Internodal cells of the giant green alga *Chara* as an expression system for ion channels

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Abstract Internodal cells of the giant green alga *Chara corallina* were utilized as an expression system for two nicotinic acetylcholine receptor subtypes (nAChR) derived from rat muscle. From *Chara* internodes that were pressure-injected with the respective cRNA, cytoplasmic droplets were formed, and functional expression of channel proteins in the membrane delineating the droplets was confirmed by patch-clamp techniques. The droplet membrane was recently identified as the original tonoplast, patch-clamp recordings on cytoplasmic droplets were easily performed and single channel activity could be measured with high resolution. The properties of the recombinant nAChR observed in this membrane were similar to those reported for the channel expressed in *Xenopus* oocytes, and for the native channel recorded in situ. The *Chara* expression system is, therefore, suitable for functional expression of animal messenger RNAs, without the risk of signal contamination by intrinsic ion channels. It offers a low-cost and practical alternative to the use of *Xenopus* oocytes for the investigation of heterologously expressed ion channels.

Key words: Acetylcholine receptor; *Characeae*; Cytoplasmic droplet; Expression system; Ion channel; Tonoplast

1. Introduction

A number of systems are utilized for functional expression of ion channels, including oocytes of *Xenopus laevis* (see [1] for the first report of heterogeneous functional expression of a nicotinic acetylcholine receptor) and human embryonic kidney cells (HEK 293, see [2], which reports the establishment of this cell line). Prerequisites to examine heterologously expressed ion channels are pressure injection of cRNA into oocytes, or the transfection of the HEK293 cells with cDNA. Disadvantages in using these expression systems include extensive preparative procedures, high costs of maintaining cell cultures, and low transformation efficiency. By using giant internodal cells of the green alga, *Chara*, most of those problems can be avoided. Giant algal cells, because of their size, have been attractive to electrophysiologists for several decades ([3] for historical survey). The internodal cells of *Characeae* contain two large compartments, the cytoplasm and the vacuole, the latter comprising about 95% of the total cell volume. The tonoplast can be accessed by preparation of vacuolar vesicles [4] or, more easily, by forming cytoplasmic droplets from internodal cells [5,6]. Cytoplasmic droplets result from effusion of cytoplasm that is surrounded by the original tonoplast.

The tonoplast-enclosed cytoplasm [7–9] of such droplets is metabolically active (indicated by light-dependent and Ca^{2+}-sensitive rotation of trapped chloroplasts), the tonoplast displays excitability [10,11], and responds electrically to illumination changes when chloroplasts are included [12]. Recently, a number of ion transport systems associated with the characean tonoplast has been demonstrated (H^+-ATPase, H^+-PPase, Cl^- channel, and K^+ channels [4,9,13–15]). To date, the Cl^- and K^+ channels remain the only identified ion channels in this membrane. This small collection of native ion channels in the tonoplast makes the use of *Chara* cells attractive for expression of exogeneous ion channels. Since secondary and final metabolic products are pooled in the vacuole, generally, it may be assumed that the tonoplast represents the major target of alien membrane proteins, in the knowledge that no receptor for the signal sequence of those membrane proteins is present in either plasma membrane.

The suitability of characean cells for functional expression of ion channels, and their electrophysiological examination in cytoplasmic droplet membranes, is demonstrated here for two heteromeric forms of the nicotinic acetylcholine receptor of rat muscle: an embryonic subtype which is expressed before innervation consisting of α-, β-, γ- and δ-subunits, and an adult subtype which is characteristic for innervated muscle being composed of α-, β-, ε- and δ-subunits (henceforth termed $nAChR_\gamma$ or $nAChR_\varepsilon$, respectively). The stoichiometric arrangement is $\alpha_2\beta\gamma$ (or ε) δ [16–18]. The procedure of cRNA injection and preparation of cytoplasmic droplets is described in detail. The compositions of all solutions are given in Table 1.

2. Materials and methods

Separated internodal cells (cell length 5–10 cm, cell diameter 0.8–1.2 mm) of *Chara corallina* were incubated prior to cRNA injection in a bathing medium of high osmolarity (injection medium) for about 15 min to induce a turgor decrease in the cells. Fully turgescent cells can not be injected since the osmotic pressure gradient across the plasmalemma is 5–10 bars. On the other hand, glass micro-pipettes cannot penetrate the relaxed cell wall of completely plasmolyzed cells; they will break and damage the cell. During incubation, turgor was reduced to a point close to plasmolysis of the cells. Single cells were taken from this medium and placed on a dry perspex plate. Injection of cRNA was accomplished using pipettes with a cannula-shaped tip of about 5 μm opening diameter. The α-, β-, γ-, δ-, and ε-specific cRNAs were synthesized with SP6 polymerase [19]. Complementary DNAs encoding the rat muscle AChR subunits [20] were cloned into a pSP64T [21] derived vector where the *NheI–AatII* and *XbaI–PrnII* fragments had been deleted. Plasmid DNAs were linearized using the *SalI* restriction site. For injection, cRNAs encoding α-, β-, γ- (or ε-), and δ-subunits were mixed in a molar ratio of 2:1:1:1 in H_2O at a final concentration of 1 $\mu g/\mu l$; the injected volume was about 100 nl per cell. After injection, cells were stored in the same medium for about 5 min and then transferred to a Petri dish containing expression medium. The injected internodal cells

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were allowed to produce nAChR for at least 24 h. In the event of cells displaying incrustations in their cell walls they were preincubated overnight in a slightly acidic solution to remove the carbonate crystals. A preincubation is favourable, because internodal cells are easily damaged by the incrustations when bent. Furthermore, the tips of injection pipettes often break on the incrustations.

1–3 days after cRNA injection, cytoplasmic droplets were isolated from *Chara* internodes. Cells were removed from the expression medium, laid on a soft tissue paper and wiped dry. Then they were placed on a support (e.g. a microscope slide), and each cell was fixed with a small droplet of vaseline such that about 1 cm of the cell protruded over the edge of the support. After a short while, wilting was observed as a shrinkage or flattening of the cylindrical cell due to water evaporation. The node of the protruding cell end was then cut. Scissors were dipped into the droplet medium before cutting to avoid an air embolism that would prevent an outflow of cytoplasm. The support was tilted such that the cut end of the cell was submerged in the droplet medium, and the cytoplasm was allowed to flow out for some 10 s (Fig. 1). The composition of the droplet medium (see Table 1) is suitable to maintain tonoplast integrity for up to several hours. Therefore, a Petri dish containing this solution may serve as a storage for droplets, and single droplets can be transferred to the experimental chamber containing test medium for patch-clamp experiments. For preparation, the droplet solution can be modified largely with respect to osmolarity (250–350 mosmol), whereas after formation droplets should be kept in approximately isotonic solutions.

Most cytoplasmic droplets are of spherical shape, however, they may also display an irregular shape with a clear contour due to an intact surrounding membrane. The sizes of cytoplasmic droplets cover a range of < 20–800 μm in diameter, with the majority exhibiting diameters of 100–200 μm. The Ca^{2+} content of the droplet or test medium is a critical factor for the integrity of cytoplasmic droplets. The Ca^{2+} concentration at the vacuolar face of the membrane should be 1 mM or higher, whereas the membrane is stable in the absence of Ca^{2+} at the cytosolic membrane face. When Ca^{2+} was added to the droplet medium or the medium in the experimental chamber, cytoplasmic droplets were stable for at least 30 min, and a number survived for several hours.

Patch pipettes were pulled from borosilicate hard glass with an internally fused filament providing quick-filling from the back (Hilgenberg, Germany). Pipettes were pulled in two stages and then fire-polished under microscopical control. Their typical electrical resistance when filled with experimental solutions was about 10–20 MΩ. Seal resistances with the droplet membrane were typically of the range of 50–100 GΩ. Single channel activity was recorded with a List EPC-7 patch clamp amplifier (List, Germany), digitized and stored on video tape. Replayed data were conditioned with an 8-pole Bessel low-pass filter set to 1 kHz corner frequency, read into a computer with a 20 kHz digitization rate, and analyzed with commercial software (pCLAMP vers. 6; Axon Instr., CA).

The definition of patch configurations on the tonoplast is analogous to that used on plasma membranes: the cytosolic face of the tonoplast is considered to be the inside surface, its vacuolar face as the outer surface. Consequently, the usual terminology of inside/outside-out configurations of excised patches applies. However, during the preparation of cytoplasmic droplets the tonoplast delineating a droplet may be inverted such that the cytosolic face of the membrane is directed towards the bathing medium (cf. scheme in Fig. 1d). This was shown by staining of the droplet interior with neutral red after addition of Mg-ATP to the bath, thus inducing ATP-fueled active transport of H^+ from the cytosolic to the vacuolar side of the tonoplast [22]. Also in some of the reported experiments here, nAChR were activated by superfusion of ostensibly inside-out patches with medium containing the agonist. Consistently, in the membrane surrounding such droplets, the agonist binding sites were all exposed to the same side. Therefore, the orientation of the agonist binding sites was decisive for the definition of inward/outward current fluctuations.

For electrophysiological investigation of nAChR channels, Cs^+ or Na^+ were used as the major salt components in the experimental solution as well as in the patch pipette in order to completely block the native 'maxi'-K^+ channel [22,23]. The test solutions applied to excised patches via a perfusion system were identical to the bathing medium, except that they contained different amounts of acetylcholine, carbamoylcholine (carbachol) or *d*-tubocurarine. In some experiments, the pipette also contained additional carbachol or acetylcholine. As a control, the same experimental procedures as those applied to cytoplasmic droplets from injected cells were also applied to cytoplasmic droplets from four non-injected cells. At least 5 droplets from each cell were examined in the cell-attached mode, as well as in inside-out and outside-out configurations, for AChR activation upon agonist perfusion. The patch pipette contained 10 μM carbachol throughout those experiments. In more than 50 trials, no response to carbachol could be detected, confirming that the tonoplast does not display endogeneous ACh-inducible channel activity.

3. Results and discussion

nAChR channel activity could be observed 24 h after injection of cRNA coding for both, the $nAChR_\gamma$ and the $nAChR_\varepsilon$, respectively. Functional expression was successful in 18 of 19 injected cells as concluded from acetylcholine- or carbachol-induced current fluctuations.

Fig. 2a displays the activity of a single nAChR detected in an outside-out patch. Current fluctuations appeared with the onset of superfusion with a solution containing 10 μM carbachol. Fig. 2b depicts another recording from an inversely oriented membrane patch with 10 μM carbachol included in the pipette solution. An ensemble of at least 6 $nAChR_\gamma$ channel copies was active. The all-points amplitude histogramm displays equidistant levels of about 50 pS (Fig. 2c). A superfusion of the excised patch with solutions containing different concentrations of carbachol, however, had no effect on channel activity. Furthermore, application of 50 μM *d*-tubocurarine did not

Table 1
Composition of applied solutions (in mM if not otherwise stated)

Components	Solutions for				
	Preincubation	Injection	Expression	Droplet	Test
KCl	1	1	1	130	–
CsCl	–	–	–	–	120
$CaCl_2$	1	1	1	5	10[a]
$MgCl_2$	–	–	–	5	1[a]
Sorbitol	–	225	–	–	–
MES	5	–	–	–	–
HEPES/Tris	–	5	5	5	5
Acetylcholine[b]	–	–	–	–	0–300 μM

[a] Test solutions contained 10 mM $CaCl_2$ only in experiments with $nAChR_\gamma$. In experiments on the $nAChR_\varepsilon$, test solutions contained 1 mM $CaCl_2$ and in addition 1 mM $MgCl_2$.
[b] Instead of acetylcholine, carbamoylcholine was also used. For partial blockade of the nAChR, *d*-tubocurarine was applied at 50 or 200 μM in the presence of agonist.

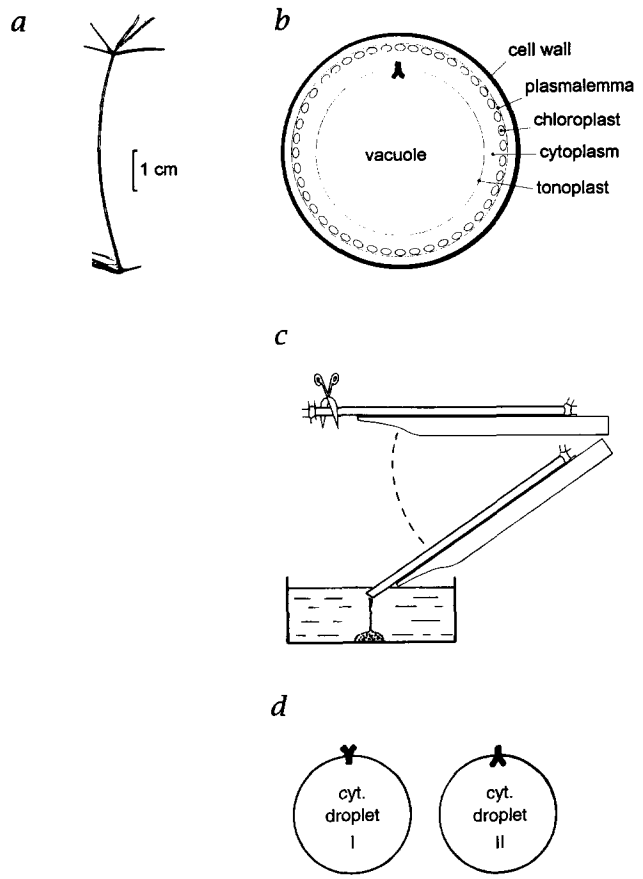

Fig. 1. Schematic drawings of a *Chara* internodal cell, its compartmentation and cytoplasmic droplet formation. (a) *Chara corallina* internodal cell with adjacent whirl cells. (b) Transverse section through internodal cell. The major compartments and their delineating membranes are depicted. An acetylcholine receptor has been drawn as residing in the tonoplast. The binding sites of the channel are turned to the vacuole. (c) An isolated *Chara* internode is placed on a support, the protruding end of the cell is cut when wilting of the cell becomes visible, and the support is tilted so that the open end of the cell is submerged in the droplet medium. Outflowing cytoplasm forms cytoplasmic droplets at the bottom of the Petri dish. (d) Cytoplasmic droplets are surrounded by the tonoplast. The orientation of the tonoplast can be right-side out, i.e. the vacuolar face of the tonoplast is directed towards the bathing medium (cyt. droplet I) or it can be inverse so that the cytosolic face is turned towards the bath (cyt. droplet II). The orientation of the channel under investigation therefore depends on the orientation of the membrane. In a right-side out configuration, the binding sites of the AChR are exposed to the bathing medium, whereas they point into the droplet interior when the droplet membrane is inversely oriented.

produce an inhibitory effect. The fact that no other channel type appeared in this recording, and that channel activity was not abolished by superfusing the patch with *d*-tubocurarine suggests that carbachol supplied from the pipette interior was the only activity-inducing factor. To exclude a participation of anion channels in the observed channel activities, choline chloride was applied to a patch drawn from the same droplet, which resulted in an immediate cessation of preceding carbachol-induced channel activity (not shown) since choline$^+$ was not conducted through the channel.

Fig. 3a shows the response of a single nAChR$_\gamma$ to different concentrations of carbachol. Here, an inside-out patch drawn from the same cytoplasmic droplet as that shown in Fig. 2b was exposed to test solutions, continuously clamped at a pipette potential V_{pip} of −40 mV. Clearly, increasing carbachol concentration led to an increased channel activity which was reduced at concentrations above 30 μM. Channel activity was blocked completely by 200 μM *d*-tubocurarine in the presence of 10 μM carbachol. The frequency of channel opening, as well as the mean lifetime of open events (Fig. 3c) turned out to depend on carbachol concentration. The data of voltage-dependent open channel amplitudes and concentration-dependent open probabilities are compiled in a current–voltage relation (Fig. 3b) and a Hill plot (Fig. 3d), revealing a channel conductance of about 50 pS and a cooperativity constant of $n = 1.95$ ($EC_{50} \simeq 20\ \mu$M). While in this recording no activity could be detected in the

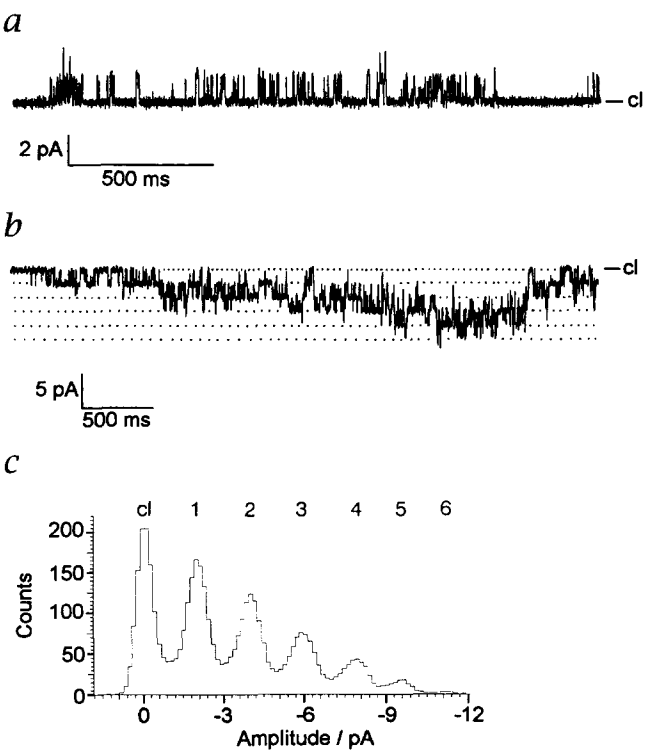

Fig. 2. Current fluctuations of AChR$_\gamma$ recorded on excised membrane patches of *Chara cytoplasmic droplets in solutions containing Cs$^+$ or Na$^+$, respectively, as the permeant ions. (a) Single channel activity in an outside-out patch upon perfusion with 10 μM* carbachol at +40 mV (referred to the vacuolar membrane face as ground), the closed state is indicated to the right-hand side of the trace (cl). Activity of the channel was recorded after 3 days of expression. The symmetrical solutions contained 120 mM CsCl, 10 mM $CaCl_2$, 5 mM Tris-HEPES, pH 7.5. Electrode resistance R_{el} was 17 MΩ, and the output signal was conditioned with a 1 kHz filter (8-pole Bessel). (b) Multiple events detected in an outside-out patch (but an inversely oriented membrane). The pipette contained additional 10 μM carbachol, the closed state is indicated (cl) to the right-hand side of the trace. Current levels that represent simultaneously open channel copies are marked by dotted lines. Current fluctuations were observed immediately after attaining the outside-out configuration, but in the absence of external carbachol, i.e. activation was due to carbachol in the pipette solution. nAChR$_\gamma$ channels were recorded 2 days after injection. Symmetrical solutions contained 120 mM NaCl, 10 mM $CaCl_2$, 5 mM Tris-HEPES, pH 7.5. R_{el} was 8.3 MΩ, and signals were filtered at 1 kHz. (c) All-points amplitude histogram of the recording in panel b. The current levels are indicated on top as cl for the closed state, and numbered continuously according to the number of detected levels. The distances between the peaks in terms of conductance increments were (1) 49.6, (2) 49.7, (3) 47.9, (4) 49.6, (5) 41.4, and (6) 41 pS.

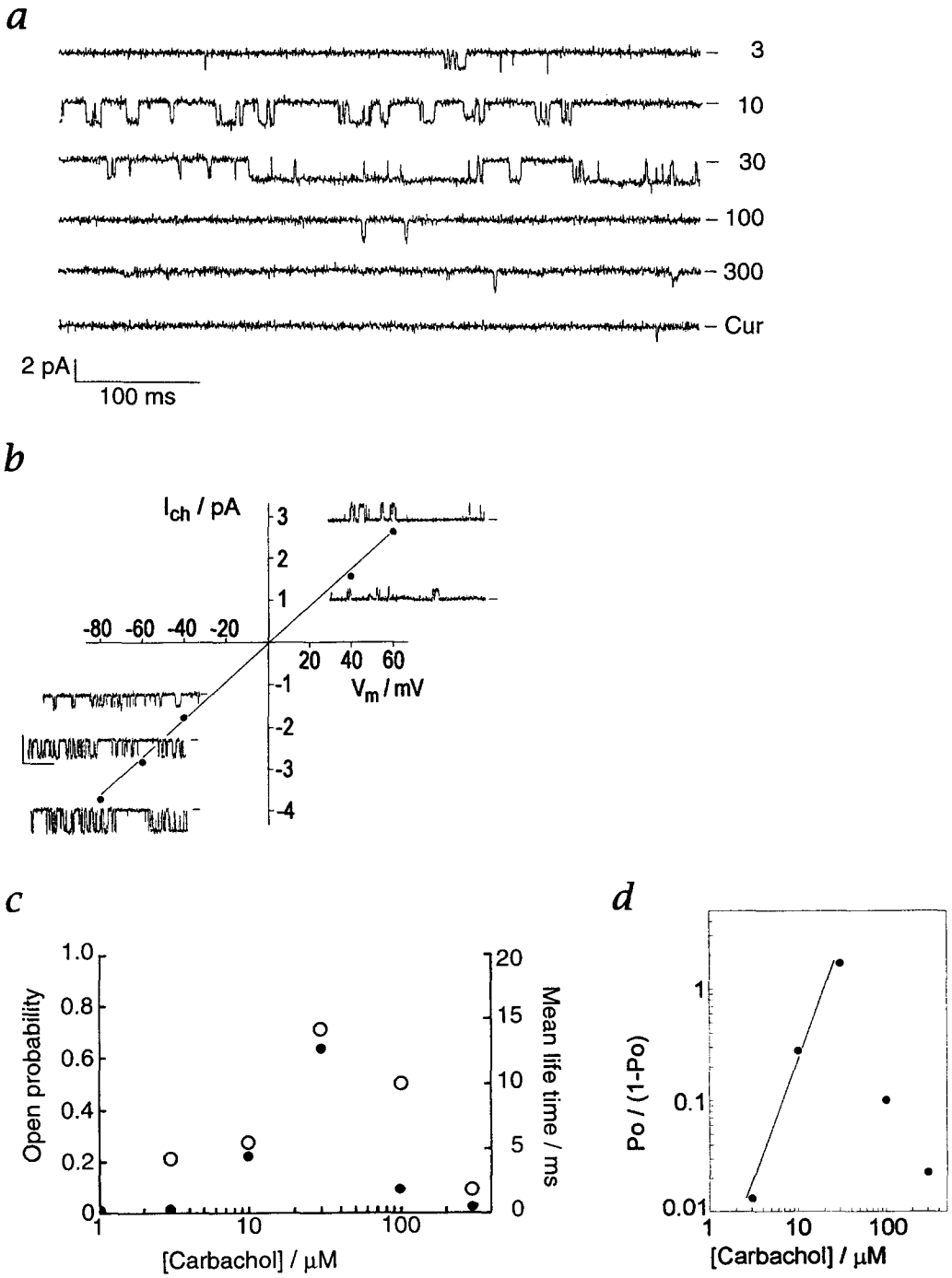

Fig. 3. nAChR$_\gamma$-mediated current fluctuations recorded on an inside-out patch of an inversely oriented membrane delineating the cytoplasmic droplet. (a) Recordings in the presence of different carbachol concentrations. The actual concentration is given at the right-hand side of each trace; the closed state is indicated by a horizontal bar. Superfusion with 200 μM *d*-tubocurarine with additional 10 μM carbachol (denoted as Cur) blocked the current fluctuations. (b) Current–voltage relation of the open channel shown in panel a, measured in identical solutions on both sides of the membrane (see below); the perfusion medium contained additional 10 μM carbachol. Short traces of channel activity are inserted at the respective voltage conditions. The calibration bars at the −60 mV trace represent 5 pA and 100 ms, respectively. The straight line was drawn by eye through the origin. (c) Dependence of open probability (●) and mean life time of the open state (○), respectively, on carbachol concentration. The decay of the open state of the channel could be fit with two exponentials. A fast time constant being of the range of 0.5 ms was not further taken into account; the slow time constant, considered here as the mean open time of the channel, ranged between 5 and 15 ms. (d) Hill plot of channel activation. The ratio of open probability P_o over $(1-P_o)$ was plotted vs. carbachol concentration, both on logarithmic scales. A straight line was drawn by eye through the points of increasing activity (3–30 μM) giving a slope of 1.95 as the cooperativity factor, and 20 μM as the concentration for half-maximal activation. The experiment was carried out after 2 days of expression. The symmetrical solutions contained 120 mM NaCl, 10 mM $CaCl_2$, 5 mM Tris-HEPES, pH 7.5. V_m was held at −40 mV throughout the experiment; R_{el} was 10 MΩ; signals were filtered to 1 kHz.

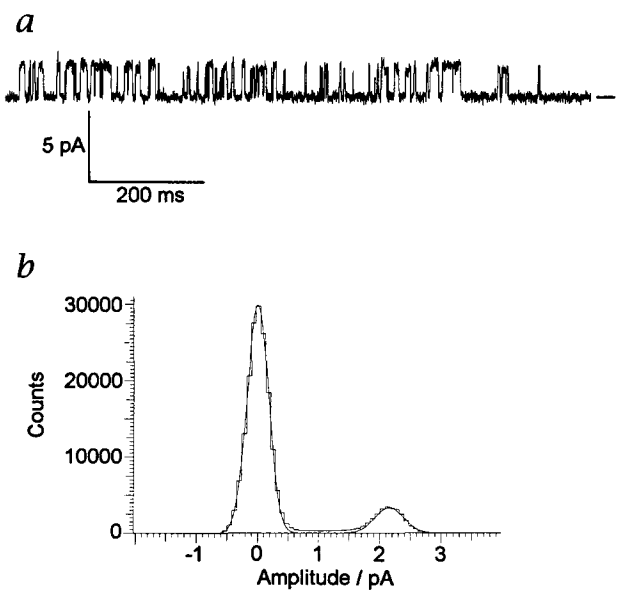

Fig. 4. Single channel recording of nAChR$_\varepsilon$ after 2 days of expression. (a) Current fluctuation in an inside-out patch employing 1 μM acetylcholine in the pipette. Symmetrical solutions contained 120 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Tris-HEPES, pH 7.5. V_m was clamped to +40 mV; R_{el} was 11 MΩ; signals were 1 kHz filtered. (b) All-points amplitude histogram for a 20 s interval of the recording in panel a, displaying the open state level at 2.16 pA (54 pS).

cell-attached mode, current fluctuations were observed immediately after perfusing the inside-out patch with carbachol-containing solutions. The AChR binding sites were apparently exposed to the cytoplasm, which is explained by an inverse orientation of the tonoplast. In other droplets the ACh binding sites were found on the extraplasmatic face of the membrane (cf. Fig. 2a).

Fig. 4 displays the activity of a nAChR$_\varepsilon$ channel, observed 2 days after injection of the cRNA into internodal cells. After preparation of cytoplasmic droplets without squeezing the internodal cells, the membranes of all examined droplets were of right-side out orientation, i.e. its vacuolar face was turned to the bathing medium as revealed by activation of the channels by external acetylcholine (here, 1 μM) or carbachol. The conductance of 54 pS of the channel should be due to the presence of divalent cations in the medium (here 1 mM $CaCl_2$ and 1 mM $MgCl_2$) which had been added to the solutions to stabilize the membrane. A conductance in the presence of divalent cations of 60 pS (at 1.8 mM Ca^{2+}) was recently reported [20]. The mean open time of the nAChR$_\varepsilon$ was determined to be about 3 ms.

In conclusion, both types of nAChR channels were shown to be functionally expressed in *Chara* internodal cells. The properties of those receptors are similar to those examined after expression of the respective cRNA in *Xenopus* oocytes [20,24–26], and also to AChR previously examined in situ [27–29]. It may be presumed that characean internodal cells may be utilized as a convenient expression system for a number of channel types. An advantage of this system is the simple and rapid preparation of cytoplasmic droplets that are surrounded by the native tonoplast. This membrane contains only 2 intrinsic channel types, a Cl^- channel of about 21 pS and a highly selective K^+ channel of about 150 pS. Another advantage of the algal system worth mentioning, therefore, is the absence of native channels competing with those functionally expressed in the tonoplast. Since it is unlikely that targeting exists for alien membrane proteins, the tonoplast is presumably a sink for those proteins. With standard patch pipettes, this membrane easily forms gigaohm seals of > 50 GΩ. In preliminary experiments, cRNA coding for the CX32 gap junction hemichannel derived from mouse liver could also be detected as being functionally expressed in the tonoplast of those internodes. Besides a simple handling of the internodal cells and the advantages the droplet membrane offers to patch clamp experiments, the costs of keeping *Chara* are almost nil. The establishment of *Characeae* cultures, cells of which are permanently transformed with cDNA coding for various ion channels, will undoubtedly also be of biotechnological interest.

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