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IMPORTANCE OF THE MEMBRANE IN LIGAND–RECEPTOR INTERACTIONS

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Abstract—NMR data that underscore the importance of the membrane in ligand–receptor interactions were obtained and analyzed. The following hypothesis for acetylcholine (ACh) binding to the acetylcholine receptor (AChR) is proposed: ACh first binds to the membrane, where it adopts its bioactive conformation, and it then rapidly diffuses along the membrane to bind to the AChR in its already-correct conformation. Data used to support this hypothesis include (a) the NMR-determined binding constant of $K_M = (2.8 \pm 0.6) \times 10^3\ M^{-1}$ for the binding of ACh to the asolectin membrane, (b) the lipid dependence of AChR activity, (c) the location of the ACh binding site close to the membrane surface, and (d) the conformation of ACh in its membrane-bound state. Additional experiments to test this hypothesis are proposed.

Although it is well known that the lipophilicity of drugs is an important modulator of pharmacological activity, the details of the interactions of lipophilic drugs with membranes are largely unknown. Many drugs interact on the intracellular and extracellular surfaces of membrane bound receptors, and others are suspected to act at receptor sites in the membrane interior [1, 2]. Furthermore, work with bioactive peptides has shown that they undergo conformational changes when added to membrane solutions [3–5]. These same conformations are thought to be the bioactive form of these peptides. For example, circular dichroism (CD) and nuclear magnetic resonance (NMR) studies have shown that endorphins adopt helical conformations in mixed solvents and when binding to membranes [6–11]. Modification of the endorphin peptide sequence to destroy helix formation simultaneously eliminates biological activity [12].

The lipid composition of membranes clearly has an important role in the activity of membrane-bound receptors. McNamee and coworkers [13] have performed an elegant series of experiments studying the lipid dependence of the activity of the acetylcholine receptor (AChR). They used lipids of defined composition and found that cholesterol and negatively charged lipids greatly enhance the ion response of vesicles [14]. They attributed this effect to vesicle size, but we note that it may also be related to interactions between the cationic acetylcholine receptor agonists and the lipid, or to membrane liquidity modulating the activity of the receptor itself. Furthermore, Zabrecky and Raftery [15] found that the optimum lipid composition for AChR activity is precisely the one found in nature, further emphasizing the importance of the membrane composition in receptor activity.

The membrane lipid composition may affect the activity of membrane-bound proteins through direct lipid–protein interactions but Schwyzer and coworker [16–18] and Herbette and coworkers [1, 19, 20] propose that ligand interactions with the membrane are important because they optimize the ligand conformation and orientation for binding to the receptor. They also argue that much faster diffusion in the membrane than in bulk solution further enhances the binding rate to the receptor. Schwyzer points out that certain polypeptide hormones interact strongly and specifically with pure lipid membranes, and that these peptides have well defined conformations and orientations when bound to the membranes. He also suggests that the accumulation of multivalent peptides near charged membranes may enhance their activity, and he has used such a model to explain the activity of opioid peptides [17]. Herbette *et al.* [20] have experimental evidence for similar partitioning of cardiac drugs where they have shown that certain drugs adopt well-defined positions in the lipid membrane, and that this positioning is different for different drugs. They explain the different pharmaceutical properties of these drugs in terms of the interactions of the drugs with the lipid bilayers. They also calculated for model systems that the approach of a drug to its membrane receptor by diffusion in the lipid bilayer is about 1000 times faster than diffusion through the aqueous medium [19]. Direct fluorescence measurements show that diffusion of drugs in lipid bilayers is significantly faster than the overall binding to the receptor [1].

We previously used proton NMR relaxation measurements to study the binding of ACh and other agonists to the AChR [21], and transferred nuclear Overhauser effect (NOE) measurements to determine the conformation of ACh bound to the receptor [22]. We found that the conformation of ACh in solution is different from the ACh conformation when bound to the high-affinity (desensitized) state of the AChR. We also found the unexpected result that the conformations of ACh in the lipid and when bound to the receptor are the same.

Our observations of ACh conformation and lipid affinity, taken together with the findings of Schwyzer

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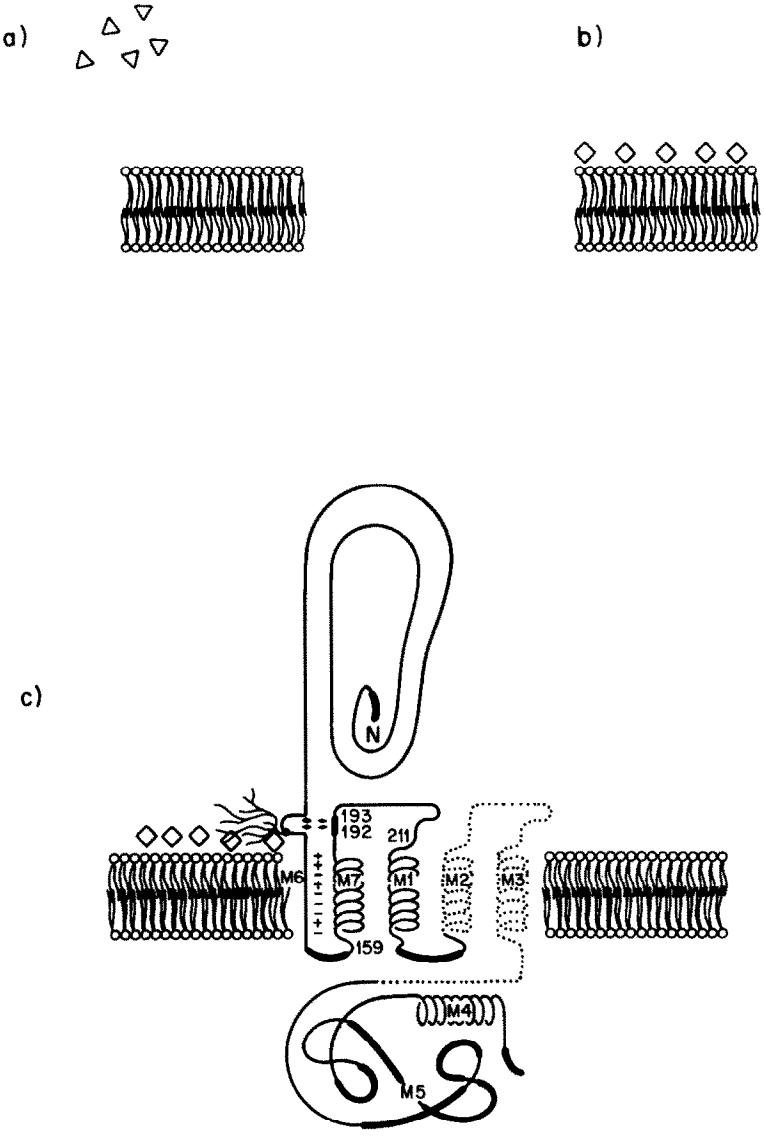

Fig. 1. Proposed steps concerning the role of the lipid in ACh binding to the AChR. (a) ACh is released from the synaptic vesicles and diffuses across the synaptic gap; (b) upon encountering the lipid, ACh undergoes a conformational change, and it undergoes two-dimensional diffusion until it binds to the AChR (c) [(c) adapted from Ref. 26].

and Herbette, lead us to propose that the lipid plays an important role in the binding of ACh to the AChR. This hypothesis is shown schematically in Fig. 1. After ACh is released from the synaptic vesicles (Fig. 1a) it diffuses across the synaptic cleft and binds to the large exposed membrane on the target cell (Fig. 1b). Once in the lipid, ACh undergoes a conformational change [22] and furthermore undergoes two-dimensional diffusion to the receptor site. Two-dimensional diffusion is much faster than three-dimensional diffusion, and this reduced dimensionality would enhance collisions between ACh and the AChR [19]. Already in its bioactive conformation, ACh then binds to the AChR (Fig. 1c). Although the exact location of the ACh binding site on the α-subunits of the AChR is unknown, it has been localised recently to approximately residues 158–216 by several groups [23–27]. This site has been localised at the membrane surface [27].

In this paper we examine the experimental data that support the hypothesis outlined in Fig. 1 and provide order-of-magnitude calculations that relate this hypothesis to relaxation data and to data in the literature. Experiments to test this hypothesis are proposed.

MATERIALS AND METHODS

Acetylcholine receptors were isolated from the electroplax organs of freshly killed *Torpedo californica* obtained from Pacific Biomarine of Venice, CA. They were purified and reconstituted into aso-

Table 1. Interproton distances in acetylcholine*

Interaction	Interproton distances (Å)			
	Crystal structure [29]	In D_2O	Bound to lipid	Bound to AChR
CH_2-CH_2	2.6	2.6	2.3	2.2
CH_2CH_2-$N(CH_3)_3$	3.3	3.5	3.1	3.2
$CH_2O(CO)CH_3$	4.3	4.1	3.0	3.1
CH_2-$N(CH_3)_3$	3.0	3.0	3.0	3.0
$CH_2CH_2O(CO)CH_3$	5.2	4.4	2.8	3.1
$N(CH_3)_3$-CH_3	5.3	5.5	3.4	3.3

* Estimated error 10% except for crystal structure data. Calculated from ^1H two-dimensional NOE measurements assuming the effective distance between the protons CH_2-$N^+(CH_3)_3$ is 3.0 Å in the bound and free states.

lectin vesicles according to the procedure described by Huganir and Racker [28] with the modifications described in Ref. 21. Characterization of vesicle size distributions and protein binding sites has been described previously [21].

NMR samples were prepared by vacuum concentrating affinity-purified receptors and dialyzing the concentrated (typically 60–100 μM in binding sites) asolectin–protein preparation against three changes of dialysis buffer [20 mM phosphate, pH 7.4, 1 mM EDTA, 5 mM dithiothreitol (DTT), and 0.4% cholate], followed by three more dialyses against the same buffer prepared with D_2O. The acetylcholine esterase activity of the protein preparation was inhibited by adding 1 μL diisopropyl fluorophosphate (Aldrich) to the NMR tube containing the receptor preparation. When needed for control experiments, a 3- to 4-fold stoichiometric excess of α-bungarotoxin was added to the NMR sample to block ACh binding to the AChR. The NMR samples were stable for several days at 23° and for longer periods at 4°. The binding activity of the receptors was destroyed by freezing the samples.

All NMR measurements on the receptor system were mader on a Bruker AM 360 MHz wide-bore spectrometer equipped with an Aspect 3000 data system. The binding of ACh to asolectin was measured on a JEOL GSX-500 by measuring selective relaxation times of the trimethylammonium protons of ACh in asolectin as a function of ACh concentration. The selective relaxation rates ($R^{sel}_{1,obs} = 1/T_{1,scl}$) were fit by a least-squares routine to the equation:

$$R^{sel}_{1,obs} = \frac{(\Delta R_M L_t)K_M}{1 + K_M[ACh]} + R^{sel}_{1,free} \qquad (1)$$

where ΔR_M is the difference between the relaxation rates of ACh in the bound and free states ($R^{sel}_{1,bound} - R^{sel}_{1,free}$), L_t is the lipid concentration (i.e. the number of lipid binding sites), and K_M is the binding constant. Nonselective T_1s were measured with a standard inversion-recovery pulse sequence. The 90° pulse width was typically 9.25 μsec. Generally, 16 scans each of 10 τ values were taken ranging from 0.2 T_1 to 2 T_1. Selective T_1s were measured by inverting the desired resonance with a 10-msec soft pulse from the decoupler. The reported T_1 values were determined by a three-parameter fit. The relaxation data were always single exponential over the entire range of τ values.

Two-dimensional NOE NMR data sets were obtained in the phase sensitive mode [29] at a temperature of 25°. The mixing time for the receptor-containing samples was 800 msec and the relaxation delay was 6 sec; for the sample of ACh in D_2O the mixing time was 2 sec and the relaxation delay was 10 sec. The samples for NOE measurements contained 10 mM ACh and 91 μM AChR. The data were processed, and peak volumes were extracted with FTNMR from Hare Research (Woodinville, WA). A complete relaxation rate analysis and subtraction techniques were used to determine the conformations of ACh in D_2O, in the lipid, and bound to the AChR. The details of this analysis have been described [22]. The cross-relaxation rates were used to determine a set of interproton distances, and these distances are repeated in Table 1 for completeness.

The distances shown in Table 1 are not sufficient to provide a unique description of the conformation of ACh. Conformational energies corresponding to four possible families of structures were calculated using the Dreiding potentials of the Biograf 1.34 program (Biodesign, Pasadena, CA) as described previously [22]. The lowest energy conformation was calculated to be 7 kcal/mol higher in energy than the crystal structure conformation.

RESULTS AND DISCUSSION

The distances obtained from analysis of the NOE results (Table 1) show that there are major conformational differences between ACh in its free state and in its lipid- and receptor-bound states. The major difference is that the arrangement of the N—C—C—O backbone goes from *gauche* in solution to nearly *trans* in both the lipid-bound and receptor-bound states. These differences are presented in Fig. 2. The solution state conformation (Fig. 2a) is in agreement with the conformation determined by X-ray crystallography [30]. The *gauche* conformation that occurs in the crystal is stabilized by the interaction of the positively charged nitrogen group with the electron-rich oxygen. We hypothesize that this inter-

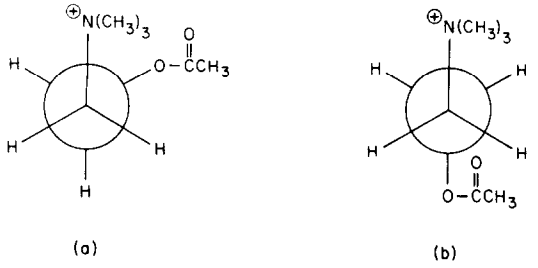

Fig. 2. Conformations of ACh. (a) The *gauche* conformation, found both in the crystalline state and in solution; and (b) the *trans* conformation, observed when ACh associates with the lipid or binds to the AChR.

Table 2. Selective spin-lattice relaxation times for ACh

Protons	Selective T_1 (sec)		
	In D_2O	In lipid*	With AChR
$CH_2O(CO)CH_3$	2.4	2.0	0.53
CH_2-$N(CH_3)_3$	2.2	1.6	0.56
$N^+(CH_3)_3$	1.7	1.4	0.77
CH_3	4.2	3.1	0.77

* This sample contains ACh, lipid, and AChR that was inhibited completely with α-bungarotoxin as the exact control, because any contribution from non-specific binding cancels out.

action is lost when the ACh molecule interacts either with the lipid or with amino acid sidechains in the active site of the receptor, allowing ACh to adopt a *trans* conformation. The similarity between the conformation of ACh interacting with the lipid and that bound to the AChR is interesting, as such a similarity has already been conjectured [31].

The selective relaxation data (Table 2) also point to an interaction between ACh and the lipid. The selective T_1s of all the ACh protons decrease in going from a D_2O environment to a lipid environment, and there is a larger decrease in going to the receptor-bound environment.

Upper bounds for the lifetimes of ACh in the lipid-associated and the receptor-bounds states can be estimated from the data in Table 2 using the equation:

$$\frac{1}{T_{1,\text{observed}}} - \frac{1}{T_{1,\text{free}}} = \frac{f_{\text{bound}}}{T_{1,\text{bound}} + \tau_{\text{bound}}}, \qquad (2)$$

where $T_{1,\text{observed}}$ is the observed relaxation time, $T_{1,\text{free}}$ is the relaxation time for the ligand in its unbound state, f_{bound} is the fraction of ligand bound, $T_{1,\text{bound}}$ is the T_1 in the bound state, and τ_{bound} is the lifetime of the ligand in the bound state [32]. The upper bound on the lifetime of ACh in its lipid-associated state is about 500 msec, and for ACh in its receptor-bound state it is 6 msec.

The data in Table 2 are more instructive if one calculates the factor decrease in the ACh T_1 in going from the water environment to the lipid environment to the receptor environment (Table 3). The overall change in the T_1 ranges from 2.2 for the choline methyl protons to 5.5 for the acetyl methyl protons (Table 3, column 1). The factor of 1.2 to 1.4 decrease in the T_1 observed when ACh associates with the lipid is fairly uniform (Table 2, column 2). The T_1 differences between the lipid environment and the AChR-bound state (Table 3, column 3) show large differences. The largest changes are seen for the $CH_2O(CO)CH_3$ and the acetyl methyl protons. These large changes may reflect specific ACh–AChR interactions at the binding state.

Together with the literature cited at the beginning of the paper, the conformational data (Table 1) and the relaxation data (Table 2) support the hypothesis that the overall binding reaction ACh + $R \rightleftharpoons$ ACh–R involves two steps:

$$\text{ACh} + M \rightleftharpoons \text{ACh}\text{–}M \quad K_M = \frac{[\text{ACh}\text{–}M]}{[\text{ACh}][M]} \qquad (3)$$

$$\text{ACh}\text{–}M + R \rightleftharpoons \text{ACh}\text{–}R + M \quad K_R = \frac{[\text{ACh}\text{–}R][M]}{[\text{ACh}\text{–}M][R]} \qquad (4)$$

where M and R refer to the membrane and the receptor respectively. This means that one can separate the free energy of ACh binding to the AChR into two main components:

$$\Delta G_{\text{total}} = \Delta G_M + \Delta G_R \qquad (5)$$

where ΔG_M is the free energy of ACh association with the membrane and ΔG_R is the free energy of ACh binding to the AChR. From Equations 2 and 3, one can write a binding expression:

$$K_{\text{total}} = K_M K_R = \frac{k_M^+ k_R^+}{k_M^- k_R^-} \qquad (6)$$

Table 3. Relative changes in T_1 as a function of lipid association and receptor binding

Protons	Overall decrease $D_2O \rightarrow$ AChR	Decrease $D_2O \rightarrow$ lipid	Decrease lipid $\rightarrow$ AChR
	$T_{1(D_2O)}/T_{1(\text{AChR})}$	$T_{1(D_2O)}/T_{1(\text{lipid})}$	$T_{1(\text{lipid})}/T_{1(\text{AChR})}$
$CH_2O(CO)CH_3$	4.5	1.2	3.8
CH_2-$N(CH_3)_3$	3.9	1.4	1.8
$N^+(CH_3)_3$	2.2	1.2	1.8
CH_3	5.5	1.4	4.0

where M and R refer to the membrane and receptor, as before, and + and − refer to the on- and off-rates respectively.

The total free energy change for binding of the ACh to the AChR is about −11 kcal/mol as measured in radiolabeled ligand binding studies [33]. This energy will be divided between binding of the ACh to the membrane (ΔG_M) and to the receptor (ΔG_R). The conformational energy difference between ACh in D_2O and ACh in lipid has been calculated *in vacuo* to be about +7 kcal/mol [22]. However, complete immersion of the ACh into the lipid is expected to be accompanied by an energy change of −9.25 kcal/mol based on surface area for ACh of 370 Å^2 (calculated using the CONGEN program [34]) and an expected energy change of 25 cal · mol^{-1} · Å^{-2} [35]. This gives a net binding energy $\Delta G_M = -2$ kcal/mol. Selective spin-lattice relaxation rate measurements of ACh interacting with pure asolectin liposomes show that $K_M = (2.8 \pm 0.6) \times 10^3\,\mathrm{M}^{-1}$, so $\Delta G_M = -4.7$ kcal/mol. ΔG_R is the remaining energy, which is −6.6 kcal/mol. Using these values of ΔG, we now estimate the lifetimes of ACh in the membrane-bound (τ_M) and receptor-bound (τ_R) states to determine if they are within the limits set by the NMR measurements ($\tau_M < 500$ msec; $\tau_R < 6$ msec).

To estimate the lifetimes of ACh in the membrane bound and receptor bound states, it is necessary to evaluate the rate constants for the binding steps. Using the steady-state approximation for the formation of the complex ACh–M, and determining the rate of formation of ACh–R, it can be shown that

$$k^+_{\mathrm{app}} = \frac{k^+_R[M]([\mathrm{ACh}]k^+_M + [\mathrm{ACh}\text{–}R]k^-_R)}{[\mathrm{ACh}](k^-_M + k^+_R[R])}. \quad (7)$$

For any reasonable choice of rates, it will be true that $k^+_M[A] \gg [\mathrm{ACh}\text{–}R]k^-_R$. It is necessary to determine what range of rate constants will fit the observed binding in the stopped flow measurements and the NMR measurements. We will choose a value of k^+_M and determine the remaining rate constants by rearranging Equation 7 to give

$$k^+_R = \frac{k^+_{\mathrm{app}}k^-_M}{(k^+_M[M] - k^+_{\mathrm{app}}[R])}, \quad (8)$$

and by using $k^-_M = k^+_M/K_M$ and $k^-_R = k^+_R/K_R$.

In the NMR experiments, the total ACh concentration is 10×10^{-3} M. The exact total lipid concentration is unknown, but it is estimated that $[M]_0 = 5 \times 10^{-3}$ M. The $[M]$ will therefore be about 3.15×10^{-4} M based on K_M. Initially, the receptor concentration, $[R]$, is 90×10^{-6} M. Using these numbers, along with $k^+_{\mathrm{app}} = 5.7 \times 10^7\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1}$ [32], the NMR-derived value for K_M, and assuming that $k^+_M = 1 \times 10^8\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1}$ (i.e. a diffusion-controlled reaction), we find that $k^-_M = 3.6 \times 10^4\,\mathrm{sec}^{-1}$, $k^+_R = 7.7 \times 10^7\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1}$, and $k^-_R = 8.7 \times 10^2\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1}$.

The lifetime of the membrane-bound ACh–M complex is:

$$\begin{aligned}\tau_M &= [k^+_M[\mathrm{ACh}] + k^-_M]^{-1} \\ &= [(1 \times 10^8\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1})(5.2 \times 10^{-3}\,\mathrm{M}) \\ &\quad + (3.6 \times 10^4)\mathrm{sec}^{-1}]^{-1} = 1.8\,\mu\mathrm{sec}. \end{aligned} \quad (9)$$

The lifetime of the receptor-bound acetylcholine is:

$$\begin{aligned}\tau_R &= [k^+_R[\mathrm{ACh}\text{–}M] + k^-_R[\mathrm{ACh}\text{–}R]]^{-1} \\ &= [(7.7 \times 10^7\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1})(4.6 \times 10^{-3}\,\mathrm{M}) \\ &\quad + (8.7 \times 10^2\,\mathrm{M}^{-1}\,\mathrm{sec}^{-1})(90 \times 10^{-6}\,\mathrm{M})]^{-1} \\ &= 2.8\,\mu\mathrm{sec}. \end{aligned} \quad (10)$$

These are well within the lifetimes required by the NMR measurements of $\tau_M < 500$ msec and $\tau_R < 6$ msec.

SUMMARY

There are several lines of evidence presented at the beginning of the paper that suggest that binding of ligands to membranes plays an important role in mediating the binding of a ligand to its receptor. The arguments by Schwyzer and coworker [16–18] and Herbette and coworkers [1, 19, 20] are persuasive, but they lack direct evidence that the ligands diffuse in the membrane to the receptor site and that the conformation of the ligand in the membrane is related to the conformation of the receptor-bound ligand. The evidence that ACh binding to the receptor is mediated by the membrane is based on the observed affinity of ACh for the membrane, the lipid dependence of AChR activity [13–15], the location of the ACh binding site close to the membrane surface [26, 27], and that the lifetimes of ACh bound to the membrane and receptor fall within the limits set by the NMR measurements. The key piece of evidence is that the conformation of ACh in the lipid is the same as the ACh conformation bound to the AChR. We suggest that the ACh first binds the membrane, adopting the conformation that will be recognized by the AChR, and then rapidly diffuses along the membrane to the AChR.

Although the arguments presented here are suggestive, they do not prove the hypothesis. Proof will require information along the following lines. Detailed kinetic models of the multiple binding steps must be consistent with observed data from stopped-flow and other measurements. There must also be direct evidence that diffusion of ACh in the membrane is important, such as observing the activity of the AChR as a function of solution viscosity, or by slowing the diffusion of the AChR in the membrane by adding large amounts of other membrane-bound proteins to interfere with the diffusion of the ACh to the AChR. The final proof, of course, would be the direct observation of membrane-bound ligand binding to the AChR.

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