Effects of Local Anesthetics and Histrionicotoxin on the Binding of Carbamoylcholine to Membrane-Bound Acetylcholine Receptor†

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ABSTRACT: The effects of local anesthetics and perhydrohistrionicotoxin on the kinetic mechanism of carbamoylcholine binding to the membrane-bound acetylcholine receptor have been studied by stopped-flow methods. Receptor-enriched membrane fragments from *Torpedo californica* were reduced and then alkylated by 5-(iodoacetamido)salicylic acid, and the agonist binding kinetics were monitored by the fluorescence changes of this bound probe. The alkylation procedures did not alter the ability of the receptor to mediate agonist-induced cation flux. Preincubation of such modified receptor preparations with saturating concentrations of lidocaine, prilocaine, or dimethisoquin did not significantly affect the equilibrium dissociation constant for carbamoylcholine binding. The multiphasic kinetic signal which accompanies the binding of the agonist was, however, much simplified in the presence of local anesthetics, and the observed kinetics could be described by a mechanism in which a single conformational change follows the formation of the initial complex. Perhydrohistrionicotoxin did not act in the same way as the local anesthetics examined since saturating concentrations did not significantly perturb the agonist binding kinetics.

Local anesthetics act at the neuromuscular junction to modulate the normal response to the release of acetylcholine. Electrophysiological studies have shown that these compounds block the increase in ionic permeability which follows the binding of the neurotransmitter to nicotinic acetylcholine receptors (AcChR)[1] present in the postsynaptic membrane (Ruff, 1976; Steinbach, 1977; Neher & Steinbach, 1978). In addition, local anesthetics have been found to increase the rate of receptor desensitization resulting from prolonged exposure to agonist (Magazanik & Vyskocil, 1973; Magazanik, 1976).

The ready availability of acetylcholine receptor enriched membrane fragments from *Torpedo* electric organs has facilitated the study of the ligand binding properties of the nicotinic receptor in vitro. Incubation of these preparations with agonists results in a time-dependent transition of the receptor to a state having higher affinity for agonists (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977; Quast et al., 1978a), and it has been suggested that this is related to the process of pharmacological desensitization. Local anesthetics affect the rate of this transition (Weiland et al., 1977; Briley & Changeux, 1978; Blanchard et al., 1979a), but the magnitude and direction of the effect depend on local anesthetic structure (Blanchard et al., 1979a). The rate of conversion to the high-affinity state can be measured indirectly by following the agonist-induced inhibition of the kinetics of association of ^{125}I-labeled α-bungarotoxin with the receptor. Using this technique, Blanchard et al. (1979a) found that at concentrations below those that directly inhibited toxin binding, some local anesthetics, namely, lidocaine and dibucaine, increased the rate of the transition while tetracaine decreased the rate.

Perhydrohistrionicotoxin (H_{12}-HTX) is also believed to modulate the ion translocation function of the receptor. It has been proposed that the effects of this alkaloid may be a result of direct blockage of the open channel when the receptor is in a conducting state (Albuquerque et al., 1973). Specific binding of [^{3}H]H_{12}-HTX to AcChR-enriched membrane fragments from *Torpedo californica* has been demonstrated (Elliott & Raftery, 1977, 1979; Elliott et al., 1979), and some local anesthetics have been shown to displace the bound toxin, although this may be an indirect effect (Blanchard et al., 1979a).

Recently, Heidmann & Changeux (1979a,b) have reported the results of a study of the kinetics of binding of the fluorescent agonist dansyl-C_6-choline and the effects of local anesthetics on this binding. The agonist association kinetics were interpreted in terms of a model in which two interconvertible forms of the receptor preexist and differ in their affinity for agonists. These authors suggested that the effect of the local anesthetics was to stabilize the receptor in the form having higher affinity.

We have recently described the use of a covalent fluorescent probe to monitor the kinetics of carbamoylcholine binding to the membrane-bound receptor from *Torpedo californica* (Dunn et al., 1980). Following reduction of a disulfide bond known to exist near an agonist binding site (Karlin, 1969), membrane preparations were labeled by alkylation with 5-(iodoacetamido)salicyclic acid (IAS). The fluorescence of this probe was shown to be sensitive to the conformational state of the receptor and permitted a stopped-flow kinetic study of carbamoylcholine binding. A kinetic model has been presented which is similar to that previously proposed by Quast et al. (1978b, 1979) to account for the observed kinetics of agonist binding as monitored by the extrinsic probe, ethidium.

In this paper, we present the results of an investigation of the effects of three local anesthetics and histrionicotoxin on the equilibrium and kinetic properties of carbamoylcholine binding to IAS-labeled membrane fragments. Incubation of the membrane-bound receptor with high concentrations of local anesthetic led to an apparent simplification of the agonist

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[1] Abbreviations used: AcCh, acetylcholine; AcChR, acetylcholine receptor; ANTS, 8-amino-1,3,6-naphthalenetrisulfonate; α-BuTx, α-bungarotoxin; Carb, carbamoylcholine; DTT, dithiothreitol; H_{12}-HTX, perhydrohistrionicotoxin; IAS, 5-(iodoacetamido)salicylic acid; Hepes, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

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binding kinetics, and the results can be interpreted in terms of a common model. H_{12}-HTX, however, at saturating concentrations, did not significantly perturb the Carb binding kinetics and therefore acts in this system in a much different way from any of the local anesthetics examined.

Materials and Methods

AcChR-enriched membrane fragments from *Torpedo californica* were prepared as previously described (Elliott et al., 1980). The concentration of α-BuTx sties was measured by the DEAE disc method of Schmidt & Raftery (1973) using [^{125}I]-α-BuTx prepared as described by Blanchard et al. (1979b). Protein concentration was determined by the method of Lowry et al. (1951). The specific activities of the membrane fragment preparations were routinely 1–2 nmol of α-BuTx sites/mg of protein. Preparations of lower purity were alkali-extracted to remove nonreceptor proteins as previously described (Neubig et al., 1979; Elliott et al., 1980). This procedure did not affect any of the experimental results presented here. The buffer used in the final stages of preparation and for storage of the membrane fragments was Ca^{2+}-free Hepes Ringers (20 mM Hepes, 250 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 0.02% NaN_3, pH 7.4).

Details of the chemical modification of the membrane-bound receptor by reduction with 50 μM DTT (Sigma Chemical Co.) and alkylation by 250 μM IAS (Molecular Probes, Inc.) have been given previously (Dunn et al., 1980). Centrifugation assays were used to determine the equilibrium dissociation constants of [^{3}H]H_{12}-HTX (Elliott & Raftery, 1979) and [^{3}H]Carb (Dunn et al., 1980). Unless otherwise stated, the buffer used in all experiments was *Torpedo* Ringers containing 4 mM $CaCl_2$.

Equilibrium fluorescence measurements were made with a Perkin-Elmer MPF-4 spectrofluorometer. Kinetic data were collected with a Durrum D-110 stopped-flow instrument equipped with a 75-W xenon lamp and PRA M303 lamp power supply (Photochemical Research Associates Inc., London, Ontario). A PRA TX-5 optical-feedback unit was used to correct for instability in the lamp output. All fluorescence experiments were carried out at 25 °C. Procedures used in the kinetic experiments and the methods of data collection and nonlinear regression analysis were as described previously (Dunn et al., 1980) with the modification that kinetic data were analyzed by using computer programs written in Fortran.

Kinetic data were fitted either to a single exponential equation

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

or to a sum of two exponentials

$$F(t) = A_0 + A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + k_0 t$$

where $F(t)$ and A_0 are the fluorescence levels at time t and equilibrium, respectively, A_1 and A_2 are the amplitudes of the two processes, and k_1 and k_2 are the corresponding rate constants. The term $k_0 t$ was used to correct for either a linear approximation of a subsequent slower exponential phase or a small linear contribution from photolysis.

The kinetics of agonist-mediated cation flux across the membrane of AcChR-enriched vesicles were measured by the thallium fluorescence quenching method of Moore & Raftery (1980). Following alkaline extraction of nonreceptor polypeptides, part of a membrane preparation was labeled by IAS. The control and IAS-labeled membrane vesicles were equilibrated in 10 mM Hepes and 35 mM $NaNO_3$, pH 7.4, and loaded with the fluorescent probe ANTS (Chem·Services).

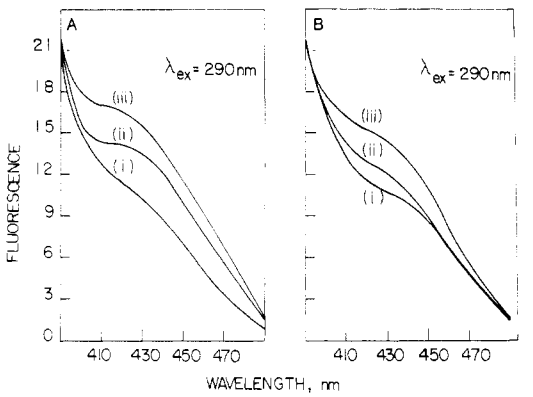

FIGURE 1: Effects of local anesthetics on fluorescence emission spectra of IAS-labeled membrane fragments and on magnitude of Carb-induced enhancement. (A) Effects of prilocaine: (i) AcChR in presence or absence of 5 mM prilocaine; (ii) spectrum after addition of 100 μM Carb to receptor–prilocaine complex; (iii) control spectrum after addition of 100 μM Carb to labeled membrane fragments (no prilocaine). (B) Effects of lidocaine: (i) Spectrum of AcChR-enriched membrane fragments [different preparation from that shown in (A)]; (ii) AcChR fluorescence spectrum after addition of 20 mM lidocaine; (iii) spectrum obtained after addition of 100 μM Carb to receptor–lidocaine complex. AcChR concentration was 0.5 μM in α-BuTx sites, and excitation was at 290 nm.

The fast kinetics of Tl^+ flux across the membrane were monitored by the quenching of ANTS fluorescence using the stopped-flow instrumentation described above. Details of these experiments have been given previously (Moore & Raftery, 1980).

Lidocaine hydrochloride was purchased from Pfaltz and Bauer, Inc., and dimethisoquin hydrochloride and prilocaine were from K and K Laboratories.

Results

Effects of Local Anesthetics on the Fluorescence of IAS-Labeled Membrane Fragments. A prerequisite for the study of the effects of local anesthetics on Carb binding to labeled membrane preparations was that the spectral properties of these compounds did not obscure the specific fluorescence changes used to monitor agonist binding. Many local anesthetics have an undesirable absorbance below 350 nm, and the resulting restriction on the concentration range available for study precluded their use. However, three local anesthetics, namely, lidocaine, prilocaine, and dimethisoquin, were suitable for the present investigation since they have neither strong absorbance nor fluorescence in the spectral region of interest.

Excitation of IAS-labeled membrane fragments at 290 nm gave rise to a fluorescence emission spectrum (Figure 1) showing a maximum at 430 nm superimposed on a membrane light-scattering component. As shown previously (Dunn et al., 1980), the binding of Carb led to a marked enhancement of this fluorescence. Following preincubation of receptor preparations with saturating concentrations of local anesthetics, the magnitude of the Carb-induced fluorescence increase was consistently reduced to 50–60% of that of the control as illustrated for prilocaine in Figure 1A.

Neither prilocaine nor dimethisoquin themselves increased the fluorescence of the labeled receptor. A few membrane preparations have, however, been found to show a fluorescence enhancement on addition of lidocaine (Figure 1B), the local anesthetic which has been the subject of our most intensive study. The magnitude of this enhancement varied in a preparation-dependent manner from 0 to 50% of the maximal Carb-induced enhancement although the amplitude of the agonist signal was unaffected by the presence of lidocaine.

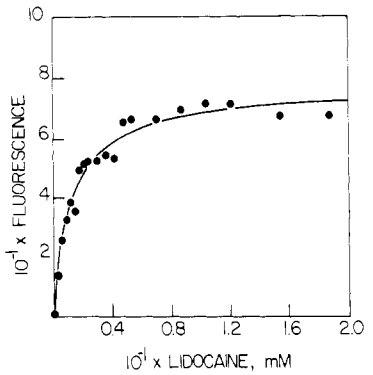

FIGURE 2: Fluorescence titration of IAS-labeled membrane fragments by lidocaine. Small volumes of lidocaine solution were added to 3 mL of labeled receptor (1 μM in α-BuTx sites), and the fluorescence level was recorded 5 min after ligand addition. Excitation and emission wavelengths were 290 and 430 nm, respectively. The data were fitted to a simple binding equation $F = F_0L/(K_d + L)$ where F is the observed fluorescence, F_0 is the fluorescence at equilibrium, and L is the total added ligand concentration. The solid line was calculated from the best-fit parameters obtained by nonlinear regression, F_0 (arbitrary units) = 76.4 ± 0.6 and K_d = 1.2 ± 0.1 mM.

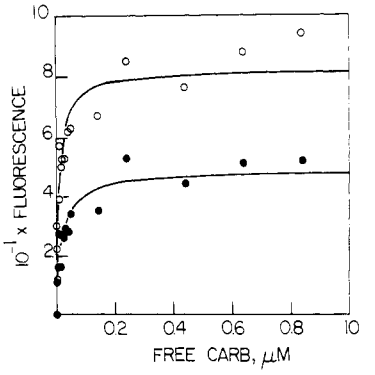

FIGURE 3: Fluorescence titration of IAS-labeled membrane fragments by Carb in the absence (○) and presence (●) of 20 mM lidocaine. AcChR concentration was 0.5 μM in α-BuTx sites, and the protocol was as described in the legend to Figure 2. Incubation time with lidocaine was 30 min prior to first Carb addition. Data were corrected for bound ligand using iterated estimates of K_d. (○) No lidocaine, F_0 = 81.9 ± 0.5, K_d = 0.10 ± 0.01 μM; (●) 20 mM lidocaine, F_0 = 48.3 ± 0.5, K_d = 0.18 ± 0.01 μM.

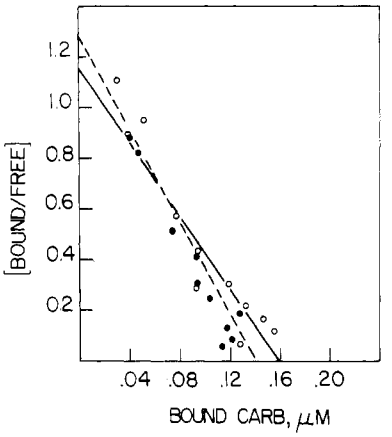

FIGURE 4: Scatchard plots of [^{3}H]Carb binding to IAS-labeled membrane fragments in absence (○) and presence (●) of 10 mM lidocaine. Linear least-squares fit gave values of (●) K_d = 0.14 ± 0.02 μM, R_0 = 0.13 ± 0.02 μM and (○) K_d = 0.10 ± 0.01 μM, R_0 = 0.16 ± 0.02 μM where R_0 is equal to the number of high-affinity Carb sites.

This variability in the lidocaine-induced signal for membrane preparations which displayed a highly reproducible Carb effect leads to the speculation that the two signals arise from different IAS-modified sulfhydryl groups. Although it is very likely that the enhancement accompanying Carb binding arises from a modified disulfide located near an agonist binding site (Dunn et al., 1980), it is possible that another receptor sulfhydryl or disulfide group which has a different susceptibility toward IAS modification is responsible for the lidocaine-induced signal. Since the extent of labeling of this less reactive group would depend on the number of sulfhydryls (including those of nonreceptor proteins) available for alkylation and thus on the purity of the membrane preparation, it is conceivable that a high level of variability be found. Despite the nonreproducibility of the lidocaine-induced signal, this enhancement could be utilized in fluorescence titration experiments to estimate the dissociation constant of the receptor–lidocaine complex as shown in Figure 2. The estimated K_d of 1.2 mM was consistent with the results from two other titrations. This value is also in good agreement with estimates obtained by Blanchard et al. (1979a) from their studies of the inhibition of the rate of [^{125}I]-α-BuTx binding (1.1 mM) and from the displacement of specifically bound [^{3}H]H_{12}-HTX in the absence (2.5 mM) or presence (0.6 mM) of 10 μM Carb.

Effect of Local Anesthetics of the Equilibrium Binding of Carb to IAS-Labeled Membrane Fragments. A fluorescence titration experiment has been used to estimate K_d values for Carb binding to labeled membrane preparations in the absence and presence of 20 mM lidocaine (Figure 3). Within the error of the experiment which is attributable to the poor signal-to-noise ratio of the fluorescence signal changes, lidocaine did not significantly alter the equilibrium constant of the complex (~0.1 μM). However, as noted previously, the amplitude of the signal change accompanying Carb binding was reduced to approximately 60% of the control value.

Centrifugation assays using [^{3}H]Carb have also been used to examine the effects of preincubation of AcChR-enriched membrane fragments with 10 mM lidocaine (Figure 4), 5 mM prilocaine, of 10 μM dimethisoquin (data not shown). In agreement with the fluorescence titration results, the local anesthetics did not significantly perturb either the number of high-affinity Carb sites or the overall K_d for Carb binding (Table II). The concentrations of local anesthetics used in these experiments were chosen because preliminary stopped-flow kinetic results had indicated that these concentrations were in excess of those required to saturate their effects on Carb binding. Furthermore, the concentrations used were considerably greater than those reported to reduce by 50% the depolarization of isolated *Electrophorus* electroplax caused by 50 μM Carb (Cohen et al., 1974).

Effect of H_{12}-HTX on the Kinetics of Carb Binding. The addition of 10 μM H_{12}-HTX, a concentration about 10-fold in excess of its K_d (Elliott & Raftery, 1979), to IAS-labeled membrane fragments had no effect on either the equilibrium fluorescence level or the magnitude of the enhancement induced by saturating concentrations of Carb. This lack of spectral interference on the binding of H_{12}-HTX permitted the study of its effects on the kinetic mechanism for Carb binding.

The association of Carb with AcChR-enriched membrane fragments follows a multiphasic path (Dunn et al., 1980). Three apparently first-order processes can be resolved, having rates of approximately 5, 0.5, and 0.05 s^{-1} for the phases we have designated fast, intermediate, and slow, respectively. Representative traces recorded at a Carb concentration of 5 μM are shown in Figure 5 where they are compared to similar traces recorded after preincubation of the membrane fragments with 10 μM H_{12}-HTX. Clearly, inclusion of H_{12}-HTX did

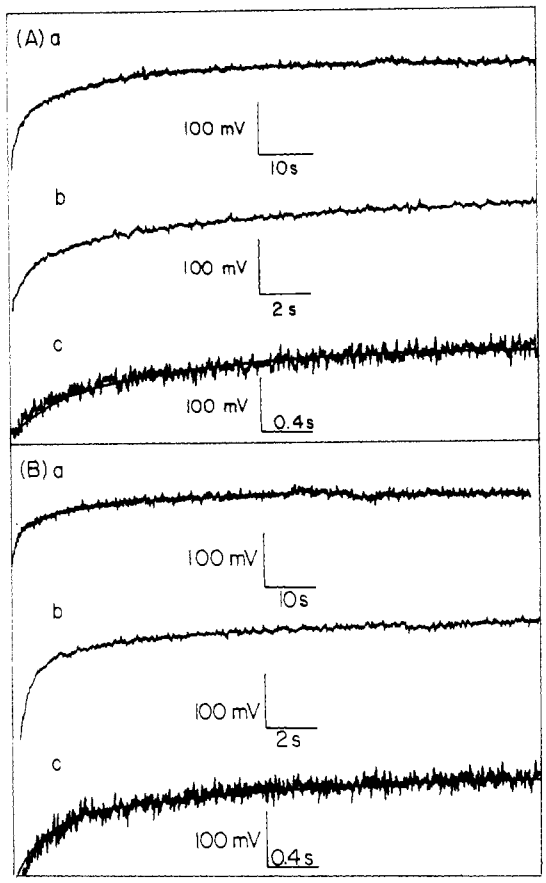

FIGURE 5: Kinetics of Carb binding to IAS-labeled membrane fragments. Stopped-flow traces were obtained by rapid mixing of 0.5 μM AcChR (in α-BuTx sites) and 4 μM Carb (final concentrations after mixing). In (B), 10 μM HTX was included in both syringes, and incubation time with membranes prior to mixing was at least 20 min. Solid lines were calculated from nonlinear regression fitting to a single or double exponential as described under Materials and Methods. Numbers in parentheses were not sufficiently reliable to be included in further analysis [see Dunn et al. (1980)]. The best-fit kinetic parameters were the following. (A) No HTX: (a) (A_1 = 96 mV), (k_1 = 0.61 s^{-1}), A_2 = 101 mV, k_2 = 0.064 s^{-1}; (b) (A_1 = 74 mV), (k_1 = 1.65 s^{-1}), A_2 = 80 mV, k_2 = 0.287 s^{-1}; (c) A_1 = 77 mV, k_1 = 3.11 s^{-1}, (A_2 = 91 mV), (k_2 = 0.317 s^{-1}). (B) +10 μM HTX: (a) (A_1 = 103 mV), (k_1 = 0.849 s^{-1}), A_2 = 72 mV, k_2 = 0.062 s^{-1}; (b) (A_1 = 186 mV), (k_1 = 3.51 s^{-1}), A_2 = 55 mV, k_2 = 0.281 s^{-1}); (c) A_1 = 124 mV, k_1 = 3.09 s^{-1}.

not have any obvious qualitative effect on the appearance of these three phases. A detailed study of the concentration dependencies of the apparent rate constants was made, and the data were fitted to the appropriate equations derived for mechanism 1 which we have previously proposed to account

$$\begin{array}{ccccccc} R + L & \overset{K_1}{\rightleftharpoons} & RL & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & R'L & \overset{K_4}{\rightleftharpoons} & R'L_2 \\ & & & & k_3 \uparrow\downarrow k_{-3} & & k_5 \uparrow\downarrow k_{-5} \\ & & & & C_1 & & C_2 \end{array} \quad (1)$$

for the Carb binding kinetics (Dunn et al., 1980). According to this model, the fast phase arises from R'L formation, the intermediate phase from C_2 formation, and the slow phase from formation of C_1. This slow phase decreases in both amplitude and rate at high ligand concentrations as a consequence of the binding of a second ligand to R'L [see Quast et al. (1979) and Dunn et al. (1980)]. The rate data and their fit to mechanism 1 for Carb binding in the presence and absence of H_{12}-HTX are shown in Figure 6, and the kinetic parameters are listed in Table I. It should also be noted that a faster phase of rate ~30 s^{-1} which was neglected in our earlier analysis (Dunn et al., 1980) and which has not yet been assigned to a mechanistic step was also unaffected by H_{12}-HTX.

Table I: Kinetic Parameters for Carb Binding to IAS-Labeled Membrane Fragments and Effect of 10 μM HTX (from Fit of Data to Mechanism 1)

parameter	no HTX	+10 μM HTX
K_1 (μM)	9.5	12.4
k_2 (s^{-1})	7.8	12.8
k_{-2} (s^{-1})	0.8	0.8
K_4 (μM)	15.6	33.5
k_5 (s^{-1})	1.5	2.5
k_{-5} (s^{-1})	0.17	0.12
k_3 (s^{-1})	0.07	0.10
k_{-3} (s^{-1})	0.04	0.03
K_1K_2 (μM)	0.95	0.79
$K_1K_2K_3$ (μM)	0.49	0.21
K_4K_5/K_3 (μM)	3.3	6.1
$K_1K_2K_4K_5$ (μM^2)	1.6	1.3

In the kinetic experiments described above, the membrane fragment preparations were diluted into Ringers containing 4 mM $CaCl_2$ approximately 1 h before use. The resulting Ca^{2+} gradient across the membrane and the possibility of ion fluxes during the course of the experiment did not contribute to any of the observed fluorescence changes. Equilibration of the membrane vesicles with Ca^{2+} by two cycles of freezing and thawing (Moore & Raftery, 1980) did not affect the kinetic parameters of any phase.

It is obvious that preincubation with H_{12}-HTX did not cause a significant change in the kinetic mechanism for the Carb–AcChR interaction. Minor perturbations in the individual rate constants such as the apparent increase in the rate of the fast phase in the presence of H_{12}-HTX (Figure 6A) were of an order that might be expected from experimental error. The possibility that the binding of H_{12}-HTX itself was affected by IAS modification of receptor-enriched membrane fragments can be excluded since centrifugation assay for the binding of [^{3}H]H_{12}-HTX showed that neither the K_d (1.3 ± 0.5 μM) nor the number of specific HTX sites (39 ± 10% of the α-BuTX sites) was altered by the labeling procedures (data not shown).

Effects of Local Anesthetics on the Kinetics of Carb Binding. Preincubation of the membrane-bound receptor with 10 mM lidocaine, 5 mM prilocaine, or 10 μM dimethisoquin prior to rapid mixing with agonist led to a dramatic departure from the control kinetic signal shown in Figure 5A. In marked contrast to the apparent lack of effect of HTX, the action of these three local anesthetics was to reduce the complex multiphasic signal characteristic of Carb binding to a single fast phase, an example of which is shown in Figure 7. At concentrations of Carb between 0.1 and 20 μM, the kinetic traces could be fitted with precision to a single exponential equation. The rapidity of this process in addition to its rather small amplitude made it difficult to obtain reliable data from any single titration. However, it was evident that the rate of the signal change increased with increasing Carb concentration, and the amplitude was saturated at Carb concentrations greater than about 2 μM. In order to improve the quality of the data, we have accumulated the results from eight separate titrations (using seven different membrane preparations) carried out in the presence of 10 mM lidocaine. Figure 8 shows the averaged values of these titrations. The amplitude data were normalized by using the saturating amplitude in each titration which was estimated from averaging all data obtained at Carb concentrations greater than 5 μM.

The rate of Carb binding to membrane fragments which had been preincubated with 10 mM lidocaine increased as the

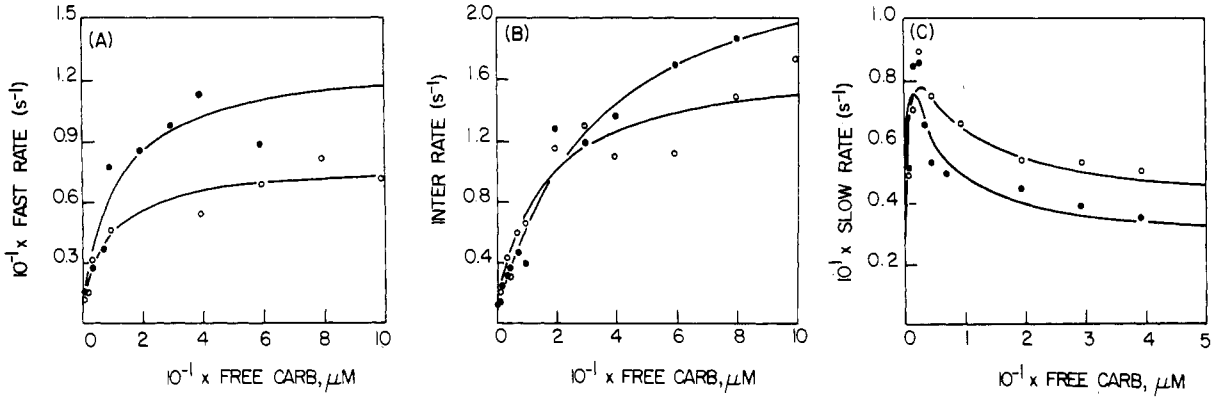

FIGURE 6: Dependence of rate constants on Carb concentration for (A) fast, (B) intermediate, and (C) slow phases in absence (O) or presence (●) of HTX. Data were fitted to mechanism 1 using procedures described by Dunn et al. (1980). The kinetic parameters used to construct the solid lines were as follows. (A) Fast phase: $\lambda_F = k_2L/(K_1 + L) + k_{-2}K_4/(K_4 + L)$, (O) $k_2 = 7.8\ s^{-1}$, $K_1 = 9.5\ \mu M$, $k_{-2}K_4 = 12.1\ \mu M\ s^{-1}$, $K_4 = 15.6\ \mu M$; (●) $k_2 = 12.8\ s^{-1}$, $K_1 = 12.4\ \mu M$, $k_{-2}K_4 = 27.6\ \mu M\ s^{-1}$, $K_4 = 33.5\ \mu M$. (B) Intermediate phase: $\lambda_I = k_{-5} + k_5[L^2/(K_1K_2K_4)]/[1 + L/(K_1K_2) + L^2/(K_1K_2K_4)]$, (O) $k_{-5} = 0.17\ s^{-1}$, $k_5 = 1.53\ s^{-1}$, $K_1K_2 = 0.95\ \mu M$, $K_1K_2K_4 = 14.8\ \mu M^2$; (●) $k_{-5} = 0.12\ s^{-1}$, $k_5 = 2.47\ s^{-1}$, $K_1K_2 = 0.79\ \mu M$, $K_1K_2K_4 = 26.3\ \mu M^2$. (C) Slow phase: $\lambda_S = k_{-3} + k_3[L/(K_1K_2)]/[1 + L/(K_1K_2) + L^2/(K_1K_2K_4K_5)]$, (O) $k_{-3} = 0.04\ s^{-1}$, $k_3 = 0.073\ s^{-1}$, $K_1K_2 = 0.95\ \mu M$, $K_1K_2K_4K_5 = 5.0\ \mu M^2$; (●) $k_{-3} = 0.03\ s^{-1}$, $k_3 = 0.101\ s^{-1}$, $K_1K_2 = 0.79\ \mu M$, $K_1K_2K_4K_5 = 2.3\ \mu M^2$.

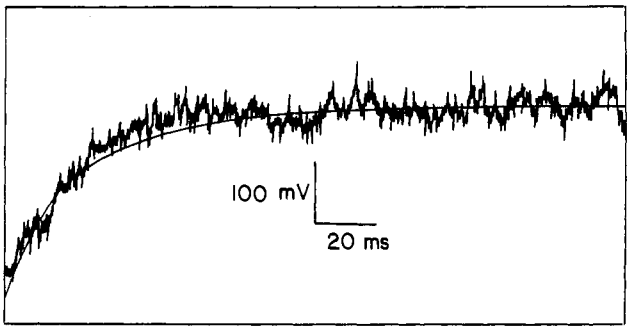

FIGURE 7: Kinetic trace for Carb binding to IAS-labeled membrane fragments after incubation with 10 mM lidocaine. Lidocaine was included in both syringes, and the final concentrations after mixing were 0.5 μM AcChR (in α-BuTx sites), 4 μM Carb, and 10 mM lidocaine. The solid line was obtained from a nonlinear regression fit to a single exponential equation with the values of the kinetic parameters being $A_1 = 303$ mV and $k_1 = 50.6\ s^{-1}$.

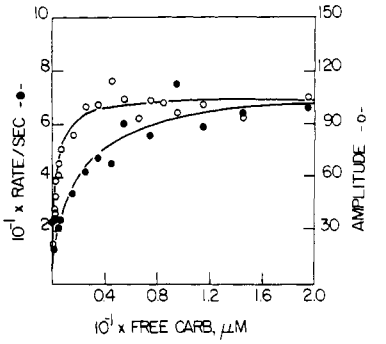

FIGURE 8: Effect of Carb concentration on rate (●) and amplitude (O) of kinetics of binding to AcChR-enriched membrane after incubation with 10 mM lidocaine. Final concentration of AcChR was 0.5 μM, and 10 mM lidocaine was included in both syringes. The figure shows the pooled results of eight titratons as described in the text. Solid lines were obtained by nonlinear regression analysis with appropriate corrections for the concentration of bound ligand. Fit of amplitude data gave $F_0 = 104.8 \pm 0.3$ (arbitrary units) and $K_1K_2 = 0.264 \pm 0.004\ \mu M$. This value for K_1K_2 was used in iterative procedures to fit rate data to the equations given in the text, and the best-fit parameters were $k_{-2} = 8.0\ s^{-1}$, $k_2 = 66\ s^{-1}$, and $K_1 = 2.2\ \mu M$.

Carb concentration was raised but reached a saturating rate of approximately $60\ s^{-1}$ at $\sim 8\ \mu M$ (Figure 8). The observed kinetics of the receptor–Carb complex formation were thus much simplified in the presence of local anesthetic, and they can be accounted for by a less complicated model than mechanism 1.

The simplest mechanism which is consistent with the ligand concentration dependencies of both the rate and amplitude is one in which a bimolecular association is followed by an isomerization of the initial complex

$$R + L \overset{K_1}{\rightleftharpoons} RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R^*L \qquad (2)$$

In this mechanism, R represents the receptor–local anesthetic complex, and K_1 is the dissociation constant of the initial complex with Carb. With the assumption that the formation of RL is a rapid preequilibrium to the isomerization step and that the signal arises from the formation of R*L, the observed rate, k_{app}, has the concentration dependence

$$k_{app} = \frac{k_2[L]}{K_1 + [L]} + k_{-2}$$

and the amplitude can be fitted to a simple binding isotherm

$$F(t) = \frac{F_0[L]}{K_1K_2 + [L]}$$

where $F(t)$ and F_0 are the fluorescence levels at time t and at equilibrium, respectively, and K_2 is equal to k_{-2}/k_2.

Table II: Effects of Local Anesthetics on Equilibrium and Kinetic Parameters for Carb Binding to IAS-Labeled Membrane Fragments[a]

local anesthetic preincubated	K_1 (μM)	k_2 (s^{-1})	k_{-2} (s^{-1})	K_1K_2[b] (μM)	K_d[c] (μM)
10 mM lidocaine	2.2	66.4	8.0	0.26	0.12
5 mM prilocaine	1.8	89.2	3.6	0.07	0.04
10 μM dimethisoquin	0.6	48.8	11.2	0.16	0.05

[a] Data were fitted to appropriate equations derived for mechanism as described in the text. [b] Determined from fit of stopped-flow amplitude data. [c] Determined from equilibrium centrifugation experiments for [^{3}H]Carb binding.

Figure 8 shows that the rate and amplitude data could be fitted to this mechanism with precision, and the parameters k_{-2}, k_2, and K_1 estimated by nonlinear regression techniques were $8.0\ s^{-1}$, $66.4\ s^{-1}$, and $2.2\ \mu M$, respectively (Table II). The value of K_1K_2 of $0.26\ \mu M$ was in good agreement with that determined in equilibrium experiments (Figures 3 and 4).

Preincubation of IAS-labeled receptor preparations with prilocaine or dimethisoquin gave quantitatively similar results to those obtained with lidocaine. Several titrations in the presence of these local anesthetics were carried out, and the

Carb concentration dependence of the kinetics was consistent with the above model. The kinetic parameters obtained are given in Table II where they are compared with equilibrium results.

A brief study was made of the effects of reducing the concentration of lidocaine used for preequilibration. These experiments showed that 1 mM lidocaine was not sufficient to saturate its effects on the Carb binding kinetics. Qualitatively, the kinetic traces accompanying Carb binding seemed to consist of a contribution from the multiphasic signal characteristic of that seen in the absence of local anesthetic and also an enhanced faster phase similar to that observed in the presence of 10 mM lidocaine. It therefore seems likely that the effective equilibrium constant for the lidocaine-induced effect is in the millimolar range, a value consistent with the lidocaine fluorescence titration shown in Figure 2.

Kinetics of Cation Flux across Vesicle Membranes of Control and IAS-Labeled Receptor Preparations. Labeling of AcChR-enriched membrane fragments with IAS does not appear to adversely affect the equilibrium ligand binding properties of the receptor (Dunn et al., 1980). In an attempt to demonstrate that such modified preparations also retain the functional capability of ion translocation, the kinetics of thallium transport (Moore & Raftery, 1980) were examined, and the rates were compared to those exhibited by unlabeled membrane preparations. When membrane vesicles were loaded with the fluorescent dye, ANTS, and then rapidly mixed with buffer containing Tl^+ (10 mM Hepes, pH 7.4, and 17 mM $TlNO_3$ final concentration after mixing), the resulting fluorescence decay had a half-time of about 10 s due to the slow leakage of Tl^+ across the membranes. However, no significant fluorescence signal change occurred on a rapid time scale as shown in Figure 9. Addition of Carb to the buffer resulted in a marked increase in the rate of this Tl^+ transport (Figure 9). This agonist-induced enhancement of membrane permeability was also displayed by IAS-labeled membrane fragments (Figure 9B). At a final concentration of 100 μM Carb, the average value of the apparent transport rate was 15.6 s^{-1} for control membranes and 19.4 s^{-1} for IAS-labeled vesicles while the corresponding values for 250 μM Carb were 55.4 and 52.9 s^{-1}. These rates are in good agreement with those previously reported (Moore & Raftery, 1980) and indicate that the IAS-labeling procedures did not alter the ability of the AcChR to mediate agonist-induced cation translocation.

Preincubation of control or IAS-labeled preparations with either 10 μM HTX or 10 mM lidocaine resulted in a complete block of the agonist-induced enhancement of the rate of ion flux. Under these conditions, the only signal change observed upon mixing of vesicles with Tl^+ and 100 μM Carb was that resulting from slow Tl^+ leakage across the membranes. Both HTX and the local anesthetic therefore have similar effects on the ion translocation properties of the receptor despite their vastly different effects on the agonist binding kinetics described above.

Discussion

Extrapolation of the kinetic results obtained with IAS-labeled membrane fragments to unmodified membrane preparations is valid only if modification of the AcChR with IAS does not alter receptor function. We have previously shown that the ligand binding properties of AcChR are not affected by labeling with IAS (Dunn et al., 1980). The rate of agonist-induced cation flux is proportional to the number of active receptors (Moore et al., 1979), and our experimental conditions result in the incorporation of IAS into receptors to an extent of >50% of the number of high-affinity agonist binding sites

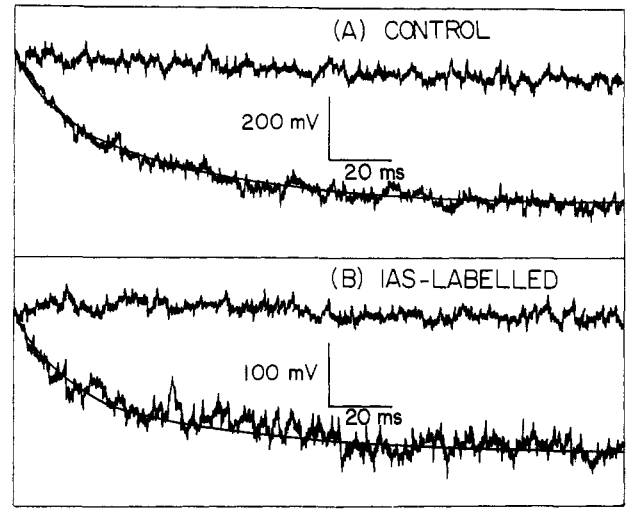

FIGURE 9: Kinetics of Carb-induced thallium flux. Control (A) or IAS-labeled (B) membrane vesicles which had been loaded with ANTS were rapidly mixed with buffer containing 17 mM (final concentration) $TlNO_3$ and no Carb (upper traces) or 100 μM Carb (lower traces). Excitation was at 370 nm, and fluorescence emission was recorded with a Corning C.S. 3-72 filter. The kinetic data were fitted to the equation $F(t) = F_0/\{1 + KT_\infty[1 - \exp(-k_1 t)]\} + A_1 + k_2 t$ where $F(t)$ and F_0 are the fluorescence levels at time t and at equilibrium, k_1 is the apparent Tl^+ flux rate, A_1 is the data base line, and k_2 is the slope of the base line (used to correct for the small leakage component occurring on this time scale). The term KT_∞ was constant and was calculated from the known final Tl^+ concentration (T_∞) of 17 mM and the Stern–Volmer constant, K, of 96 M^{-1} (Moore & Raftery, 1980). The solid lines were calculated from the following parameters obtained by nonlinear regression: (A) control F_0 = 754 mV, k_1 = 16.0 s^{-1}; (B) IAS-labeled F_0 = 305 mV, k_1 = 26.7 s^{-1}. The different amplitudes observed in (A) and (B) can be explained by the different membrane concentrations used in these experiments (not controlled during vesicle preparation).

(Dunn et al., 1980). Therefore, the rate of ion flux for IAS-labeled membrane fragments would be at least 2 times slower than that of untreated membranes if the IAS modification resulted in receptor inactivation. As shown in Figure 9, the rate of Tl^+ flux for IAS-labeled membranes did not differ from that observed for untreated membranes. Therefore, it could be concluded that the labeling procedure did not alter the ability of the receptor to mediate cation flux.

Centrifugation assays using [^{3}H]Carb showed that saturating concentrations of local anesthetics resulted in only a slight decrease in the overall dissociation constant for Carb binding (Table II). This finding is in good agreement with previous results which showed that saturating concentrations of some local anesthetics (Cohen et al., 1974) or of HTX (Kato & Changeux, 1976) resulted in only a slight (<2-fold) decrease in the K_d for [^{3}H]AcCh.

In contrast to the equilibrium results, the kinetics of Carb binding to the local anesthetic–receptor complex differed greatly from those for binding to the receptor alone. Whereas the binding of Carb to IAS-labeled membrane fragments consisted of four distinct kinetic phases, only a single phase was observed in the presence of local anesthetics (Figure 7). A qualitatively similar simplification of agonist binding kinetics was reported by Heidmann & Changeux (1979b). The binding of the fluorescent agonist dansyl-C_6-choline to AcChR-enriched membrane fragments could be resolved into three kinetic phases. However, in the presence of saturating concentrations of local anesthetic, a fast phase accounted for 90 ± 5% of the total signal change. In the presence of HTX, the increase in the amplitude of the fastest phase was less pronounced (~40% of total amplitude), and the rates of the two slower phases remained unaltered (Heidmann & Chan-

geux, 1979b). These results are similar to our finding that HTX did not alter the kinetics of Carb binding to IAS-labeled membrane fragments (Figure 6). A quantitative comparison of the results obtained by using dansyl-C_6-choline to those obtained with IAS-labeled membrane fragments is not possible since Heidmann & Changeux (1979b) did not determine the dependence of the kinetic parameters on agonist concentrations at saturating concentrations of local anesthetic and also because the agonists used in the two studies were different.

Heidmann & Changeux (1979b) interpreted their results as indicating that, in the absence of agonist, local anesthetics stabilize the receptor in a state similar to or identical with the high-affinity state observed at equilibrium in the presence of a cholinergic agonist. This interpretation is not consistent with the data obtained with IAS-labeled membrane fragments. As shown under Results, the binding of Carb to the local anesthetic–IAS-labeled AcChR complex can be described by mechanism 2, where R is the local anesthetic–receptor complex, L is Carb, K_1 is the equilibrium dissociation constant for agonist binding, and the k_2's are isomerization constants. It is clear that the final (equilibrium) receptor conformation, R*L, is reached only in the presence of bound cholinergic ligand. Thus, in our system, the receptor–anesthetic complex has a different conformation from the Carb–receptor–anesthetic complex, indicating that the prior binding of local anesthetic does not induce a receptor state having an intrinsic high affinity for agonists as suggested by Heidmann & Changeux (1979b).

The most obvious difference between the models for agonist binding in the absence of local anesthetics (mechanism 1) and in their presence (mechanism 2) is that there is no evidence for the binding of a second Carb molecule to the local anesthetic–receptor complex. Although positive correlation of an individual receptor state in the presence of local anesthetic with a particular state in its absence is not possible, it is interesting to note the similarities between mechanism 2 and the $R \rightarrow C_1$ pathway of mechanism 1. Since local anesthetics do not significantly perturb the equilibrium binding of Carb (Table II and Figure 4), the final receptor conformation of the Carb–receptor–local anesthetic complex (R*L, mechanism 2) may be similar to the equilibrium (high-affinity) state without local anesthetic (C_1, mechanism 1). The individual steps for the two pathways are also in good agreement. For instance, the initial binding constant for the first Carb in mechanism 1, K_1K_2, varies from $\sim$1 to 5.7 μM, depending on the receptor preparation used (Table I, Dunn et al., 1980). These values are very similar to those obtained for K_1 of mechanism 2 which range from 2.2 μM for the lidocaine–receptor complex to 0.6 μM for the dimethisoquin–receptor complex (Table II). The correlation of the R'L state of mechanism 1 with the RL state of mechanism 2 requires that, in the presence of local anesthetics, the isomerization corresponding to step 2 of mechanism 1 be mute. Although the corresponding states for the two mechanisms are similar, they are not identical, and the quantum yield may depend on the presence of bound anesthetic. In addition, the experimental finding that the saturating amplitude in the presence of local anesthetics is less than that observed in their absence (Figure 3) is consistent with the existence of a mute step. Although the rate constant for the isomerization of the anesthetic–receptor–Carb complex (RL, mechanism 2) is much faster than that of the receptor–Carb complex (R'L, mechanism 1), the equilibrium constants for the two processes are similar: $\sim$0.23–0.04 (range of k_{-2}/k_2 in the presence of local anesthetics, Table II) and $\sim$0.57–0.30 (k_{-3}/k_3, Table I), respectively. Therefore, the main effects of local anesthetics on the binding of Carb to the AcChR are (a) a large increase in the dissociation constant for the binding of a second Carb to the receptor and (b) an increase in the rates of the receptor isomerization which follows the binding of the first agonist molecule.

We have not attempted to identify the various receptor conformations described by mechanism 1 with any physiological states of the receptor (Dunn et al., 1980). However, the actions of local anesthetics and HTX on the rates of receptor transitions as monitored by IAS fluorescence are similar to the effects of these compounds on the agonist-induced affinity changes previously measured in vitro which have been suggested to be a correlate of desensitization in vivo: some local anesthetics increase the rate of this affinity change (Weiland et al., 1977; Briley & Changeux, 1978; Blanchard et al., 1979a) while HTX has no effect on this process (Elliott & Raftery, 1977). Although desensitization has been described as a monophasic process (Weber et al., 1975; Weiland et al., 1976; Lee et al., 1977; Quast et al., 1978a,b), recent electrophysiological studies have shown that the time course of desensitization is biphasic (Anwyl & Narahashi, 1980; Sakmann et al., 1980). It is therefore interesting to speculate that both C_1 and C_2 of mechanism 1 correspond to different desensitized states and that local anesthetics, but not HTX, increase the rate of receptor isomerization. Evaluation of the relationships of mechanism 1, if any, to desensitization in vivo will require a quantitative analysis of the biphasic kinetics recently observed for the physiological process of desensitization.

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