Walker, J. W., Lukas, R. J., & McNamee, M. G. (1981) *Biochemistry 20*, 2191-2199.
Weber, M., David-Pfeuty, T., & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3443-3447.
Weiland, G., Georgia, B., Wee, V. T., Chignell, C. F., & Taylor, P. (1976) *Mol. Pharmacol. 12*, 1091-1105.
Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., & Taylor, P. (1977) *J. Biol. Chem. 252*, 7648-7656.
Weill, C. L., McNamee, M. G., & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997-1003.
Witzemann, V., & Raftery, M. A. (1978) *Biochem. Biophys. Res. Commun. 81*, 1025-1031.
Wolosin, J. M., Lyddiatt, A., Dolly, J. O., & Barnard, E. A. (1980) *Eur. J. Biochem. 109*, 494-505.
Wu, W. C.-S., & Raftery, M. A. (1981) *Biochem. Biophys. Res. Commun. 99*, 436-444.
Wu, W. C.-S., Moore, H.-P. H., & Raftery, M. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 775-779.

Multiple Binding Sites for Agonists on *Torpedo californica* Acetylcholine Receptor†

Susan M. J. Dunn‡ and Michael A. Raftery*

ABSTRACT: The equilibrium and kinetic properties of agonist binding to the membrane-bound acetylcholine receptor from *Torpedo californica* have been measured by the fluorescence changes of a probe, 4-[[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3-benzoxadiazole, which was covalently bound to the receptor protein. Dissociation constants for the binding of several agonists have been measured in fluorescence titration experiments, and these are in good agreement with apparent equilibrium constants obtained from the concentration dependence of the cation flux response measured in quantitative in vitro kinetic experiments. These results provide evidence for the existence of a low-affinity binding site for agonists which is likely to be a functionally important site for channel activation. The kinetics of carbamylcholine and acetylcholine binding to this site have been measured in stopped-flow fluorescence experiments. Kinetic traces were recorded over a wide range of agonist concentrations, and all could be fit by a single exponential process whose rate and amplitude increased hyperbolically with the concentration of ligand. The observed signal change has been ascribed to a conformational transition of the receptor-ligand complex, and this occurs on a millisecond time scale at saturating ligand concentrations which is sufficiently fast to suggest a role for this binding site in the process of channel activation. These results indicate that in the *Torpedo* AcChR activation and desensitization may be parallel processes which are mediated by agonist association with different receptor binding sites.

The binding of acetylcholine to its nicotinic receptor at the neuromuscular junction results in the transient opening of cation-selective channels. Electrophysiological studies have shown that channel activation occurs within a few milliseconds of transmitter release, but, after prolonged exposure to agonist, the process of desensitization (Katz & Thesleff, 1957) leads to channel closing and a loss of the permeability response over a time scale of several seconds.

Much information on the ligand binding properties of the acetylcholine receptor (AcChR)[1] has come from studies of membrane-bound preparations purified from the electric organs of *Torpedo* species. *Torpedo* AcChR undergoes an agonist-induced conformational change to a state having higher affinity for these ligands, and this has been correlated with the process of pharmacological desensitization (Weber et al., 1975; Weiland et al., 1976, 1977; Lee et al., 1977; Quast et al., 1978a). Apparent dissociation constants for Carb binding to the resting and induced high-affinity states have been estimated from the time-dependent increase in Carb inhibition of the rate of α-neurotoxin binding to be $\sim$30 and 0.1 μM, respectively (Weiland et al., 1977; Quast et al., 1978a). This in vitro transition also occurs on a relatively slow time scale, and therefore to gain information on the ligand binding events leading to the functional response of channel opening, it is necessary to monitor the ligand-AcChR interaction on rapid time scales.

A variety of fluorescence techniques have recently been used for measuring the kinetics of binding of agonists to the membrane-bound AcChR. These include the monitoring of changes in the intrinsic fluorescence of the receptor protein (Bonner et al., 1976; Barrantes, 1976), the use of extrinsic probes, both noncovalent (Grunhagen & Changeux, 1976; Grunhagen et al., 1976, 1977; Schimerlik et al., 1979; Quast et al., 1978b, 1979) and covalent (Dunn et al., 1980), and the use of fluorescent analogues of acetylcholine (Heidmann & Changeux, 1979, 1980; Jürss et al., 1979). In all of these studies, conformational changes were observed, and a variety of

† From the Church Laboratory of Chemical Biology, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received February 26, 1982.* Supported by U.S. Public Health Service Grant NS-10294 and by grants from the Muscular Dystrophy Association, the Myasthenia Gravis Foundation (Los Angeles Chapter), and the Pew Charitable Trust. Contribution No. 6592.

‡ Present address: Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ, England.

[1] Abbreviations: AcChR, acetylcholine receptor; Carb, carbamylcholine; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium diiodide; IANBD, 4-[[(iodoacetoxy)ethyl]methylamino]-7-nitro-2,1,3-benzoxadiazole; α-BuTx, α-bungarotoxin; DEAE, diethylaminoethyl; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; HTX, histrionicotoxin; DTT, dithiothreitol; PTA, phenyltrimethylammonium chloride; AcCh, acetylcholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; NBD, 7-nitro-2,1,3-benzoxadiazole.

0006-2960/82/0421-6264$01.25/0

mechanisms have been proposed [see Conti-Tronconi & Raftery (1982)]. However, none of the transitions seem to be fast enough to be correlated with channel opening, and it is therefore likely that they arise from processes such as desensitization or other inactivation mechanisms.

An understanding of the role of the AcChR in synaptic transmission requires that a correlation be made between its ligand binding properties and the functional response of cation flux. Slow in vitro filtration assays based on one developed by Kasai & Changeux (1971) have been extensively used to measure agonist-stimulated $^{22}Na^+$ efflux from receptor-enriched vesicles. These techniques, however, yield limited information since their poor time resolution allows only integrated responses to be measured and activation of a small fraction of available channels may elicit a full flux response. Recently, techniques have been developed to measure ion flux on more rapid time scales. These include quench-flow filtration methods for monitoring $^{86}Rb^+$ (Hess et al., 1979) or $^{22}Na^+$ efflux (Neubig & Cohen, 1980) and a spectroscopic stopped-flow method for measuring the influx of Tl^+ by its quenching of the fluorescence of a fluorophore trapped within the vesicles (Moore & Raftery, 1980). Apparent K_d values for Carb activation estimated by using these techniques lay in the range 0.5–5 mM, which are in good agreement with those measured in electrophysiological studies of the frog neuromuscular junction (Dreyer et al., 1978; Dionne et al., 1978). However, these values do not agree well with the apparent affinity for Carb of the resting state of the AcChR prior to its conversion to the high-affinity desensitized form.

Affinity labeling techniques have shown that a high-affinity agonist or antagonist binding site exists on the AcChR subunit of M_r ~40K since, following reduction of a reactive disulfide bond near the site, it can readily be labeled by reaction with the alkylating agents bromoacetylcholine (Chang et al., 1977; Damle et al., 1978; Moore & Raftery, 1979; Lyddiatt et al., 1979; Wolosin et al., 1980) or MBTA (Weill et al., 1974; Karlin et al., 1975). It has been generally assumed that agonist binding to this 40K site leads to both channel activation and desensitization. Complicated kinetic schemes involving sequential conformational changes have therefore been proposed to account for the different affinity states of the AcChR (Hess et al., 1979; Neubig & Cohen, 1980).

Recently, preliminary evidence has been obtained for the existence of a low-affinity binding site for agonists which is present under both initial and equilibrium conditions and which is likely to be a functionally important site for channel activation (Dunn & Raftery, 1982). This site was revealed by monitoring the agonist-induced fluorescence changes of a probe, IANBD, which had been covalently reacted with the *Torpedo* AcChR.

In this paper, we describe equilibrium and kinetic properties of agonist binding to NBD-labeled AcChR from *Torpedo californica*. We present evidence for the existence of a low-affinity binding site and suggest that the receptor properties of channel opening and desensitization may be mediated by agonist binding to different binding sites on the receptor molecule.

Materials and Methods

AcChR-enriched membrane fragments were prepared from *Torpedo californica* electric organs as previously described (Elliott et al., 1980) with the exception that iodoacetamide was excluded from the initial homogenization. The preparations were routinely subjected to alkali extraction to remove nonreceptor proteins (Elliott et al., 1979; Neubig et al., 1979). [^{125}I]-α-BuTx was obtained from New England Nuclear and was isotopically diluted with α-BuTx purified by the procedures of Clark et al. (1972) before calibration by the method of Blanchard et al. (1979a). The concentration of α-BuTx sites was measured by the DEAE disc assay of Schmidt & Raftery (1973), and protein was determined by the method of Lowry et al. (1951). Specific activities of purified preparations lay in the range 2–4 nmol of α-BuTx sites/mg of protein. The buffer used in the final stages of purification and, unless otherwise stated, in each experiment was 10 mM Hepes and 35 mM $NaNO_3$, pH 7.4.

Labeling of the membrane-bound AcChR by IANBD (Molecular Probes Inc.) was achieved by first incubating the AcChR (10 μM in α-BuTx sites) with 50 μM DTT for 20 min at room temperature. IANBD, which is only sparingly soluble in aqueous solution, was then added as a finely ground powder to a final concentration of approximately 300 μM. The mixture was shielded from light and stirred for 2 h at 4 °C. Residual solid IANBD and unreacted reagent were separated by passing the mixture through a Sephadex G-25-300 column (1.7 × 24 cm) equilibrated in 10 mM Hepes and 35 mM $NaNO_3$, pH 7.4, and collecting the membrane fragments which eluted in the void volume.

Equilibrium fluorescence measurements were made by using a Perkin-Elmer MPF-4 spectrofluorometer thermostated at 25 °C. Apparent dissociation constants were estimated from fluorescence titrations in which relatively small volumes of ligand were added to 2 mL of AcChR (~2 μM in α-BuTx sites). Fluorescence levels were recorded immediately after ligand addition, and data were corrected for nonspecific effects from the results of parallel titrations of AcChR which had been preincubated with an approximately 3-fold excess of α-BuTx. Titration data were analyzed by using the nonlinear regression method described below. Membrane fragments were assayed for acetylcholinesterase activity by the method of Ellman et al. (1961), and only those preparations which were devoid of activity were used for titration by AcCh.

Kinetic data were obtained by using the stopped-flow instrumentation and data collection procedures previously described (Dunn et al., 1980, 1981), using an excitation wavelength of 482 nm and monitoring fluorescence emission with an OG-515 emission filter (Melles Griot). Unless otherwise stated, all kinetic experiments were carried out at 25 °C. Stopped-flow data were analyzed by a nonlinear regression program using the algorithm of Marquardt (1963). The data were fit by the single-exponential equation

$$F(t) = A_0 + A_1 \exp(-k_1 t) + k_0 t$$

where $F(t)$ and A_0 are the fluorescence levels at time t and equilibrium, respectively, k_1 is the rate constant and A_1 the amplitude of the signal change, and k_0 is the slope of the base line used to correct for the small linear contribution due to photolysis.

The kinetics of agonist-mediated cation flux were measured by using the method of Moore & Raftery (1980). AcChR-enriched membrane vesicles, which had not undergone preliminary labeling by IANBD, were loaded with the fluorescent probe ANTS (Chemical Services). Following removal of free ANTS by gel filtration, the kinetics of Tl^+ influx were monitored by using the stopped-flow method previously described (Moore & Raftery, 1980). Flux data were analyzed by using the following equation:

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

where the term KT_∞ was fixed by using the known final concentration of Tl^+ (T_∞) of 17 mM and the Stern–Volmer quenching constant (K) of 96 M^{-1} (Moore & Raftery, 1980).

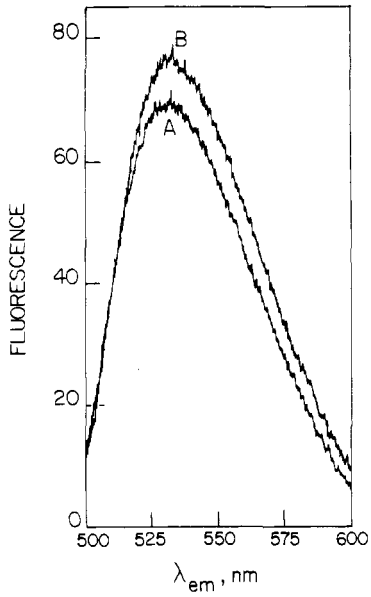

FIGURE 1: Fluorescence spectra of NBD-labeled AcChR before (A) and after (B) addition of 15 mM Carb. AcChR concentration was approximately 2 μM in α-BuTx sites, and the excitation wavelength was 482 nm.

The binding of [^{3}H]Carb or [^{3}H]HTX was measured by the centrifugation method described by Dunn et al. (1980).

Samples for sucrose gradients were prepared by solubilizing NBD-labeled membrane fragments (~4 mg/mL) in 1% Triton X-100 and incubating them with a substoichiometric concentration of [^{125}I]-α-BuTx for 30 min, at room temperature. Linear gradients (4–20% sucrose) in 10 mM sodium phosphate, pH 7.5, 50 mM NaCl, 0.2% Brij 35, and 0.02% NaN_3 were poured by using a Beckman gradient maker (volume = 13 mL). Samples of 0.25 mL were applied to each gradient, and the gradients were centrifuged for 16 h at 40000 rpm in an SW-41 rotor. Fractions of 15 drops were collected from the bottom of the tube, and each fraction was counted for ^{125}I. Corresponding fractions from four gradients were pooled, and the fluorescence of each sample was measured by using excitation and emission wavelengths of 482 and 540 nm, respectively.

Results

Labeling of AcChR-Enriched Membrane Preparations by IANBD. Following very mild reduction by DTT, reaction of AcChR-enriched membrane fragments with the alkylating reagent IANBD gave rise to a highly fluorescent membrane preparation which exhibited broad maxima for excitation at ~480 nm and emission at ~535 nm (Figure 1). The fluorescence was enhanced upon addition of Carb (Figure 1), and this enhancement was found to be saturable at high ligand concentration and to be completely blocked by prior incubation of the labeled receptor with α-BuTx, suggesting that it was specific for the AcChR. The poor solubility of IANBD in aqueous solution necessitated its addition as a solid. However, its solubility was improved in the presence of the membrane fragments, and most dissolved during the 2-h incubation period. Gel filtration on Sephadex G-25 was efficient in removing unreacted reagent, and this was an essential step in the preparation of labeled AcChR whose fluorescence was stable with time and free of artifacts arising from photolysis of free fluorophore.

Figure 2 shows sucrose gradient profiles of the NBD-labeled AcChR–[^{125}I]-α-BuTx complex. Clear overlap of the fluorescent peaks with toxin binding activity was observed, showing

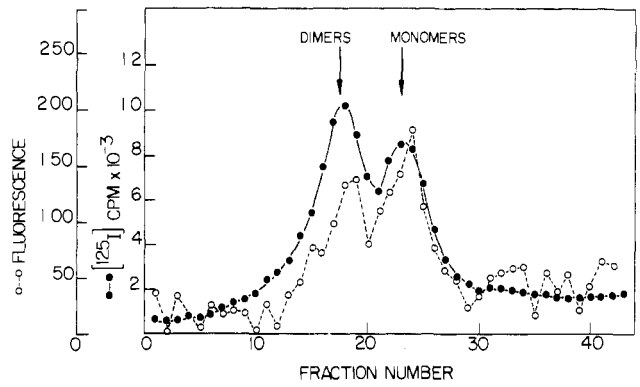

FIGURE 2: Sucrose-gradient profiles of NBD-labeled AcChR. Samples were incubated with [^{125}I]-α-BuTx and solubilized in 1% Triton before being run on sucrose gradients as described under Materials and Methods. Fractions were collected and measured for toxin binding activity (●) and fluorescence (○) by using excitation and emission wavelengths of 482 and 540 nm, respectively. Data shown represent pooled results from four gradients.

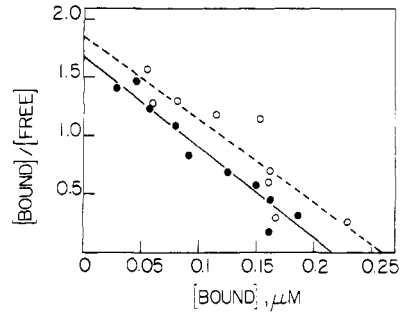

FIGURE 3: Scatchard plots of [^{3}H]Carb binding to control (○) and NBD-labeled AcChR (●). The concentration of α-BuTx sites was 0.3 ● 0.01 μM for the control and 0.30 ± 0.02 μM for the NBD-labeled preparation. Linear least-squares fit of the data gave the following: control, R_0 = 0.26 ± 0.03 μM, K_d = 0.14 ± 0.03 μM; NBD labeled, R_0 = 0.22 ± 0.03 μM, K_d = 0.13 ± 0.01 μM.

that the NBD label is associated with both the monomeric and dimeric forms of the AcChR.

Effect of NBD Labeling on AcChR Properties. A prerequisite for the use of extrinsic probes to study functional properties of the AcChR is that introduction of the probe does not itself perturb receptor function, thus validating the extrapolation of results obtained to unmodified preparations. Reaction of the membrane-bound receptor with IANBD did not appear to alter its equilibrium binding properties. Figure 3 shows Scatchard plots for [^{3}H]Carb binding to control and NBD-labeled membrane fragments, and the results indicate that neither the K_d nor the number of high-affinity binding sites for Carb was significantly affected by the modification procedures. It has previously been reported that NBD labeling did not affect the ability of the AcChR to mediate agonist-induced Tl^+ flux and that the flux response displayed the expected pharmacological behavior in that it was completely inhibited by incubation with HTX (Dunn & Raftery, 1982). Direct binding of [^{3}H]HTX was also measured by centrifugation assay, and no effect of NBD labeling on either the K_d (0.5 ± 0.1 μM) or the number of binding sites was found. It therefore seems likely that NBD labeling does not adversely affect the ligand binding or functional properties of AcChR measured in vitro.

Effect of Different Agonists on the Equilibrium Fluorescence of NBD-Labeled AcChR and on the Rate of Tl^+ Flux. Addition of agonists to NBD-labeled preparations led to a concentration-dependent increase in fluorescence which could be adequately described by a simple binding isotherm as shown

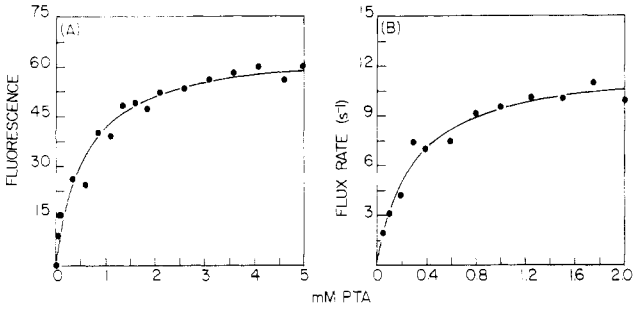

FIGURE 4: (A) Effect of PTA concentration on the fluorescence of NBD-labeled AcChR. Data were fit as described in Figure 4, and the solid line was calculated by using $F_0 = 65.8 \pm 0.7$ and $K_d = 0.59 \pm 0.03$ mM. (B) Effect of PTA concentration on the Tl^+ flux rate. Best-fit parameters were $k_{max} = 12.1 \pm 0.9$ s^{-1} and $K_d = 0.29 \pm 0.07$ mM. Data were pooled from two separate titrations.

Table I: Effects of Agonists on the Fluorescence of NBD-Labeled AcChR and on the Parameters of Tl^+ Flux

ligand	fluorescence K_d	Tl^+ flux K_d	k_{max}* (s^{-1})
AcCh	79.2 ± 11.4 μM[d]	140 μM[a]	923[a]
		44 μM[b,f]	1440[e]
Carb	0.86 ± 0.14 mM[d]	5 mM[c]	1500[b]
		1 mM[c,f]	
		0.5 mM[b,f]	
nicotine	0.88 ± 0.26 mM	0.26 ± 0.09 mM	9.1
PTA	0.47 ± 0.09 mM	0.29 ± 0.07 mM	12.1
choline	~35 mM	–	

[a] Data from Blanchard et al. (1982). [b] Data from Wu et al. (1981). [c] Data from Moore & Raftery (1980). [d] Data from Dunn & Raftery (1982). [e] Data from Dunn et al. (unpublished results). [f] Data were fitted to the model which assumed that binding of two ligand molecules was required for opening of the channel [$k_{app} = k_{max}/(1 + K_d/[L])^2$]. Other data were fitted to the single ligand binding model: fluorescence, $F = F_0[L]/(K_d + [L])$; Tl^+ flux, $k_{app} = k_{max}[L]/(K_d + [L])$.

for PTA in Figure 4A. Equilibrium dissociation constants obtained from fluorescence titrations using a number of agonists are listed in Table I. As previously reported for AcCh and Carb (Dunn & Raftery, 1982), no evidence for cooperative binding of ligands was observed in these experiments, and Hill coefficients not significantly different from 1 were found in each case.

The ability of nicotine and PTA to induce an increase in the rate of Tl^+ flux was also investigated, and the results are illustrated for PTA in Figure 4B. When membrane vesicles loaded with the fluorophore ANTS were rapidly mixed with Tl^+ in the absence of agonist, the observed signal change was a slow quench ($t_{1/2} \sim 10$ s) characteristic of the leakage of Tl^+ across the membranes. Both PTA and nicotine enhanced the rate of Tl^+ transport, and the rate increased hyperbolically with agonist concentration, allowing apparent K_d values to be estimated. In Table I, the values of dissociation constants obtained from fluorescence titration data for a number of agonists are compared with those from Tl^+ flux experiments. It is clear that good correlation exists between the direct ligand binding data and the concentration dependence of the flux response.

Although both nicotine and PTA significantly enhanced the rate of Tl^+ flux, the maximal flux rate measured at high concentrations of either of these ligands was only ~10 s. This rate is much less than that previously measured for either Carb or AcCh as agonists (Table I). The same preparations used for measuring the response to nicotine and PTA displayed normal, faster transport rates when mixed with Carb, indicating that the ability of the AcChR to mediate ion flux is strongly dependent on the nature of the agonist.

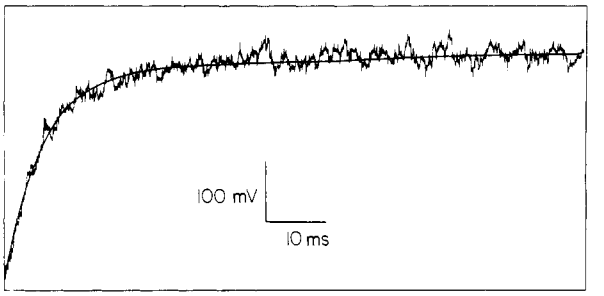

FIGURE 5: Kinetics of Carb binding to NBD-labeled AcChR. Membrane fragments (1–2 μM in α-BuTx sites) were mixed with 3 mM Carb. The solid line was calculated from data fitting of a single exponential by using $A_1 = 498$ mV and $k_1 = 152$ s^{-1}.

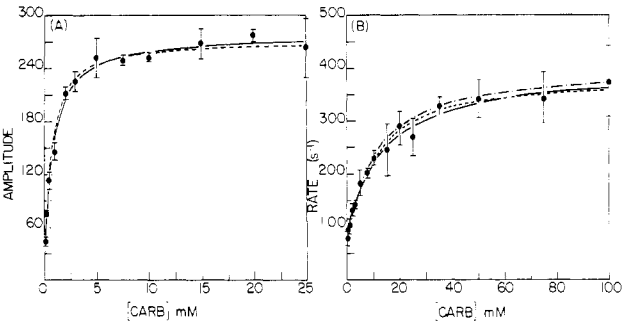

FIGURE 6: Effect of Carb concentration on the kinetics of Carb binding to NBD-labeled AcChR. (A) Amplitude: (●—●) fit of mechanisms 1 and 3 (Table IIA) to data by using best-fit parameters given in Table IIB; (●---●) fit of mechanism 2. (B) Rate: lines calculated from best-fit parameters given in Table IIB to mechanisms 1 (●—●), 2 (●---●), and 3 (●··●). Each point is the average of at least four determinations, and error bars represent the standard deviation.

Kinetics of Carb Binding to NBD-Labeled AcChR. When Carb was rapidly mixed with NBD-labeled membrane fragments, the fluorescence signal change observed was an enhancement occurring on a fast time scale as shown in Figure 5. This signal could be adequately fit by a single exponential model over a wide range of Carb concentrations. Both the rate and amplitude of this process increased hyperbolically with agonist concentration (Figure 6), the rate reaching a saturating value of ~400 s^{-1}. In this high ligand concentration range, the rate was sufficiently high that a significant portion of the signal change was lost within the dead time of the stopped-flow instrument (~2 ms). Under these conditions, in order to reduce the inevitable artifacts introduced by this effect into the data analysis procedures, it was occasionally necessary to fix the value of the amplitude (A_1, see Materials and Methods) by using the known amplitude at saturation.

The effect of Carb concentration on the amplitude of the kinetic signal (Figure 6A) closely paralleled its effect on fluorescence levels measured in equilibrium experiments, and the data could be fitted by a simple binding process having a K_d of 0.73 mM (cf. Table I). The saturation of the observed rate at high Carb concentration indicates that the signal arises not from a bimolecular reaction but rather from an isomerization of the receptor–ligand complex. Attempts to find a mechanism which adequately accounts for both the amplitude and rate data have indicated systematic deviations from simple models. Figure 7 shows the fit of the data to the three mechanisms presented in Table IIA. In each case, good fits were obtained, but, as described below and listed in Table IIB, some discrepancy exists between the overall equilibrium constants measured from the amplitude data and those calculated

Table II

(A) Kinetics of Mechanisms Considered for Interaction of Agonists and NBD-Labeled AcChR

	1	2	3
mechanism[a]	$R + L \overset{K_1}{\rightleftharpoons} RL \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} R^*L$	$R + L \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} RL \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} RL_2 \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} R^*L_2$	$R \overset{K_1}{\rightleftharpoons} RL$; $k_0 \downarrow\uparrow k_{-0}$; $k_3 \downarrow\uparrow k_{-3}$; $R' \overset{K_2}{\rightleftharpoons} R'L$
definitions	$K_2 = k_{-2}/k_2$	$k_1 = 2k_2$; $2k_{-1} = k_{-2}$	$K_0 = k_{-0}/k_0$; $K_3 = k_{-3}/k_3$
apparent rate	$k_{-2} + k_2[L]/(K_1 + [L])$	$k_{-3} + k_3[[L]/([L] + k_{-1}/k_2)]^2$	$(k_{-0} + k_{-3}[L]/K_2)/(1 + [L]/K_2) + (k_0 + k_3[L]/K_1)/(1 + [L]/K_1)$
amplitude	$\sim QR_0[L]/(K + [L])$ where $K = K_1K_2$	$\sim QR_0[[L]/(K + [L])]^2$ where $K = [k_{-3}/(k_3 + k_{-3})]k_{-1}/k_2$	$\sim QR_0[L]/(K + [L])$ where $K = (1 + 1/K_0)/[1/K_1 + 1/(K_0K_2)]$
ref	Hammes & Wu (1974)	Adams (1981); Colquhoun (1975)	Janin (1973)

(B) Kinetic Parameters Derived from Fit of Data to Above Mechanisms

mechanism	parameters	Carb (25 °C)	AcCh (25 °C)	AcCh (12.5 °C)
1	rate			
	K_1 (mM)	13.3	22.6	6.9
	k_2 (s^{-1})	317	588	178
	k_{-2} (s^{-1})	83	21.8	10
	amplitude, K (mM)	0.73	0.053	0.088
	calcd K from rate parameters (mM)	3.5	0.84	0.39
2	rate			
	k_{-1}/k_2 (mM)	4.8	6.2	2.6
	k_3 (s^{-1})	291	498	166
	k_{-3} (s^{-1})	93	31	14
	amplitude, K (mM)	0.28	0.021	0.034
	calcd K from rate parameters (mM)	1.2	0.36	0.20
3	rate			
	K_1 (mM)	9.1	17.5	6.2
	K_2 (mM)	0.11	0.05	0.013
	k_0 (s^{-1})	18	12	2.5
	k_{-0} (s^{-1})	130	24	17
	k_3 (s^{-1})	385	577	186
	k_{-3} (s^{-1})	18	2.9	2.6
	amplitude, K (mM)	0.73	0.053	0.088
	calcd K from rate parameters (mM)	0.83	0.14	0.10

[a] In each mechanism, the ligand binding steps are assumed to be too fast to be detected, and the signal change arises in the receptor isomerization step(s).

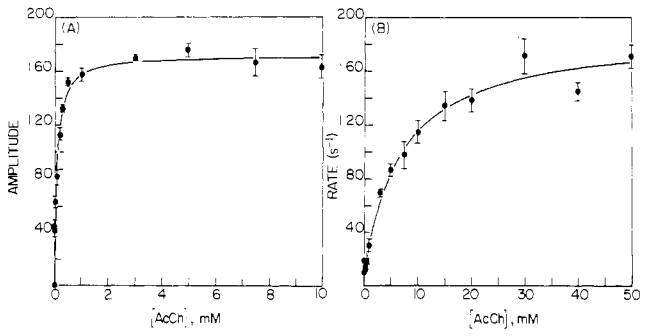

FIGURE 7: Effect of concentration on the kinetics of AcCh binding at 12.5 °C. The solid lines represent the fit of the data to mechanism 1: (A) amplitude, K = 0.088 mM, QR_0 = 171.2 ± 0.5; (B) rate, K_1 = 6.9 ± 0.1 mM, k_2 = 178 ± 1 s^{-1}, and k_{-2} = 10.0 ± 0.4 s^{-1}.

from the best-fit parameters obtained from the rate data.

In mechanism 1, the formation of the receptor–Carb complex is followed by a single conformational change. While this is the simplest mechanism which predicts a hyperbolic concentration dependence of both the rate and amplitude, the best-fit parameters listed in Table IIB indicate an approximately 5-fold discrepancy in the apparent dissociation constants (K) obtained from the two sets of data.

Mechanism 2 has frequently been invoked to describe the dose dependency of conductance changes measured in electrophysiological experiments [see Adams (1981)]. According to this model, the AcChR has two binding sites for agonists with identical affinities, but both sites must be occupied before the conformational change can occur. Since the binding sites are assumed to be independent, no cooperativity in ligand binding would be expected. For this model, as for mechanism 1, the K calculated from the best-fit rate parameters was significantly higher than that obtained from the amplitude data.

According to mechanism 3, two different conformations of receptor exist prior to addition of agonist, and the signal change arises in the receptor conformational changes R ⇌ R′ and RL ⇌ R′L. This is the two-state model often used to describe desensitization in vivo (Katz & Thesleff, 1957; Rang & Ritter, 1970) in which R represents the resting state and R′ the desensitized state. However, the physical interpretation of the two states R and R′ suggested here would be much different since both would be low-affinity receptor forms and the affinity of neither form would be consistent with the affinity of the desensitized state for agonists. The procedures described by Janin (1973) were used in the fitting of mechanism 3, and examination of Table IIB shows that this mechanism gives the best correlation between the best-fit parameters for the rate and amplitude data. However, it should be emphasized that the nature of this mechanism requires that initial estimates of the best-fit parameters for the rate data be obtained from the predetermined value of K from the concentration dependence of the amplitude [see Janin, (1973)]. Thus, the two sets of data are not analyzed independently, and, as a consequence of such force fitting, good agreement between measured and calculated dissociation constants may be misleading. Many different concentration dependencies may conform to the predictions of this mechanism, but the rate constants k_0 and k_{-0}, which determine the initial equilibrium of the two receptor

forms, should be independent of the agonist used. In the present experiments, some variability is to be expected because of the use of different receptor preparations. However, small systematic deviations have been found for different ligands.

Kinetics of AcCh Binding to NBD-Labeled AcChR. The effect of AcCh concentration on the kinetics of AcCh binding to NBD-labeled AcChR was qualitatively similar to the effect of Carb. Both the rate and amplitude increased with ligand concentration, but the saturating rate ($\sim$600 s^{-1} extrapolated) was significantly higher in the case of AcCh. These kinetic data could be adequately fit by the three mechanisms presented in Table IIA, and the kinetic parameters derived are given in Table IIB. Comparison of overall equilibrium constants, K, obtained from the amplitude and rate data shows inconsistencies similar to those described above for Carb binding.

A major consideration in interpretation of the kinetic data is that artifacts may be introduced at high concentrations of either AcCh or Carb because of the rapidity of the process which approaches the limitations of the stopped-flow technique. A titration of NBD-labeled AcChR by AcCh was therefore carried out at lower temperature, i.e., 12.5 °C, to reduce the rate of the signal change and therefore also to reduce the risk of artificial lowering of the measured rate constants at high ligand concentration. The results are illustrated in Figure 7 and show clear saturation of the observed rates even when they were well within the measurement capability of the instrumentation. These data also have been analyzed in terms of all three mechanisms, and the best-fit parameters are listed in Table IIB.

Comparison of the measured rate constants for AcCh binding at 12.5 and 25 °C also allows the calculation of the Q_{10} for the observed transition. Assuming that the process conforms to the Arrhenius law

$$\ln k_{app} = Ae^{-\Delta H/(RT)}$$

a Q_{10} of 2.5 may be calculated for the saturating rate at high ligand concentrations.

Specificity of the Ligand-Induced Fluorescence Changes of NBD-Labeled AcChR. The fluorescence enhancement occurring on the binding of ligands to NBD-labeled AcChR is likely to be agonist specific since other ligands such as curare, HTX, and lidocaine altered neither the equilibrium fluorescence level nor the agonist-induced fluorescence change (Dunn & Raftery, 1982). These inhibitory ligands do not appear to compete directly with agonists for this low-affinity site and must therefore exert their effects on receptor function in an indirect manner. Titration of NBD-labeled AcChR by Carb in the presence of saturating concentrations of HTX, lidocaine, or curare has shown that not only the equilibrium binding but also the kinetics of binding of Carb are unaffected by the presence of these ligands.

In order to investigate whether desensitization affected agonist binding to this site, we incubated NBD-labeled membrane fragments with 10 μM Carb, a concentration sufficient to induce conversion of the AcChR to its high-affinity form but low enough not to significantly bind to the low-affinity site and enhance the NBD fluorescence. The rate of binding of higher concentrations of Carb to such desensitized preparations, as monitored by the change in fluorescence, was not significantly different from that of binding to control preparations.

Discussion

Fluorescence titrations of NBD-labeled AcChR preparations from *Torpedo californica* have revealed the existence of a low-affinity agonist-specific binding site whose equilibrium properties appear to be closely correlated with the agonist concentration dependence of ion flux. This suggests that AcChR from *Torpedo*, and perhaps also from other species, has multiple binding sites for agonists and that different receptor properties such as channel activation or desensitization may be the result of parallel rather than sequential ligand binding pathways.

It has been generally assumed that the binding site located on the 40K subunit, which can be labeled by the affinity reagents MBTA (Weill et al., 1974; Karlin et al., 1975) or bromoacetylcholine (Chang et al., 1977; Damle et al., 1978; Moore & Raftery, 1979; Lyddiatt et al., 1979; Wolosin et al., 1980) by virtue of the existence of a readily reducible disulfide bond near the site, is responsible for both activation and desensitization. However, the demonstration of extensive sequence homology among all the receptor subunits supports the notion that multiple binding sites exist (Raftery et al., 1980). Other evidence for this has come from the observed labeling of more than one subunit by cholinergic affinity reagents which do not depend on disulfide bond reduction (Hucho et al., 1976; Witzemann & Raftery, 1977).

A variety of stopped-flow fluorescence techniques have been used to monitor the interaction of AcChR with agonists, but the conformational changes observed in these studies were all too slow to account for channel activation. It is likely that in these experiments binding of agonists to the previously identified binding site on the 40K subunit was monitored since apparent dissociation constants calculated from the kinetic data were in good agreement with those measured for binding to the desensitized state in equilibrium experiments in which radiolabeled ligands were used (Heidmann & Changeux, 1979; Quast et al., 1979; Dunn et al., 1980). It is quite possible that binding of agonists to another binding site, associated perhaps with a subunit other than that of M_r 40K, would not be detected by these approaches.

The fluorescent reagent IANBD has previously been used to label a thiol group in the globular head region of myosin (Haugland, 1975). The probe has low aqueous solubility and is likely to react covalently and develop appreciable fluorescence only in a hydrophobic location. It is therefore not unreasonable to propose that this probe should reflect conformational changes in parts of the AcChR which could not be monitored by using more water-soluble reagents. A mild reduction of the AcChR was carried out before the IANBD labeling reaction. This reduction step was found to make the labeling reaction highly reproducible, but in many early experiments, labeling could be achieved without reduction, suggesting that IANBD reacts with a normally free sulfhydryl group and not with a disulfide bond originally present in the AcChR.

The conditions used for labeling by IANBD are likely to have caused at least partial reduction of the reactive disulfide bond near the high-affinity agonist binding site(s) on the 40-kdalton subunit(s). It is, however, unlikely that the fluorophore reacts at this position since the number of sites available for covalent reaction with bromo[^{3}H]acetylcholine has been shown to be unaffected by prior labeling by IANBD (Conti-Tronconi et al., 1982; S. M. J. Dunn, B. M. Conti-Tronconi, and M. A. Raftery, unpublished results). This finding strongly supports the idea that IANBD reacts at a location removed from this (these) site(s) on the 40K subunit(s). Such behavior is quite different from that displayed by another covalent fluorescent probe, 5-(iodoacetamido)-salicylic acid (IAS), which has been used previously in studies

of ligand binding to the AcChR (Dunn et al., 1980, 1981; Blanchard et al., 1982). Following mild reduction of the AcChR, reaction with IAS inhibited subsequent covalent labeling by bromo[^{3}H]acetylcholine (Dunn et al., 1980), and it is likely that the usefulness of this probe as a monitor of agonist binding to the AcChR is due to its proximity to the high-affinity agonist binding site(s) by virtue of its reaction with the reduced disulfide nearby. The two fluorescent probes, IAS and IANBD, therefore react at different locations, probably as a result of their differences in size and aqueous solubility, and reflect different agonist-mediated conformational changes of the receptor.

Ligand binding and functional properties of the AcChR did not appear to be adversely affected by labeling with IANBD. The NBD chromophore has been demonstrated to be associated with the AcChR protein since sucrose gradient profiles of labeled and solubilized receptor preparations showed clear overlap of the [^{125}I]-α-BuTx binding activity with NBD fluorescence. Unfortunately, it has not yet been possible to quantitate the extent of reaction of AcChR with IANBD. It cannot, therefore, be excluded that only a small percentage of receptor molecules were modified and that the properties of ligand binding to this fraction, as reflected by fluorescence changes of bound NBD, are not characteristic of the whole receptor population. However, the high reproducibility of the labeling reaction, the magnitude and consistency of the agonist-induced fluorescence changes, and the lack of a detrimental effect on receptor properties, such as the number of high-affinity binding sites measured by using radiolabeled ligands, would indicate a reasonable extent of reaction and a lack of adverse effect of the labeling procedures.

Apparent K_d values estimated from fluorescence titrations of NBD-labeled AcChR and from the agonist concentration dependence of the Tl^+ flux response are in excellent agreement. This is convincing evidence for the existence of a functionally important agonist binding site which has lower affinity for these ligands than has previously been measured in direct ligand binding studies.

It should be emphasized that this low-affinity binding site exists under both initial and equilibrium conditions, and therefore, binding of agonist to this site is independent of receptor desensitization. This has two important consequences: (1) the site cannot represent a transient conformational state of the receptor, and therefore binding to this site must be a process parallel to the binding of agonist to another site whose occupancy leads to the increase in affinity which has been correlated with desensitization; (2) agonist binding to this site occurs under conditions where channel opening is prevented. It is possible that the observed fluorescence change directly reflects the conformational change which leads to channel opening when the receptor is originally in the resting state. This same transition may be induced even after desensitization, but under these conditions, agonist binding to the site involved in desensitization may have induced a conformational change in another, perhaps remote, part of the molecule which maintains the channel in a closed state. Activation and desensitization would therefore be independent and parallel pathways, and while agonist binding to the low-affinity site would be unaffected by occupancy of the site involved in desensitization, channel opening would only be possible at times preceding the slow transition in affinity state of this latter site.

The kinetics of binding of agonists to NBD-labeled AcChR were apparently rather simple, and the signal change observed was an exponential process whose rate and amplitude increased hyperbolically with ligand concentration. Three mechanisms (Table II) have been presented which provide adequate fits to the data although systematic deviations were found between the best-fit parameters obtained from the amplitude and rate data (see Results). No simple explanation for this discrepancy has been found, and the true binding mechanism may be more complicated than any of the models postulated. However, it is also possible that at the very high ligand concentrations used, other processes occurring in parallel such as inactivation may have caused slight distortion in the results which may be misleading. At all ligand concentrations used, the kinetic traces could be well fit by a single-exponential process, but at the very high rates approaching their saturation values, although the data were highly reproducible, it is quite possible that deviation from a monophasic process would not be detected. In this respect, it should also be noted that the rate constants measured at high ligand concentration approached the limitations of the stopped-flow method. Serious problems arising from rate artifacts under these conditions are, however, unlikely since lowering the temperature reduced the rate constants to values well within the instrumental capability but the reaction mechanism for AcCh was not significantly perturbed (see Table II).

Although the true mechanism of agonist binding to the low-affinity site may be complex, it is quite clear that the observed fluorescence change reflects a conformational transition of the receptor–ligand complex. This may be represented empirically as

$$RL \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} R^*L$$

where the rate constants ($\beta + \alpha$) at saturating concentrations of Carb and AcCh were extrapolated to be 400 s^{-1} ($t_{1/2}$ = 1.7 ms) and 600 s^{-1} ($t_{1/2}$ = 1.2 ms), respectively. These rates are in the range that may be expected for a transition involved in channel activation, which leads further credence to the identification of R*L as a functionally important state. The above scheme has been used to describe electrophysiological results, and R*L has been equated with the open-channel form [see Adams (1981)]. Although the physical significance of R*L may be different in the fluorescence experiments, the efficacy of an agonist in inducing the formation of R*L above or the open-channel form in electrophysiological experiments can be described by y, where $y = \beta/(\beta + \alpha)$. The values of y calculated from the kinetic data presented here were approximately 0.79 for Carb and 0.96 for AcCh, and these values are in qualitative agreement with the relative efficacy of these agonists in inducing channel opening [see Adams (1981)].

Titrations of NBD-labeled AcChR by AcCh at two temperatures, i.e., 25 and 12.5 °C, allowed the calculation of a Q_{10} of 2.5 for the maximal rate constant observed. This value is in excellent agreement wth those of 2.5–3.5 reported for experiments of noise analysis and relaxation kinetics at muscle fibers (Magleby & Stevens, 1972; Anderson & Stevens, 1973; Gage & McBurney, 1975; Neher & Sakmann, 1975; Dreyer et al., 1976; Fishbach & Lass, 1978) and at *Electrophorus* electroplax (Sheridan & Lester, 1975; Lester & Chang, 1977; Nass et al., 1978). In electrophysiological experiments, it has also been shown that, while the rate constants for channel activation vary with temperature, the equilibrium conductance displays little temperature sensitivity (Sheridan & Lester, 1975; Lester et al., 1980). In terms of the simplified scheme above, this would be explained if the rate constants α and β have similar temperature dependencies. In agreement with these results is the fact that the equilibrium concentration of R*L measured in fluorescence experiments was also not strongly dependent on temperature and apparent K_d values

for AcCh estimated from the concentration dependence of the kinetic amplitude were not significantly affected when the temperature was lowered from 25 to 12.5 °C.

The maximal rate of cation flux induced by the binding of nicotine or PTA was much lower than that previously measured for Carb or AcCh as agonist (see Table I). This shows that agonists vary in their capacity to elicit ion transport, which suggests that channel opening is not an all or none phenomenon and that the open-channel state of the receptor may have multiple conformations with each agonist having differing ability to induce these conformational changes. Alternatively, as multiple ligand binding sites exist, the open time of the channel and therefore the rate of ion transport may be controlled by conformational changes induced by agonist binding to a site other than that directly involved in activation. Similar low transport rates for PTA have previously been measured in rapid $^{22}Na^+$ efflux experiments using *Torpedo* vesicles (Neubig & Cohen, 1980).

Neither the equilibrium binding nor the kinetics of binding of agonists to the low-affinity site was affected by preincubation of the receptor preparations with tubocurarine, HTX, or lidocaine. It is therefore likely that the pharmacological effects of these ligands are indirect and may be the result of direct binding to the receptor when it is in its open-channel form as has been suggested for HTX (Albuquerque et al., 1973; Dolly et al., 1976) and antagonists (Colquhoun et al., 1979). These functional perturbants may also interfere with the binding of agonists to the site involved in desensitization, and local anesthetics have been demonstrated to increase the rate of desensitization both in vivo (Magazanik & Vyskocil, 1973; Magazanik, 1976) and in vitro (Weiland et al., 1977; Briley & Changeux, 1978; Blanchard et al., 1979b; Dunn et al., 1981). It is quite likely in view of the probability of multiple binding sites also for these ligands [see Conti-Tronconi & Raftery (1982)] that they have multiple actions on receptor function. Preincubation of labeled receptor preparations with α-BuTx completely inhibited the fluorescence enhancement induced by the binding of agonists. However, it has not yet been possible to establish whether α-BuTx competitively inhibits agonist binding to the low-affinity site(s) or merely inhibits the signal change induced by such binding.

In conclusion, the membrane-bound AcChR from *Torpedo californica* has a low-affinity binding site which appears to be specific for agonists. This site has been revealed by ligand-induced changes in the fluorescence of the NBD chromophore which was covalently attached to the receptor protein without an obvious adverse effect on functional properties. Equilibrium and kinetc properties of agonist binding to this site are consistent with its having a role in the process of channel activation.

References

Adams, P. R. (1981) *J. Membr. Biol. 58*, 161–174.

Albuquerque, E. X., Barnard, E. A., Chiu, T. H., Lapa, A. J., Dolly, J., Jannson, S., Daly, J., & Witkop, B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 949–953.

Anderson, C. R., & Stevens, C. F. (1973) *J. Physiol. (London) 235*, 655–691.

Barrantes, F. J. (1976) *Biochem. Biophys. Res. Commun. 72*, 479–488.

Blanchard, S. G., Quast, U., Reed, K., Lee, T., Schimerlik, M. I., Vandlen, R., Claudio, T., Strader, C. D., Moore, H.-P. H., & Raftery, M. A. (1979a) *Biochemistry 18*, 1875–1883.

Blanchard, S. G., Elliott, J., & Raftery, M. A. (1979b) *Biochemistry 18*, 1875–1883.

Blanchard, S. G., Dunn, S. M. J., & Raftery, M. A. (1982) *Biochemistry* (preceding paper in this issue).

Bonner, R., Barrantes, F. J., & Jovin, T. M. (1976) *Nature (London) 263*, 429–431.

Briley, M. S., & Changeux, J.-P. (1978) *Eur. J. Biochem. 84*, 429–439.

Chang, R. S. L., Potter, L. T., & Smith, D. S. (1977) *Tissue Cell 9*, 623–628.

Clark, D. G., Macmurchie, D. D., Elliott, E., Wolcott, R. G., Landel, A. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1663–1668.

Colquhoun, D. (1975) *Annu. Rev. Pharmacol. 15*, 307–325.

Colquhoun, D., Dreyer, F., & Sheridan, R. E. (1979) *J. Physiol. (London) 293*, 247–284.

Conti-Tronconi, B. M., & Raftery, M. A. (1982) *Annu. Rev. Biochem. 51*, 491–530.

Conti-Tronconi, B. M., Dunn, S. M. J., & Raftery, M. A. (1982) *Biochem. Biophys. Res. Commun. 107*, 123–129.

Damle, V. N., McLaughlin, M., & Karlin, A. (1978) *Biochem. Biophys. Res. Commun. 84*, 845–851.

Dionne, V. E., Steinbach, J. H., & Stevens, C. F. (1978) *J. Physiol. (London) 281*, 421–444.

Dolly, J. O., Albuquerque, E. X., Sarvey, J. M., Mallick, B., & Barnard, E. A. (1976) *Mol. Pharmacol. 13*, 1–14.

Dreyer, F., Walther, C., & Peper, K. (1976) *Pfluegers Arch. 366*, 1–9.

Dreyer, F., Peper, K., & Sterz, R. (1978) *J. Physiol. (London) 281*, 395–419.

Dunn, S. M. J., & Raftery, M. A. (1982) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1980) *Biochemistry 19*, 5645–5652.

Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1981) *Biochemistry 20*, 5617–5624.

Elliott, J., Dunn, S. M. J., Blanchard, S. G., & Raftery, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2576–2579.

Elliott, J., Blanchard, S. G., Wu, W., Miller, J., Strader, C. D., Hartig, P., Moore, H.-P. H., Racs, J., & Raftery, M. A. (1980) *Biochem. J. 185*, 667–677.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88–95.

Fishbach, G. D., & Lass, Y. (1978) *J. Physiol. (London) 280*, 527–536.

Gage, P. W., & McBurney, R. N. (1975) *J. Physiol. (London) 244*, 385–407.

Grunhagen, H. H., & Changeux, J.-P. (1976) *J. Mol. Biol. 106*, 479–516.

Grunhagen, H. H., Iwatsubo, M., & Changeux, J.-P. (1976) *C. R. Hebd. Seances Acad. Sci., Ser. D 283*, 1105–1108.

Grunhagen, H. H., Iwatsubo, M., & Changeux, J.-P. (1977) *Eur. J. Biochem. 80*, 225–242.

Hammes, G. G., & Wu, C.-W. (1974) *Annu. Rev. Biophys. Bioeng. 3*, 1–33.

Haugland, R. P. (1975) *J. Supramol. Struct. 3*, 338–347.

Heidmann, T., & Changeux, J.-P. (1979) *Eur. J. Biochem. 94*, 255–279.

Heidmann, T., & Changeux, J.-P. (1980) *Biochem. Biophys. Res. Commun. 97*, 889–896.

Hess, G. P., Cash, D. G., & Aoshima, H. (1979) *Nature (London) 282*, 329–331.

Hucho, F., Layer, P., Keifer, H. R., & Bandini, G. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2624–2628.

Janin, J. (1973) *Prog. Biophys. Mol. Biol. 27*, 77–120.
Jurss, R., Pinz, H., & Maelicke, A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 1064–1068.
Karlin, A., Weill, C. L., McNamee, M. G., & Valderrama, R. (1975) *Cold Spring Harbor Symp. Quant. Biol. 40*, 204–210.
Kasai, M., & Changeux, J.-P. (1971) *J. Membr. Biol. 6*, 1–23.
Katz, B., & Thesleff, S. (1957) *J. Physiol. (London) 138*, 63–80.
Lee, T., Witzemann, V., Schimerlik, M., & Raftery, M. A. (1977) *Arch. Biochem. Biophys. 183*, 57–63.
Lester, H. A., & Chang, H. W. (1977) *Nature (London) 266*, 373–374.
Lester, H. A., Krouse, M. E., Nass, M. M., Wassermann, N. H., & Erlanger, B. F. (1980) *J. Gen. Physiol. 75*, 207–232.
Lowry, O. H., Rosenbrough, N. J., Farr, A., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lyddiatt, A., Sumikawa, K., Wolosin, J. M., Dolly, J. O., & Barnard, E. A. (1979) *FEBS Lett. 108*, 20–24.
Magazanik, L. G. (1976) *Annu. Rev. Pharmacol. Toxicol. 16*, 161–175.
Magazanik, L. G., & Vyskocil, F. (1973) in *Drug Receptors* (Rang, H. P., Ed.) pp 105–119, Macmillan, London.
Magleby, K. L., & Stevens, C. F. (1972) *J. Physiol. (London) 223*, 173–197.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.
Moore, H.-P. H., & Raftery, M. A. (1979) *Biochemistry 18*, 1862–1867.
Moore, H.-P. H., & Raftery, M. A. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4509–4513.
Nass, M. M., Lester, H. A., & Krouse, M. E. (1978) *Biophys. J. 24*, 135–160.
Neher, E., & Sakmann, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2140–2144.
Neubig, R. R., & Cohen, J. B. (1980) *Biochemistry 19*, 2770–2779.
Neubig, R. R., Krodel, E. K., Boyd, N. D., & Cohen, J. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 690–694.
Quast, U., Schimerlik, M., Lee, T., Witzemann, V., Blanchard, S. G., & Raftery, M. A. (1978a) *Biochemistry 17*, 2405–2414.
Quast, U., Schimerlik, M., & Raftery, M. A. (1978b) *Biochem. Biophys. Res. Commun. 81*, 955–964.
Quast, U., Schimerlik, M., & Raftery, M. A. (1979) *Biochemistry 18*, 1891–1901.
Raftery, M. A., Hunkapiller, M. W., Strader, C. D., & Hood, L. E. (1980) *Science (Washington, D.C.) 208*, 1454–1457.
Rang, H. P., & Ritter, J. M. (1970) *Mol. Pharmacol. 6*, 357.
Schimerlik, M. I., Quast, U., & Raftery, M. A. (1979) *Biochemistry 18*, 1884–1890.
Schmidt, J., & Raftery, M. A. (1973) *Anal. Biochem. 52*, 349–354.
Sheridan, R. E., & Lester, H. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3496–3500.
Weber, M., David-Pfeuty, T., & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3443–3447.
Weiland, G., Georgia, B., Wee, V. T., Chignell, C. F., & Taylor, P. (1976) *Mol. Pharmacol. 12*, 1091–1105.
Weiland, G., Georgia, B., Lappi, S., Chignell, C. F., & Taylor, P. (1977) *J. Biol. Chem. 252*, 7648–7656.
Weill, C. L., McNamee, M. G, & Karlin, A. (1974) *Biochem. Biophys. Res. Commun. 61*, 997–1003.
Witzemann, V., & Raftery, M. A. (1977) *Biochemistry 16*, 5862–5868.
Wolosin, J. M., Lyddiatt, A., Dolly, J. O., & Barnard, E. A. (1980) *Eur. J. Biochem. 109*, 494–505.
Wu, W. C.-S., Moore, H.-P. H., & Raftery, M. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 775–779.