The Phe-Met-Arg-Phe-amide-activated Sodium Channel Is a Tetrramer*

(Received for publication, October 15, 1997, and in revised form, December 29, 1997)

Sylvie Coscoy‡, Eric Lingueglia, Michel Lazdunski, and Pascal Barbry§
From the Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, UPR 411, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France

The *Helix aspersa* Phe-Met-Arg-Phe-amide-activated (FMRFamide)-gated sodium channel is formed by homomultimerization of several FMRFamide-activated Na⁺ channel (FaNaCh) proteins. FaNaCh is homologous to the subunits that compose the amiloride-sensitive epithelial sodium channel, to *Caenorhabditis elegans* degenerins, and to acid-sensing ionic channels. FaNaCh properties were analyzed in stably transfected human embryonic kidney cells (HEK-293). The channel was functional with an EC₅₀ for FMRFamide of 1 μM and an IC₅₀ (25 °C) for amiloride of 6.5 μM as assessed by ²²Na⁺ uptake measurements. The channel activity was associated with the presence of a protein at the cell surface with an apparent molecular mass of 82 kDa. The 82-kDa form was formed from an incompletely glycosylated form of 74 kDa found in the endoplasmic reticulum. Formation of covalent bonds between subunits of the same complex were observed either after formation of intersubunit disulfide bonds following cell homogenization and solubilization with Triton X-100 or after use of bifunctional cross-linkers. This resulted in the formation of covalent multimers that contained up to four subunits. Hydodynamic properties of the solubilized FaNaCh complex also indicated a maximal stoichiometry of four subunits per complex. It is likely that epithelial Na⁺ channels, acid-sensing ionic channels, degenerins, and the other proteins belonging to the same ion channel superfamily also associate within tetrameric complexes.

The protein FaNaCh¹ is expressed in the *Helix aspersa* nervous system (1). It is the first identified member of a new class of ionotropic receptors that can be directly activated by peptides. FaNaCh expression in *Xenopus* oocytes generates an amiloride-sensitive Na⁺ conductance activated by the molluscan cardioexcitatory peptide FMRFamide (1). FaNaCh is related to the three homologous proteins that constitute the pore-forming subunits of the epithelial Na⁺ channel (ENaC) (2–5), the membrane proteins DEG-1, MEC-4, MEC-10, UNC-105, UNC-8, and DEL-1 from *Caenorhabditis elegans*, involved mainly in mechanosensation (6–10), and the mammalian acid-sensing ionic channels ASIC (11) (also called BNC2) (12), mammalian degenerin homologue (13) (also called BNC1) (14), and dorsal root ganglia ASIC (15). This new gene superfamily encodes ionic channels expressed in epithelial (i.e. ENaC) as well as excitable tissues (i.e. FaNaCh, ASIC, or degenerins) (16).

The proteins belonging to this family are characterized by a large extracellular domain located between two large hydrophobic zones forming the transmembrane regions. The NH₂- and COOH-terminal domains are cytoplasmic (17). Despite increasing information on the structure and function of these channels, the stoichiometry of the functional complexes remains unknown.

Biochemical handling of FaNaCh is facilitated by several properties: (i) unlike ENaC which comprises α, β, and γ subunits, active FMRFamide Na⁺ channels are formed by a homomultimerization of the FaNaCh channels; (ii) unlike ENaC, the FaNaCh channel is totally silent in the absence of FMRFamide, and therefore not toxic for cells that express it. The determination of the oligomeric arrangement of the FaNaCh channel might serve as a model for other channels within this family (ENaC, degenerins, and ASIC). After extensive functional and biochemical characterization of the channel complex, we show that four FaNaCh subunits participate in the formation of the FMRFamide-activated sodium channel.

**EXPERIMENTAL PROCEDURES**

*Constructs and Transfection—*FaNaCh cDNA structure has been previously described by Lingueglia et al. (1). The cDNA was inserted either into the plasmid pBSSK-SP6-globin (18) for efficient expression in *Xenopus* oocytes or in pRC/CMV plasmid (Invitrogen) for transfection of eucaryotic cells. A tagged FaNaCh was constructed by polymerase chain reaction, leading to the addition of the FLAG sequence (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) to the COOH terminus of the protein. This epitope, which does not alter the channel activity (not shown), can be detected by a commercial monoclonal anti-FLAG antibody (Eastman Kodak Co.) with a high specificity and a low background. Stable transformation was carried out on HEK-293 cells with the FaNaCh or FaNaCh-FLAG pRC/CMV plasmid (Invitrogen) by the Ca²⁺-phosphate technique (19). ²²Na⁺ Uptake Experiments—HEK-293 cells were seeded in collagen-coated 24-well clusters (Falcon). Confuent cells were rinsed twice with the flux buffer (140 mM chloride or N-methyl-D-glucamine chloride, 20 mM HEPES/Tris, pH 7.4, 1.8 mM CaCl₂, 0.8 mM MgSO₄) and then incubated with 200 μl of flux buffer supplemented with 2 mM ²²NaCl (0.5–1 μCi/ml Amersham Pharmacia Biotech, 12 Ci/mmol) with or without amiloride or FMRFamide. External "Na⁺" was washed out by four 1-mL washings with cold flux buffer, and trapped radioactivity was measured using a Packard 1600 CA liquid scintillation counter.

Dose-dependent activations and/or inhibitions were measured after a 150-s incubation.

**Antibodies**—The cytoplasmic COOH-terminal domain of FaNaCh (amino acids 570–625) was produced in the glutathione sulphhydryltransferase fusion protein expression vector pGEX-3X according to the

---

*This work was supported by CNRS, INSERM, and the Association Française de Lutte contre la Mucoviscidose (AFLM). Thanks are due to DGA.*

‡ Recipient of Grant 91815-48/A000 from the Delegation Générale pour l’Armement (DGA).

§ To whom correspondence should be addressed. Tel.: 33-04-93-95-77-20 or 02; Fax: 33-04-93-95-77-04; E-mail: ipmc@ipmc.cnrs.fr or pbarbry@itsa.ucsf.edu.

¹FaNaCh, Phe-Met-Arg-Phe-amide-activated Na⁺ channel; FMRFamide, Phe-Met-Arg-Phe-amide; ENaC, epithelial Na⁺ channel; ASIC, acid-sensing ionic channel; HEK, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DSS, suberic acid bis(N-hydroxysuccinimidyester); SADP, N-succinimidyl(4-azidophenyl)-1,3-dithiopropionate; NHS-ASA, N-hydroxysuccinimidyl-4-azidosalicylic acid; PCR, polymerase chain reaction.
manufacturer’s instructions (Amersham Pharmacia Biotech). The peptide was injected intradermally into a rabbit as described previously (17). Antibodies were first characterized by enzyme-linked immunosorbent assay on pure antigen, then used for immunoprecipitation experiments. Some experiments were carried out using the FaNaCh-FLAG construct alone. The FLAG was detected by the commercial monoclonal M2 anti-FLAG antibody (Kodak).

**Immunoblot Experiments**—Cells were rinsed with Hank’s balanced salt solution, scraped, and recovered in a lysis buffer containing 50 mM Tris/HCl, pH 7.4, 100 mM KCl, 5 mM EDTA, and protease inhibitors (100 μg/ml leupeptin, 1 μg/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride). After lysis in a Potter and slow centrifugation, the supernatant was centrifuged at 1 h at 30,000 g. Pellets were resuspended at about 2 mg/ml in lysis buffer and stored at −70 °C. 10–20 μg of membrane proteins were heated at 65 °C in SDS-PAGE sample buffer (2.5% SDS, 20 mM urea, bromphenol blue, with or without 4% β-mercaptoethanol) and applied to SDS-PAGE. After migration, proteins were transferred to a nitrocellulose membrane (Hybond C extra, Amersham Pharmacia Biotech). Nonspecific binding was blocked by a 1–2-h incubation in 140 mM NaCl, 20 mM Tris, 5% milk. The filter was incubated overnight in the same buffer with the monoclonal anti-FLAG antibody (dilution 1:1000, Kodak), rinsed 3 times for 10 min with 140 mM NaCl, 20 mM Tris, 0.05% Tween 20, and pelleted using protein A-Sepharose (Sigma). After washing, samples were heated in SDS-PAGE sample buffer, and applied to 6.5 or 8% SDS-PAGE. Gels were treated with 1% salicylic acid for 30 min, dried, and exposed to phosphorimager imaging plate.

**Metabolic Labeling, Cell Surface Iodination, and Immunoprecipitation**—Confluent cells were incubated in a methionine- and cysteine-deficient medium for 30 min prior to the addition of 50 μCi/ml [35S]methionine (Trans35S-LABEL, ICN, 1077 Ci/mmol). After a pulse of 2–3 h (or 30 min for pulse-chase experiments), the cells were rinsed and incubated in complete medium (plus 10% fetal calf serum) for various lengths of time. Cells were subsequently pelleted and lysed with 50 μl of chilled radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% C4E4 (Nikko Chemicals), protease inhibitors)/35-mm dish. After preclearing with pansorbin (Calbiochem), the lyase was incubated overnight with the polyclonal antibody (1/100), and pelleted using protein A-Sepharose (Sigma). After washing, samples were heated in SDS-PAGE sample buffer, and applied to 6.5 or 8% SDS-PAGE. Gels were treated with 1% salicylic acid for 30 min, dried, and exposed to a phosphorimaging plate.

**Cross-linking Experiments**—Cells were solubilized after membrane preparation by nettle surface iodination in the cross-linking assay buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM EDTA). Samples were incubated for 15 min at room temperature with DSS (sucrose bis[N-hydroxysuccinimide ester, Sigma] at pH 9.4 with SADP (N-succinimidyldi(4-azidophenyl)-1,3-dithiopropionate, Sigma) or with NHS-ASA (N-hydroxysuccinimidyldi-4-azidosalicylic acid, Pierce) at pH 7.4. Reactions were stopped with 50 mM Tris/HCl, pH 7.4. SADP- or NHS-ASA-treated samples were then exposed for 10 min to an UV source (252 nm). Following cross-linking, samples were applied to a 5% Laemmli or 3.5% Weber-Osborn polyacrylamide gel (21).

**Sucrose Gradient Centrifugation and Gel Filtration Analysis**—Membrane proteins were solubilized and centrifuged at 170,000 × g for 15 min. In some experiments, the solubilized material was also cross-linked using 500 μM DSS, or the formation of intersubunit disulfide bonds was prevented by the addition of 10 μM iodoacetamide. The solubilized material was recovered and applied to the top of a linear 5–15% sucrose gradient formed in water or in D2O (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.1% Triton X-100 or CHAPS, protease inhibitors), β-galactosidase (100 μg), catalase (100 μg), alkaline phosphatase (67 units), and cytochrome c (0.6 μg), used as markers, were also added. After a 17 h run at 39,000 rpm (190,000 × g) on a SW41-TI rotor (Beckman), 300-μl fractions were recovered. They were assayed for the presence of the standards and either immunoprecipitated overnight with the anti-FaNaCh antibodies (for metabolic and surface labeling) or assayed by Dot-blot with the anti-FLAG antibody. Gel filtration was performed on a Superdex 200 column (Amersham Pharmacia Biotech) equilibrated in a buffer containing 150 mM NaCl, 10 mM EDTA, 0.1% Triton X-100, at a flow rate of 0.4 ml/min.

The apparent sedimentation coefficients of FaNaCh in H2O and D2O were determined. From these values and the values of densities and viscosities, it was possible to determine the sedimentation coefficient at 20 °C in water (s20,w), and the partial specific volume (ε, equal to the inverse of the density) of the detergent-protein complex (22). The contribution of the detergent to the complex was deduced from that latter value, knowing the partial specific volume of Triton X-100 (equal to 0.908 cm3/g), and the partial specific volume of proteins (using a mean value equal to 0.73 cm3/g) (23).

Stokes radius was determined from gel filtration data. According to Siegel and Monty (24), the total mass of the protein-detergent complex was deduced from the Equation 1.

\[ M_{complex} = \frac{6\pi \eta_{visc} N_A s_{20,w} R_{stokes}}{1 - p_{20,w}} \]  

where \( \eta_{visc} \) is the viscosity of water at 20 °C (equal to 0.01002 Poise), \( N_A \) is the Avogadro number (6.02 1023) and \( p_{20,w} \) is the density of water at 20 °C (0.99823 g/cm3). The molecular weight of the protein complex was deduced from this value, taking into account the contribution of the detergent to the total mass of the complex.

**Results**

** Functional Expression of FMRFamide-activated Sodium Channels in FaNaCh-transfected HEK-293 Cells**—FMRFamide-activated sodium channel activity was measured after stable transfection of the FaNaCh cDNA into HEK-293 cells. Fig. 1 shows that the properties of the channel observed in FaNaCh-transfected HEK-293 cells were identical to those of the native channel recorded in snail neurons (25) and those of the channel recorded in Xenopus oocytes injected with the corresponding cRNA (1). 22Na uptake was largely increased after stimulation with 30 μM FMRFamide in transfected cells. The time course of the uptake was biphasic. After a relatively fast rise to a plateau, the 22Na uptake declined to a lower intermediate value. The initial event is consistent with the rapid development of a Na+ permeability. The decline of the signal observed after 2.5 min is likely due to the inactivation of the channel (25) combined with a sustained Na+/K+-ATPase activity. A Michaelis-Menten relationship was ob-
amiloride was 42 μM. An anti-FaNaCh antibody. A 6.5% polyacrylamide gel. 
m with 50 μM 125I-S-Met for 30 min; the chase time is indicated. B, cells were metabolically labeled for 2 h and chased for 45 min (tunicamycin) or 2 h (brefeldin A). In experiments with maturation inhibitors, cells were treated overnight and during metabolic labeling with tunicamycin (100 μg/ml) or brefeldin A (0.3 μg/ml; Sigma). C, cell surface labeling was catalyzed by lactoperoxidase in the presence of 500 μCi of 125I per 35-mm dish.

![Figure 2](https://example.com/fig2.png)

**Fig. 2.** Maturation: Immunoprecipitation by the polyclonal anti-FaNaCh antibody. Samples were analyzed by electrophoresis on a 6.5% polyacrylamide gel. A, confluent cells were metabolically labeled with 50 μCi/ml 125I-S-Met for 30 min; the chase time is indicated. B, cells were metabolically labeled for 2 h and chased for 45 min (tunicamycin) or 2 h (brefeldin A). In experiments with maturation inhibitors, cells were treated overnight and during metabolic labeling with tunicamycin (100 μg/ml) or brefeldin A (0.3 μg/ml; Sigma). C, cell surface labeling was catalyzed by lactoperoxidase in the presence of 500 μCi of 125I per 35-mm dish.

![Figure 3](https://example.com/fig3.png)

**Fig. 3.** Retention of the complex. A, membranes were prepared from cells expressing FaNaCh-FLAG in the presence or absence of 10 mM iodoacetamide, and solubilized with 1% Triton X-100 (with or without iodoacetamide). Samples were loaded on a 5.5% Laemmli polyacrylamide gel under nonreducing conditions. B, FaNaCh-FLAG membranes solubilized for 15 min with 1% Triton X-100 were cross-linked with 100 or 500 μM SADP and loaded on a 5.5% Laemmli polyacrylamide gel under nonreducing conditions. C, cells stably transfected with FaNaCh cDNA were surface labeled, solubilized with 1% Triton X-100 and 0.5% deoxycholate, cross-linked with 500 μM DSS, and immunoprecipitated with the polyclonal anti-FaNaCh antibody. The migration was performed on a 3.5% Weber-Osborn polyacrylamide gel under nonreducing conditions (21). The monomeric form was detected at an apparent molecular mass of 98 kDa (instead of 82 kDa), due to the poor resolution of this type of gel at low molecular mass. D, FaNaCh-FLAG cells were solubilized in 1% CHAPS, cross-linked with 100 or 250 μM NHS-ASA, and loaded on a 6% Laemmli polyacrylamide gel under reducing conditions. m, mature form; i, immature form.

**Fnach Is Expressed at the Cell Surface as a 82-kDa Glycoprotein**—The protein expressed in transfected cells was first characterized using a polyclonal antibody directed against the COOH-terminal domain. In pulse-chase experiments, the polyclonal antibody immunoprecipitated a 74-kDa protein, which matured after 2–3 h into a 82-kDa protein (Fig. 2A). After deglycosylation of the immunoprecipitated material with N-glycosidase F (data not shown), or pretreatment of the cells with tunicamycin (a blocker of N-glycosylation), a 67-kDa protein was detected in good agreement with a predicted molecular mass equal to 71 kDa for the polypeptide (Fig. 2B).

Fig. 2B also shows that the 74-kDa band was the only product detected after a 3 h pulse-chase made in the presence of brefeldin A, an inhibitor of intracellular transport. Since the 74-kDa form was also sensitive to endoglycosidase H (data not shown), which cleaves high mannose structures characteristic for glycosylation in the endoplasmic reticulum, it probably corresponds to an intermediate form of maturation of FaNaCh. On the contrary, the 82-kDa form, detected after longer times of metabolic labeling, was resistant to endoglycosidase H digestion (data not shown) and is thus considered to correspond to a fully mature terminally glycosylated form. Fig. 2C demonstrates that the 82-kDa protein can indeed be detected at the cell surface since it is immunoprecipitated by anti-FaNaCh antibodies after a cell surface labeling of FaNaCh-transfected cells with Na125I and lactoperoxidase. The channel complex is present in large amounts at the surface since the FaNaCh signal represented up to 10% of the total radioactive material.

**Solubilization by Triton X-100 Favors the Formation of Intersubunit Disulfide Bonds and Leads to the Formation of Covalent Tetramers**—When SDS-PAGE migration was performed under nonreducing conditions, oligomeric forms of FaNaCh were observed (Fig. 3A). These forms were sensitive to β-mercaptoethanol, suggesting the existence of disulfide bonds between subunits. When membranes were prepared in the presence of 10 mM iodoacetamide, the monomer was the only form detected (Fig. 3A). This result shows that formation of intersubunit disulfide bonds is not occurring in the native channel, but rather that it results from a redox potential alteration created by the experimental conditions during cell lysis or the following steps.

Disulfide bond formation was increased after solubilization with Triton X-100. Whereas we detected only the monomeric form (74–82 kDa) on iodoacetamide-treated membranes after solubilization with 1% Triton X-100, additional bands at 151–
Fig. 4. **Hydrodynamic measurements.** 60 μg of FaNaCh-FLAG membranes, together with standard proteins, were applied on sucrose density gradients or gel filtration column. Fractions were assayed for the presence of standards and analyzed by Dot-blot (A, B, and D) or Western blot (C) with the anti-FLAG antibody. A, B, and C, sucrose gradient sedimentation. Samples solubilized with 1% Triton X-100 or 0.8% CHAPS were layered on the top of a 5–15% sucrose gradient. Triton X-100 or 0.8% CHAPS were layered on the top of a 5–15% sucrose gradient. Samples solubilized with 1% Triton X-100 or 0.8% CHAPS were layered on the top of a 5–15% sucrose gradient. After 17 h of sedimentation at 39,000 rpm with a SW41 rotor, 300-μl fractions were collected. D, gel filtration analysis. Samples were applied on a Superdex 200 column (Amersham Pharmacia Biotech). 300-μl fractions were recovered at a 0.4 ml/min flow rate. Fractions inferior to 20 are in the void volume of the column.

164 kDa (dimer), 205–230 kDa (trimer), and 305–327 kDa (tetramer) were observed in parallel analysis with control membranes prepared in the absence of iodoacetamide (Fig. 3A). The Triton X-100 effect was time-dependent. An overnight incubation with Triton X-100 (instead of 15 min) significantly increased the percentage of tetrameric form (data not shown). In vitro intersubunit disulfide bond rearrangement or formation has been reported for a variety of other oligomeric proteins such as *Shaker* K+ channel (26), synaptophysin (27), and the brain glutamate transporter (28). In the two latter cases, the extent of rearrangement depended on the detergent used for solubilization. It was maximal with SDS for synaptophysin and with CHAPS for brain glutamate transporter. For FaNaCh, formation of tetramers was only observed after solubilization with Triton X-100 and not after solubilization with CHAPS or with deoxycholate.

**Reticulation of the Complex by Bifunctional Cross-linkers Generates Up to Tetramers—**After a lactoperoxidase labeling of the cell surface proteins with 125I and covalent reticulation with deoxycholate. Cross-linked proteins were detected on a 3.5% Weber-Osborn gel (nonreducing conditions) at apparent molecular masses of 180 (dimer), 260 (trimer), and 345 kDa (tetramer) (Fig. 3C). Most of the cross-linked material appeared as trimers, but the presence of a tetramer was detected in three out of eleven experiments as a diffuse band around 345 kDa.

When membranes were solubilized with Triton X-100 then cross-linked with the heterobifunctional reagent SADP (Fig. 3D), the tetramer was the main form detected by Western blot. Fig. 3B also shows an increase of the tetrameric form when SADP concentration was increased from 100 to 500 μM. The formation of covalent tetramers was not restricted to Triton X-100-solubilized material. They were also detected on CHAPS-solubilized material after a reticulation with the heterobifunctional cross-linker NHS-ASA (Fig. 3D).

**Hydrodynamic Analysis of Solubilized FaNaCh Demonstrates the Tetrameric Organization of the Complex—**The pool of FaNaCh proteins expressed at the cell surface was analyzed by sedimentation experiments through sucrose gradients after solubilization with 1% Triton X-100 + 0.5% deoxycholate. A unique peak characterized by an apparent sedimentation coefficient of 9.5 S was observed. This peak was not modified after cross-linking of the membrane proteins with DSS (not shown). This proved that a unique FaNaCh complex (formed by a given number of subunits) was expressed at the cell surface and was resistant to solubilization by 1% Triton X-100 + 0.5% deoxycholate.

Hydrodynamic characterization of the complex solubilized in 1% Triton X-100 was then performed. The apparent sedimentation coefficients in a linear 5–15% sucrose gradient formed in water and in D2O were determined (Figs. 4, A and B). The sedimentation coefficient at 20 °C in water ($s_{20,w}$) of the Triton X-100-protein complex was equal to 9.12 ± 0.02 S, and the partial specific volume $\tilde{v}$ was equal to 0.774 ± 0.004 cm$^3$/g (n = 2). A 25% detergent contribution to the total mass of the complex was deduced, assuming a partial specific volume of FaNaCh in the absence of detergent equal to 0.73 cm$^3$/g. The Stokes radius was equal to 73 ± 4 Å (n = 3), as determined by a chromatography on a Superdex-200 column (Fig. 4D). Results are summarized in Table I.

Using Equation 1, a value of 333 ± 24 kDa was found for the complex formed by the protein and Triton X-100, and 251 ± 18 kDa for the glycopeptidic complex in the absence of detergent. Assuming a mean molecular mass of 78 kDa for a FaNaCh monomer (due to the fact that the 74- and 82-kDa forms contribute equally to the total FaNaCh signal (Fig. 3A), a first estimate of the subunit stoichiometry equal to 3.2 was found.

Since formation of interchains disulfide bonds occurred after solubilization with Triton X-100 (Fig. 3A), two distinct control experiments were performed to ensure that the Triton X-100-solubilized material was not denatured. First, a unique peak with similar $s_{20,w}$ and $\tilde{v}$ (8.8 S and 0.806 cm$^3$/g) was detected after membranes had been treated with 10 mM iodoacetamide, which prevented the formation of covalent oligomers. Second, a similar sedimentation profile was obtained after solubilization of the complex with CHAPS (Fig. 4C). The peak was characterized by an apparent sedimentation coefficient equal to 9.7 ± 0.3 S (n = 3).

The unknown value of the partial specific volume of the protein affected the estimation of the stoichiometry (Table I). Small changes can significantly affect the evaluation of the detergent contribution to the complex. A maximal stoichiometry, however, can be obtained assuming that no detergent is bound to the complex. In that case, the total mass of the
Biochemical Characterization of FaNaCh

FaNaCh was solubilized in 1% Triton X-100 without iodoacetamide. Two independent sucrose gradient sedimentations and three gel filtration experiments were performed. Mean values and standard errors are reported. Calculations were performed assuming a partial specific volume of 0.73 or 0.76 cm$^3$/g for the FaNaCh protein.

| Sedimentation coefficient |
|--------------------------|
| $s_{20,w} = 9.12 \pm 0.02$ S |
| Stokes radius |
| $R_{	ext{stokes}} = 73 \pm 4$ Å |
| Partial specific volume |
| $\bar{v}_{\text{complex}} = 0.774 \pm 0.004$ cm$^3$/g |
| Molecular weight of the complex |
| $310$ kDa for $M_{\text{complex}} \leq 358$ kDa |
| Molecular weight of the glycoprotein complex (without detergent) |
| for $v_{\text{protein}} = 0.73$ cm$^3$/g: $233$ kDa $\leq M_{\text{protein}} \leq 268$ kDa and $3 \leq n \leq 3.4$ |
| for $v_{\text{protein}} = 0.76$ cm$^3$/g: $281$ kDa $\leq M_{\text{protein}} \leq 322$ kDa and $3.6 \leq n \leq 4.1$ |

The material sedimented through a sucrose gradient was also analyzed by Western blot. Fig. 5 shows that the covalent dimers, trimers, and tetramers formed by oxidation after Triton X-100 solubilization are co-sedimenting. This indicates that formation of disulfide bonds only occurred between subunits of the same complex. Taken together with the other data presented above, this is consistent with a tetrameric organization of the FaNaCh complex.

**DISCUSSION**

The properties of the FMRFamide-gated sodium channel expressed in HEK-293 cells are quite consistent with the properties of the channel recorded in native tissues or after expression of the cloned cDNA into Xenopus oocytes. Since FaNaCh expression in HEK-293 cells and in Xenopus oocytes is able to generate a large FMRFamide-induced inward current, it is likely that the functional channel corresponds to a homomultimeric structure, and does not need association with endogenous subunits. The transfected cells therefore represent a useful model to study the pharmacological and biophysical properties of the FMRFamide-gated sodium channel. These results also demonstrate that correct assembly of the channel complex is possible in these transfected cells, which can be used to characterize the quaternary structure of the channel.

The main purpose of this study was to analyze the maturation pattern and the quaternary structure of the FMRFamide-gated sodium channel. In transfected HEK-293 cells, FaNaCh glycosylation occurs in two steps. An immature precursor core-glycosylated form is produced into the endoplasmic reticulum and is then converted into a fully glycosylated form abundantly expressed at the cell surface, which generates the expected channel activity. Figs. 3 and 5 show that the immature form is already assembled into an oligomeric form that sediments at the level of a unique peak on the sucrose density gradient. This proves that tetrameric assembly occurs at an early step of maturation in the endoplasmic reticulum. A similar type of assembly has also been reported for the tetrameric potassium channel *Shaker* (29).

Covalent tetramers were observed after use of three distinct chemical cross-linkers, i.e. DSS, SADP, and NHS-ASA. They were also detected after oxidation of the protein through the formation of interchain disulfide bonds. Triton X-100 increased the latter covalent bonding, but tetramers were also observed after solubilization in the presence of other detergents such as CHAPS, followed by chemical cross-linking (Fig. 3). It is therefore unlikely that tetramers only represent artifactual denaturation due to Triton X-100. Thus, cross-linking experiments provided a minimal stoichiometry of the complex equal to 4, and hydrodynamic properties of the solubilized FaNaCh complex provided a maximal stoichiometry strictly below 5. Taken together, these results led us to the conclusion that the FaNaCh complex is composed of four subunits. Since the FMRFamide-gated sodium channel is a member of the same structural family as ENaC, degenerins, and ASIC, a stoichiometry of 4 can also be expected for these channels.

McNicholas and Canessa (30) analyzed the properties of epithelial Na$^+$ channels generated after coexpression of $\alpha$ENaC and $\beta$ENaC, or $\alpha$ENaC and $\gamma$ENaC. Using different ratios of injected cRNAs, they showed an optimal signal after injection of equal amounts of $\alpha$ENaC + $\beta$ENaC and $\alpha$ENaC + $\gamma$ENaC. This would also be consistent with a tetrameric organization in which two $\alpha$ subunits would be associated with one $\beta$ and one $\gamma$ subunit. The same type of conclusion is also suggested by the results of Gründer et al. (31). These authors have analyzed the residual ENaC current generated after mutation of a highly conserved region located before the first transmembrane region of $\alpha$, $\beta$, or $\gamma$ENaCs. As assessed by the residual current recorded after expression of a mutant subunit with two other wild-type chains, the rule of the three subunits was not strictly equivalent. A stronger effect was observed after mutation of $\alpha$ENaC than after mutation of $\beta$ or $\gamma$ENaCs. A tetrameric organization of the ENaC complex with two copies of $\alpha$ENaC, one copy of $\beta$ENaC, and one copy of $\gamma$ENaC might suggest that the dominant negative effect would directly depend upon the number of mutated subunits participating in the complex.

Gain-of-function mutations affecting MEC-4 and MEC-10 degenerins, which are likely subunits of a common complex expressed in the six touch-receptor neurons of *C. elegans*, cause neurodegeneration of these cells. Modulation of MEC-4 and/or MEC-10-induced degenerations by a supplementary loss-of-function mutation in *trans* was analyzed for MEC-10 (8) and
MEC-4 (32). It was concluded that there are at least two copies of MEC-10 and two copies of MEC-4 in the complex. A tetrameric organization of the MEC-4/MEC-10 complex formed by two copies of MEC-10 and two copies of MEC-4 would then imply that the other mec gene products that build up the complex such as MEC-6 (8) are not degenerins.

This work is the first direct demonstration that one member of the ENaC/FaNaCh/ASIC/degenerins superfamily can form tetramers. It is interesting to note that the family of inward rectifying K+ channels, in which each of the subunits is also characterized by the presence of only two transmembrane domains, but which displays no homology with proteins of the ENaC/FaNaCh/ASIC/degenerins family, also has a tetrameric organization (33). It might be tempting to speculate that the P2x subtype of purinergic receptors, which also displays a structural organization (34) also forms tetramers.

Acknowledgments—We are very grateful to Dr. Guy Champigny for initial electrophysiological characterization of FaNaCh transfected HEK-293 clones, to Dr. Joëlle Bigay for help with FPLC experiments, to Dr. Amanda Patel for careful reading of the manuscript, and to Valérie Friend, Danièle Moinier, Franck Aguila, and Dahyva Doume for expert technical assistance.

REFERENCES
1. Lingueglia, E., Champigny, G., Lazdunski, M., and Barbury, P. (1995) Nature 378, 730–733
2. Canessa, C. M., Horisberger, J. D., and Rossier, B. C. (1993) Nature 361, 467–470
3. Lingueglia, E., Voilley, N., Waldmann, R., Lazdunski, M., and Barbury, P. (1995) FEBS Lett. 318, 95–99
4. Canessa, C., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D., and Rossier, B. C. (1994) Nature 367, 463–467
5. Lingueglia, E., Renard, S., Waldmann, R., Voilley, N., Champigny, G., Plass, H., Lazdunski, M., and Barbury, P. (1994) J. Biol. Chem. 269, 13736–13739
6. Chalfie, M., and Wolinski, E. (1990) J. Gen. Physiol. 95, 99–107
7. Driscoll, M., and Chalfie, M. (1991) Neuron 7, 1173–1177
8. Huang, M., and Chalfie, M. (1994) Nature 367, 467–470
9. Liu, J., Schrank, B., and Waterston, R. (1996) Science 273, 361–364
10. Tavernarakis, N., Schild, L., Wang, S., and Driscoll, M. (1997) Neuron 18, 224–229
11. Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C., and Lazdunski, M. (1997) Nature 386, 173–177
12. Price, M. P., Snyder, P. M., and Welsh, M. J. (1996) J. Biol. Chem. 271, 7879–7882
13. Waldmann, R., Champigny, G., Voilley, N., Lauritzen, I., and Lazdunski, M. (1996) J. Biol. Chem. 271, 10433–10436
14. Garcia-Añoveros, J., Derfler, B., Neville-Golden, J., Hyman, B., and Corey, D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1459–1464
15. Waldmann, R., Bassilana, F., de Wille, J. R., Champigny, G., Heurteaux, C., and Lazdunski, M. (1997) J. Biol. Chem. 272, 20975–20978
16. Barbury, P., and Hofman, P. (1997) Am. J. Physiol. 273, G571–G585
17. Renard, S., Lingueglia, E., Voilley, N., Lazdunski, M., and Barbury, P. (1994) J. Biol. Chem. 269, 12081–12086
18. Waldmann, R., Champigny, G., and Lazdunski, M. (1995) J. Biol. Chem. 270, 11735–11737
19. Barbury, P., Champ, M., Chassande, O., Munemitsu, S., Champigny, G., Lingueglia, E., Maes, P., Frelin, C., Tartar, A., Ulrich, A., and Lazdunski, M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7347–7351
20. Dalemans, W., Barbury, P., Champignon, G., Jallat, S., Dett, K., Dreyer, F., Crystal, R. G., Pavirani, A., Leceq, J.-P., and Lazdunski, M. (1991) Nature 354, 526–528
21. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
22. Clarke, S. (1975) J. Biol. Chem. 250, 5459–5469
23. Davis, A. (1984) in Molecular and Chemical Characterization of Membrane Receptors (Venter, C. J., and Harrisson, L. C., eds) Vol. 3, pp. 161–178, Alan R. Liss, Inc., New York
24. Siegel, L. M., and Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346–362
25. Green, K. A., Falconer, S. W., and Cottrell, G. A. (1994) Pflugers Archiv. 428, 232–240
26. Schwartz, C. T., Nagaya, N., and Papazian, D. M. (1996) Biochemistry 35, 12133–12140
27. Johnston, P. A., and Sudhof, T. C. (1990) J. Biol. Chem. 265, 8869–8873
28. Haug, K. E., Ullensvang, K., Levy, L. M., Chaudhry, F. A., Honore, T., Nielsen, M., Lehre, K. P., and Danbolt, N. C. (1996) J. Biol. Chem. 271, 27715–27722
29. Nagaya, N., and Papazian, D. M. (1997) J. Biol. Chem. 272, 3022–3027
30. McNicholas, C. M., and Canessa, C. M. (1997) J. Gen. Physiol. 109, 681–692
31. Gruner, S., Fiszov, D., Chang, S., Jaeger, N., Gautschi, I., Schild, L., Lifton, R., and Rossier, B. (1997) EMBO J. 16, 899–907
32. Hong, K., and Driscoll, M. (1994) Nature 367, 470–473
33. Yang, J., Jan, Y. N., and Jan, L. Y. (1995) Neuron 15, 1441–1447
34. Surprenant, A., Buell, G., and North, R. A. (1995) Trends Neurosci. 18, 224–229
