J. Membrane Biol. 185, 117–127 (2002)
DOI: 10.1007/s00232-001-0118-x

The Journal of
Membrane
Biology

Protein Orientation Affects the Efficiency of Functional Protein Transplantation into the *Xenopus* Oocyte Membrane

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Received: 11 July 2001/Revised: 17 September 2001

Abstract. *Xenopus* oocytes incorporate into their plasma membrane nicotinic acetylcholine receptors (nAChRs) after intracellular injection of lipid vesicles bearing this protein. The advantage of this approach over the classical oocyte expression system lies in the transplantation of native, fully processed proteins, although the efficiency of functional incorporation of nAChRs is low. We have now studied the incorporation into the oocyte membrane of the *Torpedo* chloride channel (ClC-0), a minor contaminant protein in some nAChR preparations. nAChR-injected oocytes incorporated functional ClC-0: i) in a higher number than functional nAChRs; ii) retaining their original properties; and iii) with a right-side-out orientation in the oocyte membrane. In an attempt to elucidate the reasons for the low efficiency in the functional incorporation of nAChRs into the oocyte membrane, we combined electrophysiological and [^{125}I]α-bungarotoxin-binding experiments. Up to 3% of injected nAChRs were present in the oocyte plasma membrane at a given time. Thus, fusion of lipoproteosome vesicles to the oocyte plasma membrane is not the limiting factor for an efficient functional transplantation of foreign proteins. Accounting for the low rate of functional transplantation of nAChRs is their backward orientation in the oocyte membrane, since about 80% of them adopted an out-side-in orientation. Other factors, including differences in the susceptibility of the transplanted proteins to intracellular damage should also be considered.

Key words: ClC-0 — Nicotinic receptors — Lipoproteosomes — *Xenopus* oocytes — Electroplaque membranes — Functional transplantation

Introduction

Nicotinic acetylcholine receptors (nAChRs) purified from *Torpedo* electroplaques and reconstituted in asolectin lipid vesicles become incorporated into the *Xenopus* oocyte membrane after being injected intracellularly, and they retain their functional properties (Morales et al., 1995; Gal, Ivorra & Morales, 2000). Some water-channel proteins purified and reconstituted in an artificial lipid matrix have also been functionally incorporated into the *Xenopus* oocyte membrane (Le Cahèrec et al., 1996). This approach constitutes an extension to the use of either mRNA (Miledi, Parker & Sumikawa, 1989) or plasma membranes (Marsal, Tigy & Miledi, 1995) to transplant foreign membrane proteins to this convenient host cell. Injection of plasma membranes or purified and reconstituted proteins has as main advantage over the classical use of oocytes as a cellular expression system that the properties of the incorporated protein are not modified by an altered post-translational processing of the protein (such as cleavage, acetylation, glycosylation or phosphorylation), or by modifications in the assembly of oligomeric receptor/channel complexes. In this sense, it is important to mention that, for instance, *Torpedo* nAChRs expressed in oocytes display an altered pattern of glycosylation (Buller & White, 1990) and neuronal nAChRs do not exhibit the properties of native receptors, likely because oocytes fail to assemble correctly their different subunits (Sivilotti et al., 1997). The use of purified and reconstituted proteins, instead of fragments of cellular membranes, allows the

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functional study of specific molecular entities and can even be used to study proteins that are not very abundant in the cellular membrane. However, either the purification and reconstitution in artificial lipid matrices or the incorporation into the oocyte membrane may affect the activity of the incorporated protein. For this reason, it is interesting to know whether or not other membrane proteins, besides nAChR, retain their native functional properties when transplanted after their purification and reconstitution. Another important point regarding this approach is that although several million of nAChRs can be functionally incorporated into the oocyte membrane, this amount is actually small as compared to the number of injected molecules (Morales et al., 1995). Thus, another aim of this work was to determine whether the low efficiency in the functional transplantation of exogenous proteins into the oocyte membrane was either due to a rare fusion of the injected vesicles with the oocyte plasma membrane or, alternatively, to a small proportion of the transplanted proteins retaining their functional activity. We have used for this study a few preparations of purified and reconstituted nAChRs (prAChRs) that contained *Torpedo* chloride channels (ClC-0) as a copurifying protein. The ClC-0 protein belongs to a family of voltage-dependent chloride channels, which are structurally and functionally very different from ligand-gated ion-channel receptors, such as nAChRs (Jentsch et al., 1999; Maduke, Miller & Mindell, 2000). Preliminary results have been published elsewhere (Ivorra et al., 1996; Gal et al., 1998).

Materials and Methods

Electroplaque Plasma Membrane (epm) Isolation and nAChR Purification and Reconstitution

The electric organ of Torpedo marmorata was used to prepare epm fractions highly enriched in nAChR (Artigues et al., 1987). A few aliquots of these epms were resuspended in 140 mM KCl at a protein concentration of 5 mg/ml, and the remaining sample was used to purify the nAChR by affinity chromatography in the presence of asolectin lipids, as described by Jones, Earnest & McNamee (1987). Briefly, cholate-solubilized membranes (2 mg/ml protein and 1% cholate in a buffer containing: NaCl, 100 mM; 3-(N-morpholino)propanesulfonic acid (MOPS $NaNO_3$), 10 mM; ethylenediaminetetraacetic acid (EDTA), 0.1 mM; $NaNO_3$ 0.02%; pH 7.4) were applied to a bromoacetylcholine-derivatized Affi-gel 401 column and the nAChR was eluted with 10 mM carbamylcholine in the presence of 1 mg/ml asolectin lipids. The purified nAChR had specific activities of approximately 8 nmol of α-bungarotoxin (α-Btx) bound per mg of protein, exhibited an apparent molecular weight of 270 kDa and contained the characteristic 40, 50, 60 and 65 kDa subunits in a 2:1:1:1 stoichiometry, as determined by various methods (Paraschos, González-Ros & Martínez-Carrión, 1982). Plain lipid vesicles used for reconstitution were prepared from asolectin lipids, at ~40 mg/ml, by a CHAPS dialysis procedure. These dialyzed samples were resolubilized in 4% sodium cholate and used immediately for reconstitution. Reconstituted nAChR samples were prepared by mixing aliquots of purified nAChR with the solubilized lipid vesicles from above. The reconstitution was accomplished by dialysis (NaCl, 100 mM; Tris-ClH 10 mM; pH 7.4) at 4°C for about 50 hr. The degree of purification was determined by SDS-PAGE on 9% polyacrylamide gels. Protein concentration was determined by the Lowry method (Lowry et al., 1951) and [^{125}I]α-bungarotoxin ([^{125}I]α-Btx) binding was measured by using a DEAE-cellulose filter disk assay (Artigues et al., 1989). Agonist-mediated cation translocation through lipoproteosome vesicles bearing prAChR was monitored using a "stopped-flow/fluorescence quenching" assay of Tl^+ influx (Fernández et al., 1993). Samples of prAChR were aliquoted at a final protein concentration of 0.5–1 mg/ml, and injected immediately into oocytes or stored in liquid nitrogen until the time of injection.

Oocyte Preparation, Microinjection and Recording

Fully-grown *Xenopus* oocytes were isolated and collagenase-treated as previously described (Morales et al., 1995; Ivorra & Morales, 1997). Cells were kept at 15–16°C in a modified Barth's solution (MBS, in mM; NaCl, 88; KCl, 1; $Ca(NO_3)_2$, 0.33; $CaCl_2$, 0.41; $MgSO_4$, 0.82; $NaHCO_3$, 2.40; HEPES, 10; pH, 7.4) supplemented with 100 IU/ml penicillin and 0.1 mg/ml streptomycin until used for electrophysiological recordings.

Freeze-thawed samples of epm or prAChR were homogenized before injection of 100 nl into each oocyte. The recording methodology has been previously described (Morales et al., 1995; Ivorra & Morales, 1997). Briefly, oocytes were placed in a small chamber and continuously superfused (3–9 ml/min) with a Ringer solution to which 0.5 μM atropine sulphate was eventually added to block any muscarinic response. To test the functional incorporation of nAChRs, membrane currents elicited by acetylcholine (ACh) were recorded with a high-compliance two-electrode voltage-clamp system (TurboTEC-10CD, npi) at room temperature (21–25°C), while holding oocyte membrane potential (V_h) at −60 mV.

Incorporation of ClC-0 was assessed in every cell recorded by applying to the oocyte membrane hyperpolarizing-depolarizing voltage ramps (5 mV/sec), from a V_h of −20 to −120 and back to −20 mV. The reversal potential of ClC-0 current was measured either by applying voltage pulses to different values at the end of the ramp protocol, or by plotting the I/V relationship for currents evoked by the hyperpolarizing and depolarizing ramps, in order to determine the intersection point. The anion selectivity of the channel was estimated by giving depolarizing-hyperpolarizing voltage ramps from a V_h of −120 up to +20 and back to −120 mV (50 mV/sec) in oocytes bathed in normal Ringer (NR; in mM: NaCl, 115; KCl, 2; $CaCl_2$, 1.8; HEPES, 5; pH 7.0) or in a Ringer in which 115 mM Cl^- was substituted by equimolar amounts of other anions. Functional activity of the fast and slow gates of the incorporated channels was tested in some oocytes by applying short-(80 msec) or long-(25 sec) duration hyperpolarizing and depolarizing pulses from a V_h of −20 mV. The current records obtained with these voltage protocols were fitted to single-exponential curves to estimate the instantaneous I/V relationship.

Determination of nAChR Orientation

The number of functional nAChRs incorporated into the oocyte membrane was estimated from the ACh current. The total number of incorporated receptors with their ligand-binding site facing up the extracellular or intracellular sides was determined by [^{125}I]α-Btx-binding experiments. For this, ACh currents were recorded in prAChR-injected oocytes and afterwards part of these cells were

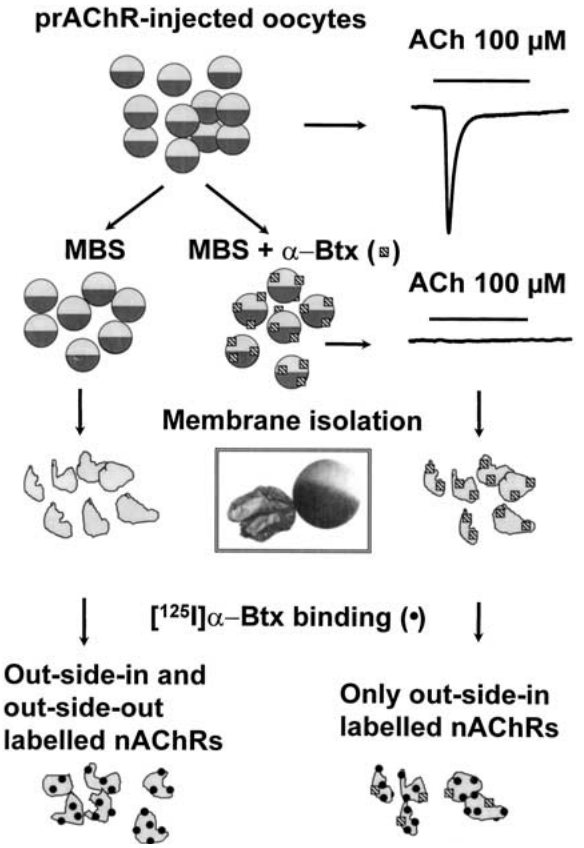

Fig. 1. Scheme showing the steps followed for quantitation of total and out-side-in oriented nicotinic acetylcholine receptors (nAChRs) incorporated into the oocyte membrane. The inset shows an oocyte and the plasma membrane, stained with a dark blue ink, isolated from another oocyte.

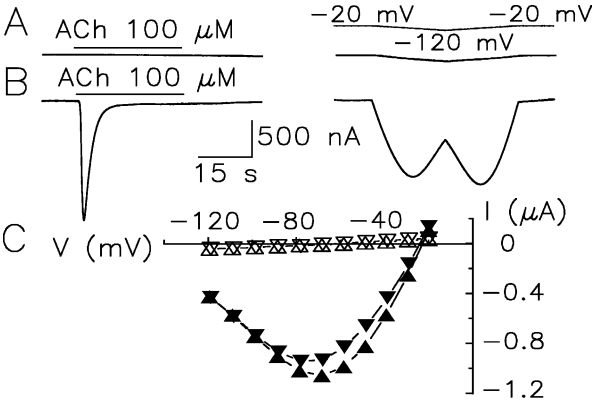

Fig. 2. Functional incorporation of nAChRs and voltage-dependent channels in prAChR-injected oocytes. Membrane currents elicited either by acetylcholine (ACh; *left*) or by the hyperpolarizing-depolarizing voltage ramp (*right*, upper record) in a noninjected (*A*) and in a prAChR-injected oocyte (*B*). (*C*) *I*/*V* relationship for the currents elicited by the voltage ramp in the uninjected (open symbols; downward triangles for the hyperpolarizing ramp and upward triangles for the depolarizing one) and in the injected (same symbols, filled) cells. Note the ohmic response in the uninjected oocyte in contrast to the marked outward rectification and the different values for hyperpolarizing and depolarizing potentials in the prAChR-injected oocyte. In this and following figures, the holding potential (V_h) for ACh currents was −60 mV, downwards deflections denote inward currents and bars indicate ACh application.

incubated for 30 min with α-Btx (100 nM), to irreversibly block the out-side-out oriented nAChRs. After that time, these oocytes were again kept in MBS until their new electrophysiological recording, to confirm the α-Btx blockage of the ACh-elicited current, and the subsequent membrane isolation (see scheme of Fig. 1). Batches of 3–10 plasma membranes from non-injected oocytes, prAChR-injected oocytes and prAChR-injected oocytes incubated with α-Btx were then manually isolated, as previously described (Sadler & Maller, 1981; Aleu et al., 1997), and incubated for 4 hr with [^{125}I]α-Btx. The average number of nAChRs incorporated into the membrane of each injected oocyte was determined from the difference in radioactivity counts between injected and non-injected cells and considering the number of plasma membranes used in each case. The number of receptors oriented out-side-in was given by the counts obtained in those oocytes previously incubated with non-radioactive α-Btx (*see* scheme in Fig. 1). Hence, the number of nAChRs oriented out-side-out was estimated by subtracting the number of receptors showing an out-side-in orientation from the total number of incorporated nAChRs.

Solutions

To examine the ion selectivity of the incorporated channels, the NaCl in NR was substituted by equimolar amounts of NaI, NaBr or NaSCN. Ca^{2+}-free solutions were prepared without $CaCl_2$ and with 10 mM $MnCl_2$ added. ACh (100 μM), 4,4′-di-isothiocyanatostilbene-2,2′-disulphonic acid (DIDS, 0.1 to 1 mM) and α-Btx (100 nM) were freshly made from their stock solutions prior to application. [^{125}I]α-Btx and radioactive calibration compounds were purchased from New England Nuclear (Boston, MA). All other products were obtained from Sigma (St. Louis, MO).

Statistics

Unless otherwise specified, values given in the text correspond to the mean ± SEM. When comparing two-group means of normally distributed data, the Student's *t*-test was used. Otherwise the Mann-Whitney rank sum test was applied. Among groups differences were determined by the analysis of variance and the group means were compared using the Student-Newman-Keuls (SNK) test. A significance level of $p < 0.05$ was adopted in all comparisons.

Results

Incorporation of nAChRs and ClC-0

Oocytes injected with prAChRs and exposed to ACh (100 μM) elicited a characteristic nicotinic current (Fig. 2*B*, *left*; Morales et al., 1995). This current was attributable to the incorporation of the purified receptors, since it could not be evoked in uninjected cells (Fig. 2*A*, *left*). *Xenopus* oocyte membrane potential has some variation between donors, but cells injected with prAChRs present similar values as un-

Table 1. Membrane properties of control, epm-injected and prAChR-injected oocytes

	E_m (mV)	E_{rev} ClC-0 (mV)	g (−40 to −20, Cl^-) (μS)	g (−40 to −20, I^-) (μS)	g (Cl^- − I^-) (μS)	I_{ACh} (100 μM) (nA)
Control	−43 ± 2 (106)	—	4.6 ± 0.4 (106)	6.4 ± 1.6 (10)	−2.2 ± 2.7 (10)	0 (37)
epm-Injected	−34 ± 1* (43)	−28 ± 1 (12)	95.9 ± 10.4* (43)	19.7 ± 2.7* (19)	71.4 ± 16.9* (19)	215 ± 56* (40)
prAChR-Injected	−35 ± 1* (89)	−27 ± 1 (15)	20.5 ± 1.7*,# (89)	8.0 ± 1.0# (21)	14.3 ± 2.8*,# (21)	417 ± 76*,# (63)

The reversal potential of ClC-0 current was measured as indicated in Methods. Membrane conductance figures were obtained from the depolarizing phase of the ramp protocol (−40 to −20 mV), in NR or after substituting most Cl^- by I^- ions. Values are the mean ± SEM and the number of observations is given in parentheses. *Indicates significant differences ($p < 0.05$) with control group; # indicates significant differences between epm- and prAChR-injected oocytes.

injected oocytes from the same toad (AM, unpublished observations). Unexpectedly, oocytes injected with prAChRs from two preparations exhibited a significantly lower resting potential and membrane resistance than uninjected cells of the same batch (see Table 1), which was apparently not due to oocyte membrane damage caused by microinjection. We applied to these cells a hyperpolarizing-depolarizing ramp protocol to assess whether they had additional voltage-dependent conductances that account for their low membrane potential and input resistance. The membrane current elicited by the voltage ramp was markedly different in uninjected (Fig. 2*A*, *right*), and prAChR-injected (Fig. 2*B*, *right*) cells. In control oocytes the I/V relationship was ohmic, whereas in injected cells it followed a complex behaviour, including a high membrane conductance at V_h, a marked outward rectification at potentials more negative than −60 mV and an increased membrane conductance after hyperpolarization (Fig. 2*C*; note the different I/V relationships for the hyperpolarizing and depolarizing phases of the voltage ramp). The reversal potential of this current was measured in a few oocytes by stepping the membrane potential to different voltages during the slow current tail that follows the ramp protocol (Fig. 3*A*). The reversal potential value obtained was confirmed by determining, from the I/V curve, the point at which the current from hyperpolarizing and depolarizing ramps intersected (Fig. 3*B*). Both methods gave a similar value, close to −25 mV (Table 1), which corresponds to the equilibrium potential for chloride in oocytes (Kusano, Miledi & Stinnakre, 1977), suggesting that prAChR-injected oocytes had incorporated in the plasma membrane voltage-dependent chloride channels besides nAChRs. Most likely, these channels were present in the epm and were copurified with nAChRs. To confirm this, we injected some oocytes with epm and others, from the same toad, with a sample of prAChR obtained from the same epm preparation. Cells injected with the sample of prAChRs showed a typical ACh response and a voltage-dependent conductance similar to that described above (Fig. 4*B right*, *upper* and *lower* records, respectively). Compared with these cells, epm-

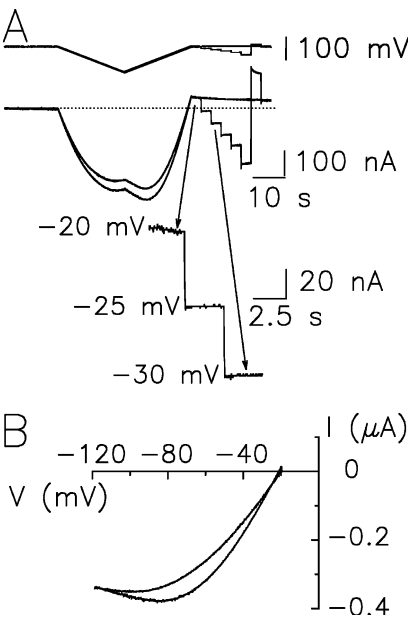

Fig. 3. Reversal potential of the voltage-dependent current evoked in prAChR-injected oocytes. Two voltage (*A*, *upper*) and current (*A*, *middle*) records are superimposed, one corresponding to the normal ramp protocol (from a V_h of −20 mV to −120 and back to −20 mV) and a similar one, but stepping back the membrane potential to different voltages during the long current tail that follow the ramp protocol. Arrows signal 2 of 3 such steps, at an expanded scale, to show current-reversal potential (−25 mV) in this cell. Current-reversal potential was also estimated from the I/V curve (*B*), as the point in which membrane currents from the hyperpolarizing and depolarizing ramp intersected. For this figure, the membrane current at the holding potential was considered as 0 (dotted line in *A*).

injected oocytes showed smaller ACh currents (Fig. 4*B left*, upper record and Table 1), and a larger voltage-dependent conductance (ranging from 15.8 to 383.4 μS; Table 1) with characteristics similar to those observed in prAChR-injected oocytes and also to those mediated by ClC-0 channels incorporated into the oocyte membrane after injection of epm (Marsal et al., 1995). Other membrane properties of epm-injected oocytes, such as the resting potential or the I_{ClC-0} reversal potential, were not significantly different to those found in prAChR-injected oocytes

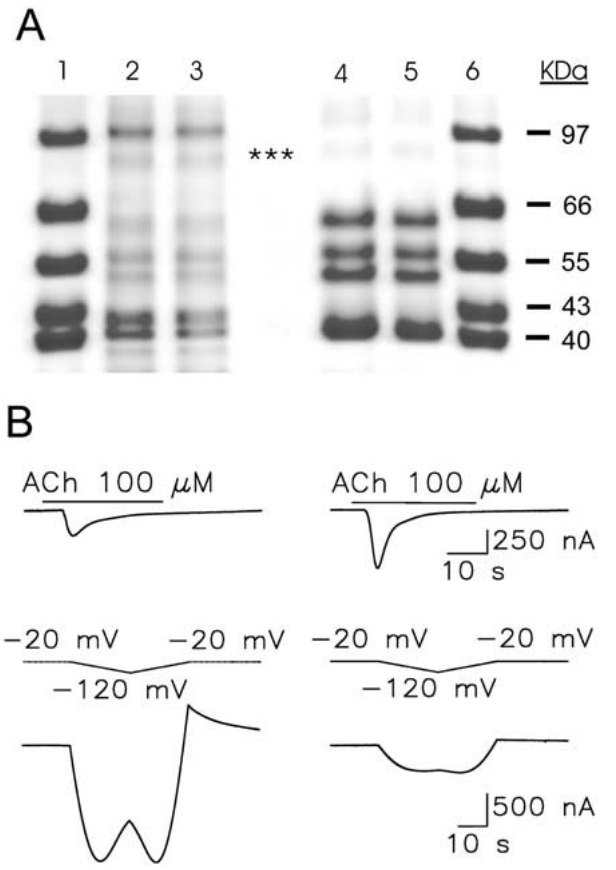

Fig. 4. Gel electrophoresis (*A*) showing the presence of nAChR and *Torpedo* chloride channel (ClC-0) in electroplaque plasma membrane (epm; lanes *2* and *3*) and prAChR (lanes *4* and *5*) samples. Molecular weight markers (*see* numbers on the right) were loaded in lanes *1* and *6*. Asterisks were placed to signal a band of about 90 kDa, which corresponds to the molecular mass of the ClC-0 protein. Note in lanes *4* and *5* the strong 4 bands due to the different subunits of nAChR and the weak ClC-0 band, and the difference in intensity between them and their corresponding bands in the epm sample. The amount of protein loaded was 12 μg in lanes *2* and *4* and 8 μg in lanes *3* and *5*. (*B*) Membrane currents elicited by ACh (upper record) and the voltage ramp protocol (middle and lower records) in an epm-injected oocyte (*left*) and in a prAChR-injected cell (*right*). Both oocytes were from the same toad. The epm- and prAChR-injected samples were the same ones shown in (*A*).

(Table 1). Thus, the voltage-dependent conductance observed in oocytes injected with these samples of prAChRs was very likely due to incorporation of ClC-0. The relative amount of this copurifying protein in the prAChR sample was estimated from SDS-PAGE of epm and prAChRs samples. Electrophoresis analysis of the prAChR sample (Fig. 4*A*, lanes *4* and *5*) showed four marked bands, corresponding to the four different subunits of this protein, but also present was a faint band of about 90 kDa. This band, which corresponds to the molecular mass of ClC-0 (Jentsch, Steinmeyer & Schwarz, 1990), was more evident in the sample of epm (Fig. 4*A*, lanes *2* and *3*). It was estimated from scanning densitometry that less than 3% of the total protein present in the prAChR sample corresponds to the ClC-0 band.

Functional Properties of Incorporated ClC-0

The membrane of *Xenopus* oocytes is rich in Ca^{2+}-dependent Cl^- channels, which display an outward rectification at hyperpolarized potentials (Miledi & Parker, 1984). To rule out that the high membrane conductance observed after prAChR injection was due to activation of Ca^{2+}-dependent Cl^- channels, we applied in a few prAChR-injected oocytes the double-ramp protocol while bathing the cells first in NR and later in a Ca^{2+}-free solution. There was no difference in the elicited membrane current when Ca^{2+} removed from the bath (*not shown*), as it would be expected for ClC-0 channels (White & Miller, 1979).

The presence of ClC-0 in the sample of prAChRs allowed to assess whether the purification, reconstitution or incorporation into the oocyte membrane processes had any influence on the functional properties of this protein, structurally very different to the nAChR. ClC-0 have a high selectivity for chloride over other anions (White & Miller, 1979), something unusual for the so-called chloride channels. The channel permeability to different anions was investigated by giving fast depolarizing-hyperpolarizing ramps, from a V_h of −120 mV up to +20 mV, to a few uninjected (control) and prAChR-injected oocytes while bathed with either NR or Ringer in which 115 mM Cl^- was replaced by equimolar amounts of I^-, Br^-; or SCN^-. In control oocytes, both SCN^- and I^- had higher permeability through endogenous chloride channels than Br^- or Cl^- (Fig. 5*A*). In contrast, prAChR-injected oocytes showed a higher membrane conductance in NR than when bathed in SCN^- or Br^- solutions, and the current was even smaller when the cells were bathed with I^- (Fig. 5*B*). In fact, the membrane conductance of prAChR-injected oocytes bathed in I^- was not significantly different ($p > 0.05$, SNK test; Table 1) from that of uninjected cells (*compare* records in Fig. 5*A* and *B*). The effect of the different anions on the oocyte membrane conductance is better seen in the I/V relationship obtained for each of them in control and in prAChR-injected oocytes (Fig. 5*C*, *left* and *right*, respectively). It is noteworthy that at negative potentials both I^- and SCN^- blocked this conductance, preventing Cl^- from leaving the cell through ClC-0 channels. This blocking effect has been previously reported for ClC-0 expressed in oocytes (Bauer et al., 1991), and seems to be related to differences in the gating of the channel by the external permeant anion (Pusch et al., 1995).

ClC-0 channels are also characterized by the presence of two kinetically different gates: a fast gate opened by depolarization and a slow gate that opens

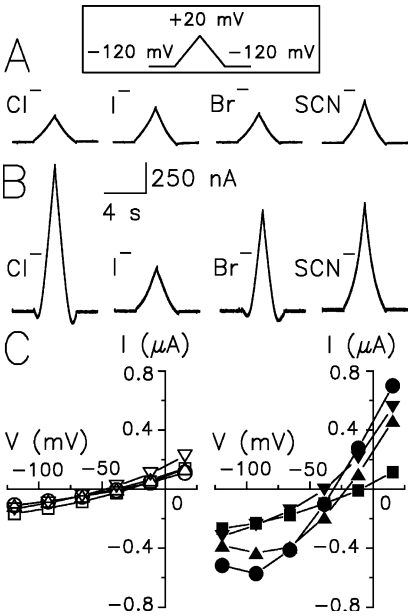

Fig. 5. Anion permeability of ClC-0 transplanted to oocytes. A depolarizing-hyperpolarizing ramp (50 mV/sec) from a V_h of −120 mV (*see* inset) was given to a noninjected (*A*) and to a prAChR-injected oocyte (*B*), while bathed in NR (Cl^-) or in a Ringer in which 115 mM Cl^- was substituted by equimolar amounts of I^-, Br^- or SCN^-. The corresponding *I/V* relationships (*C*) show that prAChR-injected oocytes (*right*), in contrast to noninjected ones (*left*), had a higher permeability for Cl^- (circles). I^- (squares) and, at negative potentials, SCN^- (downward triangles) blocked the Cl^- conductance; Br^- (upward triangles) only shifted the *I/V* curve to the right. Both oocytes came from the same toad. Baseline values of records shown in *A* and *B* are arbitrary.

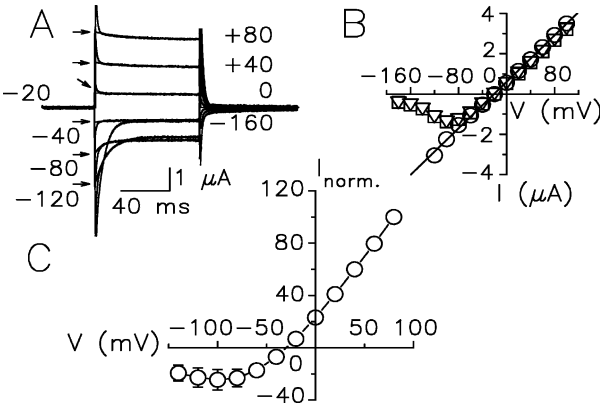

Fig. 6. Time and voltage dependence of the ClC-0 fast gate. Membrane currents elicited by brief depolarizing and hyperpolarizing pulses, from a V_h of −20 mV, in a prAChR-injected oocyte (*A*). Voltage pulses were given in 20 mV, steps, but for clarity, some records are skipped. Membrane currents were fitted by single-exponential curves, which are superimposed to the records. Capacitative transients have been truncated. (*B*) *I/V* relationship for the stationary (squares) and instantaneous (circles) currents from *A*. The instantaneous current values (arrowheads shown in *A*) were obtained from the exponential function; stationary current values were also well fitted by these exponential curves (triangles). Note the linear relationship for the instantaneous values. To compare this *I/V* relationship in different oocytes, steady-state current amplitude was normalized in each cell as a percentage of the value obtained at +80 mV. In *C* is shown the normalized *I/V* curve for 6 oocytes; bars correspond to the SEM.

with hyperpolarization (Miller, 1982). The function of both voltage gates in transplanted ClC-0 was studied in a few cells by applying fast or slow voltage pulses. From a holding potential of −20 mV, fast depolarizing potentials elicited an ohmic response, but hyperpolarizing pulses over −60 mV originated a marked outward rectification (Fig. 6 *A* and *B*). To compare the *I/V* relationships obtained with this protocol in different oocytes, the current values for each cell were normalized as a percentage of the current at +80 mV (Fig. 6*C*). Since the instantaneous *I/V* relationship, estimated from exponential fitting, was fairly linear (Fig. 6*B*), the outward rectification can be attributed to the closure of the fast gate by hyperpolarization. The slow gate was tested by applying long (25 sec) hyperpolarizing pulses from a V_h of −20 mV and comparing the initial current values (I_0) to those obtained 20 sec later (I_{20}; Fig. 7*A*). Low hyperpolarizing potentials elicited a small decrease in the I_{20}/I_0 ratio, likely due to closure of the ClC-0 fast gate (Fig. 7*B*). However, hyperpolarizations over −80 mV caused a slow current increase that should correspond to the opening of the slow gate. When the membrane potential was stepped at very negative potentials, i.e. −140 or −160 mV, the I_0 values decreased markedly (Fig. 7*B*), as it would be expected for a large proportion of protochannel fast gates being closed.

In bilayer experiments, both pH and DIDS, an unspecific chloride channel blocker, affect ClC-0 function (White & Miller, 1979; Hanke & Miller, 1983). Thus, in order to test the effect of these factors on ClC-0 transplanted channels, prAChR-injected oocytes were challenged using the ramp protocol with Ringer solutions at different pH values (5.5 to 8; 4 cells) or containing DIDS (100 μM; 2 cells). Neither pH changes nor DIDS did appreciably modify the current shape or amplitude observed in NR in the same cells (*not shown*). In two additional cells we injected intracellularly a "puff" of 10–20 nl of 1 mM DIDS, which resulted in a reduction in the tail current amplitude to a 63% and 68% of their values in NR (*not shown*).

Quantitation of the Number of ClC-0 and nAChRs Incorporated

The number of functional ClC-0 and nAChRs incorporated into the oocyte membrane can be estimated from electrophysiological recordings. Hence, the increase in the oocyte membrane conductance, due to incorporation of ClC-0, was calculated by

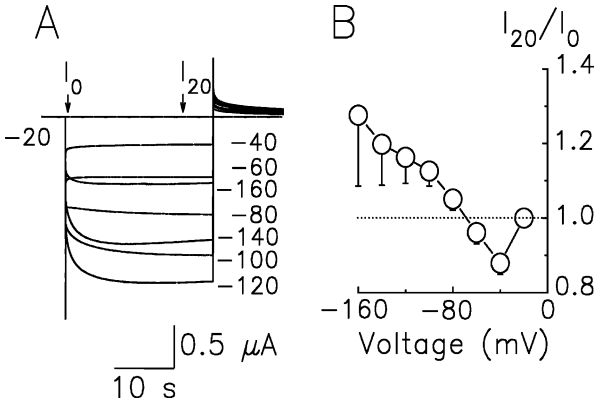

Fig. 7. Time and voltage dependence of the ClC-0 slow gate. Membrane currents elicited by long hyperpolarizing pulses given from a V_h of −20 mV in a prAChR-injected oocyte (*A*). Numbers correspond to the stepped voltage values. Note the slow current activation evoked by pulses at potentials more negative than −80 mV, and the small initial current (I_0) at highly negative potentials. The slow activation by hyperpolarization was normalized by dividing the current value at 20 seconds (I_{20}) by its initial value (I_0). (*B*) Plot of the average I_{20}/I_0 ratio versus membrane potential for 6 oocytes; bars correspond to the SEM.

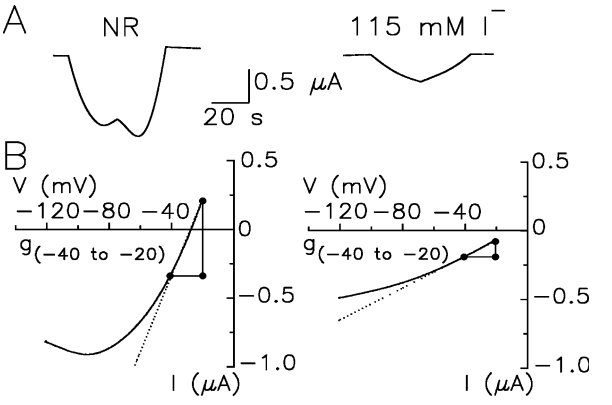

Fig. 8. Quantitation of the number of transplanted ClC-0. (*A*) Membrane currents elicited by the hyperpolarizing-depolarizing ramp in a prAChR-injected oocyte bathed in NR (*left*) or when NaCl was substituted by NaI (*right*). Note that in NR, but not in I^-, the membrane conductance was higher during the depolarizing phase following the membrane hyperpolarization. (*B*) *I*/*V* relationships for the depolarizing phase of the above records. The slope conductance was maximum and constant in the range from −40 up to −20 mV in both conditions (dotted line). The difference in slope conductance in NR and I^- was used as an estimation of the change in oocyte membrane conductance due to ClC-0 incorporation.

subtracting the slope value in the linear region of the *I*/*V* curve obtained from the ramp protocol in oocytes bathed in 115 mM I^- from the corresponding value obtained in normal Ringer, since I^- almost fully blocks ClC-0 (Figs. 5 and 8 and Table 1). In control oocytes, the difference between the membrane conductances in Cl^- and I^- was negative, indicating a higher membrane permeability to I^- (Table 1). However, in prAChR-injected oocytes this difference was positive, of about 14 μS (Table 1), which indicates that at least 1–2 × 10^6 ClC-0 molecules, assuming an elementary conductance of 10 pS (Miller, 1982), were incorporated into the oocyte membrane. As expected, epm-injected oocytes showed the highest difference in membrane conductance when comparing the values obtained for Cl^- and I^- (*see* Table 1), even though the membrane conductance in I^- is higher in these cells than in control or prAChR-injected cells.

The number of functional nAChRs can be deduced from the peak of the ACh current. Assuming a reversal potential of −5 mV, a single-channel conductance of 40 pS (Tank, Miller & Webb, 1983), and that the 100 μM ACh current amplitude is a 66% of the maximum response (Morales et al., 1995), then an ACh current of 417 nA (average response obtained in prAChR-injected oocytes, *see* Table 1) should correspond to, at least, 3 × 10^5 functional incorporated receptors. ACh currents in epm-injected oocytes were smaller, on average about half the amplitude of those recorded in prAChR-injected cells (Table 1), and hence there was a lower number of functional incorporated receptors, even though the total protein concentration in the epm sample was fivefold.

Therefore, prAChR-injected oocytes incorporated into their membrane a higher number of ClC-0 than nAChRs, although the major protein in the prAChR lipoproteosome vesicles corresponded, by far, to the latter (Fig. 3*A*). Since ClC-0 and nAChRs were likely carried to the oocyte membrane by the same vesicles, it was interesting to know whether the membrane of injected oocytes contained a higher number of nAChRs that, for any reason, were silent, i.e., non-functional.

To estimate the total number of nAChRs incorporated into the oocyte membrane and the number of functional receptors, we carried out electrophysiological and [^{125}I]α-Btx-binding experiments in the same cells (Fig. 1). Oocytes were injected with two additional preparations of prAChR and cells were classified into three groups: small, medium and large (*S*, *M*, *L*, respectively) according to their ACh current amplitudes (Table 2). Some of the recorded oocytes from each group were later incubated for 30 min with α-Btx to irreversibly block the receptors with an outside-out orientation, i.e., with their ligand-binding side oriented towards the extracellular space. Thereafter, a few of them were again challenged with ACh to verify that the ACh current was completely blocked (*see* scheme Fig. 1, and Gal et al., 2000). Manually isolated plasma membranes from oocytes of each group and kept either in MBS or α-Btx were later on incubated with [^{125}I]α-Btx. The number of nAChRs incorporated into the oocyte membrane, determined from the radioactivity counts of bound [^{125}I]α-Btx (*see* Methods), was found to vary between groups (Table 2). On average, each *S* group oocyte

Table 2. Results from combined [^{125}I]α-Btx binding and electrophysiological experiments to determine the number of transplanted and functional nAChRs and their orientation in the oocyte membrane (mb)

	I_{ACh} (100 μM) (nA)	nAChRs/mb* (ng)	nAChRs/ mb	Functional nAChRs/oocyte**	Incorporated/ Injected (%)***	Functional/ Incorporated	α-Btx-incubated/ MBS (%)
S	23 ± 11 (22)	0.50 ± 0.28 (3)	1.20×10^9	1.57×10^4	0.60	1.30×10^{-5}	56
S + Btx	61 ± 16 (5)	0,28 (1)					
M	476 ± 83 (23)	1.18 ± 0.06 (6)	2.84×10^9	3.26×10^5	1.42	1.15×10^{-4}	67
M + Btx	595 ± 155 (7)	0.79 ± 0.17 (2)					
L	2284 ± 345 (25)	2.23 ± 0.15 (5)	5.37×10^9	1.56×10^6	2.69	2.90×10^{-4}	107
L + Btx	3226 ± 1012(6)	2.38 (1)					

Three groups of prAChR-injected oocytes were considered according to their response to ACh: *S* (small; <100 nA), *M* (medium; 100–1000 nA) and *L* (large; >1 μA). After being recorded, some oocytes were incubated with α-Btx (0.1 μM) to quantitate the percentage of transplanted nAChRs showing out-side-in orientation (*see* text for details). The ACh current amplitude in Btx-treated oocytes indicates the values obtained in these cells before α-Btx incubation.

(*) Values were obtained from radioactivity counts of bound [^{125}I]α-Btx from 3–10 oocyte membranes. The number of determinations is given in parentheses.

(**) A reversal potential of −5 mV and a 66% of the maximal response at 100 μM ACh was considered (*see* Morales et al. 1995).

(***) 2×10^{11} was considered as the number of injected nAChRs (from the sample protein concentration and volume injected into the oocyte).

had about 1×10^9 nAChRs, the number increased in the *M* group and was even higher for the *L* group oocytes, although the increase was not proportional to the size of the recorded currents (Table 2). Since the number of nAChRs injected per oocyte was about 2×10^{11}, estimated from the protein concentration in the sample and the volume injected per oocyte, the proportion of incorporated to injected nAChRs was in the range of 0.60–2.69% (Table 2). Unexpectedly, the proportion of functional nAChRs of those incorporated into the oocyte membrane was fairly small, even for *L* group oocytes it was only circa 3×10^{-4} (Table 2). Interestingly, in those oocytes previously incubated with α-Btx a sizeable amount of [^{125}I]α-Btx bound to their isolated plasma membrane was found, indicating that a high number of nAChRs were not actually exposed to α-Btx in the intact oocyte. The radioactivity counts from these membranes were about 60 to 100% of those obtained in membranes from non α-Btx preincubated oocytes of their corresponding groups (Table 2).

Discussion

In this work it is shown that in addition to nAChRs, a different membrane protein from the *Torpedo* electroplaque, ClC-0, can be incorporated into the oocyte membrane, maintaining its functional properties and with a higher efficiency than that obtained for nAChRs.

The plasma membrane of electrocytes contains a large density of nAChRs in the innervated face, and it is also very rich in ClC-0, located in the opposite face. Thus, the *Torpedo* electroplaque has been commonly used as the source tissue for purification of both nAChRs (*see* Popot & Changeux, 1984) and ClC-0 (Middleton, Pheasant & Miller, 1994) and for many functional studies on these proteins (Huganir, Schell & Racker, 1979; White & Miller, 1979; Nelson et al., 1980; Tank et al., 1982, 1983; Sumikawa et al., 1984; Jentsch et al., 1990, 1999; Marsal et al., 1995). The density of ClC-0 in the electric organ of *Torpedo* is 2–4% of the membrane protein (Goldberg & Miller, 1991), while nAChRs constitute up to 40–50% of electrocyte membrane protein (Popot & Changeux, 1984). These values are roughly in good concordance with the profile observed in SDS-PAGE analysis of epm samples. Besides a band of about 90 kD, attributable to ClC-0, and the four corresponding to the different subunits of the nAChR, some others were evident, including that corresponding to the Na^+/K^+-ATPase, a protein slightly lighter than ClC-0. In the two preparations of prAChR used in this study, over 97% of the total protein was nAChRs and, hence, at most the remaining 3% represented ClC-0. Since the nAChR contains 5 subunits, each represents approximately 19% of the total protein and hence this must be the proportion of assembled nAChRs. This implies that the number of nAChRs in the sample of prAChR is, at least, 6 times higher than that of ClC-0 monomers.

The presence of ClC-0 in the oocyte membrane was also detected electrophysiologically. Oocytes injected with some preparations of prAChR showed a high membrane conductance, which was markedly voltage-dependent, as deduced from the pronounced rectification of the I/V curve. This conductance was fairly selective for chloride and was not due to activation of oocyte endogenous channels, but to the incorporation of electroplaque ClC-0. Since transplanted ClC-0 were, in fact, a "contaminant" protein in some preparations of prAChRs, they were subjected to the same protocol followed to incorporate

nAChRs into the oocyte membrane, i.e., ClC-0 were exposed to detergents for purification, reconstituted in an artificial lipid matrix and incorporated into the oocyte membrane by unknown steps following their intracellular injection. Therefore, it is noteworthy that transplanted ClC-0 maintained well-preserved their functional properties. So, their ionic selectivity and voltage dependence of their fast and slow gates were similar to those previously described for ClC-0 expressed in oocytes from injected mRNA (Sumikawa et al., 1984) or cDNA (Jentsch et al., 1990, 1999) or for purified ClC-0 incorporated either in liposomes (Tank et al., 1982) or planar lipid bilayers (White & Miller, 1979).

The number of functional ClC-0 incorporated into the oocyte membrane was, on average, 1.6×10^6 (16.4 μS, 10 pS per channel; *see* Table 1), while the average number of functional nAChRs was 2.9×10^5 (*see* Table 2). Hence, the ratio between ClC-0 and nAChR was about 5.5, which indicates that there were roughly 5–6 more functional ClC-0 than nAChRs, even though the injected proteoliposomes contained about 6 times more nAChRs. This difference in functional incorporation of ClC-0 and nAChRs can be due, at least in part, to a better protein orientation in the oocyte membrane by the former.

We have not determined how ClC-0 are oriented in the oocyte membrane, but a correct orientation can be inferred from: i) The I/V relationship, which only showed outward rectification at negative potentials (*see* Fig. 6). In fact, at positive potentials there was a fairly linear I/V relationship at least up to +160 mV (AM, II and BG, unpublished results). ii) The lack of blocking effect of DIDS on ClC-0 current when applied extracellularly, and the partial, but evident blockage of ClC-0 upon intracellular injection of DIDS (it can be estimated that, if DIDS was uniformly distributed into the oocyte, its intracellular concentration would be about 10–20 μM, although a considerable proportion of the injected DIDS could be bound to other intracellular proteins). This is in agreement with data obtained in bilayers where DIDS blocked ClC-0 function with an apparent $K_{0.5}$ of approximately 10 μM, and with an orientation-specific effect, affecting the channel activity only when it was added to the cis side (White & Miller, 1979). iii) Changes in extracellular pH in the range 5.5–8 hardly affected the I/V relationship of the $I_{ClC\text{-}0}$. This also indicates that ClC-0 are out-side-out oriented, since in bilayers ClC-0 gating is affected by pH changes only when these are imposed on the cis side (Hanke & Miller, 1983). All these data point out that ClC-0 incorporated into the oocyte membrane with a correct orientation, at least those with functional activity. However, we cannot fully discard the existence of some out-side-in oriented ClC-0, which could be silent. In this regard it is interesting that ClC-0 are inserted into bilayers asymmetrically, with the extracellular side facing the trans chamber, as it is also observed for native *Torpedo* membranes (Miller & Richard, 1990). Likely, something similar happened to ClC-0 transplanted to oocytes.

[^{125}I]α-BTX-binding experiments lead to additional interesting conclusions: i) The number of nAChRs incorporated was not directly proportional to the amplitude of the recorded currents, although oocytes showing large responses carried a higher number of incorporated nAChRs. ii) The percentage of nAChRs incorporated into the oocyte plasma membrane of 1–3% of those injected indicates a relatively efficient fusion of injected proteoliposome vesicles with the oocyte plasma membrane. Furthermore, this value likely underestimates the total number of nAChRs that reached the oocyte membrane, since it was estimated at a given time point, and the incorporation process takes place for a prolonged period (Gal et al., 2000). A relatively efficient fusion of lipoproteosomes with the oocyte membrane would also justify the large ClC-0 currents recorded in prAChR-injected oocytes in spite of the low proportion of ClC-0 injected. iii) There was a large difference between the number of incorporated and functional nAChRs. Thus, even in the L group only about 3 out of 10000 transplanted nAChRs were functional (*see* below). iv) Radioactivity counts from oocytes preincubated with non radioactive α-Btx represented a high percentage, the average for the three groups was of 77 ± 13%, with respect to that obtained in MBS-preincubated oocytes. This value is coherent with the percentage of nAChR with a right-side-out orientation in proteoliposomes with a different phospholipid composition (70–90%; Criado, Eibl & Barrantes, 1982). Therefore, after fusion of lipoproteosome vesicles to the oocyte membrane, this proportion becomes inverted, and only about 1 out of 4–10 nAChRs adopted the right orientation in the oocyte membrane. nAChRs out-side-in oriented cannot be activated by bath application of ACh, and likely they have lost their functional activity, since no responses could be evoked by intracellular ACh injection (Morales et al., 1995). It is also likely that a similar proportion of out-side-in nAChRs were present in epm-injected oocytes, since ACh currents were markedly smaller than those mediated by ClC-0, although the number of injected ClC-0 was not higher than that of nAChRs.

Other factors should contribute, in addition, to the low efficiency in functional transplantation of prAChRs, since the wrong orientation of this protein only accounted for a decrease of about one order of magnitude in the ratio between functional and incorporated nAChRs. Among these factors is probably a large desensitization of the purified nAChRs, as well as a high susceptibility of this protein to structural damage, either before or after reaching the cell membrane, which would result in silent receptors. In

this sense, structural differences between ClC-0 and nAChRs may explain differences in the susceptibility to intracellular damage of these proteins. Anyway, additional experiments are needed to ascertain the contribution of these and other factors to the functional activity of the transplanted proteins.

In conclusion, we show in this work that fusion of lipoproteosome vesicles with the oocyte plasma membrane is not a rare process, and that the orientation of the protein in the oocyte membrane plays an important role in the functional transplantation of exogenous proteins. These results extend the use of this methodology to the functional study in oocytes of foreign proteins that are not necessarily predominant in the original plasma membrane.

We thank Dr. José Martín-Nieto for helpful comments on the manuscript and Mr. Simón Moya for technical assistance. This work was partially supported by a grant from the Spanish MEC (PB97-0121). B.G. was the recipient of a predoctoral fellowship from the País Vasco Regional Government.

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