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Analysis of the Native Quaternary Structure of Vanilloid Receptor 1*[S]

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Vanilloid receptor subtype 1 (VR1) is a ligand-gated channel that can be activated by capsaicin and other vanilloids as well as by protons and heat. In the present study, we have analyzed the oligomeric state of VR1. Co-immunoprecipitation of differently tagged VR1 molecules indicated that VR1 can form oligomers. Using two different heterologous VR1 expression systems as well as endogenous VR1 expressed in dorsal root ganglion cells, we analyzed oligomer formation using perfluorooctanoic acid polyacrylamide gel electrophoresis. Results were confirmed both with chemical cross-linking agents as well as through endogenous cross-linking mediated by transglutaminase. Our results clearly show that VR1 forms multimers in each of the expression systems with a homotetramer as a predominant form. The oligomeric structure of VR1 may contribute to the complexity of VR1 pharmacology. Finally, differences in glycosylation between the systems were observed, indicating the need for caution in the use of the heterologous expression systems for analysis of VR1 properties.

Pain-producing stimuli are detected by specialized primary afferent neurons called nociceptors, which can be activated by different noxious chemical (acid, irritants, inflammatory mediators) or physical stimuli (heat, cold, pressure). Capsaicin, the pungent ingredient in hot peppers, and structurally related vanilloids such as the ultrapotent irritant resiniferatoxin (RTX)[1] bind to specific receptors on nociceptors (1–3), causing cation influx, triggering action potentials, and inducing the sensation of burning pain. Prolonged or repeated exposure to capsaicin leads to pronounced tachyphylaxis and desensitization, in which nociceptors become insensitive to capsaicin and to other noxious stimuli. This phenomenon underlies the seemingly paradoxical use of capsaicin as an analgesic agent in the treatment of various painful disorders (4–6).

A functional vanilloid receptor termed vanilloid receptor type 1 (VR1) has been cloned recently. Both when expressed endogenously in cultured dorsal root ganglion cells or when expressed heterologously in transfected human cells or frog oocytes, VR1 can be activated by vanilloids and by noxious heat (>43 °C) and can be activated or potentiated by protons (extracellular pH <6) (6–8). VR1 encodes a protein of 838 amino acids with a predicted molecular mass of 92–95 kDa (9–10). It is a nonselective cation channel with high Ca^{2+} permeability and belongs to the superfamily of cation channels with six transmembrane segments including the Shaker-related voltage-gated K^+ channels, the hyperpolarization-activated cyclic nucleotide-gated cation channels, the cyclic nucleotide-gated cation channels, the polycystin channels and the transient receptor potential (Trp) family of ion channels. These proteins all contain six transmembrane domains, a pore-forming loop between the fifth and sixth transmembrane domains, and cytoplasmic C- and N-terminal domains (6, 7, 11). Within this superfamily, VR1 is most closely related to the Trp family of ion channels. These channels include *Drosophila* Trp and TrpL, which play crucial roles in phototransduction, and the mammalian homologues TrpC1–7, which are candidates for store-operated calcium channels.

Understanding of the function, gating, regulation and quaternary structure of Trp family members is emerging rapidly. The *Drosophila* Trp and TrpL have been shown to form heteromultimeric channels associated in a signaling complex with a receptor (rhodopsin), an effector (phospholipase C), regulators (protein kinase C and calmodulin), and the scaffolding protein INAD (12). Similarly, hTrp1 is localized in a caveolin-scaffolding lipid raft domain in plasma membrane, where it interacts directly or indirectly with a complex of Ca^{2+} signaling proteins such as inositol 1,4,5-trisphosphate receptor and $G\alpha_{q/11}$ (13). Trp3 and Trp1 form hetero-oligomers with nonselective cation permeability (14, 15), and Trp4 coexpressed with Trp1 was proposed to form store-operated calcium channels in adrenocortical cells through homo- or heteromultimers (16). However, their actual subunit organization has not been determined. More distant members of the superfamily, *e.g.* voltage-gated K^+ channels or cyclic nucleotide-gated channels, are known to form tetramers of the same or different subunits (17).

The first direct experiments evaluating the quaternary structure of VR1 were performed by Szallasi and Blumberg (18) using radiation inactivation analysis. This technique, which gives the functional size of a receptor, yielded a value for the vanilloid receptor, detected by [^{3}H]RTX binding, of 270 ± 25 kDa; this value compares with a monomer mass for VR1 of 92

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[S] The on-line version of this article (available at http://www.jbc.org) contains Supplementary Figs. 1–6.

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[1] The abbreviations used are: RTX, resiniferatoxin; BS^3, bis[sulfosuccinimidyl]suberate; sulfo-BSOCOES, bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone; DRG, dorsal root ganglion; INAD, inactivation no afterpotential D; PFO, perfluoro-octanoic acid; PKC, protein kinase C; PNGase F, peptide-*N*-glycosidase F; PPAHV, phorbol 12-phenylacetate 13-acetate 20-homovanillate; PAGE, polyacrylamide gel electrophoresis; VR1, vanilloid receptor type 1; PBS, phosphate-buffered saline; Trp, transient receptor potential; eGFP, enhanced green fluorescence protein.

kDa, strongly arguing for a functional size greater than the monomer. Further evidence for oligomerization is the dominant negative behavior of a mutant VR1 which does not respond to capsaicin (19). Finally, a Hill-coefficient of ~2 is found for VR1 interaction with different agonists, indicating positive cooperativity (6, 20–22). Together with the similarities in molecular structure between the members of the six transmembrane domain channel superfamily, it thus seems highly probable that the active VR1 channel is composed of more than one subunit, forming homomultimeric or heteromultimeric ion channels (16, 23, 24). Potential partners include the VR1 homologues SIC, VRL-1, and VR5'sv (25–27). Multimeric channels could contribute to the functional heterogeneity and complex pharmacology of VR1 seen in binding or calcium uptake experiments in DRGs and in different heterologous systems (28–31).

The goal of the present paper is to evaluate the native quaternary structure of VR1, as the complex structure of the active channel could provide insights into channel regulation and the design of new analgesic agents. Using three different biochemical approaches, we clearly show that the VR1 channel forms multimers both in DRGs and in heterologous expression systems, with a tetramer as a predominant form.

EXPERIMENTAL PROCEDURES

Transfection of Cells and Cell Culture—Cos7 cells were cultured in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). Cos7 cells were transiently transfected with VR1ϵ plasmid and/or VR1eGFP plasmid using LipofectAMINE Plus (Life Technologies, Inc.) according to the procedure recommended by the manufacturer. VR1ϵ and VR1eGFP plasmids were constructed as described previously (22).

The selected stable CHO cell clone expressing pUHG102 VR1 and pTet-Off Regulator plasmids (Tet-Off induced CHO-VR1 cells) is the generous gift of James E. Krause and Daniel N. Cortright (Neurogen Corp., Branford, CT). These cells were cultured in F-12 medium supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 500 μg/ml Geneticin, 10 mM HEPES (pH 7.4) (Life Technologies, Inc.), and 5 μg/ml tetracycline (Sigma). For the optimal level of VR1 expression the cells were cultured in tetracycline-free medium containing 10 μM sodium butyrate (Calbiochem, San Diego, CA) for 36–48 h.

Western Blot Analysis—The cell cultures were rinsed two times with ice-cold phosphate-buffered saline (PBS), and then cells were harvested in ice-cold PBS containing protease inhibitors (1 tablet/10 ml of PBS) (Roche Molecular Biochemicals), and sonicated. Protein content was measured by a micromethod using the Bio-Rad protein assay. SDS-PAGE was performed on a mini-slab apparatus using precast 4–12% SDS-polyacrylamide gels (Novex, San Diego, CA) by the method of Laemmli. Twenty μg of lysates were mixed to equal volumes of 2× SDS sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.7 M β-mercaptoethanol, and bromphenol blue), boiled for 5 min, and subjected to SDS-PAGE, followed by electrotransfer onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). For immunostaining, anti-eGFP monoclonal antibody (Roche Molecular Biochemicals) was used at 0.4 μg/ml concentration, anti-VR1 polyclonal antibody was diluted 1:1000 and anti-PKCϵ polyclonal antibody (Life Technologies, Inc.) was diluted 1:1000. The anti-VR1 polyclonal antibody was generated in rabbits against the extreme C terminus of rat VR1, namely peptide E824-K838 (Amgen, Thousand Oaks, CA) and showed specific recognition of VR1. Nonspecific binding of antibodies to the membranes was blocked by a 60-min incubation with 5% milk in PBS. The membranes were probed overnight at 4 °C with the different antibodies diluted in 5% milk in PBS. Following the incubation, the membranes were washed three times for 10 min in PBS containing 0.05% Tween 20 and then incubated for 60 min with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Bio-Rad). After the membranes were rinsed three times for 10 min in PBS containing 0.05% Tween 20, immunostaining was visualized by ECL (Amersham Pharmacia Biotech). BenchMark prestained Protein Ladder (Life Technologies Inc.) was used as molecular size marker.

Co-immunoprecipitation—Plasmids encoding VR1ϵ and VR1eGFP were transfected into Cos7 cells either together or individually using LipofectAMINE Plus (Life Technologies, Inc.) according to the procedure recommended by the manufacturer. 48 h after transfection, 3 ml of ice-cold radioimmune precipitation buffer without SDS (pH 7.5) containing 50 mM Tris-HCl, 150 mM NaCl, 1% IGEPAL CA-630 (Sigma), 0.5% sodium deoxycholate (Sigma), and protease inhibitors (1 tablet/10 ml of PBS) (Roche Molecular Biochemicals) was added to the cells and incubated for 10 min on ice. The lysates were centrifuged at 3,000 × *g* for 10 min to remove cellular debris. Subsequently, 10 μl of anti-PKCϵ polyclonal antibody (Life Technologies, Inc.), 5 μl of anti-eGFP monoclonal antibody (Roche Molecular Biochemicals), or 10 μl of non-immune serum was added to 1 ml of supernatant containing 0.4 mg of protein and rotated for 4 h at 4 °C. Then, 20 μl of Protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the immune complexes and rotated at 4 °C overnight. The immunoprecipitates were washed four times with ice-cold radioimmune precipitation buffer without SDS, eluted with 2× SDS sample buffer, and analyzed by Western blotting on 4–12% SDS-polyacrylamide gels.

Preparations of DRG Lysates—Cell membranes from rat DRGs were prepared as described previously (29). Female Harlan Sprague-Dawley rats weighting 200–250 g were decapitated under CO_2 anesthesia. The spinal columns were opened, and DRGs from all levels were collected in ice-cold physiological saline. DRGs were homogenized in ice-cold buffer (pH 7.4) containing 5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM sucrose, and 10 mM HEPES using a tissue homogenizer type PRO 200 (PRO Scientific Inc., Monroe, CT) followed by sonication. Tissue homogenates were centrifuged for 10 min at 3,000 × *g* (4 °C) to remove cellular debris and the supernatant was centrifuged again for 30 min at 35,000 × *g* (4 °C) to obtain a partially purified membrane fraction (pellet). After solubilization in PBS containing 2% *n*-dodecyl β-D-maltoside (Sigma), the membrane proteins were separated on PFO-PAGE and analyzed by Western blotting.

PFO-PAGE—Proteins were fractionated by PFO-PAGE as described by Ramjeesingh *et al.* (32). Freshly poured 6% Tris-glycine gels without SDS were used. Total cell lysates were prepared from Tet-Off induced CHO-VR1 cells 48 h after tetracycline withdrawal, from Cos7 cells expressing VR1eGFP 48 h after transfection, and from rat DRGs as described above. In some experiments cell lysates from CHO-VR1 cells were treated with peptide-*N*-glycosidase F (New England Biolabs) for 1 h at 37 °C. Twenty μg of lysates were mixed with doubly concentrated sample buffer (100 mM Tris base, 2–8% NaPFO (Oakwood Products Inc., West Columbia, SC), 20% glycerol, 0.7 M β-mercaptoethanol, and 0.005% bromphenol blue, pH 8.0), the final PFO concentration being in the range of 0.8–3.2%. After 30 min of incubation at room temperature the samples were subjected to electrophoresis. The running buffer contained 25 mM Tris, 192 mM glycine, 0.5% NaPFO, pH 8.5, adjusted with NaOH. The electrotransfer and immunostaining were performed as described above. As molecular weight standard, cross-linked phosphorylase *b* (Sigma) was separated on the same gels, electroblotted, and stained with Amido Black.

Chemical Cross-linking—The following water-soluble, homobifunctional *N*-hydroxysuccimide esters were used for protein cross-linking studies: the non-cleavable bis[sulfosuccinimidyl]suberate (BS^3) and the base-cleavable bis[2-(sulfosuccinimidooxycarbonyloxy)ethyl]sulfone (sulfo-BSOCOES; Pierce). Both reagents were dissolved in PBS immediately before use. The lysates prepared from Tet-Off induced CHO-VR1 cells 48 h after tetracycline withdrawal and from Cos7 cells expressing VR1eGFP 48 h after transfection were incubated with different concentrations of cross-linkers (1 μM to 10 mM) or without any cross-linker for 30 min at room temperature. To stop the reaction, an equal volume of 1 M glycine was added to the mixtures and incubated for an additional 20 min. The quenched samples were subsequently mixed with 4× concentrated Nu-PAGE sample buffer (564 mM Tris base, 424 mM HCl, 292 mM lithium dodecyl sulfate, 2 mM EDTA, 0.7 M β-mercaptoethanol, 0.88 mM Serva Blue G250, 0.70 mM Phenol Red, pH 8.5) according to the description provided by the manufacturer (Novex, San Diego, CA), incubated at 70 °C for 15 min, and then subjected to electrophoresis. The samples were separated on 3–8% gradient Nu-PAGE Tris acetate gels and analyzed by Western blotting. Cross-linking experiments were also performed on living Cos7 cells transiently expressing VR1eGFP. The cells were incubated for 30 min at 37 °C in PBS containing different concentrations of BS^3 (0.01–10 mM) followed by preparation of cell lysates and their Western blot analysis.

RESULTS

Co-immunoprecipitation—To determine if two or more VR1 subunits form a complex, VR1ϵ and VR1eGFP were coexpressed in Cos7 cells. These differently tagged VR1 proteins

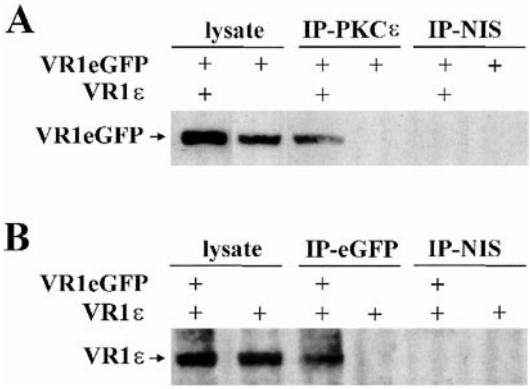

FIG. 1. **Co-immunoprecipitation of VR1ϵ and VR1eGFP.** Plasmids encoding VR1ϵ and VR1eGFP were transfected into Cos7 cells either together or individually. Cell lysates were processed for Western analysis or used for immunoprecipitation. The result shown is from one of two independently performed experiments. *A*, cell lysates were immunoprecipitated with anti-PKCϵ polyclonal antibodies (*IP-PKCϵ*) or non-immune serum (*IP-NIS*) and Western blots of total cell lysates (*lysate*), and the immune complexes were probed with anti-eGFP antibodies. *B*, cell lysates were immunoprecipitated with anti-eGFP monoclonal antibodies (*IP-eGFP*) or non-immune serum (*IP-NIS*) and Western blots of total cell lysates (*lysate*) and the immune complexes were probed with anti-PKCϵ antibodies.

were immunoprecipitated with anti-eGFP or anti-ϵ tag antibodies and Western blots containing the immune complexes were probed with the reciprocal antibodies. Transfection with just one construct as well as immunoprecipitation with non-immune serum were used as controls. The results shown in Fig. 1 demonstrate that VR1eGFP co-immunoprecipitated with VR1ϵ and *vice versa*, suggesting the existence of at least two VR1 molecules in a protein complex.

PFO-PAGE—The first method used to determine the nature of the VR1 channel complex was PFO-PAGE. In this method, PFO is used in place of SDS in gel electrophoresis, as it is less disruptive of interactions within protein oligomers and thus permits molecular mass determination of multimeric proteins. VR1eGFP-transfected Cos7 cell lysates, Tet-Off induced CHO-VR1 cell lysates, and partially purified DRG membranes were separated on PFO-PAGE and analyzed by Western blotting (Fig. 2). The pattern of protein migration depended on time and temperature of incubation with sample buffer containing PFO; a longer incubation time and higher temperature resulted in a greater degree of subunit dissociation from the protein complex (see Supplementary Figs. 1–6, available in the on-line version of this article). Subunit dissociation was also highly dependent on the ratio of PFO to protein and/or lipid (Fig. 2*A*). CHO-VR1 cell lysates were incubated with different PFO concentrations (0.4–3.2%) for 30 min at room temperature and separated on 6% PFO-PAGE. Using 3.2% and 1.6% PFO, VR1 migrated as monomers and some dimers. With 0.8% PFO, VR1 was separated into multiple bands representing VR1 monomers, dimers, trimers, and tetramers; using 0.4% PFO, VR1 migrated mainly as tetramer, but higher molecular weight protein aggregates were also seen, probably due to insufficient solubilization of membrane proteins (Fig. 2*A*). Based on these results in each system, we varied the PFO concentration to find the appropriate conditions for detecting oligomers.

In CHO-VR1 cells VR1 monomer is present in two different forms: a non-glycosylated form with apparent molecular mass of 95 kDa and a glycosylated form with calculated molecular mass of 114 kDa as shown in Fig. 2 (*A*, *B*, and *F*). Treatment for 1 h at 37 °C with peptide-*N*-glycosidase F, an enzyme that cleaves *N*-glycosidic bonds between the sugar portion and asparagine of glycoproteins, reduced or eliminated the upper band of VR1 monomer representing the glycosylated form of the protein. On SDS-PAGE, VR1 migrated as a double band without and as a single band after PNGase F treatment (Fig. 2*F*). On PFO-PAGE the upper band of VR1 monomer and some upper bands of the dimers and trimers were markedly reduced with PNGase F treatment, and there also was a shift in the mobility of VR1 tetramer (Fig. 2*B*). Based on these observations, we assume that the multiple bands corresponding to VR1 multimers are due to the different combinations of the glycosylated and non-glycosylated monomers, with predicted apparent molecular masses ranging from 190 to 220 kDa for dimers (three possible bands of 190, 205, and 220 kDa) and from 285 to 330 kDa for trimers (four possible bands of 285, 300, 315, and 330 kDa). The failure to resolve multiple bands of VR1 tetramers (five possible bands with predicted apparent molecular masses of 380, 395, 410, 425, and 440 kDa) can be explained by the decreased resolution of the gel in the higher molecular weight region; the increased mobility of VR1 tetramer after glycosidase treatment is still apparent. In the treated sample, a band with higher molecular mass and some protein aggregates stacked at the top of the separating gel are also seen. Based on our present studies, their nature and importance cannot be determined.

In the other heterologous system used, VR1eGFP migrated as four distinct bands on 6% PFO gels (Fig. 2*C*) with estimated molecular masses of 119, 236, 345, and 464 kDa, respectively (Fig. 2*D*). Molecular mass determination of the observed protein species was based on the linear relationship of the logarithm of the molecular masses of cross-linked phosphorylase *b* subunits used as molecular weight standards *versus* their migration distance. Based on the obtained molecular weights, we assume that these four bands represent the VR1eGFP monomers, dimers, trimers, and tetramers (predicted molecular mass for VR1eGFP of 124, 248, 372, and 496 kDa). VR1eGFP migrated as a single band on SDS-PAGE as shown on Fig. 2*F*, although some glycosylation (10–15%) was seen in some experiments, resulting in the appearance of a double band when higher exposure times were used for ECL development.

After treating the cells expressing VR1eGFP for 30 min with the VR1 agonist RTX at doses activating the channel, VR1eGFP was separated as monomer, dimer, trimer, and tetramer on PFO-PAGE (Fig. 2*C*) and as monomer, dimer, and multimer on SDS-PAGE (Fig. 3*A*), showing that some covalent bonds are formed between the subunits of the complex. Some higher molecular weight bands running in the PFO-stacking gels could be visualized with higher exposure times (see supplemental material, available on line) possibly because some other proteins or another preformed complex are covalently cross-linked to VR1 tetramer.

When partially purified DRG membranes were subjected to PFO-PAGE using optimized conditions (solubilization of membrane proteins with 2% *n*-dodecyl β-D-maltoside and incubation with sample buffer containing PFO at a final concentration of 0.8% for 30 min room temperature), VR1 migrated as a major band representing VR1 tetramer and as a minor band representing VR1 monomer (Fig. 2*E*). When higher PFO concentrations were used, VR1 monomers, dimers, trimers, and tetramers could be also seen (see supplemental material, available on line). VR1 monomer migrated as a single band both on PFO and SDS-PAGE showing the lack of the glycosylated form of VR1 in DRG (Fig. 2, *E* and *F*). In accordance with this observation, VR1 tetramer from DRG had a slightly higher mobility than the VR1 tetramer from CHO-VR1 cells, as the tetramer of the latter is a combination of the glycosylated and non-glycosylated form of VR1 resulting in a complex with higher molecular weight.

Endogenous Cross-linking of VR1 by Transglutaminases

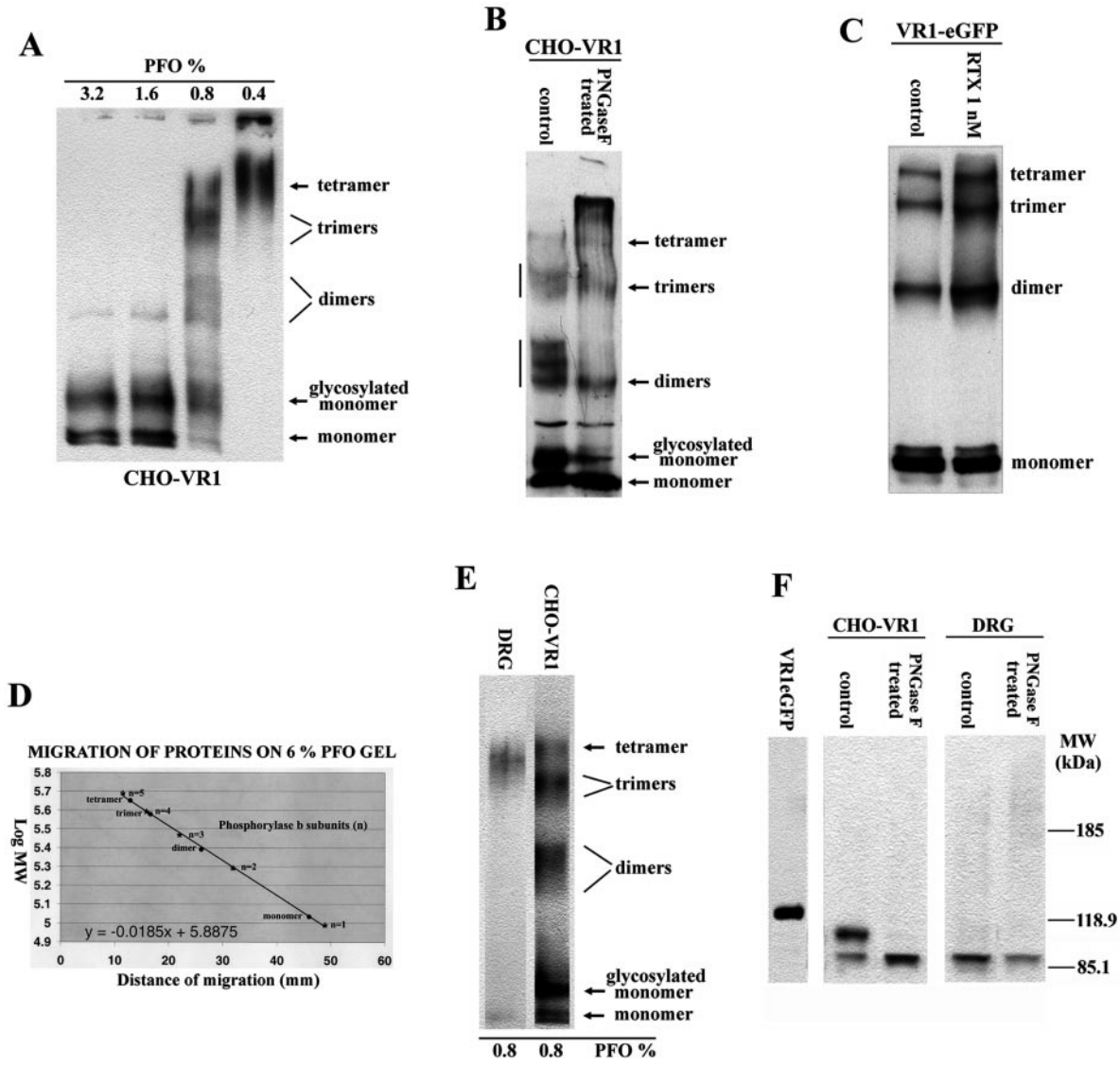

FIG. 2. **Determination of VR1 oligomeric structure using PFO-PAGE.** Total cell lysates prepared from VR1eGFP-transfected Cos7 cells, Tet-Off induced CHO-VR1 cells treated or not with PNGase F, and partially purified DRG membranes solubilized with 2% *n*-dodecyl β-D-maltoside were separated on 6% PFO-PAGE, electrotransferred to nitrocellulose membranes, and immunostained with appropriate antibody. In parallel, cross-linked phosphorylase *b* was separated on the same gel, electrotransferred, and visualized by Amido Black staining for molecular weight determination purposes. These results are representative of at least three independent experiments. *A*, CHO-VR1 lysates were incubated with sample buffer containing different PFO concentrations for 30 min at room temperature before electrophoresis; the Western blot was probed using anti-VR1 antibody. *B*, CHO-VR1 cell lysates were treated or not with peptide-*N*-glycosidase F for 1 h at 37 °C; the Western blot was probed using anti-VR1 antibody. *C*, separation of VR1eGFP (with or without agonist activation) on 6% PFO gel. Anti-eGFP monoclonal antibody was used for probing. *D*, the molecular weights of the different VR1eGFP protein species were calculated using the linear relationship between the distance of migration (*x* axis) and the logarithm of molecular weight (*y* axis) of cross-linked phosphorylase *b*. *E*, separation of VR1 from DRG and CHO-VR1 on 6% PFO-PAGE; anti-VR1 antibody was used for probing. *F*, separation of VR1eGFP, and VR1 from induced CHO cells and DRG on 4–12% SDS-PAGE. Anti-eGFP monoclonal and anti-VR1 polyclonal antibody were used for probing.

upon Vanilloid Treatment of Cells—Upon VR1 activation by agonists such as capsaicin or its ultrapotent analog RTX, VR1 became covalently cross-linked as detected by SDS-PAGE. The cross-linking depended on time, on ligand concentration, and on the presence of extracellular calcium. Treatment of Cos7 cells expressing VR1eGFP with different doses of RTX (0.001–1 nM) for 30–60 min resulted in formation of VR1 dimers and multimers; the cross-linking was not seen when 1 mM EGTA was added to the extracellular fluid (Fig. 3*A*). The lowest RTX concentration causing cross-linking was 0.01 nM. This ligand concentration is sufficient to activate the channel and induce a sustained increase in intracellular Ca^{2+} concentration (see supplemental information, available on line). Cross-linked VR1eGFP showed a pattern similar to control VR1eGFP without agonist treatment when separated on PFO-PAGE (Fig. 2*C*).

The same multimerization was observed when cells were treated with other VR1 agonists (capsaicin, olvanil, PPAHV) at doses known to activate the VR1 channel but was not observed with the competitive inhibitor capsazepine at concentrations up to 30 μM. Likewise, pretreatment with 10 μM capsazepine could prevent the covalent bond formation between VR1 monomers (Fig. 3*B*). Treatment of cells with 1–10 μM ionomycin, an agent that increases intracellular Ca^{2+}, did not result in the same degree of cross-linking, although some formation of dimers and a very low amount of higher oligomers could be seen (see supplemental information, available on line). We surmise that covalent cross-linking of VR1 reflects the Ca^{2+}-dependent activation of endogenous transglutaminases. Consistent with this interpretation, pretreatment with 20 mM cysteamine or 250 μM mono-dansylcadaverine, two transglutaminase inhibitors, prevented the covalent cross-linking (Fig. 3*B*). Similar cross-linking of VR1 in response to VR1 agonists

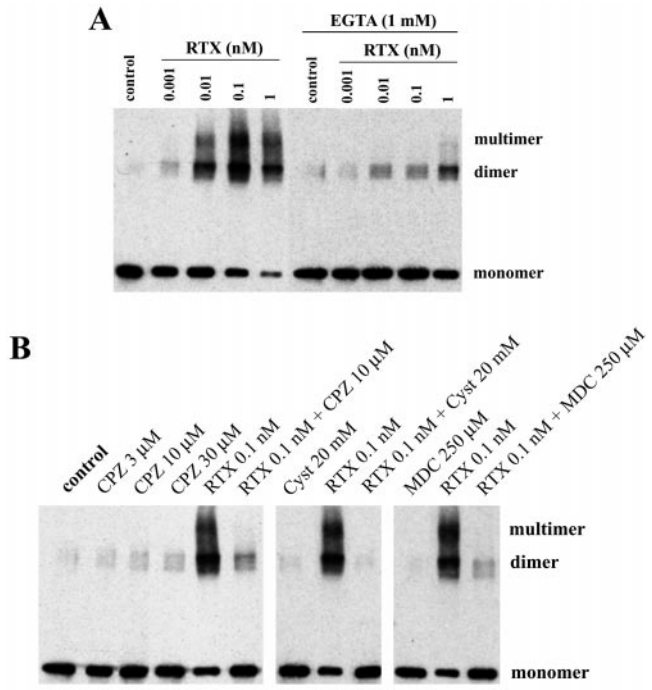

FIG. 3. **Cross-linking of VR1 by transglutaminase upon VR1 channel activation.** Cos7 cells expressing VR1eGFP were treated with different doses of RTX for 30 min in the absence or presence of 1 mM EGTA, or after pretreatment with the competitive VR1 antagonist capsazepine or inhibitors of transglutaminase. The total cell lysates were analyzed on 4–12% SDS-PAGE, followed by electrotransfer and immunostaining with anti-eGFP monoclonal antibodies. Similar results were obtained in three to five independent experiments for each agent. *A*, immunoblot of cross-linked VR1 upon treatment with different doses of RTX in the absence or presence of EGTA. *B*, 1-h pretreatment with capsazepine (10 μM), with cysteamine (20 mM) or with mono-dansylcadaverine (250 μM) prevented the formation of VR1 multimers in response to 0.1 nM RTX treatment.

was seen in CHO-VR1 cells as well (see supplemental information, available on line).

Chemical Cross-linking—A third method to explore VR1 oligomerization and to potentially reveal associated proteins in complex with VR1 was cross-linking with chemical cross-linking agents. Two water-soluble cross-linkers were examined in detail: the non-cleavable cross-linker BS^3 with a spacer arm of 1.14 nm and the base-cleavable cross-linker sulfo-BSOCOES with a spacer arm of 1.3 nm. Total cell lysates from VR1eGFP-transfected Cos7 cells and from induced CHO-VR1 cells, treated or not treated with PNGase F, were incubated with different concentrations of the cross-linkers for different times at room temperature. The formed complexes were separated on Nu-PAGE Tris acetate gels for better separation of high molecular protein complexes and were analyzed by Western blotting. Fig. 4 (*A* and *B*) shows the immunoblot of the reaction products when different concentrations of BS^3 were used as cross-linkers with a 30-min incubation time at room temperature. As shown in Fig. 4*A*, the amount of VR1eGFP monomers decreased with increasing concentration of BS^3, whereas the amount of the oligomeric forms increased concomitantly; first dimers, then trimers and tetramers were formed. Incubation with 300 μM BS^3 resulted in formation of VR1eGFP dimers, trimers, and tetramers, but no other protein species of intermediate molecular weights, indicating the presence of other proteins in the VR1 complex, were found in this system. At higher cross-linker concentrations, multimers with calculated molecular weights corresponding to VR1 hexamers were also formed. When the cross-linking experiments were performed on CHO-VR1 cell lysates, a more complex pattern of protein separation was seen, partly because dimers, trimers, and tetramers with different molecular weights can be formed from glycosylated and non-glycosylated VR1 monomers. To prevent this phenomenon, PNGase F-treated VR1 was used. Using different BS^3 concentrations up to 1 mM, formation of VR1 dimers, trimers, and tetramers was seen (Fig. 4*B*). At higher cross-linker concentrations, multimers with calculated molecular weights corresponding to VR1 hexamers and octamers were also formed. The molecular weight determination of the higher molecular weight complexes is problematic, however, because of the decreased resolution of Nu-PAGE in this range as well as the lack of good high molecular weight markers. When sulfo-BSOCOES was used as a cross-linker, similar cross-linking patterns were observed, although sulfo-BSOCOES was somewhat less efficient than was BS^3 (see supplemental information, available on line).

Cross-linking experiments were also performed on living Cos7 cells expressing VR1eGFP, when the chemical cross-linker can form covalent bonds only between accessible primary amines sitting on the outer surface of cell membranes. The cells were incubated with different concentrations of BS^3 for 30 min at 37 °C; cell lysates were prepared and analyzed by Western blotting. As shown on Fig. 4*C*, at higher BS^3 concentrations (1–10 mM), VR1eGFP migrated as four sets of bands representing monomers, dimers, trimers, and tetramers without any other, intermediate molecular weight or high molecular weight complexes being present except some highly cross-linked polymers not able to enter the gel. The VR1eGFP dimers run as double or multiple bands; we assume that dimers are formed between the glycosylated and non-glycosylated forms of VR1eGFP, which can be observed with the higher exposure times needed to visualize trimers and tetramers present at lower amount.

DISCUSSION

Analysis of the native, multimeric structure of membrane proteins is problematic. SDS-PAGE, although a powerful technique for determination of monomer molecular weights, disrupts protein-protein interactions. Sucrose density gradient centrifugation and gel filtration after solubilization of membrane complexes with relatively mild detergents can be used, assuming that adequate conditions for solubilization can be established (32–34); however, these methods require large amounts of recombinant or purified proteins and lack the resolving power of PAGE.

To evaluate the quaternary structure of VR1, we used three different approaches. First, we coexpressed two differently tagged versions of VR1 in Cos7 cells. Immunoprecipitation of either caused co-immunoprecipitation of the other, implying the presence of at least two subunits of VR1 in the receptor complex. These results complement the findings of Oxford *et al.* (19), who demonstrated that a mutant of VR1 could have dominant negative activity on channel function.

To characterize the composition of the VR1 receptor complex, we used PFO-PAGE, a novel method for quaternary structure determination of membrane proteins (32). We analyzed VR1 in two different heterologous expression systems, Cos7 cells transiently expressing VR1eGFP and a Tet-Off inducible CHO-VR1 cell line, and we examined native VR1 in rat dorsal root ganglion membranes. In the two heterologous systems, we detected VR1 monomers, dimers, trimers, and tetramers, although by lowering the PFO concentration we were able to decrease the relative ratio of the lower oligomers and find conditions where tetramer was the major form of VR1. In each system the optimal protein/PFO ratio had to be determined to yield a PFO percentage that did not result in disaggregation of subunits from the protein complex but did give adequate solubilization.

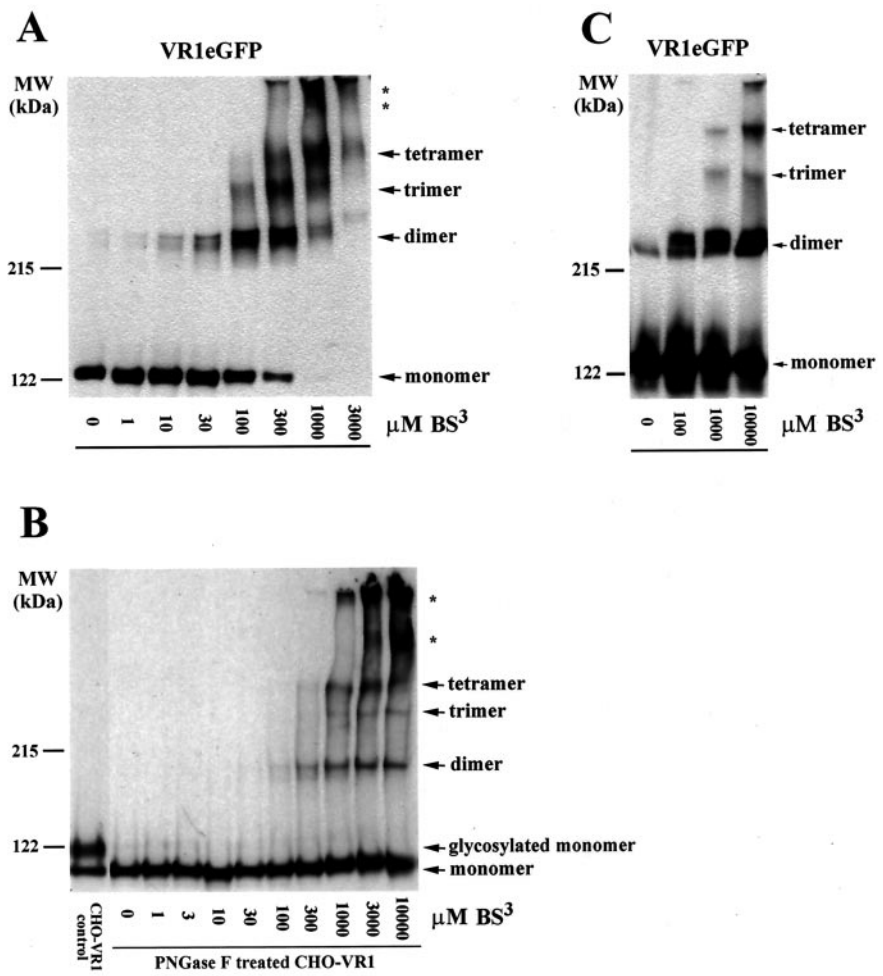

FIG. 4. **Chemical cross-linking of VR1 by BS^3.** Total cell lysates of Cos7 cells expressing VR1eGFP and of induced CHO-VR1 cells treated or not with PNGase F were incubated with different concentrations of BS^3 (1 μM-10 mM) for 30 min at room temperature. After quenching the reaction, the cross-linked proteins were separated on 3–8% Nu-PAGE, electroblotted, and immunostained with appropriate antibodies. In living cell experiments, Cos7 cells expressing VR1eGFP were treated with different concentrations of BS^3 for 30 min at 37 °C followed by Western blot analysis of the lysates. The molecular mass of the protein complexes was determined by cross-linked phosphorylase *b* separated on the same gels, electroblotted and stained with Amido Black and by SeeBlue pre-stained protein standard. *A* and *B* are from a representative experiment repeated an additional two times; the experiment illustrated in *C* was repeated once with similar results. *A*, BS^3 cross-linked VR1eGFP probed with anti-eGFP monoclonal antibody (*asterisks* indicate oligomers higher than tetramer). *B*, BS^3 cross-linked CHO-VR1 after PNGase F treatment probed with anti-VR1 polyclonal antibody (*asterisks* indicate hexamers and octamers). *C*, cross-linking experiments on living cells. The Western blot was probed with anti-eGFP monoclonal antibody.

When partially purified DRG membranes were analyzed by PFO-PAGE using optimized conditions, VR1 was present mainly as tetramer and some monomer but without evident dimers, trimers, or higher molecular weight protein aggregates being present, although at higher PFO concentrations the oligomers smaller than tetramer became detectable. VR1 in DRG was non-glycosylated in contrast to VR1 in CHO cells, which were highly glycosylated (~ 60%). We cannot distinguish whether the existence of oligomers smaller than the tetramers represent artifacts of disaggregation by the PFO or are present in the native membranes. Kir 2.1, an inwardly rectifying potassium channel known to exist as tetramers in membranes, was shown to migrate as a single band with a molecular weight corresponding to the tetramer on PFO-PAGE. On the other hand, the GABA-A receptor complex, which is thought to be a heteropentameric oligomer formed from three different subunits, was found to migrate as trimers and pentamers on PFO-PAGE, the trimeric form being the predominant band (32). We conclude that VR1 is indeed a multimer, most possibly a homotetramer. We observe modest differences in post-translational modification between native VR1 and VR1 expressed in heterologous systems, just as somewhat different behavior of endogenous and heterologously expressed VR1 has been seen in calcium uptake, binding, and other experiments (28–31). These differences emphasize the need for caution in interpretation of results using heterologous expression systems.

In the course of our studies, we found that VR1 becomes covalently cross-linked spontaneously in the presence of VR1 agonists. The pattern of cross-linking, which was analyzed mostly by SDS-PAGE, was similar to that found for VR1 by PFO-PAGE in the absence of agonists, namely multimers up to tetramer and, to a much lower extent, some higher polymers but with no bands indicative of the formation of hetero-oligomers between VR1 and protein subunits of a different size. The VR1 agonist dependence, the inhibition by the VR1 competitive antagonist capsazepine, and the inhibition in the absence of extracellular calcium all fit with the model that the covalent cross-linking reflects elevated intracellular calcium in response to VR1 activation. The probable mechanism responsible to this protein cross-linking is through transglutaminase, since pretreatment of the cells with known transglutaminase inhibitors could prevent VR1 oligomerization. Transglutaminases are a family of structurally and functionally related Ca^{2+}-dependent enzymes that catalyze cross-linking reactions of proteins by transferring the γ-carboxy group from protein-bound glutamine to the ϵ-amino group of protein-bound lysine residues or other primary amines (35, 36). The cross-linking of proteins by transglutaminases has been shown to play a variety of physiologically important functions in different systems. For example, transglutaminases are involved in cross-linking of cornified envelope proteins during terminal differentiation of keratinocytes (36–39) and in programmed cell death (40, 41). In the current study, we have explored the endogenous cross-linking of VR1 as a reporter for its state of association. Studies are in progress to evaluate its possible contribution to VR1 desensitization or DRG toxicity in response to vanilloid treatment.

Our third approach to characterize VR1 subunit structure was to use chemical cross-linking agents. This method has been widely used in the nearest neighbor analysis of membrane proteins. The bifunctional chemical cross-linkers used in the present study produce covalent bonds between primary amines

of proteins resulting in intra- or intermolecular cross-linking. The cross-linked oligomers subsequently can be separated on SDS-PAGE and their structures deduced from the sizes of the complexes. This technique has helped demonstrate the hexameric nature of bacterophage T7 primase/helicase (42), the oligomeric structure of human immunodeficiency virus-1 reverse transcriptase (43), and the stoichiometry of subunits in the native atrial G-protein-gated K^+ channel, I_{KACh} (44). Using BS^3 or sulfo-BSOCOES, a non-cleavable or a base-cleavable water soluble cross-linking agent, we have found that VR1eGFP expressed in Cos7 cells can be cross-linked to form dimers, trimers, and tetramers, as well as higher molecular weight polymers. We did not observe other associated proteins. The higher molecular weight polymers (more than tetramer) were not observed when the cross-linking experiments were performed on living cells, conditions where the water-soluble agents can form covalent bonds only between the accessible primary amines sitting on the outer surface of cell membranes. In CHO-VR1 cells the same pattern of oligomerization was found, but occasionally some bands with intermediate molecular weight were also seen, albeit at a much lower level. This suggests the possibility that proteins can be associated with VR1. The *Drosophila* Trp and TrpL proteins are known to be associated with proteins such as phospholipase C or protein kinase C via the scaffolding protein INAD (12). Likewise, hTRP1 was shown to be associated with G-proteins and caveolin (13). On the other hand, some other members of the superfamily are known to form homo- or heterotetramers without being in tight complexes with other proteins (17, 44). An unresolved issue is the origin of the bands with mobilities corresponding to a hexamer or octamer. Based on the expectation of a tetrameric structure, we surmise that we have cross-linked subunits of adjacent tetrameric channels. *Drosophila* Trp and TrpL proteins are known to be clustered in multichannel complexes (12). In any case, the role of such clustering in the regulation of VR1 and its pharmacology remains to be assessed.

In summary, the experiments presented here using multiple independent methods demonstrate that the VR1 channel is a multimer and that the VR1 channel is most likely made up of four identical subunits. Variation in the individual subunits of the tetramer, whether through splice variants or through post-translational modification, could provide a mechanism to generate complexity.

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