Ligand-Induced Changes in Membrane-Bound Acetylcholine Receptor Observed by Ethidium Fluorescence. 3. Stopped-Flow Studies with Histrionicotoxin†

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ABSTRACT: Rapid kinetic studies of histrionicotoxin interactions with membrane-bound acetylcholine receptor showed a conformational change in the receptor–histrionicotoxin complex as reflected by a decrease in fluorescence intensity of the extrinsic probe ethidium. The simplest kinetic mechanism consistent with the observed data is one in which a rapid preequilibrium exists between receptor and toxin (K = 3.33 μM), followed by a slow conformational change ($k_1 \simeq 2 \times 10^{-2}$ s^{-1} and $k_{-1} \simeq 1.5 \times 10^{-3}$ s^{-1}). The overall equilibrium constant (K_{ov}) determined from a fit of the amplitude dependence on toxin concentration had a value of 0.25 μM. The data preclude kinetic mechanisms where histrionicotoxin acts as an effector, shifting equilibria between preexisting, discrete, and slowly interconverting receptor forms.

The alkaloid HTX,[1] originally isolated by Daly et al. (1971), causes a progressive decrease in the amplitude of the end-plate potential upon tetanic stimulation of frog sartorius muscle (Albuquerque et al., 1973a, 1974). The microiontophoretic application of short pulses of AcCh was found to strongly potentiate HTX antagonism of the observed change in end-plate potential in mammalian skeletal muscle (Albuquerque et al., 1973b). For these reasons, HTX is thought to interact with the "ion conductance modulator" of the AcChR in vivo. Kato & Changeux (1976) have observed that HTX acts noncompetitively and reversibly to block the steady-state depolarization produced by Carb on *Electrophorus* electroplax, and they suggested that it behaves like a potent local anaesthetic. The in vitro characterization of this toxin (Elliott & Raftery, 1977) has shown that there was a saturable component of HTX binding to AcChR-enriched membrane fragments from *Torpedo californica*, having a dissociation constant of 0.3–0.5 μM and a stoichiometry of 1 mol of HTX sites/4 mol of α-BuTx sites.

Although the equilibrium properties of HTX binding to AcChR-enriched membrane fragments (Elliott & Raftery, 1977) are fairly well established, little is known regarding the kinetic mechanism by which these interactions take place. The fluorescent probe Eth has been used in both equilibrium (Schimerlik & Raftery, 1976; Schimerlik et al., 1979) and kinetic (Quast et al., 1978, 1979) studies to show conformational changes in the AcChR–Eth complex upon the binding of cholinergic ligands. Because HTX does not displace [^{3}H]Eth from AcChR-enriched membrane fragments (Schimerlik et al., 1979), at concentrations of HTX up to 30 μM, and since Eth did not displace [^{3}H]HTX in the converse experiment at Eth concentration up to 25 μM (Elliott & Raftery, 1979), it seemed to be a suitable indicator for rapid kinetic studies of the mechanism of AcChR–HTX interaction. In this communication we present evidence that HTX causes a conformational change in the membrane-bound AcChR–Eth complex reflected by fluorescence changes in bound Eth. The final state of the AcChR–HTX–Eth complex was different from that of the AcChR with cholinergic ligands (Quast et al., 1979), and the data were fit by a much simpler kinetic mechanism.

Experimental Section

Materials

The membrane preparation, characterization, and buffering conditions were identical with those reported in the first communication in this series (Schimerlik et al., 1979). Perhydrohistrionicotoxin (H_{12}-HTX) was the generous gift of Professor Y. Kishi, and the sources of all other chemicals have been given (Schimerlik et al., 1979).

Methods

The experimental protocol used in the stopped-flow studies of the Eth–AcChR complex was presented in the previous communication (Quast et al., 1979). The exact experimental conditions used are given in the figure legends. Equilibrium titration of the decrease in Eth fluorescence was done as described in the first communication in this series (Schimerlik et al., 1979).

Data Fitting. Observed rate constants and amplitudes obtained under pseudo-first-order conditions were calculated from a nonweighted least-squares fit to ln $(F_t - F_\infty)$ vs. time, where F_∞ is the fluorescence intensity at equilibrium and F_t is the fluorescence intensity at time t. Where HTX was not in excess over HTX sites, the amplitude of the fluorescence change was measured from the kinetic trace ($\Delta F = F_0 - F_\infty$).

The dissociation constant for HTX was calculated from the reaction amplitudes (Figure 3) by means of a weighted least-squares fit to a double-reciprocal plot of $[HTX]_{bound}^{-1}$ vs. $[HTX]_{free}^{-1}$, where the weighting factors were computed according to Bevington (1969, sections 6.4 and 9.3) by using the errors obtained from the least-squares fit to the semilogarithmic plots. This method was also used to determine the dissociation constant for HTX at various Eth concentrations by steady-state fluorescence titration using a Perkin-Elmer MPF-4 spectrofluorimeter (Table I).

The fit to eq 3 was done by the method of weighted least-squares where the weighting factors were renormalized according to Bevington (1969, section 9.3). Using an initial

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[1] Abbreviations used: α-BuTx, α-bungarotoxin; AcChR, acetylcholine receptor; Carb, carbamylcholine; Eth, ethidium; HTX, histrionicotoxin; H_{12}-HTX, perhydrohistrionicotoxin.

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Table I: Effect of Ethidium on the Apparent Dissociation Constant for HTX

Eth concn (μM)	dissociation constant for HTX (μM)	method
0	0.43 ± 0.19	*a*
	0.3	*b*
0.5	0.60 ± 0.40	*c*
1.0	0.16 ± 0.01	*c*
2.0	0.25 ± 0.06	*d*
5.0	0.43 ± 0.03	*c*

[a] Direct ligand binding studies with [^{3}H]HTX (Elliott & Raftery, 1977). [b] Competition of HTX vs. [^{3}H]HTX (Elliott & Raftery, 1977). [c] Equilibrium fluorescence titration. [d] This stopped-flow study.

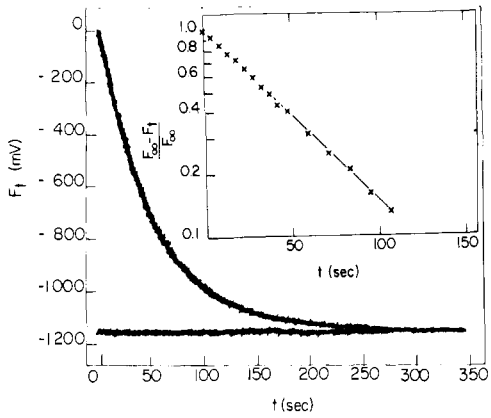

FIGURE 1: Stopped-flow trace after mixing of HTX with membrane-bound AcChR. Final concentrations were Eth, 2 μM; HTX, 20 μM; and α-BuTx sites, 0.36 μM (corresponding to 0.09 μM HTX sites; see text). F_t and F_∞ represent the fluorescence intensities at $t = t$ and at equilibrium, respectively, given in mV. The insert shows the replot of the data in semilogarithmic form. The line was calculated from a nonweighted least-squares fit to the data, giving the parameters $k = (1.82 \pm 0.02) \times 10^{-2}$ s^{-1} and the ordinate intercept $(F_\infty - F_0)/F_\infty = 1.01 \pm 0.01$ where F_0 equals the fluorescence intensity at time zero (correlation coefficient = 0.999).

estimate of k_{-1} from an extrapolation of a plot of k_{obsd} vs. HTX concentration to [HTX] = 0, we calculated k_1 and K from the weighted semireciprocal fit (Figure 4, insert). A new value of k_{-1} was then calculated from these results and the value of $K_{ov} = K(k_{-1}/k_1)$ was independently determined from the the dependence of the reaction amplitudes on HTX concentration (Figure 3). This iterative procedure converged rapidly.

Results

Kinetic Observations and Characterization of Ethidium as an Indicator. The kinetic trace (Figure 1) showed a fluorescence decrease upon mixing 40 μM HTX and 2 μM Eth from one syringe with 2 μM Eth plus AcChR-enriched membrane fragments (0.72 μM in α-BuTx sites) in the second syringe. Under these conditions (HTX in large excess over HTX sites), the reaction followed pseudo-first-order kinetics, and a fit of a semilogarithmic plot of the data to a straight line (Figure 1, insert), taking into consideration well over 90% of the reaction, gave a correlation coefficient of 0.999.

Since HTX does not displace either specifically or nonspecifically bound [^{3}H]Eth (Schimerlik et al., 1979), the fluorescence decrease could be attributed to a change in quantum yield of one or both classes of dye bound to the membrane fragments. After incubation of the membrane fragments with a fivefold molar excess of α-BuTx (thus displacing the specifically bound dye), the fluorescence decrease at 15 μM final HTX was diminished by over 90%. Since it has been previously shown (Elliott & Raftery, 1977)

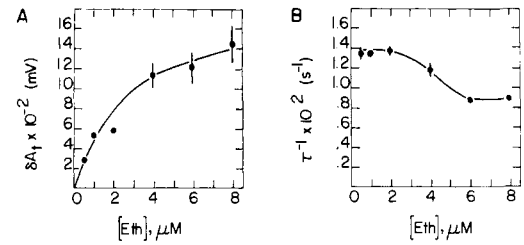

FIGURE 2: (A and B) Dependence of the amplitude and relaxation time on Eth concentration. Final concentrations were 5 μM HTX and AcChR and 0.45 or 0.225 μM (at two different photomultiplier tube voltages) in α-BuTx sites where the values for δA_t are normalized to the same photomultiplier tube voltage. The curve in Figure 2A was calculated from the law of mass action (Eth >> Eth sites) with a dissociation constant $k = 2.54 \pm 0.28$ determined by a weighted linear least-squares fit to the data. The curve in Figure 2B has no theoretical significance. The error bars in Figure 2A and 2B represent the standard deviation of three determinations.

that HTX and α-BuTx bind independently to the AcChR in *Torpedo* membrane fragments, the simplest interpretation of this experiment was that the observed fluorescence decrease was almost completely attributable to a change in the environment of that class of Eth that was displaced by the α-BuTx.

Figure 2 shows the dependence of the observed rate constant and amplitude on Eth concentration. The amplitude dependence (Figure 2A) was that expected for a simple titration curve, indicating that the response was directly related to the occupancy of a saturable class of Eth binding sites: $\Delta F = \Delta F_\infty E_0/[E_0 + K]$, where ΔF is the fluorescence increase, ΔF_∞ is the fluorescence increase at saturation, E_0 is the total Eth concentration (in excess over Eth sites), and K is the dissociation constant for Eth binding to the AcChR. From a weighted least-squares fit to these data, a dissociation constant of 2.54 ± 0.11 μM was calculated for Eth. The agreement of this value with the dissociation constant for the specifically bound dye (Schimerlik et al., 1979) further supports the hypothesis that the fluorescence decrease results from a conformational change that causes a decrease in fluorescence enhancement of the population of Eth molecules bound to the AcChR. The value of the observed rate constant (Figure 2B) (5 μM HTX, final) was not affected at Eth concentrations up to 4 μM and decreased by only 20–25% at 6–8 μM Eth. These data provide a further argument against a displacement of Eth by HTX, in which case a strong decrease in observed rate constant should have been observed as the Eth concentration was increased. They also indicate that the increase in *total* Eth bound to the membrane did not strongly affect the membrane-bound AcChR. The absence of an effect with increasing Eth concentration on the observed dissociation constant for HTX (Table I) indicates that the equilibrium properties of the system also remained unperturbed by the dye.

HTX Concentration Dependence of the Observed Fluorescence Decrease. The dependence of the amplitude of the fluorescence decrease upon total HTX concentration is shown in Figure 3. These data were fit by the law of mass action (see Methods) with an overall dissociation constant $K_{ov} = 0.25 \pm 0.06$ μM, assuming that the concentration of HTX sites equals one-fourth the concentration of α-BuTx sites (Elliott & Raftery, 1977, 1979).

The observed relaxation time τ_1^{-1} (Figure 4) increased with increasing HTX concentration, reaching a limiting value of about 2×10^{-2} s^{-1} at high HTX concentrations. A simple combination of HTX with the Eth–AcChR complex would result in a linear increase of τ_1^{-1} with HTX concentration and would be expected to be much faster than the observed rate. Therefore, the mechanism

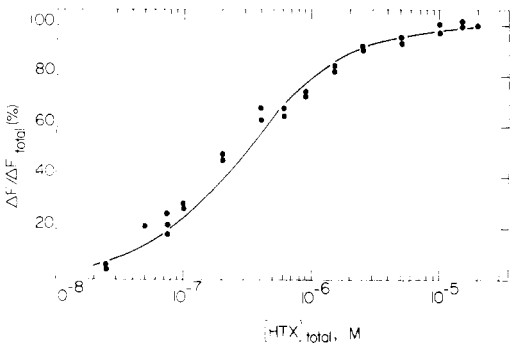

FIGURE 3: Amplitude dependence on HTX concentration. The final concentrations were Eth, 2 μM, and AcChR, 0.36 μM in α-BuTx sites. The data were fitted by a weighted least-squares methods to give a dissociation constant of 0.25 ± 0.06 μM for HTX. The curve in the plot of normalized amplitude vs. total HTX shown above was calculated from the law of mass action with the above dissociation constant and HTX sites equal to one-fourth of the α-BuTx sites.

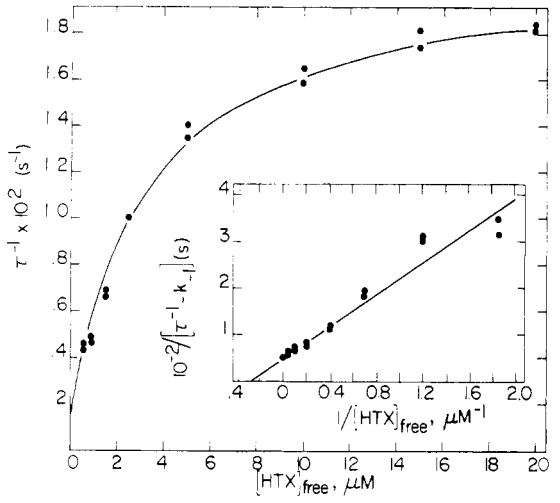

FIGURE 4: Dependence of τ^{-1} on HTX concentration. Final concentrations of Eth and AcChR are the same as in Figure 3. The curve was calculated according to eq 2 (see text) with $k_{-1} = 1.47 \times 10^{-3}$ s^{-1}, $k_1 = 1.96 \times 10^{-2}$ s^{-1}, and $K = 3.33$ μM. (Insert) Weighted least-squares fit to eq 3 which permitted calculation of $k_1 = (1.96 \pm 0.04) \times 10^{-2}$ s^{-1} and $K = 3.33 \pm 0.02$ μM with the estimate of k_{-1} given above (see Experimental Section).

$$R + T \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RT$$

where T is HTX and R is the receptor–Eth complex with the fluorescence decrease resulting from the formation of RT, is not compatible with the data. The simplest mechanism that is consistent with the observed amplitude and rate dependence on HTX concentration is

$$R + T \overset{K}{\rightleftharpoons} RT \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RT' \qquad (1)$$

The HTX (T) is considered to be in rapid equilibrium with the AcChR–ethidium complex (R) with $K = [R][T]/[RT]$. After the binding of the HTX, a slow conformational change from RT to RT′ occurs with first-order rate constant k_1 in the forward direction, the decrease in fluorescence corresponding to the formation of RT′. If we assume that $K_1 = k_{-1}/k_1 \ll 1$ (see below), $K_{ov} = [R][T]/([RT] + [RT'])$ is approximately equal to KK_1. In general, mechanism 1 has two relaxation times (Quast et al., 1974); however, in the case that the binding step is unobservably fast, only the slow relaxation time remains and can be written

$$\tau^{-1} = k_{-1} + k_1 T/(T + K) \qquad (2)$$

(Hammes & Wu, 1974). This concentration dependence agrees with the experimental observations (Figure 4). Since the observed reaction amplitude δA_t remains constant with increasing saturation of the fast preequilibrium, the observed fluorescence signal can be assigned solely to the slow RT ⇌ RT′ isomerization step. If part of the signal were to arise in the binding step, δA_t would decrease in the concentration range where $[T] > K$ since the fast step is unobservable. However, at $[HTX]_{total} = 6K$, the fit of the data yields a value of $F_\infty - F_0/F_\infty$ equal to 1 (Figure 1, insert), indicating that the total fluorescence signal change was observed in the amplitude of the slow relaxation. Only monophasic kinetics were seen at all HTX concentrations.

The parameters in eq 2 were obtained by a weighted linear least-squares fit to the linearized form of eq 2

$$(\tau^{-1} - k_{-1})^{-1} = (K/k_1)(1/T) + 1/k_1 \qquad (3)$$

(see Methods). The fit is shown in Figure 4, insert. Using an estimated value of k_{-1} equal to 1.47×10^{-3} s^{-1}, k_1 was calculated from the intercept of the double-reciprocal plot to equal $(1.96 \pm 0.4) \times 10^{-2}$ s^{-1}, and K was calculated to equal 3.33 ± 0.02 μM. The value of K_1 could then be calculated according to $K_1 = K_{ov}/K = (7.5 \pm 1.8) \times 10^{-2}$ μM. The fit of the experimental dependence of the relaxation rate on free HTX concentration to eq 2, with the parameters described above, is shown in Figure 4.

In the presence of 100 μM Carb, such that the AcChR was essentially all in the AcChR–Carb complex, different kinetics were observed after mixing with HTX. At least two exponentials were seen, the fastest about 10-fold faster and the slowest about 10-fold slower than the relaxation observed in the absence of Carb. Neither the rate nor amplitude of either kinetic phase appeared to depend on HTX concentration in the region where the kinetics were pseudo first order. With increasing time of exposure of the membranes to Carb (in the presence of 4 mM Ca^{2+} at room temperature), the kinetic behavior of the different phases changed in that the slower phase (1) increased in amplitude at the expense of the faster phase (2). These difficulties prevented a rigorous evaluation of the observed HTX kinetics in the presence of Carb.

Discussion

The kinetic studies reported here indicate that HTX induces a slow conformational change in the membrane-bound AcChR which is reflected by a decrease in the quantum yield of the Eth specifically bound to the receptor. This assignment of the signal stems from the fact that over 90% of the fluorescence decrease is eliminated when these molecules of dye are displaced by α-BuTx (which does not interfere with HTX binding). It is further supported by the dependence of the amplitude on Eth concentration. Neither the observed rate constant nor the overall dissociation constant for HTX was greatly affected by Eth at concentrations up to 5–8 μM. The results indicate that HTX does not displace Eth from the receptor or alter the equilibrium properties of the system. We therefore conclude that the fluorescence probe Eth accurately reflects the interactions of HTX with the membrane-bound AcChR.

The HTX concentration dependence of the amplitude can be fit to the law of mass action by using an equilibrium constant $K_{ov} = 0.25$ μM and assuming the number of HTX sites equals one-fourth of the α-BuTx sites. These values agree well with those previously determined in equilibrium studies with [^{3}H]HTX in this laboratory [Table I and Elliott & Raftery (1977)]. The dependence of the observed relaxation rate, τ_1^{-1}, on HTX concentration was not consistent with a

$$\begin{array}{ccc} R + T \underset{\text{fast}}{\overset{k}{\rightleftharpoons}} RT \\ \text{slow } k_1 \uparrow\downarrow k_{-1} \\ R' \end{array} \quad (4)$$

simple bimolecular combination of HTX with the Eth–AcChR complex as discussed above.

These data are also inconsistent with mechanism 4 which includes two slowly equilibrating receptor forms, only one of which, R, binds HTX. The signal due to the decreased quantum yield of bound Eth to the R and RT forms would come from the slow isomerization step $R' \rightleftharpoons R$. This mechanism predicts an increase in amplitude but a decrease in the observed rate, τ^{-1}, as ligand concentration increases (Hammes & Wu, 1974). Therefore, the observations described above argue against any mechanism that assumes two preexisting interconvertible states for the membrane-bound AcChR where HTX acts as an allosteric effector favoring the R over the R′ state.

The simplest kinetic mechanism consistent with our observations is given in eq 1 and fitted to the experimental data in Figures 3 and 4. The concentration dependence of the reaction amplitude, δA, agrees with mechanism 1 assuming that the change in quantum yield of the ethidium–AcChR complex occurs exclusively from the formation of RT′. In this case the reaction amplitude is given by eq 5, where γ, γ_1, and

$$\delta A = F_\infty - F_0 \approx (\gamma_2 - \gamma)\overline{RT'} \approx (\gamma_2 - \gamma)\frac{R_0 T}{T + kk_1} \quad (5)$$

γ_2 are the quantum yields of bound dye in complexes R, RT, and RT′ and the bar denotes equilibrium concentrations. In addition to the above assumption that $\gamma = \gamma_1$, it also has been assumed that $(\gamma_1 - \gamma)\overline{RT} << (\gamma_2 - \gamma)\overline{RT'}$. Since $k_1 \ll 1$, the latter assumption is correct as long as $\gamma_1 - \gamma$ is not significantly greater than $\gamma_2 - \gamma$. The experimental data (Figure 3 and Table I) support the above assumptions since both stopped-flow and equilibrium data are fit by the law of mass action with a single dissociation constant. If the ligand binding step was accompanied by a change in quantum yield ($\gamma \neq \gamma_1$) that is too fast to be observed, the expression for the reaction amplitude becomes

$$\delta A \approx (\gamma_2 - \gamma)(\overline{RT'}) = (\gamma_1 - \gamma)RT|_{t=0} \quad (6)$$

where $t \approx 0$ is the earliest time at which experimental observations are possible (~2 ms) and $RT|_{t\approx 0} = R_0T/k + T$. The data in Figure 3 and Table I can only be fit to the law of mass action with one dissociation constant as long as $\gamma_1 = \gamma$. The high calculated value for the second-order rate constant for HTX binding to the AcChR also argues against a fast, unobservable reaction that results in an increase in quantum yield of bound dye. At [HTX] equal to 1 μM, assuming $k = 3.3$ μM, $|\gamma_1 - \gamma| \approx |\gamma_2 - \gamma|$ and that RT is formed within 2 ms, the forward rate constant for the fast process is calculated to be greater than 10^9 M^{-1} s^{-1}. This approaches the upper limit for a diffusion controlled encounter and is 1 order of magnitude faster than the value reported for AcCh binding to the solubilized AcChR (Neumann & Chang, 1976) and 50 times faster than the forward rate constant for suberyldicholine binding to AcChR-enriched membranes (Barrantes, 1978). Thus, both the amplitude dependence on HTX concentration and the high value of the estimated second-order rate constant argue in favor of the fluorescence increase arising exclusively in the slow isomerization in eq 1. The values of $k_1 = 1.96 \times 10^{-2}$ s^{-1} and $k_{-1} = 1.42 \times 10^{-3}$ s^{-1} obtained from the data evaluation indicate that the conformational change induced by HTX was much slower than that caused by high Carb concentrations where observed rates of about 3 s^{-1} were seen (Quast et al., 1979). This conformational change results in a 13-fold tighter binding of HTX ($K_{ov} = 0.25$ μM) than in the precomplex ($K = 3.33$ μM).

In a recent study Sobel et al. (1978) found a 15% reduction of the total quinacrine fluorescence signal, measured by energy transfer, in AcChR-enriched membranes. At the HTX concentration reported in their study (5 μM), they found a half-time of 15 min. The half-time that we observed at 5 μM HTX calculated from the data in Figure 4 is slightly less than 1 min. Since they did not report the HTX concentration dependence of their observed rate constant, the reason for this 15-fold difference remains unclear.

The fact that the fluorescence signal of the dye has the opposite sign in the Eth–AcChR–HTX ternary complex from in the Eth–AcChR–Carb complex strongly indicates that the final conformation of the receptor is different for each of these ligands. The finding that the observed kinetic mechanisms are different depending on whether the cholinergic ligand was an antagonist, agonist (Quast et al., 1979), or HTX further supports the existence of different final states for the AcChR. These studies indicate that the final conformation of the membrane-bound AcChR may, in fact, depend on the number of ligands bound per receptor molecule (Quast et al., 1979) as well as the specific structure of the ligand. This type of "induced fit" mechanism (Koshland, 1970) that we have observed in the interaction of cholinergic ligands with the membrane-bound AcChR is at variance with the concept of discrete, interconvertible, preexisting receptor states in vitro as described by Grünhagen & Changeux (1976).

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