonists, the membrane-bound *Torpedo* AcChR undergoes a slow conformational change (time scale of seconds) to a state of higher affinity for these ligands (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978). This increase in affinity for agonists and the relatively slow time scale of the transition ($t_{1/2}$ ~80 s at 1 μM carbamylcholine) allowed its correlation with the phenomenon of desensitization observed in vivo.

Both muscle (Conti-Tronconi et al., 1982b) and electric organ (Raftery et al., 1980; Conti-Tronconi et al., 1982a) AcChRs are pseudosymmetric complexes of four structurally related subunits of molecular weights 40000, 50000, 60000, and 65000 in *Torpedo californica*, present in the AcChR molecule in the ratio 2:1:1:1. Affinity labeling techniques have shown that the subunit of lowest M_r (commonly referred to as "α") carries a high-affinity binding site(s) for cholinergic agonists and/or antagonists, since, following reduction of a reactive disulfide bond near this (these) site(s), it can be alkylated by the agonist bromoacetylcholine (Chang et al., 1977;

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[1] Abbreviations: AcChR, acetylcholine receptor; DTT, dithiothreitol; α-BTx, α-bungarotoxin; [^{125}I]-α-BTx, ^{125}I-labeled α-bungarotoxin; IANBD, 4-[(iodoacetoxy)ethylmethylamino]-7-nitro-2,1,3-benzoxadiazole; MBTA, (4-maleimidobenzyl)trimethylammonium chloride; BrAcCh, bromoacetylcholine; AcCh, acetylcholine; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Tetram, *O,O'*-diethyl *S*-2-(diethylamino)ethylphosphorothioate; EDTA, ethylenediaminetetraacetic acid; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; HTX, histrionicotoxin; NBD, 7-nitro-2,1,3-benzoxadiazole; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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Damle et al., 1978; Moore & Raftery, 1979; Lyddiatt et al., 1979; Wolosin et al., 1980) or by the affinity reagent MBTA (Reiter et al., 1972; Karlin & Cowburn, 1973; Weill et al., 1974; Karlin et al., 1976; Froehner et al., 1977). It has been generally assumed that agonist binding to this (these) high-affinity site(s) on the 40-kdalton subunit(s) leads to both channel activation and desensitization, despite the frequent lack of agreement between apparent dissociation constants measured for agonist binding to the resting state and values obtained from the ligand concentration dependence of the flux response [reviewed in Conti-Tronconi & Raftery (1982)].

Recently, evidence has been obtained for the existence in *T. californica* AcChR of a distinct binding site of low affinity for agonists which is present under both initial and equilibrium conditions (Dunn & Raftery, 1982a,b). This site was revealed by monitoring the agonist-induced fluorescence changes of a probe (IANBD) covalently bound to the AcChR. The equilibrium and kinetic properties of agonist binding to this site as well as the very fast (millisecond) time scale of the ligand-mediated conformational change of AcChR suggest that this site may be important for channel activation. In *Torpedo* AcChR, activation and desensitization might therefore be parallel processes, mediated by agonist binding to different sites on the AcChR oligomer. In the studies presented here, we have investigated various aspects of the covalent attachment of [^{3}H]BrAcCh to the membrane-bound AcChR from *Torpedo californica* and have examined the effects of such labeling on the agonist-induced cation flux and on the binding of agonist to the low-affinity *agonist-specific* site as monitored by changes in the fluorescence of bound NBD.

Materials and Methods

AcChR-enriched membrane fragments were prepared from *Torpedo californica* electric organs as described previously (Elliott et al., 1980) and were routinely subjected to alkali extraction to remove nonreceptor proteins (Neubig et al., 1979; Elliott et al., 1979). $NaDodSO_4$ gel electrophoresis patterns (Laemmli, 1970) of these preparations showed that the four receptor subunits were the only major protein components. The concentration of α-BTx sites was measured by the DEAE disk assay of Schmidt & Raftery (1973) by using [^{125}I]-α-BTx obtained from New England Nuclear, for which the specific activity was measured by using the procedures of Blanchard et al. (1979). Protein concentrations were determined by the method of Lowry et al. (1951).

Bromoacetyl[^{3}H]choline perchlorate (68 mCi/mmol) was synthesized from [^{3}H]choline (New England Nuclear), and its specific activity was determined as described by Moore & Raftery (1979). The concentration of [^{3}H]BrAcCh was estimated by the hydroxylamine–ferric chloride method of ester determination (Hestrin, 1949) using unlabeled bromoacetylcholine perchlorate as standard.

Acetylcholinesterase was inhibited by addition of 10^{-5}–10^{-4} M Tetram, and only those preparations which were devoid of esterase activity as measured by the method of Ellman et al. (1961) were used.

Labeling of AcChR-enriched membrane fragments by [^{3}H]BrAcCh was carried out under an argon atmosphere by using Aldrich AtmosBags, and all buffers were thoroughly degassed before use. Membrane fragments were diluted to 1 μM in α-BTx sites in the pH 8 buffer used by Wolosin et al. (1980), i.e., 15 mM Tris-HCl, 150 mM NaCl, 4.5 mM NaN_3, and 1.5 mM EDTA, pH 8. DTT was added, and reduction was allowed to proceed for 45 min at room temperature. Membrane fragments were then pelleted to remove DTT by using tightly capped centrifuge tubes and either an Eppendorf microfuge for 15 min or, for larger volumes, a Sorvall SS34 rotor for 45 min at 18000 rpm. The tubes were returned to an argon atmosphere, the supernatants were discarded, and the pellets were resuspended in the above buffer, whose pH had been adjusted to 7. Following removal of a small volume for assay of the concentration of α-BTx sites, appropriate aliquots were taken, and half of each aliquot was incubated with a 3–60-fold excess of α-BTx for 20 min at room temperature. [^{3}H]BrAcCh was added, and after 15 min, duplicate aliquots of 50 μL were pipetted onto DE-81 disks, and unreacted ligand was washed away with 10 mM sodium phosphate–50 mM NaCl, pH 7.4, containing 0.1% Triton X-100 as described by Moore & Raftery (1979). The disks were transferred to scintillation vials and were extracted with 0.8 mL of 1 M NaCl containing 1% Triton for approximately 1 h before addition of 10 mL of Aquasol 2 and counting for ^{3}H content by using a Beckman scintillation counter. Specific labeling with [^{3}H]BrAcCh was calculated by subtraction of nonspecific labeling measured in the presence of excess α-BTx. For investigation of the relationship between the extent of labeling by [^{3}H]BrAcCh and the amount of residual α-BTx binding activity, the remainder of each sample was pelleted as before and resuspended in an appropriate volume of the pH 7 buffer described above. Duplicate 50-μL aliquots were assayed for bound [^{3}H]BrAcCh as described above, and the remaining sample was assayed for [^{125}I]-α-BTx binding by using the DEAE disk assay.

$NaDodSO_4$ gel electrophoresis was performed according to Laemmli (1970) by using 1-mm-thick slab gels containing 8.75% polyacrylamide. For determination of the labeling pattern of receptor subunits, gel slices (1 mm) were cut, digested, and counted as previously described (Blanchard & Raftery, 1979).

The kinetics of agonist-mediated cation transport were investigated by using the stopped-flow method described by Moore & Raftery (1980) in which the influx of Tl^+ is monitored by the quenching of the fluorescence of a probe, ANTS, loaded within the vesicles. Samples for these experiments were prepared by reacting membrane fragments with DTT alone or with DTT followed by unlabeled BrAcCh exactly as described above. The extent of reaction was monitored by a parallel experiment in which [^{3}H]BrAcCh was used, and the concentrations of bound BrAcCh and α-BTx sites were measured as described above. Following these treatments, membrane fragments were centrifuged in an SS 34 rotor for 45 min at 18000 rpm, and the pellets were resuspended in 10 mM Hepes–35 mM $NaNO_3$, pH 7.4, before being loaded with ANTS. This and subsequent steps were carried out in the room atmosphere, but degassed buffers were used.

Flux data were fitted to the equation

$$F(t) = A_0 + A_1/\{1 + KT_\infty[1 - \exp(-k_1 t)]\} + k_0 t$$

where A_1 and A_0 are the fluorescence levels at time t and equilibrium, respectively, k_1 is the flux rate constant, k_0 is the slope of the base line, and the term KT_∞ was fixed as a constant by using the known final Tl^+ concentration (T_∞) of 17 mM and the Stern-Volmer quenching constant (K) of 96 M^{-1} (Moore & Raftery, 1980).

The AcChR was labeled by the fluorescent probe IANBD (Molecular Probes Inc.) as previously described (Dunn & Raftery, 1982a,b). In these experiments, membrane fragments which had been prepared without iodoacetamide in the initial homogenization steps were used. These were diluted to 10 μM in α-BTx sites in 10 mM Hepes–35 mM $NaNO_3$, pH 7.4, and were reacted with 50 μM DTT for 20 min at room tempera-

ture. Solid IANBD was added to give a nominal concentration of 300 μM, and after being stirred for 2 h at 4 °C, unreacted agent was removed by gel filtration on Sephadex G-25-300.

Fluorescence measurements of NBD-labeled AcChR were made at 25 °C by using a Perkin-Elmer MPF-4 fluorometer and excitation and emission wavelengths of 482 and 540 nm, respectively. Agonist-induced fluorescence enhancements were measured by titration in which small aliquots of concentrated AcCh were added to 2 mL of membrane fragments ($\sim$2 μM in α-BTx sites), and the fluorescence level was recorded immediately after ligand addition. Data were corrected for nonspecific effects by parallel titration of the sample reacted with an approximately 3-fold excess of α-BTx. Titration data were fit by using the nonlinear regression program previously described (Dunn et al., 1980), and dissociation constants were estimated from the equation

$$F_1 = \frac{F_0[L]}{K_d + [L]}$$

where F_1 and F_0 are the observed fluorescence and the fluorescence level at saturating ligand concentration, respectively.

To assess the extent of AcChR labeling by IANBD, labeled membrane fragments prepared as described above were centrifuged in an SS34 rotor for 45 min at 18 000 rpm. The pellets were resuspended in 1 mL of 50 mM sodium phosphate, pH 7.5, and extracted with 1% Triton for 1 h at 4 °C. Following centrifugation to remove nonsolubilized material, the extract was diluted to give a Triton concentration of 0.1% and applied to a DE-52 column (1.4 × 3 cm) equilibrated in 50 mM sodium phosphate, pH 7.5, containing 0.1% Triton. After the column was extensively washed in the same buffer, the AcChR was eluted with 0.5 M NaCl. Fractions of 1.3 mL were collected and assayed for [^{125}I]-α-BTx binding activity and for bound NBD by measuring the absorbance at 490 nm by using an extinction coefficient of 24 mM^{-1} cm^{-1} (Lancet & Pecht, 1977).

For double labeling of the AcChR by IANBD and by [^{3}H]BrAcCh, membrane fragments were first reacted with IANBD as described above, recovered by centrifugation, and resuspended in 15 mM Tris-HCl, 150 mM NaCl, 4.5 mM NaN_3, and 1.5 mM EDTA, pH 8. Following assay for α-BTx binding activity, the sample was divided into three aliquots. Each aliquot was diluted to 1 μM under argon, and one was used as a control, one was reacted with 0.3 mM DTT, and the third was reacted with 0.3 mM DTT followed by 40 μM [^{3}H]BrAcCh as described above. For each sample, a control which had been incubated with excess α-BTx was treated in parallel. Samples were centrifuged and resuspended in 10 mM Hepes–35 mM $NaNO_3$, pH 7.4, to a final AcChR concentration of approximately 3 μM. The extent of [^{3}H]BrAcCh labeling was estimated as described above.

Results

Dependence of [^{3}H]BrAcCh Labeling on Reduction Conditions. The conditions used for reduction of the membrane-bound AcChR were rigorously controlled, and all labeling procedures were performed under an argon atmosphere in order to achieve reproducible results at low concentrations of DTT, possibly by preventing reoxidation of the reduced receptor. Figure 1 portrays the dependence of the extent of labeling on the concentration of DTT at [^{3}H]BrAcCh concentrations of 4 and 40 μM. In each case, a similar hyperbolic dependence on the reducing agent concentration was observed, and a plateau was reached at approximately 100 μM DTT. Covalent labeling of the AcChR by [^{3}H]BrAcCh was completely inhibited by prior incubation with excess α-BTx. The data shown (Figure 1) were corrected for nonspecific binding by using the results of parallel titrations of AcChR preparations which, after removal of the reducing agent by centrifugation, had been preincubated with excess α-BTx. Nonspecific labeling determined in this way represented a small fraction of the total binding (<10%) and was not significantly affected by the extent of reduction. For each sample, the number of α-BTx sites was measured before reaction with [^{3}H]BrAcCh, and this allowed quantitation of the relative numbers of binding sites for these ligands. As shown in Figure 1, a plateau was reached at a 1:1 ratio of sites for both concentrations of [^{3}H]BrAcCh.

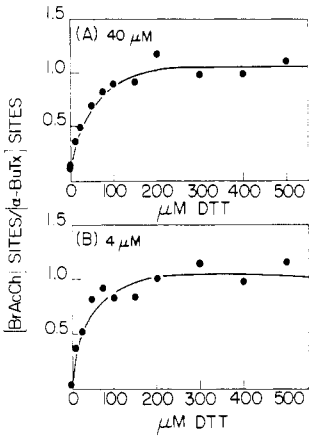

FIGURE 1: Effect of DTT concentration on the extent of labeling by (A) 40 and (B) 4 μM [^{3}H]BrAcCh. Data shown were corrected for nonspecific labeling by parallel titration in which membrane fragments were preincubated with excess α-BTx.

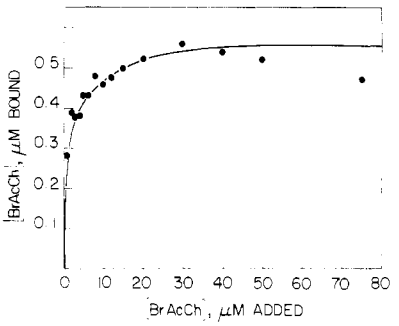

FIGURE 2: Effect of [^{3}H]BrAcCh concentration on the extent of labeling. AcChR-enriched membrane fragments were reduced with 0.3 mM DTT and then reacted with varying concentrations of [^{3}H]BrAcCh.

[^{3}H]BrAcCh Concentration Dependence of AcChR Labeling. Following reduction of AcChR preparations with 0.3 mM DTT, the extent of labeling measured 15 min after addition of [^{3}H]BrAcCh was concentration dependent below 10 μM, after which saturation of the labeling occurred (Figure 2). Such experiments gave no indication of heterogeneity in reaction sites: i.e., an apparently simple concentration dependence was observed. The amount of covalently bound [^{3}H]-BrAcCh using a concentration of 4 μM was only about 20% lower than that at 40 μM, and the results were therefore consistent with the similar extent of reaction achieved by these two concentrations of [^{3}H]BrAcCh at high DTT levels (Figure 1).

The effect of covalent labeling by [^{3}H]BrAcCh on the binding of α-BTx was also investigated. Prior to addition of [^{3}H]BrAcCh, aliquots of each sample were removed and assayed for [^{125}I]-α-BTx binding sites. The results obtained were compared with those observed after BrAcCh labeling. Loss

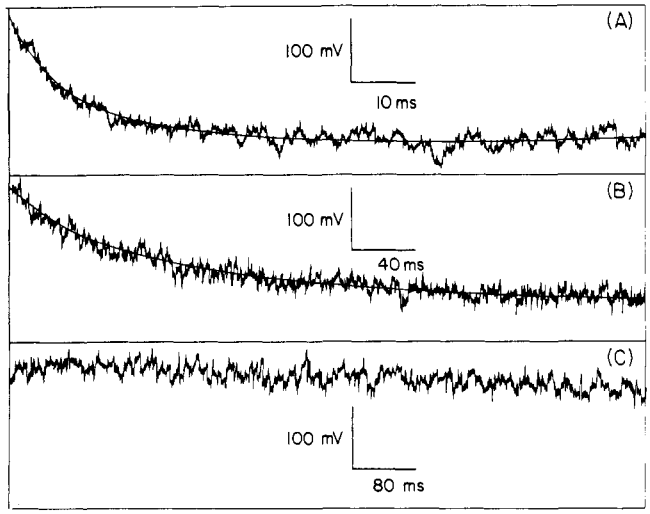

FIGURE 3: Kinetics of agonist-mediated Tl^+ flux. AcChR-enriched membrane vesicles loaded with the fluorophore ANTS were mixed with 10 μM AcCh and 17 mM $TlNO_3$ (final concentrations after mixing). Data were fit by a single exponential equation as described under Materials and Methods, and solid lines were calculated from the following best-fit parameters: (A) control membrane vesicles; A_1 = 352 mV, k_1 = 41.7 s^{-1}; (B) vesicles reduced with 0.3 mM DTT; A_1 = 273 mV, k_1 = 5.8 s^{-1}; (C) vesicles reduced with 0.3 mM DTT and alkylated by reaction with 40 μM BrAcCh.

of binding sites by centrifugation and resuspension were controlled by directly comparing bound [^{3}H]BrAcCh and [^{125}I]-α-BTx binding in the resuspended pellet. In these experiments, it was found that maximal [^{3}H]BrAcCh labeling inhibited only about 50% of the binding of [^{125}I]-α-BTx.

Effects of Reduction and BrAcCh Labeling on Cation Flux. When control (untreated by DTT, IANBD, or BrAcCh) membrane vesicles loaded with the fluorescent probe ANTS were mixed with Tl^+ in the absence of agonist, the resulting fluorescence change was a slow quench ($t_{1/2}$ ~10 s) due to leakage of Tl^+ across the membrane. Addition of AcCh led to a marked enhancement of ion flux as illustrated for 10 μM AcCh in Figure 3A. For AcChR preparations that had been reduced with 0.3 mM DTT before the vesicles were loaded with ANTS, a similar agonist-induced increase in cation flux was observed, although at the same AcCh concentration (10 μM) this rate was significantly slower than that measured for the control preparation (Figure 3B). In contrast, when reduction was followed by labeling with BrAcCh, the flux response was abolished (Figure 3C). The extent of BrAcCh labeling in this preparation was monitored by a parallel, smaller scale, reaction using radiolabeled ligand, and it was found that [^{3}H]BrAcCh labeled a number of binding sites equivalent to the number of α-BTx sites in the membrane preparation.

The effect of AcCh concentration on the rate of ion transport was measured for both control membrane preparations and those which had been reduced by 0.3 mM DTT. In each titration, at high agonist concentration the rate became too fast to be accurately measured by the stopped-flow technique: under these conditions, it was therefore necessary to inactivate a proportion of the AcChR molecules by incubation with 1–3 μM histrionicotoxin (HTX) (Moore & Raftery, 1980) to reduce the rate. True rates were then calculated by normalization to rates obtained for fully active preparations. The results of these titrations are show in Figure 4. For each preparation, the rate of cation influx increased with AcCh concentration, and the data could be fit by a simple model which assumed that binding of a single agonist molecule led to channel opening. Reduction of the AcChR preparation by

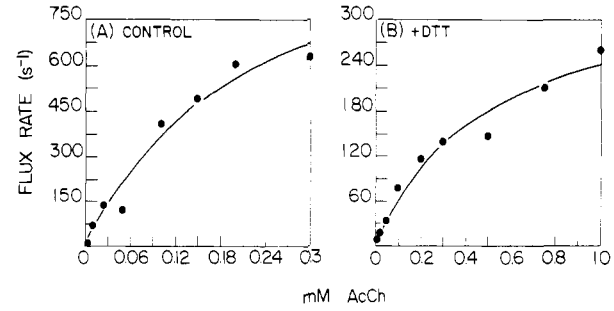

FIGURE 4: Effect of AcCh concentration on the rate of Tl^+ flux for (A) control vesicles and (B) preparations which had been reduced with 0.3 mM DTT. Solid lines were calculated from the fit of the data to a model where $k_{app} = k_{max}[L]/(K_d + [L])$, and best-fit parameters were as follows: (A) k_{max} = 1440 s^{-1}, K_d = 0.21 mM; (B) k_{max} = 356 s^{-1}, K_d = 0.48 mM.

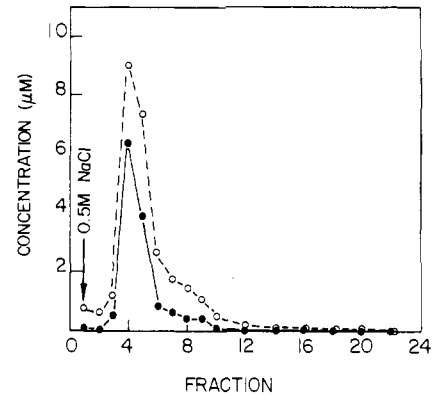

FIGURE 5: Elution of NBD-labeled AcChR from DE-52 cellulose. Fractions of 1.3 mL were collected and assayed for [^{125}I]-α-BTx sites (●) and for NBD absorbance (○) at 490 nm ($\epsilon \simeq 24\,000$ M^{-1} cm^{-1}).

DTT caused both a slight reduction in the maximal rate of ion transport (~3-fold) and a slight increase in the effective dissociation constant for AcCh.

Effect of Reduction and [^{3}H]BrAcCh Labeling on Agonist-Induced Changes in the Fluorescence of NBD-Labeled AcChR. The specificity of [^{3}H]BrAcCh labeling was ascertained by $NaDodSO_4$–polyacrylamide gel electrophoresis of a labeled sample. Gels were sliced and counted for ^{3}H, and it was shown that, in agreement with previous results (Chang et al., 1977; Damle et al., 1978; Moore & Raftery, 1979; Lyddiatt et al., 1979; Wolosin et al., 1980), only the 40K subunit was covalently labeled. It was of interest to investigate whether prior NBD labeling adversely affected the binding of BrAcCh to this site on the 40K subunit and whether BrAcCh labeling affected the properties of agonist binding to the low-affinity site. For this purpose, AcChR preparations were first labeled with the fluorescent probe IANBD following reduction with 50 μM DTT and reaction with 300 μM IANBD. This gave rise to a fluorescent receptor preparation that responded to the binding of agonists with a saturable fluorescent enhancement (at high ligand concentrations) that was completely blocked by incubation with excess α-BTx.

The extent of receptor modification by IANBD was investigated by solubilizing an NBD-labeled membrane preparation in Triton and adsorbing it to a column of DE-52 cellulose. After the column was extensively washed to remove all free fluorescent ligand, the absorbed NBD–AcChR was eluted by 0.5 M NaCl, and fractions were assayed for [^{125}I]-α-BTx binding activity and for absorbance at 490 nm, i.e., the absorbance maximum of the NBD chromophore. The elution profiles illustrated in Figure 5 show clear overlap of α-BTx binding activity and NBD absorbance and show that

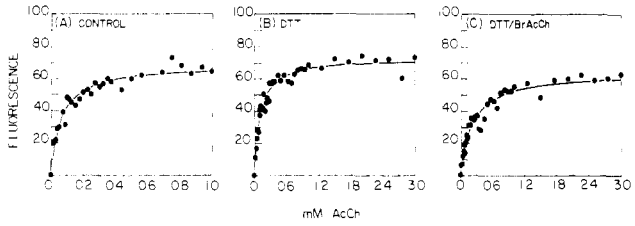

FIGURE 6: Effect of AcCh concentration on fluorescence changes of NBD-labeled membrane fragments. AcChR was first labeled with IANBD and then reduced or reduced and alkylated as described under Materials and Methods. AcChR ($\sim$2 μM in α-BTx sites) was titrated with AcCh, and the fluorescence level was recorded by using excitation and emission wavelengths of 482 and 540 nm, respectively. Data were fit by a simple binding isotherm, $F_1 = F_0[L]/(K_d + [L])$. (A) Control preparations: $F_0 = 68.7 \pm 0.4$; $K_d = 63 \pm 1$ μM. (B) AcChR reduced with 0.3 mM DTT: $F_0 = 73.0 \pm 0.3$; $K_d = 110 \pm 10$ μM. (C) AcChR reduced with 0.3 mM DTT and reacted with 40 μM [^{3}H]BrAcCh: $F_0 = 63.7 \pm 0.4$ μM; $K_d = 220 \pm 10$ μM.

approximately 1.5 molecules of NBD were bound per α-BTX binding site; i.e., an average of three molecules of NBD were covalently attached to each molecule of the AcChR.

Prior labeling with IANBD had no effect on the extent of labeling with 40 μM [^{3}H]BrAcCh upon reduction of the AcChR with 0.3 mM DTT; i.e., the ratio of bound [^{3}H]-BrAcCh to the measured number of [^{125}I]-α-BTX binding sites was 1:1, showing that covalently attached NBD does not inhibit BrAcCh labeling of its site on the 40K subunit. After the double labeling procedures, about 50% of the [^{125}I]-α-BTx binding activity was retained, in agreement with the results given earlier for preparations unlabeled by IANBD and labeled by [^{3}H]BrAcCh.

For investigation of the effects of BrAcCh labeling on the fluorescent changes induced by agonists on NBD-labeled AcChR, fluorescence titrations of NBD-labeled AcChR by AcCh were carried out by using untreated preparations, preparations which had been reduced with 0.3 mM DTT, and preparations which had been labeled by reaction with 40 μM [^{3}H]BrAcCh following reduction. In each case, enhancement of the NBD fluorescence was observed upon the addition of agonist, and this increase was completely absent in preparations which had been preincubated with excess α-BTx. The fluorescence enhancement always had a simple hyperbolic dependence on agonist concentration. In Figure 6, a typical experiment is shown where the apparent dissociation constants estimated for the control, the reduced, and the reduced–BrAcCh-alkylated preparations were 63, 110, and 220 μM, respectively. Maximal labeling of the AcChR by BrAcCh did not therefore significantly perturb agonist binding to its low-affinity site as revealed by changes in the fluorescence of covalently bound NBD, indicating that the two classes of sites for agonists are distinct.

Discussion

A major goal in elucidation of the function of the AcChR has been to mechanistically define the events mediated by ligand binding and leading to channel activation and to correlate such events with structural transitions of the AcChR. Various attempts have been made to correlate studies of direct ligand binding in vitro with the agonist concentration dependence of conductance changes measured both in vivo by electrophysiological techniques and in vitro by rapid kinetic measurements [reviewed in Conti-Tronconi & Raftery (1982)]. A major discrepancy has stemmed from the lack of agreement between apparent dissociation constants obtained for binding to the resting or to the high affinity desensitized states and those characterizing the permeability response. This has led to proposals of complex ligand-binding mechanisms involving sequential transitions of affinity states (Hess et al., 1979; Neubig & Cohen, 1980). The complexity of these mechanisms may sometimes arise from efforts to fit limited data to models conforming to preconceived notions of limited numbers of binding sites.

An alternative possibility is that the AcChR has multiple binding sites of greatly differing affinities for agonists and that different functional responses of the receptor may be mediated by agonist binding to different sites. This is suggested by the demonstration that the AcChR from all species and tissues studied so far is formed from homologous subunits (Raftery et al., 1980; Conti-Tronconi et al., 1982a,b), which makes it likely that homologous binding domains exist on some or all of the subunits. The data presented here strongly support such a possibility by showing that the sites labeled by BrAcCh are distinct from the low-affinity site(s) recently identified in *T. californica* AcChR (Dunn & Raftery, 1982a,b).

Before investigating the relationship between agonist binding sites, it was necessary to characterize the covalent labeling of the AcChR by BrAcCh. In agreement with Wolosin et al. (1980), using similar experimental conditions when maximal labeling with [^{3}H]BrAcCh was achieved, the number of sites labeled were equivalent to those present for α-BTx, and this labeling was specific for the 40-kdalton subunit. Wolosin et al. (1980) reported some indication of binding site heterogeneity in that the extent of labeling had a biphasic dependence on [^{3}H]BrAcCh concentration. In the present study, no such heterogeneity was found and, within the error of the experiments, a simple hyperbolic concentration dependence was observed. The extent of labeling was dependent also on the concentration of DTT, and therefore, the final stoichiometry of BrAcCh and α-BTx binding sites under submaximal conditions may be variable since it is determined by the balance between the concentration of the two reagents.

Preincubation of the AcChR with excess α-BTx completely inhibited specific alkylation by [^{3}H]BrAcCh. On the contrary, when all the available sites were labeled by BrAcCh, approximately 50% of α-BTx binding activity was consistently retained. This result is at variance with the complete inhibition of α-BTx binding reported by Wolosin et al. (1980). The origin of this discrepancy has not been established, but in view of the relative sizes of the two ligands and the likelihood that α-BTx (M_r 8000) has multiple points of interaction, it is not unreasonable that some toxin binding activity is preserved even when all the high-affinity sites on the 40K subunits are alkylated by BrAcCh.

In the recent past, a complex interaction of α-neurotoxin with AcChR has been proposed. Leprince et al. (1981) have claimed that α-BTx association with *T. californica* AcChR enriched membrane particles is composed of two kinetic processes as distinct from earlier studies of Blanchard et al. (1979), who described the association as a monophasic process. These different experimental results are readily explained by virtue of the fact that Leprince et al. (1981) used *Torpedo* membranes prepared by the method of Sobel et al. (1977), which yields degraded AcChR preparations in which only the lightest AcChR subunit retains its integrity. Such degraded AcChR preparations might not surprisingly yield complex kinetic patterns.

A low-affinity binding site which is specific for agonists has been revealed in *T. californica* AcChR by monitoring agonist-induced changes in the fluorescence of a probe (IANBD) which was covalently bound to the receptor protein (Dunn & Raftery, 1982a,b). Good correlation was found between

dissociation constants obtained in fluorescence titration experiments and those describing the concentration dependence of the flux response. These results suggested its involvement in channel activation. However, in these previous reports (Dunn & Raftery, 1982a,b), no estimate of the extent of the reaction of the AcChR with IANBD was made. It could not, therefore, be excluded that only a small percentage of receptor molecules was modified and that this fraction displayed abnormal binding behavior. Quantitation of the extent of receptor modification has now shown that for a labeled preparation displaying such typical fluorescence properties, approximately 1.5 molecules of NBD were incorporated per α-BTx site, i.e., $\sim$3 per AcChR monomer. The extent of this reaction indicates that the agonist binding properties revealed by fluorescence changes of the bound probe are indeed characteristic of the whole receptor population.

Prior labeling of the AcChR by IANBD had no effect on the number of sites available for labeling by [^{3}H]BrAcCh. The same site, therefore, is not labeled by both reagents, and this indicates that IANBD reacts at a location(s) removed from the high-affinity site on each of the two 40K subunits. When all available sites were occupied by [^{3}H]BrAcCh, the fluorescence enhancement occurring on agonist binding was unaltered, and neither reduction by DTT nor subsequent alkylation by BrAcCh affected the dissociation constant ($\sim$100 μM) for AcCh binding to the low-affinity site. This provides strong support for the notion that the two classes of agonist binding sites, i.e., the high-affinity sites on the 40K subunit labeled by BrAcCh and the low-affinity site revealed by NBD fluorescence, are distinct.

When *T. californica* AcChR was maximally alkylated by BrAcCh, the flux response was completely inhibited, and no permeability increase to Tl^+ was elicited by addition of AcCh at concentrations up to 1 mM. The only signal observed when vesicles loaded with the fluorophore ANTS were mixed with Tl^+ in the presence of agonist was a slow quench which is characteristic of the leakage of Tl^+ across the membrane. These results show that in *T. californica* covalently bound BrAcCh does not persistently activate the channel, as suggested by early electrophysiological experiments in *Electrophorus* electroplax (Silman & Karlin, 1969), but instead prevents the channel from opening, possibly by blocking the AcChR in a desensitized state. This complete inhibition of flux can be interpreted as being suggestive that both the high-affinity BrAcCh binding sites believed to exist on the AcChR molecule, i.e., on each of the two 40K subunits, were fully labeled, in concert with the conclusions drawn by Delegeane & McNamee (1980), who found that when only half of the number of toxin binding sites were labeled by BrAcCh an apparently normal slow flux of $^{22}Na^+$ was induced by agonists.

The inhibition of the flux response is a result of the alkylation by BrAcCh and not of the preliminary DTT reduction since after reduction alone the response was retained, although there was a slight reduction in the maximal flux and a slight increase in the concentration of acetylcholine eliciting a half-maximal response. This increase in the apparent dissociation constant agrees with results previously obtained by using a slow $^{22}Na^+$ filtration assay (Schiebler et al., 1977; Delegeane & McNamee, 1980; Walker et al., 1981). The decrease in maximal flux resulting from DTT reduction could not be detected in earlier studies because of the lack of temporal resolution of the filtration assay utilized.

Following maximal labeling by BrAcCh, when the permeability response to agonists was completely inhibited, agonist binding to the low-affinity sites was apparently unaltered as determined by the fluorescence response of NBD–AcChR to agonist. This result is consistent with the previous finding that the low-affinity site exists under equilibrium conditions, when the AcChR is presumably desensitized and no cation flux response is possible (Dunn & Raftery, 1982a,b). One interesting and highly attractive possibility is that the conformational transition revealed by changes in NBD fluorescence is a direct reflection of the conformational change which normally leads to the opening of the ion channel but that the presence of bound AcCh at another, perhaps remote site on the AcChR maintains the channel in a closed state despite AcCh occupancy of the low-affinity site.

The results we present show that there is a low-affinity binding site(s) for agonists in *T. californica* AcChR which is distinct from the well-documented site on the 40-kdalton subunit. Binding of agonists to each of these sites could trigger parallel, independent processes such as activation and desensitization. The equilibrium and kinetic properties of binding to the low-affinity site suggest that this site is involved in channel opening and that, on the other hand, the site labeled by BrAcCh could be involved in inactivation processes such as desensitization. Support for this interpretation comes from the results of previous studies in which the kinetics of agonist binding were monitored by a variety of fluorescence techniques [reviewed in Conti-Tronconi & Raftery (1982)]. In such studies, it is likely that binding to the high-affinity 40K site(s) was monitored since overall equilibrium constants calculated from the kinetic data were in good agreement with those measured in equilibrium measurements of agonist binding to the desensitized state of the AcChR (Heidmann & Changeux, 1979; Quast et al., 1979; Dunn et al., 1980) and the conformational transitions leading to the final state were all too slow to be correlated with channel opening. It is therefore likely that the transitions observed in such studies were related to desensitization or to other inactivation mechanisms.

Registry No. Acetylcholine, 51-84-3; bromoacetylcholine, 17139-54-7.

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Modification of Pyruvate, Phosphate Dikinase with Pyridoxal 5′-Phosphate: Evidence for a Catalytically Critical Lysine Residue†

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ABSTRACT: Pyruvate, phosphate dikinase from *Bacteroides symbiosus* is strongly inhibited by low concentrations of pyridoxal 5′-phosphate. The inactivation follows pseudo-first-order kinetics over an inhibitor concentration range of 0.1–2 mM. The inactivation is highly specific since pyridoxine and pyridoxamine 5′-phosphate, analogues of pyridoxal 5′-phosphate, which lack an aldehyde group, caused little or no inhibition even at high concentrations. The unreduced dikinase–pyridoxal 5′-phosphate complex displays an absorption maxima near 420 nm, typical for Schiff base formation. Following reduction of the Schiff base with sodium borohydride, N^6-pyridoxyllysine was identified in the acid hydrolysate. When the enzyme was incubated in the presence of pyridoxal 5′-phosphate and reducing agent, the ATP/AMP, P_i/PP_i, and pyruvate/phosphoenolpyruvate isotopic exchange reactions were inhibited to approximately the same extent, suggesting that the modification of the lysyl moiety causes changes in the enzyme that affect the reactivity of the pivotal histidyl residue. Phosphorylation of the histidyl group appears to prevent the inhibitor from attacking the lysine residue. On the other hand, addition of pyridoxal 5′-phosphate to the pyrophosphorylated enzyme promotes release of the pyrophosphate and yields the free enzyme which is subject to inhibition.

Pyruvate, phosphate dikinase (EC 2.7.9.1) catalyzes the reversible formation of phosphoenolpyruvate from pyruvate, a reaction which is dependent on ATP and inorganic phosphate.

$$\text{pyruvate} + \text{ATP} + P_i \rightleftharpoons \text{phosphoenolpyruvate} + \text{AMP} + PP_i \quad (1)$$

The overall reaction catalyzed by enzyme preparations from *Bacteroides symbiosus* and *Propionibacterium shermanii* in-

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