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Activation and desensitization of *Torpedo* acetylcholine receptor: Evidence for separate binding sites

(carbamoylcholine/low affinity/multiple sites/parallel mechanisms)

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ABSTRACT **The acetylcholine receptor from *Torpedo californica* was labeled by reaction with the fluorescent probe 4-[*N*-(iodoacetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole without apparent effect on its *in vitro* ligand binding and functional properties. Addition of acetylcholine or carbamoylcholine to the labeled-receptor preparations enhanced the fluorescence of the bound probe, and this effect was specific for agonists and inhibited by prior incubation with excess α-bungarotoxin. Equilibrium fluorescence titrations gave apparent dissociation constants of 0.86 ± 0.14 mM for carbamoylcholine and 79 ± 11 μM for acetylcholine, in good agreement with the dissociation constants measured for the permeability response of the receptor. Stopped-flow experiments showed that the fluorescence change was a single exponential process whose rate increased with ligand concentration, reaching a saturating value for carbamoylcholine of approximately 400 s^{-1}. The equilibrium binding of carbamoylcholine was not significantly affected by prior incubation of the receptor with *d*-tubocurarine or histrionicotoxin and the dissociation constant was only slightly increased in the presence of lidocaine. These inhibitory ligands do not, therefore, compete directly with agonists for this low-affinity binding site, suggesting that their mode of action may be indirect.**

The *Torpedo* acetylcholine (AcCho) receptor (AcChoR) has been shown to undergo a time-dependent increase in agonist affinity on exposure to these ligands and this has been correlated with *in vivo* desensitization (1–6). This transition occurs on a relatively slow time scale ($t_{1/2} \approx 80$ s; ref. 6), and therefore rapid kinetic techniques are necessary to monitor ligand-induced conformational changes occurring prior to receptor inactivation. Recently, a variety of stopped-flow techniques have been used to monitor the interaction of agonists and the membrane-bound AcChoR (7–16). Complex ligand binding kinetics were found in each study, and these have been interpreted in terms of a variety of mechanisms with, as yet, no agreement as to a common model. In all cases, conformational changes of the receptor–ligand complexes were observed but the rate constants of these processes appear too slow to account for channel opening.

Agonist-mediated $^{22}Na^+$ flux using AcChoR-enriched membrane vesicles has been extensively studied by slow filtration assays but the limited time resolution of these methods allows only integrated responses to be measured and gives no information on the rate of ion transport. Recently, techniques have been developed to monitor cation flux on more rapid time scales. These involve quench-flow methods for monitoring $^{86}Rb^+$ (17) or $^{22}Na^+$ efflux (18) and a rapid spectroscopic stopped-flow technique based on Tl^+ quenching of the fluorescence of a fluorophore loaded within the vesicles (19). In these studies, the apparent K_d values measured for carbamoylcholine (CarbCho)-induced ion flux were in good agreement and ranged from 0.5 to 6 mM, depending on the model used to fit the data. These values are consistent with those measured in electrophysiological studies (20, 21) but they are, however, inconsistent with those obtained for CarbCho binding to the resting state (30 μM) or to the high-affinity desensitized state (0.1 μM) *in vitro* (4, 6). Complicated kinetic models involving sequential ligand binding steps have been proposed to account for this discrepancy (17, 18).

In this communication, we describe the modification of the membrane-bound AcChoR by reaction with the fluorescent probe 4-[*N*-(iodoacetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD). Fluorescence changes of the bound fluorophore were used to monitor the equilibrium and kinetic properties of agonist binding and the results provide evidence for the existence of a low-affinity binding site for agonists.

MATERIALS AND METHODS

AcChoR-enriched membrane fragments were prepared as described (22, 23) with the exception that iodoacetamide was excluded from the initial homogenization. Unless otherwise stated, the buffer used in the final stages of preparation and the experiments was 10 mM Hepes/35 mM $NaNO_3$, pH 7.4.

Labeling of the membrane-bound receptor by IANBD (Molecular Probes) was achieved by first treating the membranes [10 μM in α-bungarotoxin (α-BTX) sites] with 50 μM dithiothreitol for 20 min at room temperature in Hepes buffer flushed with argon. IANBD was added as a finely ground powder to give a nominal concentration of 300 μM. The reaction mixture was shielded from light and stirred for 2 hr 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) and collecting the membrane fragments that eluted in the void volume.

Fluorescence spectra were recorded on a Perkin-Elmer MPF-4 spectrofluorimeter thermostatted at 25 ± 1°C. Equilibrium dissociation constants were obtained from fluorescence titration experiments using excitation and emission wavelengths of 482 and 540 nm, respectively. Small volumes of concentrated ligand solution were added to 2 ml of AcChoR (1 to 2 μM in α-BTX sites) and the fluorescence level was recorded immediately after ligand addition. The data were corrected for nonspecific effects from the results of a parallel titration of membrane fragments previously incubated with excess α-BTX. Prior to titration with AcCho, acetylcholinesterase activity was measured using acetylthiocholine as substrate (24) and only those prepa-

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Abbreviations: AcCho, acetylcholine; AcChoR, acetylcholine receptor; α-BTX, α-bungarotoxin; CarbCho, carbamoylcholine; HTX, histrionicotoxin; IANBD, 4-[*N*-(iodoacetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole; NBD, 4-[*N*-(acetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole.

rations that were devoid of esterase activity were used.

Kinetic data were obtained by using the stopped-flow instrumentation and data collection systems described in refs. 16 and 25.

At high ligand concentrations, the rate of the signal change was so fast that a significant fraction of the process was complete within the instrument dead time (approximately 2 ms). To reduce the inevitable artifacts introduced by this effect into the rate constants derived from the data-fitting procedures, it was occasionally necessary to fix the value of A_1, the signal amplitude (as defined in ref. 16), during the fitting procedure using the observed amplitude at saturating concentrations of ligand.

The kinetics of agonist-mediated cation flux for control and 4-[*N*-(acetoxy)ethyl-*N*-methyl]amino-7-nitrobenz-2-oxa-1,3-diazole (NBD)-labeled membrane preparations were measured by loading the vesicles with the fluorescent probe 8-aminonaphthalene-1,3,6-trisulfonic acid (Chemical Services) and monitoring the kinetics of Tl^+ translocation by the stopped-flow method described in ref. 19.

RESULTS

Spectral Properties of the NBD-Labeled AcChoR. After reduction of the AcChoR-enriched membrane fragments by low concentrations of dithiothreitol, reaction with the sulfydryl-selective alkylating reagent IANBD gave a highly fluorescent receptor preparation having broad fluorescence maxima for excitation at 480 nm and emission at 535 nm, which are characteristic of the properties of the bound fluorophore NBD (26, 27). Addition of CarbCho or AcCho led to a fluorescence enhancement that was saturable at high ligand concentrations. This was completely abolished by prior incubation of the receptor with α-BTX, showing that it was specific for the AcChoR. Preliminary studies of NBD labeling of the membrane-bound receptor showed that specific fluorescence labeling could be achieved even in the absence of dithiothreitol reduction. The results, however, were variable and reproducibility was achieved by introducing the reduction step.

Effect of NBD Labeling on the Properties of the AcChoR. A major problem with the use of fluorescent probes to monitor the ligand binding properties of the AcChoR is that introduction of the probe may lead to perturbation of function. The ability of the NBD-labeled preparation to mediate cation flux was therefore studied by a quantitative rapid kinetic assay (19). Representative Tl^+ flux traces obtained for control and labeled AcChoR preparations using a CarbCho concentration of 0.1 mM are shown in Fig. 1. The labeling procedures apparently did not affect the cation flux properties of the receptor and the labeled preparation displayed the expected pharmacological response in that the agonist-induced enhancement of the rate of Tl^+ transport was completely blocked by prior incubation of the receptor preparation with histrionicotoxin (HTX) (Fig. 1*B*). NBD labeling also did not affect either the dissociation constants for [^{3}H]HTX or [^{3}H]CarbCho binding or the number of high-affinity binding sites for these ligands. All these results indicate that the labeled preparations retain their characteristic ligand binding and functional properties.

Equilibrium Binding of Agonists to the NBD-Labeled AcChoR. After alkali extraction and NBD-labeling of the receptor-enriched membrane fragments, the preparations were frequently found to be devoid of acetylcholinesterase activity. Equilibrium fluorescence titrations with AcCho could therefore be carried out in the absence of added esterase inhibitors, which interfered with the binding of or the fluorescence changes induced by agonists (or both).

Addition of AcCho led to a concentration-dependent increase in fluorescence that could be fitted with precision by a simple binding process characterized by an apparent K_d of 88 ± 2 μM (Fig. 2*A*). The Hill coefficient, n, calculated from these data (28) was 0.95 ± 0.11, emphasizing noncooperative ligand binding and clear deviation from the cooperative two-ligand binding mechanism that is frequently invoked to describe the dose dependency of conductance changes measured in electrophysiological experiments (see ref. 29) (Fig. 2*A*).

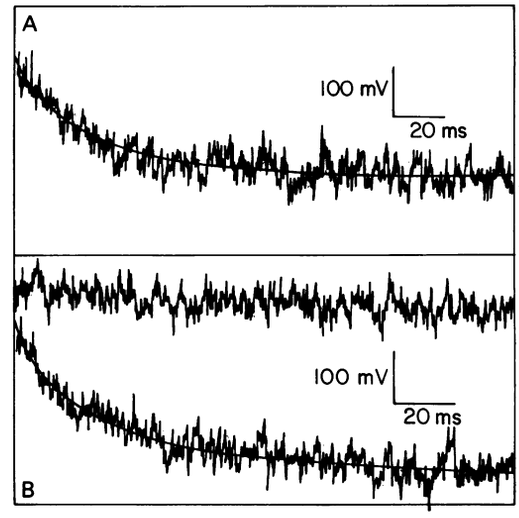

FIG. 1. Kinetics of CarbCho-induced Tl^+ flux of control (*A*) and NBD-labeled AcChoR (*B*). 8-Aminonaphthalene-1,3,6-trisulfonic acid-loaded vesicles were mixed with 100 μM CarbCho and 17 mM Tl^+ (final concentrations after mixing). The best-fit kinetic parameters were: control, k_1 = 15.7 s^{-1} and A_1 = 412 mV; NBD labeled, k_1 = 22.0 s^{-1} and A_1 = 420 mV. The upper trace in *B* was recorded after mixing 100 μM CarbCho with NBD-labeled AcChoR previously incubated with 10 μM HTX.

Similar simple binding curves were obtained using CarbCho as ligand and the results in Fig. 2*B* show that the apparent K_d for CarbCho is about 0.96 mM (n = 0.99). The K_d values measured by equilibrium titration of NBD-labeled preparations are in good agreement with the effective dissociation constants measured by Tl^+ flux experiments (refs. 19, 30; unpublished work) and these values compared in Table 1.

Effects of HTX, *d*-Tubocurarine, and Lidocaine. The ligand-induced enhancement of the fluorescence of the NBD-labeled AcChoR was agonist specific since the fluorescence level was unaltered by HTX, by the antagonist *d*-tubocurarine, and by lidocaine, a local anesthetic, even when these ligands were added in concentrations considerably in excess of their known affinities for the receptor. Prior incubation of the AcChoR with an excess of any of these ligands also had no dramatic effect on the binding of CarbCho, either with respect to the measured K_d (Table 2) or the magnitude of the observed fluorescence enhancement. Only lidocaine was found to cause a slight (approximately 2-fold) increase in K_d; this effect is shown in Fig. 2*B*.

Kinetics of Agonist Binding to NBD-Labeled AcChoR. When either CarbCho or AcCho was rapidly mixed with the NBD-labeled AcChoR, the resultant signal change appeared to be a single exponential process and was completely blocked by prior incubation with excess α-BTX, as shown for the binding of 1 mM AcCho in Fig. 3. Both the rate and amplitude of the CarbCho-induced signal change increased with the concentration of agonist and showed saturation. The concentration dependence of the amplitude paralleled the changes measured in equilibrium titrations, and the data could be fit by a simple binding process with a K_d of 0.73 mM (Fig. 4*A*). The rate also increased hyperbolically with CarbCho concentration and had limiting val-

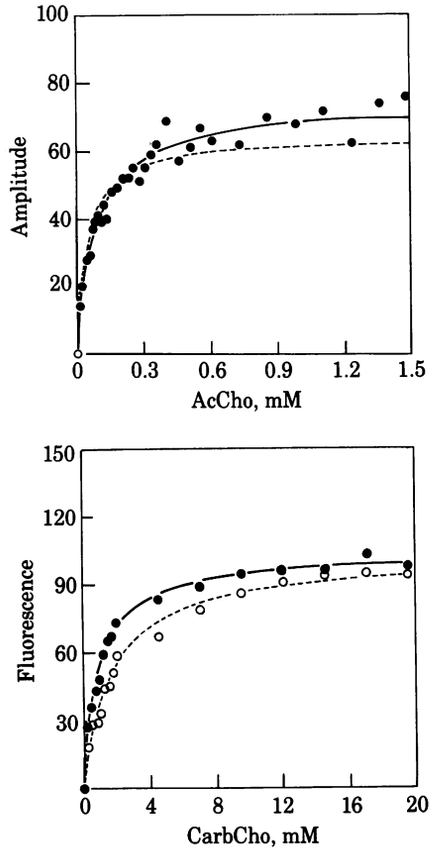

FIG. 2. Fluorescence titration of NBD-labeled AcChoR by agonists. (*A*) AcCho. ——, Best-fit line to the simple binding isotherm $F1 = F_0[L]/(K_d + [L])$, where $F1$ and F_0 are the observed and equilibrium fluorescence levels; $F_0 = 74.0 \pm 0.4$ and $K_d = 88 \pm 2$ μM. – – –, Fit to the two-ligand binding mechanism $F1 = F_0/(1 + K_d/L)^2$ with $F_0 = 63.9 \pm 0.3$ and $K_d = 23 \pm 1$ μM. (*B*) CarbCho. ——, Curve calculated by assuming $F1 = F_0[L]/(K_d + [L])$ and using the best-fit parameters $F_0 = 103.8 \pm 0.9$ and $K_d = 0.96 \pm 0.02$ mM. – – –, Fluorescence titration after incubation of AcChoR with 20 mM lidocaine. Best-fit parameters were $F_0 = 102.7 \pm 1.8$ and $K_d = 1.78 \pm 0.04$ mM.

ues at zero and high concentrations of 83 s^{-1} (extrapolated) and 317 s^{-1}, respectively (Fig. 4*B*).

DISCUSSION

One conceptual problem in the study of the AcChoR has been the frequent lack of agreement between the apparent dissociation constants describing agonist-mediated permeability responses and those measured in direct ligand binding experiments. This has led to proposals of complex kinetic mechanisms involving sequential ligand binding steps and many different receptor conformations having different affinities for agonists (17, 18). The results presented here for the binding of CarbCho and AcCho to NBD-labeled preparations from *Torpedo* electroplax suggest the existence of a low-affinity binding site for agonists for which the measured equilibrium constants are in agreement with the dependence on the concentration of agonist measured in rapid *in vitro* ion flux experiments using *Torpedo* (refs. 18, 19, and 30; unpublished work) or *Electrophorus* (17) AcChoR and in electrophysiological studies using the frog neuromuscular junction (20, 21).

These results appear to contradict the demonstration that, in intact BC3H-1 cells, good correlation exists between an agonist-induced affinity change of the AcChoR and both the activation and desensitization responses (31, 32). For this species, the results reported suggest that occupation of one binding site by agonist leads to both functional responses.

Affinity labeling techniques have previously shown that an agonist binding site is located on the receptor subunit of M_r ≈40,000 (33–37) and it is generally assumed that binding of agonist to this site leads to channel activation. However, the recent finding that all four subunits (M_r 40,000, 50,000, 60,000, and 65,000) have extensive sequence homology (38) supports the hypothesis that multiple binding sites of different affinities and perhaps different functional properties may coexist. Such a possibility is further supported by the observations that the affinity label 4-azido-2-nitrobenzyltrimethylammonium fluoroborate (39) and the photoaffinity label (3-azidopyridinium)-1,10-diiododecane (40) labeled other receptor subunits in addition to that of M_r 40,000. It is not therefore unreasonable to consider that functional properties of the AcChoR such as channel activation and desensitization may be the results of parallel rather than sequential ligand binding pathways for the *Torpedo* system.

The equilibrium fluorescence titration data show that the binding of AcCho or Carb has a simple hyperbolic dependence on concentration and thus provide no evidence for multiple low-affinity binding sites. Electrophysiological studies of the dose dependence of conductance changes of *Electrophorus* elec-

Table 1. Comparison of apparent K_d values for agonist binding obtained by fluorescence titration and by cation flux experiments

Ligand	Apparent K_d: Binding to NBD-labeled AcChoR	Tl^+ influx	Ref.
AcCho	79.2 ± 11.4 μM	44 μM*	30
		140 μM	Unpublished
CarbCho	0.86 ± 0.14 mM	5 mM	19
		1 mM*	19
		0.5 mM*	30

* Data were fit by a model that assumed that binding of two ligand molecules was required for channel activation [$k_{app} = k_{max}/(1 + K_d/L)^2$].

Table 2. Effects of previous incubation of NBD-labeled membrane fragments with cholinergic ligands on apparent K_d for CarbCho binding

Ligand	K_d, mM
None	0.80 ± 0.19
d-Tubocurarine (10 μM)	0.85 ± 0.20
HTX (12.5 μM)	0.98 ± 0.04
Lidocaine (20 mM)	2.2 ± 0.4

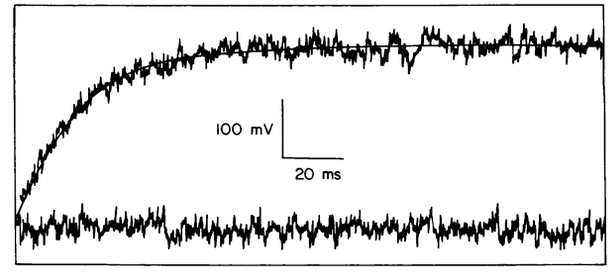

FIG. 3. Kinetics of AcCho binding to NBD-labeled AcChoR. Membrane fragments (1 to 2 μM in α-BTX sites) were mixed with 1 mM AcCho before (upper trace) or after (lower trace) incubation with 3 μM α-BTX for 30 min. ——, Curve calculated by using the best-fit parameters $k_1 = 47.5$ s^{-1} and $A_1 = 302$ mV.

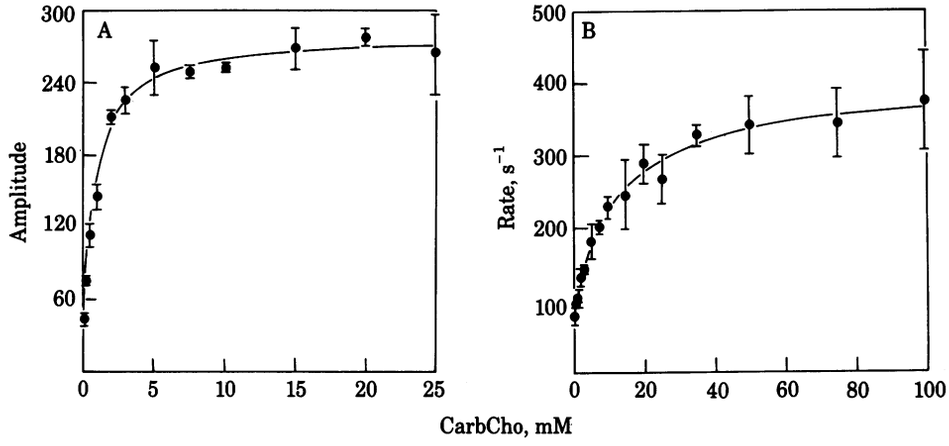

FIG. 4. Effect of CarbCho concentration on kinetics of binding to NBD-labeled AcChoR. Each point represents mean ± SD of at least four determinations. (*A*) Effect of CarbCho concentration on the amplitude of the signal change. ——, Best-fit line to the equation $F1 = F_0[L]/(K_d + [L])$, where $F_0 = 278.8 \pm 0.5$ (arbitrary units) and $K_d = 0.73 \pm 0.01$ mM. (*B*) Effect of CarbCho concentration on apparent rate constant. Data were fitted to the mechanism shown in Eq. 1. ——, Curve calculated by using the parameters $K_1 = 12.6 \pm 0.1$ mM; $k_2 = 317 \pm 1\ s^{-1}$ and $k_{-2} = 82.6 \pm 0.5\ s^{-1}$.

troplaque and muscle endplates have suggested that the channels usually open only when the receptor has two ligands bound (21, 29, 41, 42). Although the fluorescence data do not conform to the predictions of a sequential cooperative two-ligand binding mechanism, other more-complicated models involving two ligand-binding steps may be found to describe adequately the observed behavior. Another possibility is that if, as is likely, the observed signal change arises not from channel opening but rather from a conformational change preceding activation, the response itself may display cooperativity that is not necessarily the consequence of cooperativity in ligand binding. In this respect, it should be noted that the observed low-affinity binding also occurs under equilibrium conditions when, presumably, the receptor is desensitized and thus channel opening is unlikely.

The equilibrium binding of CarbCho to the low-affinity site was unaffected by prior incubation of the receptor with either the antagonist, *d*-tubocurarine, or HTX and was only slightly perturbed by the presence of lidocaine. It has previously been proposed that the blocking action by HTX (43, 44) and local anesthetics (45) may be due to a direct block of the open channel when the receptor is in its conducting state. The binding of agonists to a receptor site responsible for activation may therefore be insulated from such an action. *d*-Tubocurarine does not compete for the low-affinity site for agonist because it neither itself affected the fluorescence of NBD-labeled AcChoR nor inhibited the binding of CarbCho, suggesting that the effect of antagonists may be indirect, possibly due partially to a direct block of the open channel as previously suggested (46).

Stopped-flow fluorescence experiments showed that the signal change accompanying the binding of CarbCho or AcCho to the NBD-labeled AcChoR is an apparently first-order process occurring on a rapid time scale. The concentration dependence of the amplitude paralleled that measured in equilibrium titrations, indicating not only that no unobservably fast process was occurring but also that desensitization, which is unlikely to occur within the time of the kinetic experiments, does not affect the equilibrium constant for binding to this site. The rate of the signal change also had a hyperbolic dependence on ligand concentration and, thus, the process cannot be attributed to a bimolecular step but rather is likely to reflect a conformational change of the receptor–ligand complex. The nonlinear regression fitting of the data to a simple binding mechanism

$$R + L \underset{}{\overset{K_1}{\longleftrightarrow}} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R^*L, \qquad [1]$$

where $k_{app} = k_{-2} + k_2[L]/(K_1 + [L])$, is shown in Fig. 4*B*. It should, however, be noted that this mechanism does not adequately account for the data because the overall dissociation constant obtained from the fit of the amplitude (0.73 mM) and calculated from the rate data ($k_1k_2 = 3.3$ mM) are inconsistent. The true binding mechanism therefore must be more complicated, but a simplistic representation of the conformational change(s) may be considered [assuming that the isomerization(s) and not the binding step(s) is rate limiting] as

$$RL \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} R^*L, \qquad [2]$$

where the fraction of R*L at equilibrium (y) is given by $\beta/(\beta + \alpha)$ which, for CarbCho is approximately 0.77. The rate constants β and α for CarbCho as agonist are approximately 317 s^{-1} and 83 s^{-1}, respectively, yielding an apparent rate constant of about 400 s^{-1} at saturating ligand concentration ($t_{1/2} = 1.7$ ms). This rate is considerably faster than the isomerization rate constants previously measured in rapid kinetic experiments (7–15, 25) and is indeed in the range that might be expected for a rate-limiting step in the process of activation of the population of channels present in the membrane vesicle preparations.

The above simplified scheme has previously been invoked to explain the results of electrophysiological experiments in which a variety of techniques were used (29, 47, 48). In these cases, R*L would denote the open channel form of the receptor and y would be a measure of the efficacy of an agonist to activate the channel. Although this scheme empirically describes the present fluorescence data, it is unlikely that R*L in this case can be equated with the open channel state since the appearance of this receptor conformation is unaffected by desensitization.

In summary, the results provide evidence for the existence of a low-affinity agonist binding site that is likely to be directly involved in channel activation because (*i*) its affinity for agonists is closely correlated with the apparent dissociation constants measured for the permeability response and (*ii*) the rate constants for ligand binding to the site are sufficiently high that they are consistent with the rapidity of conductance changes measured *in vivo*. The low-affinity site exists under equilibrium conditions and this suggests that desensitization and activation

may be parallel processes and that the loss of response is not due to perturbation of ligand binding to this site.

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