A Method for Determining the Unitary Functional Capacity of Cloned Channels and Transporters Expressed in Xenopus laevis Oocytes


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Abstract. The Xenopus laevis oocyte is widely used to express exogenous channels and transporters and is well suited for functional measurements including currents, electrolyte and nonelectrolyte fluxes, water permeability and even enzymatic activity. It is difficult, however, to transform functional measurements recorded in whole oocytes into the capacity of a single channel or transporter because their number often cannot be estimated accurately. We describe here a method of estimating the number of exogenously expressed channels and transporters inserted in the plasma membrane of oocytes. The method is based on the facts that the P (protoplasmic) face in water-injected control oocytes exhibit an extremely low density of endogenous particles (212 ± 48 particles/μm2, mean, sd) and that exogenously expressed channels and transporters increased the density of particles (up to 5,000/μm2) only on the P face. The utility and generality of the method were demonstrated by estimating the “gating charge” per particle of the Na+/glucose cotransporter (SGLT1) and a nonconducting mutant of the Shaker K+ channel proteins, and the single molecule water permeability of CHIP (Channel-like Intramembrane Protein) and MIP (Major Intrinsic Protein).

We estimated a “gating charge” of -3.5 electronic charges for SGLT1 and -9 for the mutant Shaker K+ channel from the ratio of Qmax to density of particles measured on the same oocytes. The “gating charges” were 3-fold larger than the “effective valences” calculated by fitting a Boltzmann equation to the same charge transfer data suggesting that the charge movement in the channel and cotransporter occur in several steps. Single molecule water permeabilities (pfs) of 1.4 × 10^-14 cm^3/sec for CHIP and of 1.5 × 10^-16 cm^3/sec for MIP were estimated from the ratio of the whole-oocyte water permeability (Pf) to the density of particles. Therefore, MIP is a water transporter in oocytes, albeit ~100-fold less effective than CHIP.

Key words: Freeze-fracture — Plasma membranes — Heterologous expression

Introduction

After the work of Barnard, Miledi & Sumikawa (1983), a large variety of channels, receptors and transporters and their genetically engineered and naturally occurring mutants have been expressed in Xenopus laevis oocytes and characterized by either electrophysiological or biochemical methods. Such studies have been highly effective in revealing overall functional differences between mutated versions of ion channels and transporters. However, the understanding of their mechanisms of action requires that functional measurements recorded in whole oocytes be normalized as the functional capacities of single channel, transporter or receptor. Often this transformation is hampered by difficulties in estimating the number of channels and transporters in the oocytes on which the functional measurements have been made. Methods used to estimate this number are either model-dependent or require specific, high-affinity ligands which are unavailable for many channels and transporters.

This paper describes a method that estimates the number of exogenously expressed cloned channels and transporters from the density of intramembrane particles inserted in the plasma membrane (oolemma) of Xenopus oocytes. The method is based on the facts that water-injected control oocytes exhibit a surprisingly low den-
sity of endogenous proteins inserted in the oolemma and that exogenous channels and transporters appear on the P (protoplasmic) fracture face. An important feature of the method is that both the density of intramembrane particles and functional properties of channels and transporters, such as charge transfer, water permeability or conductance, can be measured on the same oocyte.

The effectiveness and generality of the method was demonstrated by estimating the “gating charge” per particle of the Na+/glucose cotransporter (SGLT1) and of a nonconducting mutant of the Shaker K+ channel, and the single-molecule water permeability of CHIP (Channel-like Intrinsic Protein) and MIP (Major Intrinsic Protein). For SGLT1 and the mutant Shaker K+ channel, the “gating charge” was estimated from the ratio of the maximum number of charges translocated across the oolemma in response to voltage pulses (Qmax) to the density of intramembrane particles. The value of the gating charge estimated with this method was ~3-fold larger than that calculated by fitting a Boltzmann equation to the same charge transfer data. Thus, the number of channels and transporters estimated from the density of intramembrane particle provides independent cross checks and constraints on models of channel function based on analysis of charge transfer data.

For MIP, CHIP and SGLT1, the density of particles and the water permeability (P) were measured in oocytes from the same batch. Comparison of the estimated values of the water permeability per molecule (p) of a bona fide water channel (CHIP) and SGLT1 indicated that MIP is a water transporter, albeit 100-fold less effective than CHIP. Thus, freeze-fracture electron microscopy provides accurate estimates of the number of exogenous channels and transporters inserted in the oolemma. From the ratio of functional properties and the density of particles, both measured on the same oocyte, a variety of single-molecule capacities of channels and transporters can be estimated.

Materials and Methods

OOCYTE PREPARATION

Stage VI X. laevis oocytes were removed from female frogs and processed in one of two ways. At UCLA, they were defolliculated by treatment with collagenase B as described previously (Parent et al., 1992). At UCI, they were defolliculated by treating them twice, for 90 min, with 2 mg/ml collagenase B. The oocytes were washed with Ca2+-free saline solution (in mM: 82.5 NaCl, 2.5 KCl, 1 MgCl2, 5 HEPES) and allowed to recover overnight in ND96 medium (in mM: 96 NaCl, 2.5 KCl, 1.8 CaCl2, 1 MgCl2, and 5 HEPES, pH 7.4) plus supplements (2.5 mM sodium pyruvate, 10 μg/ml aminophenylalkanilide and 10 μg/ml streptomycin) at 18°C.

Control oocytes were injected with 50 nl of distilled water. Oocytes expressing SGLT1 and the mutant Shaker K+ channels were injected with 50 nl of a solution containing 1 μg/ml of either rabbit SGLT1 cRNA (Parent et al., 1992) or cRNA coding for the W434F mutant of the H4-1R Shaker K+ channel (Perozo et al., 1993). Oocytes expressing MIP and CHIP were injected with 50 nl of a solution of cRNA coding for bovine CHIP and human CHIP flanked by Xenopus β-globin 5' and 3' untranslated regions (Gorin et al., 1984; Preston & Agre, 1991; Preston et al., 1992; Mulders et al., 1995). RNAs were produced from Bluescript plasmids (provided to us by Dr. P. Agre). CHIP and MIP plasmids were linearized with XbaI and the capped RNA synthesized with T3 RNA polymerase. The cRNAs were precipitated in ethanol, resuspended in DEPC treated water and stored at −70°C. All injections were performed using positive-displacement micropipettes.

ELECTRON MICROSCOPY

Fixation

After the appropriate functional property was measured, oocytes were fixed sandwiched between two glass slides separated by 200 μm thick spacers (Fig. 2, panels a and b). This procedure flattened oocytes into oblates, a geometry that greatly simplified obtaining large areas of fracture faces of the oolemma. The fixative solution was 3–3.5% glutaraldehyde in 0.2 M Na cacodylate pH 7.4. After 15 min, the oocytes were removed from the slides and immersed in the same fixative solution for 1 hr, at room temperature (Fig. 2, panel d).

Thin Sectioning

The flattened oocytes were postfixed in 1% OsO4 in 0.2 M Na cacodylate buffer for 90 min at room temperature. They were washed in 0.1 M Na acetate buffer pH 5.0 and block stained in 0.5% uranyl acetate in 0.1 M Na acetate buffer pH 5.0 overnight at 4°C. The oocytes were dehydrated in ethanol, passed through propylene oxide and embedded in Epon 812. Sections were cut in a Sorval MT5000 ultramicrotome, collected on single-hole, formvar-coated grids and stained on the grids with uranyl acetate and lead (Zampighi et al., 1988).

Freeze Fracture

The oblate-shaped oocytes were infiltrated with 25% glycerol in 0.2 M Na cacodylate buffer pH 7.4 for 1 hr at room temperature. They were cut first in halves and then into 4–6 smaller pieces which were placed on Balzers specimen holders with the external surfaces (i.e., the vitelline membrane) facing upward. This precaution greatly increased the chances of fracturing P instead of E faces (Fig. 2, panel e). The specimens were frozen by immersion in liquid propane cooled in a liquid nitrogen bath. The frozen oocytes were transferred into a Balzers 400K frozen-fracture-etch apparatus, fractured at either −150°C or −120°C and at 1 × 10−7 mbar of partial pressure. The fractured surfaces were coated with platinum at 80°C and carbon at 90°C. Shadowing at 45°, instead of 45° produced intramembrane particles with shorter shadows which facilitated their quantification, particularly in replicas from oocytes that exhibited the highest densities (~5,000/gm2). The replicated specimens were coated with 0.5% collodion in amyl acetate (to avoid fragmentation of the replica) and cleaned in a solution of bleach. The replicas were washed in distilled water and deposited on formvar-coated copper grids. The collodion was removed by immersion in amyl acetate (Zampighi et al., 1988).

Sampling

The large total area of the oolemma (3–6 × 107 μm2) introduces sampling problems because freeze-fracture and morphometric methods can