

Different channel properties of *Torpedo* acetylcholine receptor monomers and dimers reconstituted in planar membranes

(single channel/cooperativity/protein-protein interaction/cation selectivity/cholinergic agonist)

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ABSTRACT It is demonstrated that the monomeric and dimeric structures of the nicotinic acetylcholine receptor of *Torpedo californica* electric tissue, reconstituted in planar lipid bilayers, are functionally different. The native dimer D of M_r 500,000 (heavy-form) exhibits a "single" channel conductance about twice as large as that of the monomer M of M_r 250,000 (light form). Under conditions where monomers aggregate, the conductance changes from the level of the monomer M to that of dimers M_2 . The dimer conductances (D and M_2) seem to result from synchronous opening and closing of the two channels in the dimer, giving the impression of "single channel" activity. This channel cooperativity is apparently mediated by noncovalent interactions between the two monomers, since it requires no disulfide linkage between monomers. Both the monomers M and the dimers D and M_2 show at least one substate of lower conductivity. The relative population of the two conductance levels depends on the ion type (Na^+ and K^+), indicating ion-specific channel states. Since the channel conductance of isolated dimers resembles those obtained from unextracted microsacs, the dimer with two synchronized channels appears to be the *in vivo* predominant gating unit. In the linear association of dimers, observed in the native membrane, channel synchronization may extend to more than two channels as suggested by oligomeric channel cooperativity in associations of monomers and dimers.

The nicotinic acetylcholine receptor (AcChoR) of *Torpedo* fish electric tissue can be isolated as a monomer, M, [or light (L) form] of the relative molar mass $M_r \approx 250,000$ or as a dimer, D, [or heavy (H) form] of $M_r \approx 500,000$. The ratio of the two macromolecular forms depends on the isolation procedure (1-3). The "native" dimer, consisting of two disulfide-linked monomers, is the predominant form when alkylating agents are present in the first tissue homogenization step (1).

Electronmicrographs of postsynaptic membranes of *Torpedo* electric organs show the AcChoR as rosette-like structures (4, 5). Rosettes most often occur in doublets that have been assigned to receptor dimers (6-9). Extensive double rows of lined-up rosette doublets also have been described in intact electrocytes (10) and receptor-rich membranes (6). The functional significance of this receptor topology is not yet known. Vesicle flux studies and planar lipid bilayer reconstitution experiments of the isolated and purified receptor proteins have been interpreted in terms of functional equivalence of the dimers and the monomers (refs. 11-16; see, however, ref. 17).

Recently it was shown that isolated AcChoR monomers have an inherent tendency to specific, noncovalent association in vesicular lipid bilayers as well as in solution (3). Monomer associations to dimers and higher oligomers might

be one of the reasons why no characteristic functional differences between monomers and dimers have been observed so far.

In the present study, conditions were developed for stable AcChoR monomer-lipid systems, and the functional significance of the dimer relative to the monomer was reinvestigated by using the assays in planar membrane reconstitution (18). It appears that the channel activity of the dimer represents the synchronized and concerted gating of the two monomer channels constituting the AcChoR dimer. The cooperativity observed with the dimers extends to higher oligomers of channels of associated monomers and dimers.

MATERIALS AND METHODS

Preparation of AcChoR Dimers. Frozen tissue (50 g) of electroplax from *T. californica* (Pacific Biomarine, Venice, CA) was broken up into small pieces and homogenized in 200 ml of buffer I (10 mM *N*-ethylmaleimide/20 mM Pipes, pH 6.8). Homogenization, centrifugation cycles, and affinity purification were carried out as described by Chang and Bock (1). The receptor protein was eluted from the affinity column with 10 mM Pipes containing 50 mM NaCl, 1% cholate, 2 mg of phospholipid (asolectin) per ml, and 50 μM Flaxedil (pH 6.8). Subsequently, the receptor-containing fractions were diluted to a protein concentration of 50 $\mu\text{g}/\text{ml}$ with elution buffer (minus Flaxedil).

Preparation of AcChoR Monomers. *With dithiothreitol.* The preparation scheme for the AcChoR monomers was similar to that of the dimer preparation. However, the homogenization buffer contained 5 mM dithiothreitol, 20 mM Pipes, 3 mM Ca^{2+} , 0.1 mM phenylmethanesulfonyl fluoride, and 10 mM benzamidine (pH 7.0).

Without dithiothreitol. The procedure used was the same as that for dimer preparation with the exception of buffer I and the isolation steps by ultracentrifugation. The homogenization buffer consisted of 20 mM Pipes, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 3 mM Ca^{2+} (pH 6.8). Finally, the receptor protein was purified by affinity chromatography and identified (see "Assays"), and 500 μl of the trace-labeled sample was layered on top of a linear 4-18% sucrose gradient in 10 mM Pipes/50 mM NaCl/1% cholate/1 mg of asolectin per ml, pH 6.8. The gradients were centrifuged for 12 hr at $250,000 \times g$ and fractionated. Monomer-containing peak fractions of six sucrose gradients were pooled (protein concentration, 10-50 $\mu\text{g}/\text{ml}$) and used immediately in the reconstitution experiments.

Assays. Receptor-containing fractions were identified by toxin binding with ^{125}I -labeled α -bungarotoxin (α -BTX; New England Nuclear) and by applying the DE-81 (Whatman) filter assay. Fresh receptor preparations revealed 9 nmol of toxin binding sites per mg of protein. Equilibrium binding of

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Abbreviations: AcChoR, acetylcholine receptor; H, heavy; L, light; SubCho₂, suberyldicholine; α -BTX, α -bungarotoxin.

[³H]acetylcholine (Amersham) to the AcChoR was determined by dialysis (Diachema, Zürich). Acetylcholine concentrations were checked by the test of Hestrin (19). The relative amounts of monomers and dimers were determined by ultracentrifugation for each preparation (see above).

Preparation of Vesicles. Lipid vesicles. Soybean lipid (Sigma), partially purified (20), and cholesterol (16 mol%) were mixed in hexane (4 mg of lipid per ml). Lipid (20 mg) was dried under a stream of nitrogen to a thin film in a round-bottom flask (1 dm³). The film was resuspended into 20 ml of buffer A (20 mM Hepes/0.5 M KCl or NaCl, pH 7.4) by swirling the flask in the presence of glass beads of 2-mm diameter. The resulting turbid vesicle suspension was filtered through a 0.4- μ m polycarbonate filter (Bio-Rad).

Lipid-AcChoR vesicles (fast dilution technique). The procedure was as for lipid vesicles except that a small volume ($\approx 5 \mu$ l) of receptor sample was added to buffer A prior to peeling off the lipid film. Just before adding receptor to buffer A, soybean lipid (≈ 1 mg) was added to the buffer from a fresh suspension through an 80-nm polycarbonate filter. The final vesicle solution was dialyzed against 0.5 dm³ of buffer A for at least 2 hr, yielding ratios of detergent to lipid of $< 10^{-5}$ and an efficiency of AcChoR insertion into vesicles of about 25% (21). The volume of the receptor sample to be added to buffer A was calculated from the desired receptor-to-lipid ratio, usually 10^{-8} to 10^{-9} . Alternatively, conventional cholate dialysis (14, 15) was applied for AcChoR incorporation in vesicles. Dialysis was performed for 48 hr applying four changes of 1000 sample volumes of dialysis buffer (10 mM Pipes/100 mM NaCl/0.01% NaN₃, pH 7.4).

Formation of Planar Bilayers From Vesicles. This was performed as described (18). AcChoR-containing vesicles were added to the *cis* compartment of the bilayer cell, whereas the *trans* compartment contained only lipid vesicles. The bilayer area was 2×10^{-4} cm². The number of receptors per bilayer was estimated (21, 22) from the ratio of receptor to lipid in the vesicles and the number of lipids in one layer of the planar bilayer (about 3×10^{10} at 65×10^{-20} m² per lipid).

Histograms. Each conductance histogram (see Figs. 2–4) is based on at least 10 min of recording time ($\geq 12 \times 10^6$ samples and several thousand single-channel events).

RESULTS

The relative purity of the AcChoR monomer and dimer preparations, used to compare channel conductances in planar lipid bilayers in the presence of NaCl and of KCl, was better than 95% (Fig. 1). Dialysis binding studies in the presence of 0.1% Lubrol PX revealed a 1:2 equilibrium (≈ 5 hr of dialysis time) stoichiometry of high-affinity ($K = 5 \pm 1 \times 10^{-9}$ M) acetylcholine to α -BTX sites both for purified dimers and monomers. Reconstitution involved reincorporation of AcChoR into lipid vesicles and formation of planar bilayers from these vesicles (18). For reincorporation into vesicles, a novel technique was applied (fast dilution technique) because conventional cholate dialysis caused inactive or perturbed receptor channels and receptor aggregation, apparent from conductance traces observed in planar bilayers derived from such vesicles (see also refs. 3 and 21). Planar bilayers contained about 50 dimers or monomers as estimated from the receptor-to-lipid ratio in the vesicle suspensions.

Channel of the AcChoR Dimer and Monomer. Receptor dimers and monomers were studied at membrane conditions optimal for normal AcChoR channel function (22)—i.e., in planar bilayers adjusted to cohesive pressure values between 30 and 32 mN/m and containing cholesterol at 16 mol% of the total lipid. In the absence of agonist, no channels were observed, and the steady membrane conductance was as low as that of pure lipid bilayers: 1–2 pS at ± 100 -mV applied voltage.

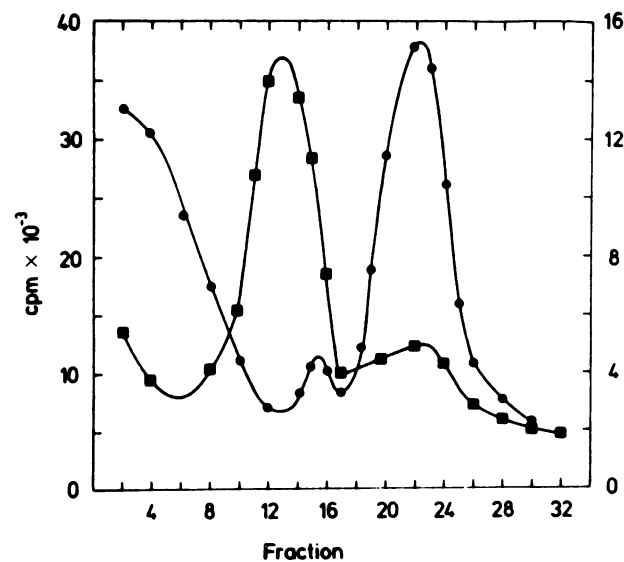


FIG. 1. Distribution of *Torpedo* AcChoR monomers and dimers on a sucrose gradient. ●, Dimer preparation in the presence of *N*-ethylmaleimide; ■, monomer preparation in the presence of dithiothreitol.

Channel Conductance of the AcChoR Dimer. When the agonist suberyldicholine (SubCho₂) was added to the *cis* compartment (0.5 μ M) of a planar bilayer containing AcChoR dimers, single channel activity appeared (Fig. 2, trace a). Channels with mean open-times of about 10 ms occurred in bursts with a mean duration of about 0.4 s. Bursts were grouped in well-separated clusters of 5- to 10-s duration. The frequency of occurrence of conductance levels is shown in conductance histograms (*Insets* to the right of the traces). The most probable conductance level of the dimer is 39.5 pS for 0.5 M NaCl (Fig. 2, trace a *Inset*). The mean channel conductance values were independent of voltage between +150 mV and -150 mV. Addition of SubCho₂ to the *trans* side of the bilayer did not induce channel conductivity, indicating complete sidedness of functional AcChoR binding sites.

Channel activity also was induced by the natural activator acetylcholine and by carbamoylcholine, whereas the antagonist Flaxedil and α -BTX inhibited channel activity. The amplitude and time characteristics of the channels formed by the isolated AcChoR dimers in lipid bilayers were almost quantitatively the same as for unextracted receptor (microsacs) from a different *Torpedo* species: *Torpedo marmorata* (22, 23).

Channel Conductance of the AcChoR Monomer. When the planar bilayer contained AcChoR monomers instead of dimers (same conditions otherwise), SubCho₂ elicited channel conductances as shown in Fig. 2, trace b. Mean open times of the channels were 5–10 ms, and clustered channel activity lasted, on the average, 1–5 s. The most striking difference between the two channel traces in Fig. 2 is that the channel conductance of monomers is about half as large as that of dimers. The conductance histogram (Fig. 2, trace b *Inset*) shows, besides the main peak at 21 pS, another population at 9 pS, both independent of voltage between +200 and -200 mV. Eight independent AcChoR monomer preparations [using disulfide reduction (six preparations) and using ultracentrifugation (two preparations)] gave the same conductance values within experimental accuracy: 20 ± 2 pS and 10 ± 2 pS (0.5 M NaCl). No higher conductance levels were observed except during chance overlaps of different channels. Thus, the contamination of monomers by AcChoR dimers was negligibly small. In order to correlate the monomer and

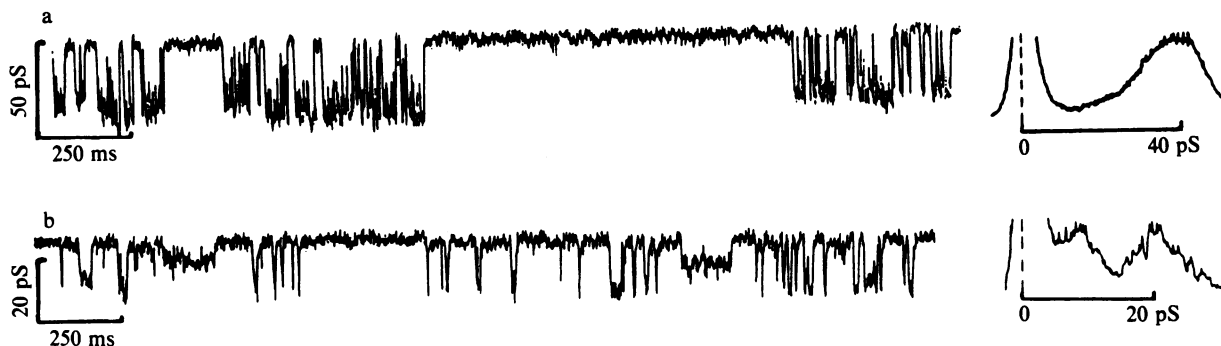


FIG. 2. Channel traces of isolated AcChoR and channel-conductance histograms (*Insets* to the right) at 0.5 M Na⁺ (buffer A) at 20°C. The bilayer was composed of soybean lipid containing 16 mol% cholesterol at a cohesive pressure of 32 mN/m. Time resolution was 1 ms. Histograms (*Insets*) were done on a 400-point probability analyzer (Honeywell, Sai-43A) with a sampling interval of 50 μ s. Traces: a, AcChoR dimers (≈ 50), recording was at 100-mV 10 min after bilayer formation and 5 min after addition of SubCho₂ (0.5 μ M) at the *cis* side; b, AcChoR monomers (≈ 50), recording was at 150 mV, with other conditions as in trace a.

dimer channel conductances, it was attempted to dissociate dimers into monomers within the planar bilayer. Furthermore, the monomers were studied under conditions where they may form dimers or oligomers.

Channel Conductance and AcChoR Association. Monomer channel conductance observed with dithiothreitol-treated dimers. The disulfide reducing agent dithiothreitol was added to a final concentration of 10 mM to the *cis* side of a planar bilayer containing dimers. Within about 1 min, the dimer channel traces (Fig. 2, trace a) started to show conductance levels (20 pS) of monomer channels (Fig. 2, trace b) as exemplified in Fig. 3, trace a and histogram. After about 10 min, the appearance of channels became undefined; within 1 hr, the channel activity dropped to zero. These late observations may relate to inhibitory effects of dithiothreitol on ligand binding and receptor activity (24, 25). When dithiothreitol was added to the *trans* side of the bilayer, no change of the dimer-channel activity was observed, confirming the functional sidedness of the AcChoR in the planar bilayer and the apparent *cis* location of the δ - δ disulfide bridge (26, 27).

Dimer channel conductance observed with oligomerized

monomers. Purified AcChoR monomers and dimers in planar bilayers give rise to an apparently synchronized opening and closing of up to several dimeric channel units (Fig. 3, trace b) caused by protein aggregation. In detail, it has been found that the AcChoR aggregation started in the bilayers at cohesive pressures at >35 mN/m (22, 24). When the cohesive pressure of a bilayer with single channels as in Fig. 2, trace b was raised from 32 to 40 mN/m by the addition of CaCl₂ to a final concentration of 0.5 mM to both compartments, large conductances developed that were grouped in clusters of multiple dimer levels (Fig. 3, trace b) but were separated by longer periods of no conductance events. The conductance histogram of such a cluster (Fig. 3, trace b *Inset*) exhibits peak values at the monomer channel conductance (20 pS) and at multiples of it. Most remarkably, only even multiples of 20 pS are populated corresponding well to dimers (40 pS) and multiples thereof (80, 120, and 160 pS); peaks at uneven multiples do not occur.

Both the multiple-channel patterns in isolated clusters and the population of only even multiples of the basic conductance unit (20 pS) reflect positive cooperativity between

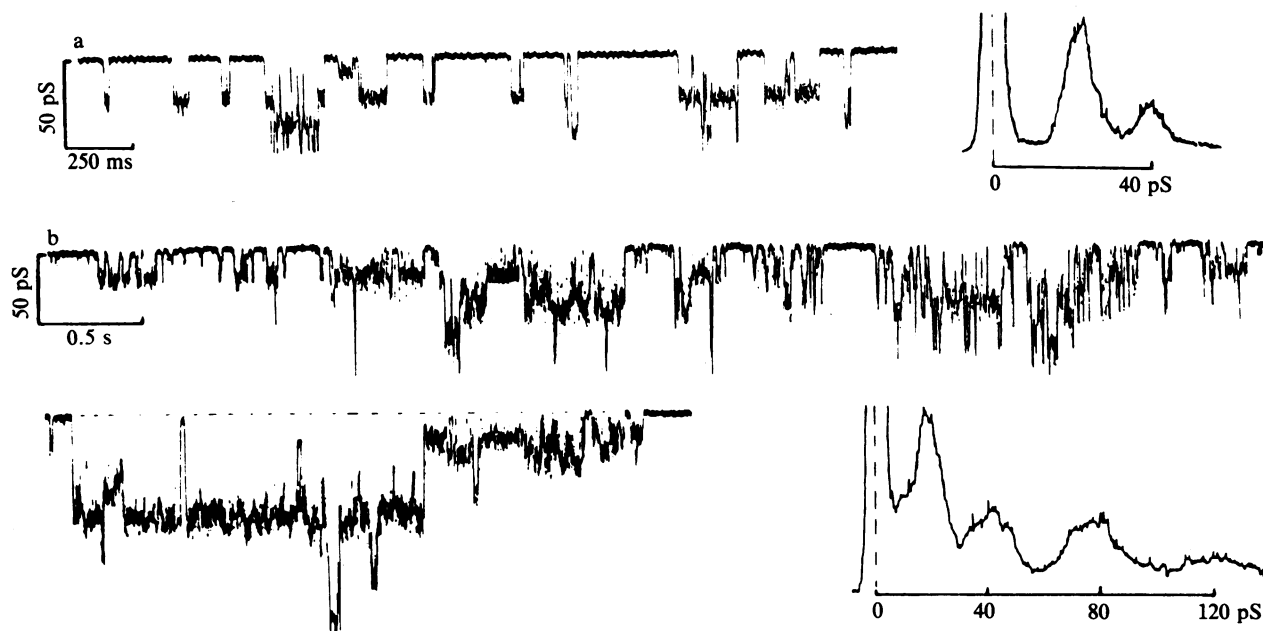


FIG. 3. Channel conductance traces and their histograms (*Insets*) of dithiothreitol-treated AcChoR dimers (trace a) and of aggregated AcChoR monomers (trace b). Trace a, recorded 5 min after addition of dithiothreitol (10 mM) to the *cis* side, shows the dithiothreitol-induced change of dimer patterns (Fig. 2, trace a) to that of monomers (20 pS). Trace b shows the effect of Ca²⁺ addition (0.5 mM): after 15 min, the monomer channels (20 pS) change to isolated clusters of multiple-channel activity (40, 80, and 120 pS).

channels. Oligomerization of AcChoR molecules was also indicated (data not included) by the time of channel appearances. This time interval decreased with inverse proportionality to the increasing number of AcChoR monomers present in the bilayer, as expected for diffusion-controlled association starting from a random distribution of monomers.

The number of receptors in the "conductance oligomers" may be estimated from the maximum conductance during single-cluster activity, which never exceeded 200 pS and amounted to at least 80 pS. Thus, under certain conditions stable oligomers may contain at least tetrameric units and may extend to decamers.

Cation Dependence of Open-Channel States. The AcChoR monomer channels always exhibit two different conductance levels for Na^+ (Fig. 2, trace b) and for K^+ (Fig. 4, trace b); they are, within experimental accuracy, of the same magnitude: 9 ± 2 , 20 ± 2 pS for 0.5 M NaCl and 9 ± 2 , 19 ± 2 pS for 0.5 M KCl. The relative population of the two levels is, however, cation dependent [see the histograms (trace b *Inserts*) of Figs. 2 and 4]. The population ratio (higher-to-lower level) was, on average, 2 for NaCl and 0.15 for KCl. Substitution of Na^+ by K^+ in the presence of AcChoR dimers revealed an average channel conductance of 31 ± 2 pS (Fig. 4, trace a), which compares to 40 ± 3 pS for Na^+ (Fig. 2, trace a). In view of the cation effect on the two-conductance-state population of monomer channels, dimer channels were reinvestigated at mixed salt conditions. Trace c in Fig. 4 refers to the presence of 0.3 M NaCl and 0.2 M KCl. The two main conductance levels are at 30 ± 2 and 40 ± 2 pS; these are just the values found for only K^+ (31 ± 2 pS) and only Na^+ (40 ± 3 pS). Reinspection of the traces in the presence of one cation type (traces a in Figs. 2 and 4) also revealed these two conductance levels; for only Na^+ there was 20% of 30-pS events and for only K^+ there was 6% of 40-pS events. In summary, it may be concluded that the different permeability of the AcChoR monomers and dimers for Na^+ and K^+ ions are apparently due to the existence of two open-channel states, whose relative populations are dependent on the type of cation present.

DISCUSSION

In comparison with previous investigations (11–16), the present study provides evidence of different channel properties of the dimeric and monomeric forms of the AcChoR. Most

pronounced, the AcChoR monomer conductance level is about half as large as that of the dimer form. It is unlikely that this difference is due to irreversible structural changes of the receptor during purification or reincorporation. Remarkably, the channel characteristics of the dimer equal those obtained from unextracted membrane fragments (22, 23). The problem of potential deterioration of the monomer function was investigated along three different experimental lines. (i) Two different procedures, with and without the use of reductant, were applied to isolate AcChoR monomers; both yielded the same channel conductances, excluding the possibility that cleavage of the disulfide bridge near the active site (24) changes the single-channel conductance. Both preparations exhibited agonist-induced gating patterns characteristic of AcChoR channels. (ii) At high values of cohesive membrane pressure, aggregation of the initially randomly distributed monomers led to multiple-channel conductances in single clusters of activity. During aggregation, the initial monomer channel pattern changed into a pattern characteristic of dimers and aggregates of dimers. (iii) Similarly, disulfide bond reduction of dimers within the reconstituted planar bilayer led to the appearance of conductance levels characteristic of monomer channels. Facts *ii* and *iii* directly link the two-channel conductances of AcChoR dimer and monomer to the organization of receptor molecules in the membrane. Contact formation between receptor molecules alone leads to changes of the channel characteristics, especially of the channel conductance. The effect of the monomer–monomer interactions in the dimer does apparently not depend on the intactness of the δ - δ disulfide bridge. The channel conductance of two aggregated monomers in the dimer M_2 equals that of the native dimer D containing the native δ - δ disulfide bridge. If dimerization or higher aggregations would leave the monomer channels unchanged and independent, no distinction would be possible between the channel traces of monomers, dimers, and oligomers. Among the extreme possibilities of interpretation, dimerization may either lead to higher open-state conductances of the constituent channels (model 1) or, alternatively, to a synchronous opening and closing of the two otherwise unmodified channels (model 2). Three basic observations may serve to differentiate between these two models. (i) The conductance levels of the dimer channels are larger by a factor of about 2 compared to those of the monomer channels, both for NaCl

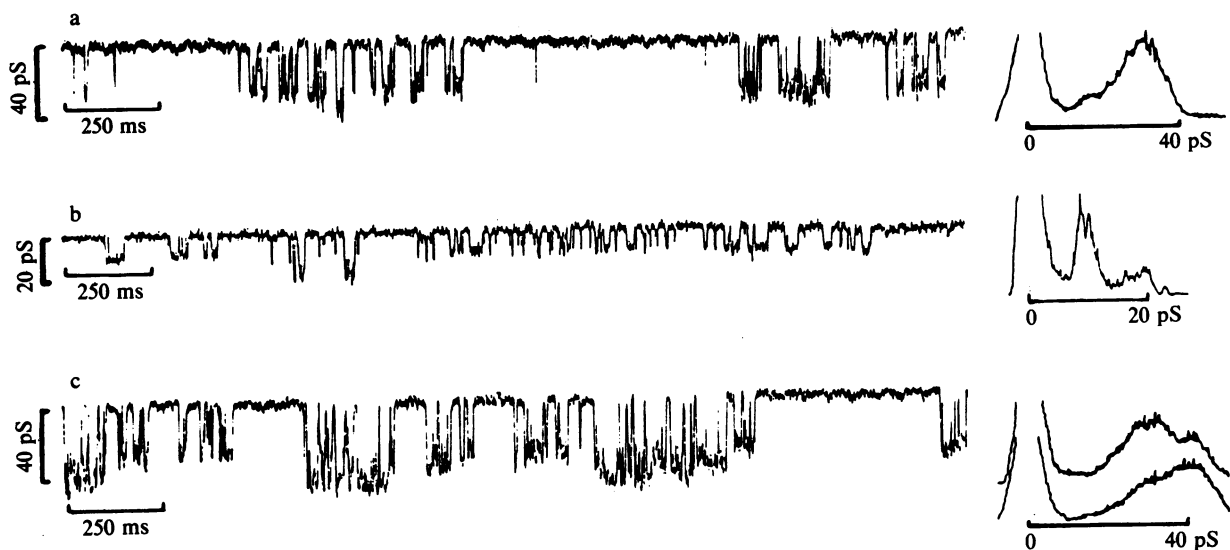


Fig. 4. Effect of Na^+ and K^+ on AcChoR-channel states. Traces: a, dimers at 0.5 M KCl (cf. Fig. 2, trace a, NaCl); b, monomers at 0.5 M KCl (cf. Fig. 2, trace b, NaCl); c, dimers in the presence of both KCl (0.3 M) and NaCl (0.2 M). The two histograms of trace c refer to two individual clusters; the average of eight such clusters revealed that, at this salt composition, the levels 30 pS and 40 pS are equally probable.

as well as for KCl. Model 2 provides a natural explanation for the particular factor 2, whereas in model 1 other factors are equally likely, *a priori*. (ii) The channel activity of the monomer aggregates indicates positive cooperativity between the constituent units of large channel oligomers. The conductance histograms of single bursts exhibit peaks at the dimer conductance level and multiples of it, indicating positive cooperativity between dimeric units within the oligomers. The almost complete lack of uneven multiples of the monomer channel conductance within a burst caused by monomer aggregates suggests strong positive cooperativity between the two constituent channels of the dimer species, consistent with model 2. Consistency with the observed cooperativity and model 1 would require no (or strictly negative) cooperativity between the two monomers of a dimeric unit but positive cooperativity between the channels of adjacent dimeric units within a monomer associate, a very unlikely construction. The model of two independent channels within a dimeric unit was also inconsistent with Li⁺ flux data of *Torpedo marmorata* microsacs (28). (iii) If model 1 applies, a 2-fold conductance increase would result from dimerization and no further increase upon oligomerization. On the other hand, model 2 provides a simple explanation also for the multiplicity of the conductance levels of the oligomers. Therefore, the operationally defined "dimer channel" with apparent "single-channel" characteristics has to be visualized as two monomeric channels that act fully in concert. The fact that the same channel conductances were observed with isolated dimers and with unextracted microsacs (22, 23) suggests that the basic functional units of the observable AcChoR channels in the biomembrane are, in reality, synchronized double channels. At present it is not known if this principle applies to other cholinergic systems as well, although corresponding patch-clamp data for "single-channel" conductances (29, 30) are in closer correspondence to the channel conductance of the reconstituted dimer than to that of monomers. However, the comparison is obscured by the different ionic conditions applied besides possible species-dependent differences.

There is clear evidence of two conductance states of both the isolated AcChoR monomer and dimer channel. The relative population of the two levels is dependent on whether Na⁺ or K⁺ ions are present. Therefore, the channel structure is also affected by ion-receptor interactions. Multiple conductance states of single AcChoR channels have been observed in patch-clamp studies on cultured embryonic muscle cells (30); the analysis of channel conductances for different cations, unfortunately, did not include K⁺.

Two distinct cooperative interactions within receptor aggregates are apparent from the corresponding multiple-state conductance patterns of reconstituted AcChoR: (i) strong (short range) cooperativity between pairs of channels both in the absence and presence of the disulfide linkage between the δ - δ subunits of two monomers, and (ii) weak (long range) cooperativity between several pairs of channels. The extent of the "pair cooperativity" is smaller in monomer associates (on the average between two pairs of channels) than for dimer associates (on the average between four pairs of channels) (cf. refs. 22 and 23). Thus, the disulfide bridge in the dimer species enhances long-range cooperativity probably because it allows for a particular arrangement of receptors, different from that in monomer associates. Since the AcChoR in the native postsynaptic membrane seems to occur in double rows of dimers (6, 9), it appears likely that oligomeric channel cooperativity is the functional counterpart of this structural organization in double rows. Long-range

channel cooperativity may render synaptic transmission more effective than merely cooperativity within a macromolecule. It is also intriguing to suggest that receptor aggregation in the native membrane is also controlled, as in the planar bilayer, by the physical state of the membrane (assayed by the cohesive pressure), which critically depends on ionic conditions and membrane composition. In addition, the presence of surface proteins, for instance the M_r 43,000 protein, may alter the interactions between receptor molecules in yet unknown directions.

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