THE DIMER OF TORPEDO ACETYLCHOLINE RECEPTOR: SYNCHRONIZED DOUBLE CHANNEL

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Introduction

Recent biochemical and biophysical observations on the acetylcholine receptor channel protein may possibly lead to a re-evaluation of classical data on gated ion flow in bioelectricity (1).

It is well known that the nicotinic acetylcholine receptor (AcChR) of electric fish can be isolated in two macromolecular forms, depending on the experimental conditions of isolation (2-4). When sulfhydryl-alkylating agents are present in the first tissue homogenization step the dimer (D), consisting of two disulfid-linked monomers (M), is the predominant species. The monomeric form prevails when reducing agents such as dithiothreitol (DTT) are used in the homogenization medium. As outlined in various summaries (see, e.g., ref. 4) the monomer or light (L) form has a subunit stoichiometry of two α-chains, one β-, one γ- and one δ-chain ($\alpha_L\beta\gamma\delta$). The relative molar mass of the monomer is M_r 290,000; the dimer or heavy (H) form is twice as large.

It is still disputed which of the two isolation products, the D-species or the M-molecule is the native and functionally

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operative unit in the biomembrane. Electronmicrographs of intact electrocytes (5) and of receptor rich postsynaptic membranes show the receptor proteins as rosette-doublets lined-up in rows (6). The functional significance of this topology: doublets assigned to receptor dimers and the dimer rows, is not yet understood.

In this context two specific questions of the receptor organization are discussed: (i) Are there functional differences between the D- and the M-species? More explicitely, does it matter whether the monomer is functioning as an isolated monomer or as a part of the dimer? (ii) Which of the two macromolecular forms is the in vivo functional unit?

Vesicle flux studies and planar lipid bilayer reconstitution experiments of the isolated and purified receptor proteins have so far been interpreted in terms of functional equivalence of the reconstituted monomers and dimers (4, 7-13). Recently a combined biochemical and biophysical approach to the questions raised above has provided new information for a possible resolution of this particular structure/function problem of the AcChR channel proteins. In the present account some key data of this biophysicochemical study on the AcChR monomer/dimer problem are summarized. The actual observations suggest an extension and re-interpretation of classical electrophysiological and biophysical data on the gating of ion transport by the nicotinic acetylcholine receptor, in terms of a concerted and synchronized opening and closing of the dimer double-channel unit.

The main conclusions are: (i) Measured single channel events must not necessarily reflect one single channel protein; rather, a single channel event may result from two or more cooperatively synchronized channel proteins. (ii) Positive cooperativity in dose-response curves must not necessarily be caused by the binding of two (or more) agonist or activator molecules to one

channel protein; rather, a Hill coefficient of two can reflect a pair (or more) of channels like that of the AcChR dimer. (iii) Different conductance states may simply result from differences in the transient binding of different types of ions to the open channel conformers of the AcChR protein.

AcChR Oligomerization in Solution

In the course of studies to reconstitute the isolated and purified AcChR proteins in lipid bilayers it was found that the rereptor monomers exhibit an inherent tendency to specific noncovalent association in vesicular lipid bilayers as well as in solution (1,14,15). In particular the reversible formation of stable dimers according to $2M \rightleftharpoons M_2$, i.e. without reformation of the δ-δ sulfide bond, readily occurs in the presence of phospholipids (14,15). Noncovalent aggregations of monomeric receptors to dimers [and higher oligomers (16)] might be one of the reasons why, until recently (1), no characteristic functional differences between monomers (M) and dimers (D) have been observed.

Due to the intrinsic tendency to noncovalent dimer formation in the presence of phospholipids many monomer preparations may have actually dealt with dimers of the type M_2. Although phospholipids are not a necessary condition to form M_2 the stability of the noncovalent dimer strongly increases in the presence of lipids ($K_d \approx 10^{-10}$ M) compared to that in the absence of lipids ($K_d \approx 10^{-6}$ M); see ref. 14,15. Several lines of evidence support the specificity of this dimer formation and exclude artefactual crosslinking: (i) Gel electrophoresis and the reversibility of complex formation observed on the time scale of ultracentrifugation experiments ($\approx$ 24 h) confirms the noncovalent nature of the dimeric associates. (ii) The stoichiometry of the complex is 1:1; no aggregates of variable size

occur under the given conditions. (iii) The dimerization is independent of inhibitors of free radical lipid oxidation, thus any oxidative crosslinking by detergent impurities was absent. (iiii) The dimerization is hardly affected by agents which modify sulfhydryl groups. However, chemical modification of carboxylate groups of the (acidic) receptor protein reduces the stability of the dimers (14,15).

Crosslinking (14,15). Under experimental conditions where the formation of dimers M_2 is favoured crosslinking with glutardialdehyde leads to a spectrum of oligomers that, after sucrose gradient centrifugation, is consistent with the presence of dimers and specific oligomers of the dimer (of lower stability): tetramers $(M_2)_2$, hexamers $(M_2)_3$, etc. Therefore, in addition to the specificity of the stable dimer formation there is some kind of 'long range specificity' for oligomers of the dimer excluding random association of monomers to species with uneven numbers of monomers.

The crosslinking data support the results from experiments at very low detergent concentrations (0.05% Lubrol PX) where the AcChR proteins occur as M_2 species and larger aggregates. By addition of an excess of phospholipid/cholate mixture the large aggregates dissolve and, concomitantly, the amount of receptors at the dimer position increases.

Single Channel Events of Dimers and Monomers

The functional reconstitution of the isolated and purified AcChR proteins from Torpedo californica electric tissue involved reincorporation of the channel proteins into lipid vesicles, formation of monolayers and finally of planar bilayers from these vesicles (17). For the protein reincorporation into vesicles a novel technique was applied (fast dilution method)

because conventional cholate dialysis caused perturbed and irregular channels and receptor aggregations.

Provided the planar bilayers are adjusted to cohesive pressures of 30 and 32 mN/m, 16 mole per cent cholesterol of the total lipid, both the dimer and the monomer exhibit single channel events when agonists such as AcCh, carbamoylcholine or suberoyldicholine are added (Fig. 1). As depicted in Figure 1

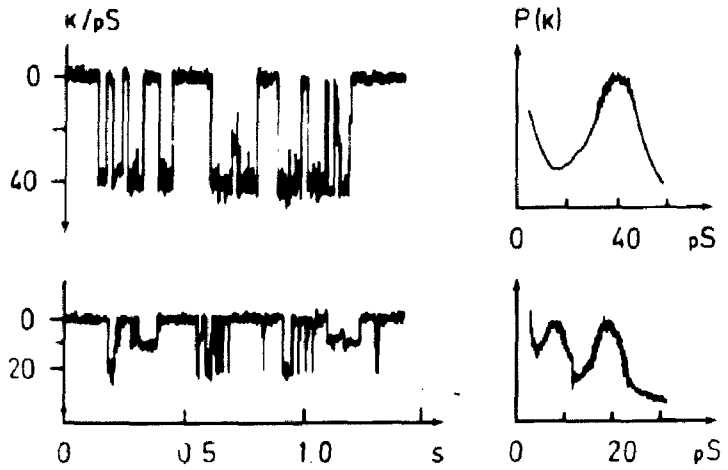

Fig. 1 Conductance κ/time traces and channel histograms P(κ) (probability of occurrence) of reconstituted Torpedo californica AcChR in planar lipid bilayer (16 mole % cholesterol), 5 min after addition of suberoyldicholine (0.5 μM); 20°C, 0.5 M NaCl. Upper trace: the native dimer (D) at 100 mV; lower trace: monomer (M) at 150 mV.

the most striking difference between the channel events is that the maximum channel conductance (≈20 pS) of monomers (M) is about half as large as that (≈40 pS) of dimers (D). Furthermore, the amplitude and time characteristics of the channels formed by the isolated dimers (≈40 pS) in lipid bilayers are almost quantitatively the same (≈40±10 pS) as for unextracted receptors (microsacs) from a different Torpedo species: T. marmorata (18).

Effect of reducing agents on the dimer (1). When the disulfide reducing agent DTT was added to a planar bilayer containing

dimers (D), the initial dimer channel events started to change: at first conductance levels characteristic of the monomeric receptor occurred at the expense of dimer events; after about 10 min the channel events became undefined and within 1 h the channel activity disappeared. It thus seems that disulfide reduction first abolishes one of the two channels within a dimer and with progressing time the second one also disappears. These observations may relate to inhibitory effects of DTT on ligand binding and on receptor activity (19,20). In view of the dimer conductance level being twice as large as that of the monomer the dimer appears to cause a "double-channel" with apparent "single channel" characteristics.

AcChR Channel Oligomerization

The isolated and purified AcChR monomers (M) and dimers (D) reconstituted in planar bilayers may give rise to a synchronized opening and closing of up to several dimeric channel units caused by protein association (1,21). For instance, when the cohesive pressure of a bilayer with monomer channels was raised from 32 to 40 mN/m by the addition of $CaCl_2$ to a final concentration of 0.5 mM, large conductance peaks developed at the expense of the monomer levels; they are grouped in activity clusters of multiple dimer levels. The conductance histogram of such a cluster is shown in Figure 2. Most remarkably, only even multiples of the monomer level (20 pS) are populated corresponding well to the dimer level (40 pS) and multiples thereof (80,120 and 160 pS); peaks at uneven multiples do not occur.

The specific channel oligomerization in multiples of dimers corresponds well with the oligomerization of receptor proteins in solution where, after crosslinking, the dimer complexes $(M_2)_n$, $n = 1,2,3...$ may be identified (14,15). The channel characteristics of the dimer M_2 is hardly distinguishable from that of the (native) dimer D.

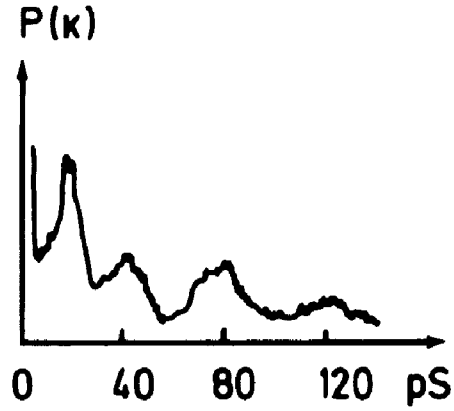

Fig. 2 Probability of occurrence $P(\kappa)$ of channel conductances κ of AcChR monomers (M) and oligomers at 0.5 M NaCl, 15 min after increase in cohesive membrane pressure from 32 to 40 mN/m by 0.5 mM $CaCl_2$. Conductance oligomers are multiples of the dimers (M_2): $(M_2)_2$,$(M_2)_3$,$(M_2)_4$; no intermediate oligomers of M occur.

Cation Dependence of Open-Channel States

The AcChR monomer channels always exhibit two different conductance levels for Na^+ ions (Figure 1) and for K^+ ions: a maximum conductance of 20±2 pS and a substate of 9±2 pS. The relative population of the two levels is, however, dependent on the type of cation which is transported (1).

The dimers also show substates of lower conductance. For Na^+ ions the main level is 40±3 pS; a substate of 30 pS occurs to 20%. For K^+ ions there is only 6% of the 40 pS events, the larger fraction are 30 pS events. The data are summarized in Table I.

It may be concluded that the different conductance levels of

Table I

Channel Conductance Levels (and Relative Populations) of Torpedo Californica Acetylcholine Receptor (AcChR) Proteins Reconstituted in Planar Lipid Bilayers. (Cohesive membrane pressure of 32 mN/m, 0.5 μM suberoyldicholine, pH 7.4, 20°C.)

AcChR protein	Conductance levels (pS)	
	0.5 M KCl	0.5 M NaCl
	low , high	low , high
Dimer (D; H-form):	31±2 , 40±3 (12:1)	30±1 , 40±1 (1:5)
Monomer (M; L-form):	9±2 , 19±2 (7:1)	9±2 , 20±2 (1:2)
Dimer (D):	31±2 , 40±3	30±1 , 40±1
+ 10 mM DTT (≈5 min):	9±2 , 19±2	9±2 , 20±2
Dimer (D) at 0.3M Na^+/0.2M K^+:	30±2 , 40±2	
Monomer at 40 mN/m :	9±2 , 19±2	9±2 , 20±2
after 10 min:	9±2 , 31±2	40±1
>> 10 min:	multiples of 31,	of 40±1
Torpedo marmorata microsacs (18):		40±10

the receptor monomers and dimers are due to the existence of two open-channel states. The transient associations of the Na^+ and K^+ ions with these channel states are different. K^+ usually favors the receptor state of lower conductance.

Multiple conductance states in single channel activity of AcChR have been observed in patch-clamp studies on cultured embryonic muscle cells, but unfortunately did not include K^+ ions (22). A direct comparison with the data of Torpedo AcChR (summarized in Table I) is thus limited to the conductance values for Na^+ ions.

Double Channel and Pair Cooperativity

Recalling the oligomerization tendency of the isolated AcChR monomers in solution and in the planar lipid bilayer it appears that contact formation between the monomers alone leads not only to stable complexes but also to pronounced changes in the channel characteristics, especially in the channel conductance. The effect of the monomer-monomer interactions within the dimer does apparently not depend on the intactness of the δ-δ disulfide bridge. The channel conductance of two noncovalently associated monomers in the dimer M_2 equals that of the isolated dimer D containing the (native) disulfide bridge.

The factor of 2 in the conductance difference between monomers and dimers and the occurrence of multiples of dimers in the protein complexes as well as in the channel conductances can be consistently interpreted in terms of a simple model: synchronous opening and closing of two monomeric channels within a dimer (D and M_2). Therefore, the operationally defined "dimer-channel" with apparent "single-channel" characteristics has to be visualized as two cooperative monomeric channels that act fully in concert.

The fact that the same channel conductances were observed with isolated dimers (D and M_2) and with unextracted microsacs (18, 21) suggests that the basic functional units of the observable AcChR channels in the biomembrane are, in reality, synchronized double-channels.

At present it is not known if the principle of cooperative double-channel gating applies to other cholinergic systems as well. Remarkably corresponding patch-clamp data for "single-channel" conductances (22,23) are closer correlated to the channel conductance of the reconstituted Torpedo dimer than to that of the monomers. However, the comparison is obscured by the different ionic conditions applied besides possible species-dependent differences.

Cooperativity. Two distinct cooperative interactions within AcChR complexes are apparent from biochemical studies in solution as well as from the channel oligomerization of reconstituted monomers and dimers: (i) Strong (short range) cooperativity between pairs of monomers and monomeric channels both in the absence and presence of the disulfide linkage between the δ-subunits of two monomers. (ii) Weak (long range) cooperativity between several pairs of dimers and dimer-channels.

The extent of this "pair cooperativity" is, however, smaller in monomer associates, on the average between two pairs of channels, compared to dimer(D) associates under otherwise the same membrane conditions, on the average between four pairs of channels (18,21). Thus, the S-S bridge in the (native) dimer species enhances long range cooperativity probably by allowing a more rigid arrangement of the dimers(D) compared to that in monomer associates (M_2).

Since the AcChR in the native postsynaptic membrane can be visualized in double rows of dimers (6), it appears likely that

the oligomeric channel cooperativity (consistent with the receptor oligomerization in solution) is the functional counterpart of the native organization in double rows. This feature and the enhancement of long range cooperativity by the intact disulfide bridge between the monomers of the D-species together with the effect of reducing agents on the ratio of monomers and dimers in the protein isolation (1), suggest that the disulfide-bridged dimer D is the native and functionally operative channel unit.

Channel Gating Scheme of the AcChR Dimer

If the "dimer-channel" is indeed the functional conductance unit in the biomembrane, the Hill coefficient of about 2 for the channel activation (for review see ref. 4) may be interpreted in a different manner compared to previous analyses. The dimer channel activation may result from the successive association of two agonist molecules (A) with the two monomeric low affinity conformers R_ℓ of the dimer species $R_\ell \cdot R_\ell$; i.e. each monomer channel within the dimer binds only one agonist molecule. The functionally relevant step is then the concerted transition of the complex $AR_\ell \cdot AR_\ell$ to the conducting conformer $AR_\ell^* \cdot AR_\ell^*$.

$$2A + R_\ell \cdot R_\ell \rightleftharpoons AR_\ell \cdot R_\ell + A \rightleftharpoons \begin{matrix} AR_\ell \cdot AR_\ell \\ \Downarrow \\ AR_\ell^* \cdot AR_\ell^* \end{matrix} \qquad (1)$$

The Hill coefficient of 2 is thus related to the highly cooperative, practically synchronous transition of the receptor dimer, in its low affinity state for agonist binding. The occupation of only one of the α-subunits within a monomeric part of the dimer compares well with the channel activation by irreversibly binding agonists. It is sufficient for channel acti-

vation when only one "irreversible agonist" binds per monomer after reduction of disulfide bridges in the vicinity of a binding site for AcCh on one of the two α-subunits (24-26); see also ref. 27. The covalent agonist labeling of the second α-subunit requires much higher concentration of irreversible agonists (28).

There are still other arguments in favor of the activation scheme(1) in terms of two agonist molecules and the dimer. Recent dialysis studies have confirmed that the final equilibrium state for the binding of [^{3}H]AcCh to membrane-bound receptors (microsacs) and to purified dimers is associated with only one AcCh molecule bound per monomer ($K \approx 5$ nM, 4°C, Torpedo cal.), independent of whether detergent is present or not; there is no positive cooperativity to this final high affinity state (29). If the channel activation would require two agonist molecules per receptor monomer, then the final slow phase of receptor inactivation (associated with a Hill coefficient of one) leading to one agonist bound per monomer would have to release one of the two previously bound agonist molecules. Although such a process could occur via allosteric structural transitions (including the two α-subunits of R) the sequence $2A + R_\ell = R_\ell A_2 = R_\ell^* A_2 = R_h A + A$ appears very unusual. Furtheron, according to Neubig et al. (1982) the functional unit of $^{23}Na^+$ efflux gating in Torpedo membranes (microsacs) comprises two α-bungarotoxin (α-Bgt) sites (30). Since in the absence of detergents there is only one α-Bgt molecule bound with high affinity per monomeric AcChR (29), the functional unit of ion transport gating must be the dimer (D).

Receptor inactivation procedes in at least two phases (see e.g. ref. 4), a fast cooperative one (31,32) and a slow noncooperative one (30,32). Recent dialysis data show that at low AcCh concentrations (≤ 50 nM) the slow transition to the final equilibrium state of very high affinity denoted by R_{vh} ($K \approx 5$ nM) is induced by agonist binding (29). The preexisting high affi-

nity receptor state R_h to which the agonists may directly bind is of smaller affinity ($K_h \approx 0.1$ μM for AcCh).

In order to facilitate a comparison with schemes developed previously, the following correspondences to ref. 4 hold:
$R_\ell \hat{=}$ R of ref. 4; activatable, low AcCh affinity ($K_\ell \approx 10^{-4}$ M).
$R_\ell^* \hat{=}$ A of ref. 4; activated, conducting ($K_\ell^* \approx 10^{-5}$ M).
$R_h \hat{=}$ I of ref. 4; inactivated, high AcCh affinity ($K_h \approx 10^{-7}$ M).
$R_{vh} \hat{=}$ D of ref. 4; fully desensitized, very high AcCh affinity ($K_{vh} = 5 \times 10^{-9}$ M).

Channel activation from desensitized AcChR. Channel activity is also observed under conditions where the AcChR protein is usually assumed to be inactivated or desensitized (23,1). Whereas the response immediately after agonist addition are frequently occurring short single channel events, longer exposure to agonists gives rise to occasional bursts of repetitive opening and closing events (23,12,13,1): "Nachschlag" or flickering (23). See Fig. 3. The occasional dimer-channel activation under desensitizing conditions requires an extension of the receptor activation scheme by the cooperative coupling (Hill coefficient two) of the open conformation $AR_\ell^* \cdot AR_\ell^*$ to inactivated states according to

$$AR_\ell^* \cdot AR_\ell^* \rightleftharpoons AR_h \cdot AR_h \qquad (2)$$

The reaction scheme depicted in Fig. 3 is an abbreviated form of the possibly more complicated reaction network of AcChR activation and inactivation processes. In detail, the inactive (intermediate) state AR_h may be populated either via the open receptor states or by direct binding of activator to the R_h conformers. The slow noncooperative phase of inactivation (30) to the final state AR_{vh} of very high AcCh affinity involves structural transitions of the type $AR_h \rightleftharpoons AR_{vh}$, where each of

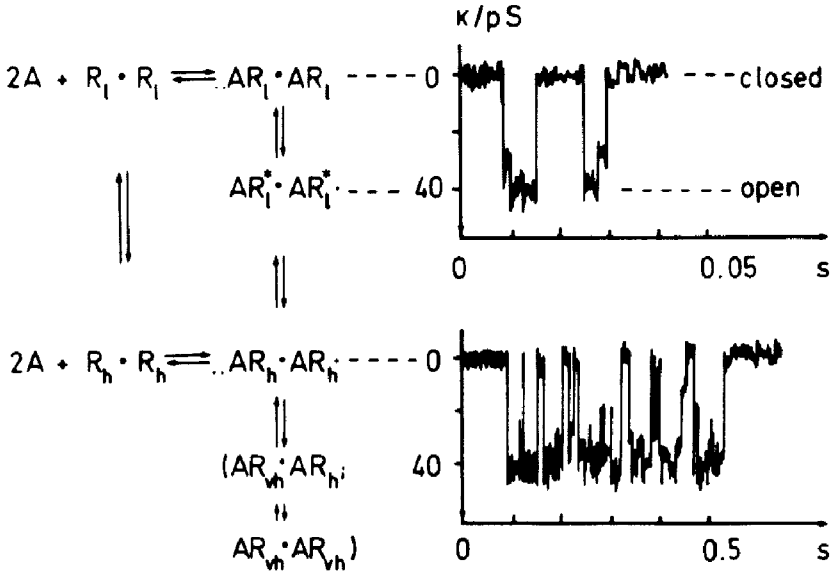

Fig. 3 Reaction scheme (short form) for the AcChR dimer channel activation. Upper part: normal low affinity pathway of activation leading to "single-channel" events according to $AR_\ell \cdot AR_\ell$ (closed) ⇌ $AR_\ell^* \cdot AR_\ell^*$ (open). Lower part: burst of repetitive opening and closing events under desensitizing conditions, modelled by $AR_h \cdot AR_h$ (closed) ⇌ $AR_\ell^* \cdot AR_\ell^*$ (open).

the two AR_h conformers within the dimer $AR_h \cdot AR_h$ can independently convert to the AR_{vh} state (Hill coefficient one, ref. 30,32).

Monomer. The isolated AcChR monomer (fragment) in detergent solution (27) as well as reconstituted in planar lipid bilayers (1) appears to have a more loose structure compared to the dimer. The high affinity AcCh binding to the M-species is a factor of about ten ($K \approx 5 \times 10^{-8}$ M) less stable (2,29) than to the dimer ($K \approx 5 \times 10^{-9}$ M). The hydrodynamic properties of the M-fragment suggest a more extended, perhaps partially unfolded structure (27). As seen from Table I the occurrence of the sub-

states of lower conductance are much more frequent for the monomers than for the dimers. The monomer channels are thus more "flexible" than the dimer channels. The more loose structure of the monomer may also be the reason why membrane fragments in the presence of detergents (0.1% Triton X-100) bind two α-Bgtx molecules per monomer compared to only one high affinity bound α-Bgt per monomer in the absence of detergents (29).

In the absence of detergents high affinity toxin binding to the second α-subunit of the monomer part does not occur because of sterical hindrance (33); or the high affinity occupation of one α-subunit turns the second α-subunit allosterically into a state of very low affinity for the binding of agonists and inhibitors. Unusually high concentrations may then be necessary to also occupy the second α-subunit.

Acknowledgements

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