Aquaporin-4 (AQP4) can assemble into supramolecular aggregates called orthogonal arrays of particles (OAPs). In cells expressing single AQP4 isoforms, we found previously that OAP formation by AQP4-M23 requires N terminus interactions just downstream of Met-23 and that the inability of AQP4-M1 to form OAPs involves blocking by residues upstream of Met-23. Here, we studied M1/M23 interactions and regulated OAP assembly by nanometer-resolution tracking of quantum dot-labeled AQP4 in live cells expressing differentially tagged AQP4 isoforms and in primary glial cell cultures in which native AQP4 was labeled with a monoclonal recombinant neuromyelitis optica autoantibody. OAP assembly was assessed independently by Blue Native gel electrophoresis. We found that OAPs in native glial cells could be reproduced in transfected cells expressing equal amounts of AQP4-M1 and M23. Mutants of M23 that do not themselves form OAPs, including M23-F26Q and M23-G28P, were able to fully co-associate with native M23 to form large immobile OAPs. Analysis of a palmitoylation-null M1 mutant (C13A/C17A) indicated palmitoylation-dependent OAP assembly only in the presence of M23, with increased M1 palmitoylation causing progressive OAP disruption. Differential regulation of OAP assembly by palmitoylation, calcium elevation, and protein kinase C activation was found in primary glial cell cultures. We conclude that M1 and M23 co-assemble in AQP4 OAPs and that specific signaling events can regulate OAP assembly in glial cells.

Aquaporin-4 (AQP4) is the predominant water channel in the mammalian central nervous system, where it is localized mainly in endfoot processes of pericapillary glial cells and in ependymal cells lining the ventricles. AQP4 provides the principle pathway for water entry into the brain and spinal cord through an intact blood-brain barrier in cytotoxic edema and hydrocephalus. In addition to its central role in maintaining brain water balance, AQP4 is also involved in neuroexcitation and glial cell migration. AQP4 also acts as a receptor for autoantibodies found in serum from patients with the severe demyelinating autoimmune disease neuromyelitis optica (NMO). Freeze-fracture electron microscopy of glial cell endfoot membranes has revealed densely packed square arrays of intramembrane proteins known as orthogonal arrays of particles (OAPs). Our laboratory first proposed that AQP4 forms OAPs based on its expression in the specific cell types where OAPs are seen. The involvement of AQP4 in OAP formation was proven by showing OAPs in AQP4-transfected cells, the absence of OAPs in tissues from AQP4 knock-out mice, and OAP labeling by AQP4 antibodies. Biological relevance of AQP4 assembly into OAPs is at present unknown. It has been speculated that OAPs might enhance AQP4 water permeability, stabilize AQP4 polarization in glial cell foot-processes, and enhance glial cell-cell adhesion. The presence of OAPs has been correlated with neurological and neuromuscular diseases. The AQP4 transcript contains at least two alternate translation initiation sites, yielding a “long” (M1) isoform of ~34 kDa, and a “short” (M23) isoform of ~31 kDa. Both AQP4 isoforms form stable tetramers and function as water channels, with brain expressing both isoforms and tissues outside of the central nervous system expressing predominantly AQP4-M23. A recent study of the rat brain genome suggested the presence of additional AQP4 isoforms, including a larger 39-kDa isoform (AQP4e) with 41 extra residues at the AQP4-M1 N terminus that forms functional water channels but is expressed in very low abundance. Freeze-fracture electron microscopy of cells transfected with AQP4-M23 shows large OAPs, sometimes containing >100 particles, whereas OAPs are largely absent in cells transfected with AQP4-M1. OAP size is reduced when AQP4-M1 is co-transfected with AQP4-M23, suggesting an interaction between these two isoforms in vivo.
cellular loop of AQP4 for quantum dot labeling and showed free, rapid diffusion of AQP4-M1 and slow, highly restricted diffusion of OAP-associated AQP4-M23. Analysis of single molecule trajectories allowed determination of the fraction of AQP4 molecules in OAPs and their diffusion characteristics. We also investigated the molecular determinants of OAP formation by measuring the diffusion and assembly state of a series of AQP4 truncation mutants, point mutants, and chimeras (35). By expressing single AQP4 isoforms, we found that OAP formation by AQP4-M23 requires N-terminal hydrophobic interactions at residues 24–26 (just downstream of Met-23) and that the absence of OAP formation by AQP4-M1 involves non-selective blocking of these hydrophobic interactions by seven residues just upstream of Met-23. In support of the conclusion that OAP formation by AQP4-M23 is stabilized by hydrophobic intermolecular interactions involving N-terminal residues, we found that AQP1, an AQP4 homolog that does not form OAPs, could be immobilized when its N-terminal domain was replaced with that of AQP4-M23.

Here, we investigated a different set of questions about whether and how the M1 isoform of AQP4 can disrupt M23 OAPs and whether AQP4 OAP assembly is regulated in native brain glial cells by lipid modification or phosphorylation. For these studies, a two-color tracking approach was developed in which two co-expressed AQP4 isoforms were labeled with different color quantum dots. We also developed an approach to track native AQP4 molecules in primary glial cell cultures using a purified, recombinant antibody against an extracellular epitope(s) on native AQP4. The single-molecule tracking data together with Blue Native gel electrophoresis suggest that M1 and M23 co-assemble in AQP4 tetramers with differential abilities to further assemble into OAPs. We also show that OAP assembly in glial cells can be regulated by post-translational modification of N-terminal cysteines in AQP4-M1 and by phosphorylation signaling.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs, Cell Culture, and Transfections—**DNA constructs encoding rat AQP4 (M1 and M23 isoforms and mutants thereof) and human AQP1, each containing a 10-residue Myc epitope (NH$_2$-EQKLISEEDL-COOH) in the second extracellular loop, were previously described (35). M23 with a hemagglutinin (HA) epitope (NH$_2$-YPYDVPDYA-COOH) in place of the Myc epitope was generated by PCR amplification using rat M23 as template. A C-terminal serine residue was included to introduce a cloning restriction site and to generate the epitope tags of equal length (Fig. 1A). Constructs encoding non-epitope-tagged AQP4-M1 and M23 from rat, mouse, and human were PCR-amplified using whole-brain mRNA as template. All PCR fragments were ligated into mammalian expression plasmids and transfected into COS-7 cells.

Primary cultures of astrocytes (glial cells) from the neocortex of wild-type and AQP4 knock-out mice (36) were generated. All PCR fragments were ligated into mammalian expression vectors and transfected into COS-7 cells. AQP4 isoforms were amplified by PCR using whole-brain mRNA as template.

**NMO Patient Serum and Recombinant AQP4 Autoantibody—**NMO serum was obtained from an NMO-IgG seropositive individual who met the revised diagnostic criteria for clinical disease (37). Control (non-NMO) human serum was purchased from the University of California, San Francisco cell culture facility. Recombinant NMO antibody (rAb-53) was generated from a cloned expanded cerebrospinal fluid blast clone as described by Bennett et al. (38). Briefly, heavy- and light-chain constructs were co-transfected into HEK293 cells, and the supernatant was harvested, centrifuged to remove any cells and debris, and then incubated overnight with protein A-Sepharose (Sigma) at 4 °C. The rAb was eluted in 0.1 M glycine, 1 M NaCl (pH 3.0) and immediately adjusted to pH 7.5 by the addition of 0.1 M Tris-HCl (pH 8.0). Recombinant IgG was subsequently exchanged and concentrated in storage buffer (PBS plus 0.1% IgG/protease-free bovine serum albumin) using Ultracel YM-30 microconcentrators (Millipore, Billerica, MA). Antibody integrity was confirmed by gel electrophoresis, and IgG concentration was determined by a human IgG capture enzyme-linked immunosorbent assay. Purified rAb-53 showed specific binding to human and mouse AQP4 in multiple independent assays (38).

**Immunostaining—**COS-7 cells transfected with Myc- and/or HA-tagged AQP4 isoforms were fixed with 4% paraformaldehyde and incubated for 20 min in blocking buffer (PBS containing 6% glucose, 1% bovine serum albumin, and 1% goat serum) and then for 20 min in 70% ethanol. The cells were rinsed extensively with PBS, and cover glasses were mounted with VECTA-MOUNT (Vector Laboratories, Burlingame, CA) containing 4% 6-diamino-2-phenylindole to stain nuclei.

Quantum Dot Labeling and Cell Treatments—Before labeling of AQP4 with quantum dots (Qdots), cells were washed with 2 ml of PBS containing 6% glucose and 1% bovine serum albumin.
Aquaporin-4 Orthogonal Arrays

(GP buffer) and incubated for 5 min in blocking buffer. For two-color labeling of COS-7 cells co-transfected with Myc- and HA-tagged AQP4 isoforms, cells were incubated for 8 min with 70 ng/ml mouse anti-Myc (Covance) and rabbit anti-HA (Zymed Laboratories Inc.) antibodies in blocking buffer. Cells were then rinsed 5 times with GP buffer and incubated for 5 min with 0.1 μM goat F(ab′)2 anti-mouse IgG-conjugated Qdot 605, and goat F(ab′)2 anti-rabbit IgG-conjugated Qdot 655 (Invitrogen) in blocking buffer. Qdot labeling of non-epitope-tagged AQP4 isoforms in COS-7 or glial cell cultures was done using the same method. Cells were incubated for 8 min in 5 μg/ml rAb-53 in blocking buffer, rinsed 5 times with GP buffer, and incubated the 5 min with goat F(ab′)2 anti-human IgG-conjugated Qdot 655 (Invitrogen) in blocking buffer. After incubation with Qdots, cells were rinsed extensively and maintained throughout experiments in GP buffer.

In some experiments S-palmitoylation was disrupted by incubation for specified times with 100 μM 2-bromopalmitate (Sigma) in cell culture medium beginning 6–12 h after transfection. To produce Ca2+- or kinase-mediated signaling, cells were incubated with 1 μM thapsigargin (Sigma), 20 μM forskolin (Sigma), or 0.2 μM phorbol 12-myristate-13-acetate (Sigma) in GP buffer for 10 min at 37 °C after Qdot labeling just before measurements.

Single Particle Tracking—SPT was done on a Nikon Eclipse TE2000S inverted epifluorescence microscope (Nikon, Melville, NY) equipped with a Nikon 100× TIRF oil immersion objective (numerical aperture 1.45) and a deep-cooled CCD camera (Hamamatsu EM-CCD, Bridgewater, NJ), with additional technical specifications described previously (35). Qdot fluorescence was excited using an E460SPUV excitation filter and 475DCXRU dichroic mirror and detected through D605/40m or D655/40m emission filters (Chroma, Rockingham, VT). Data were acquired continuously at 11 ms per frame (91 Hz) for 6 s at 37 °C within 30 min of the final wash step. For 2-color SPT, cells expressing many Qdots of each color were identified, then two successive 6-s acquisitions were made at each wavelength set.

Image sequences were analyzed, and trajectories were constructed as described previously (39). Briefly, the mean-squared displacement as a function of time (\( \langle r^2(t) \rangle \)) was constructed for each trajectory, and the diffusion coefficient \( D \) and offset (due to noise) were determined by a linear fit of the first three time steps of the mean-squared displacement,

\[
\langle r^2(t) \rangle_{1-3} = 4Dt + \text{offset} \tag{1}
\]

The offset was subtracted from each point, and the first quarter of the mean-squared displacement curve was fitted using a weighted nonlinear least-squares fitting algorithm (39). The range of an individual particle at selected time \( t \) was computed as

\[
\text{range}(t) = \langle r^2(t) \rangle_{1/2}^{1/2} \tag{2}
\]

Only trajectories greater than 200 steps were analyzed, with data sets composed of at least 200 trajectories from at least 20 different cells. Diffusion data are primarily reported in the form of the cumulative distribution of ranges at 1 s, where \( P(\text{range}) \) is defined as the probability that a particle range is less than or equal to a given distance at \( t = 1 \) s. The fraction of M23 and mutants assembled in OAPs was estimated as \( P(79 \text{ nm}) \), the range at which \( P(\text{range}) = 0.95 \) for pure M23 in COS-7 cells, as described (35).

Histograms of ranges of native AQP4 in glial cell cultures were fitted to a sum of distributions, each of the form \( f(x; A, α, β) \),

\[
f(x; A, α, β) = \frac{Aβ^α}{Γ(α)}x^{α-1}e^{-βx} \tag{3}
\]

where \( x \) is the range at 1 s, and \( Γ \) is the gamma function. In fitting the sum, shape parameters \( α \) and \( β \) in the component corresponding to tetramer (non-OAP) diffusion were fixed at those values determined for pure M1. The remaining fitted parameters describe the distribution of AQP4 diffusion in OAPs. Statistical significance (\( p \) value) for differences between distributions after deconvolution was determined using Pearson’s \( χ^2 \) test.

Electrophoresis and Immunoblotting—Whole brains were removed from a euthanized adult mouse and a juvenile rat. A 1-cm³ fragment of human adult male brain cortex was obtained from the University of California, San Francisco tissue core. Brain tissues were homogenized in 10 mM Tris buffer containing 250 mM sucrose and protease inhibitors. Homogenized brain as well as COS-7 and glial cell cultures were lysed with NativePAGE sample buffer (Invitrogen) containing 0.5% dodecyl-β-d-maltoside (EMD Chemicals, Gibbstown, NJ) for 10 min on ice. Lysates were centrifuged at 20,000 × g for 30 min at 4 °C, and the pellet was discarded. Total protein content was determined by Bradford assay. For Blue Native gel electrophoresis (BN-PAGE), 1–5 μg of protein was mixed with Coomassie G-250 at a detergent:Coomassie ratio of 4:1, loaded onto a NativePAGE 3–12% Bis-Tris gel along with NativeMARK molecular weight markers, and run with NativePAGE running buffers according to the manufacturer’s protocol (Invitrogen). For SDS-PAGE, the same cell lysates were denatured with NuPAGE lithium dodecyl sulfate sample buffer, loaded onto a NuPAGE 12% Bis-Tris gel gel along with SeeBlue Plus2 molecular weight markers, and run with NuPAGE MES SDS running buffer at 200 V (Invitrogen). Proteins were blotted onto polyvinylidene difluoride membranes (Millipore). After transfer, native proteins were fixed by soaking membranes for 15 min in 8% acetic acid and destained with methanol. Membranes were blocked with 5% nonfat milk and incubated with rabbit anti-AQP4 (Santa Cruz), rabbit anti-Myc (Santa Cruz), or rabbit anti-HA (Zymed Laboratories Inc.) primary antibodies at 4 °C overnight. Membranes were then rinsed, incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), rinsed extensively, and labeled proteins were detected by ECL Plus enzymatic chemiluminescence kit (Amersham Biosciences).

RESULTS

Two-color SPT Indicates Strong Interactions between M1 and M23 AQP4 Isoforms—We previously found that the diffusion of AQP4-M1 was slowed when co-expressed with AQP4-M23 (34), suggesting possible M1/M23 interactions. Also, Furman et al. (33) and Silberstein et al. (21) reported that co-transfection
of M1 and M23 produced smaller OAPs than transfection with M23 alone. However, a limitation of these prior studies to characterize M1/M23 interactions has been the inability to distinguish M1 from M23 in the membrane. To overcome this limitation, we generated AQP4 constructs containing Myc or HA epitopes inserted in the second extracellular loop of AQP4 (Fig. 1A). We found previously that insertion of a Myc tag at this position did not affect AQP4 plasma membrane trafficking or water channel function (34).

FIGURE 1. Labeling strategy for two-color single particle tracking. A, a AQP4 schematic shows Met-1 and Met-23 (black circles) in the cytoplasmic N terminus, transmembrane helices (gray), the positions of inserted Myc or HA sequence (orange) in the second extracellular loop, and consensus protein kinase A (cyan) and protein kinase C (yellow) phosphorylation sites. The expanded blue box shows the N-terminal sequence of AQP4 with potential sites of palmitoylation in M1 (green) and sites of mutation that reduce OAP formation in M23 (red). B, immunofluorescence of COS-7 cells transfected with M1.Myc (top), M23.HA (middle), or both (bottom) and stained with anti-Myc (green) or anti-HA (red) antibodies is shown. Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Bar, 50 μm. C, shown are representative trajectories of Qdot-labeled COS-7 cells at 37 °C transfected individually with M1.Myc (green) or M23.HA (red). Bar, 1 μm. D and E, shown is combined mean-squared displacement and cumulative distribution of the range at 1 s from COS-7 cells transfected with M23.HA and labeled with 655 nm Qdots (black), M23.HA labeled with 655 nm Qdots (red), M1.Myc labeled with 655 nm Qdots (gray), and M1.Myc labeled with 605 nm Qdots (green).
Aquaporin-4 Orthogonal Arrays

FIGURE 2. OAP modulation by co-expression of M1 and M23 isoforms in COS-7 cells. A, shown is the cumulative distribution of the range of AQP4 isoforms in COS-7 cells transfected with M23 only (black) or M1 only (gray) or co-transfected with M23 (red) and M1 (green) at M23-to-M1 ratios of 3:1 (top), 1:1 (middle), or 1:3 (bottom). B, shown are the combined distributions comparing all AQP4 diffusion when M1 and M23 were transfected together (solid) or separately (dashed), derived by summing P(range) curves in panel A weighted by the relative amounts. C, shown are immunoblots after BN-PAGE (top) and SDS-PAGE (bottom) of lysates from COS-7 cells transfected with AQP4 at indicated M23-to-M1 ratios and labeled with anti-AQP4. D, shown are immunoblots after BN-PAGE of the same cell lysates as in panel C but labeled with anti-HA (left) or anti-Myc (right) to identify individual AQP4 isoforms.

BN-PAGE was done to further investigate the co-mingling of M1 and M23 (Fig. 2, C and D). In cells expressing M23 alone, a large diffuse band migrating at >1200 kDa was visible by AQP4 immunoblot (Fig. 2C, top) along with a band at ~300 kDa corresponding to AQP4 tetramers. With increasing M1, the larger bands disappear, and the smaller bands and the tetramer band become more prominent. SDS-PAGE (lower panel) confirmed that the altered M23-to-M1 transfection ratio produced approximately proportionate changes in M1 and M23 protein expression. To identify AQP4 isoforms individually on native gels, BN-PAGE was done using Myc and HA antibodies to probe immunoblots (Fig. 2D). As probed by anti-HA antibody to visualize M23 (left panel), a reduced M23-to-M1 ratio resulted in the appearance of M23-containing OAP bands of smaller molecular size. Probing with anti-Myc antibody to visualize M1 (right panel) showed M1 presence in the OAP bands as well as more M1 in the tetramer band with a reduced M23-to-M1 ratio. M1, therefore, partitions at least partially into OAPs in the co-transfected cells.

OAP Structure Is Modulated by Palmitoylation of N-terminal Cysteines in AQP4-M1—Previous studies have suggested that OAP disruption by M1 might involve post-translational S-palmitoylation at Cys-13 and Cys-17 in the M1 N terminus. Suzuki et al. (40) found that the palmitoylation-null mutant M1-C13A/C17A, when expressed alone in transfected cells, formed OAPs in freeze-cleaved membranes. We found recently that this mutant expressed alone does not form OAPs in live cells at 37 °C but can form OAPs at reduced temperatures (41). The involvement of S-palmitoylation in OAP assembly was found only at reduced temperature, where incubation of native M1 with 2-bromopalmitate (BrPA) resulted in OAP formation at 10 °C. We postulated that palmitoylation is only partially destabilizing to OAPs, and although it has no effect on the diffusion of M1 (when expressed alone) at 37 °C, it might affect the ability of M1 to disrupt OAPs (at 37 °C) when M1 and M23 are co-expressed, as in native glial cell membranes.

Fig. 3A shows the effect of (de)palmitoylation of M1 on the diffusion of AQP4 in the presence of equimolar M23 at 37 °C. Maximum effect was achieved after 8–16 h of BrPA exposure, which resulted in slowed diffusion of both M1 and M23. BrPA had no effect on the diffusion of M1 or M23 when each was expressed alone (Fig. 3B). We then tested the ability of M1 cysteine mutants to disrupt M23 OAP formation. Single cysteine mutants M1-C13A and M1-C17A had a similar ability to disrupt M23 OAPs as native M1 (Fig. 3C), and as seen with native M1, BrPA treatment reduced this ability (Fig. 3D). After exposure to BrPA, the median range of AQP4 displacement at 1 s decreased from 84 to 49 nm with M1-C13A and from 87 to 43 nm with M1-C17A, as compared with a reduction from 130 to 48 nm with native M1 (Fig. 3E). These data implicate palmitoylation as a critical factor in M1-mediated regulation of OAP formation. As a key control, SPT was done in cells co-expressing M23 with the double cysteine (palmitoylation-null) mutant M1-C13A/C17A (Fig. 3C, bottom) at 37 °C. When expressed alone, the diffusion of M1-C13A/C17A was nearly identical to that of native M1. Remarkably, when co-expressed with M23, the M1-C13A/C17A mutant became largely immobilized, as seen for M23 alone. The palmitoylation-null mutant of M1 is, thus, unable to disrupt OAP formation by M23 but, instead, fully associates with M23 in large immobile OAPs.

We previously characterized point mutants in the AQP4-M23 N terminus with diminished ability to form OAPs (35, 41). When expressed in COS-7 cell membranes, <20% of M23-F26Q and <10% of M23-G28P tetramers assembled into arrays at 37 °C, whereas nearly all (non-mutated) M23 is present in assays. Interestingly, like the double cysteine (palmitoylation-null) mutant of M1 studied above, the M23-F26Q and M23-G28P M23 mutants completely lack the ability to actively disrupt array formation but fully assemble in immobile OAPs when co-expressed with native M23 (Fig. 4, A and B). As a control, we tested the effect of a non-OAP forming aquaporin, AQP1, when co-expressed with native AQP4-M23. As antici-
Aquaporin-4 Orthogonal Arrays

We developed a numerical method to resolve in native glial cells the number of AQ4P molecules in OAPs versus non-OAPs and the apparent size distribution of OAPs. This method is an extension of the threshold range analysis, as done in Fig. 4 and in prior studies (35, 41), which provides a good description of one-component systems. However, a simple threshold analysis provides limited biological insight in complex systems where M1 and M23 co-expression produce broad distributions of AQ4P tetramers in OAPs.

We began by examining the distributions of pure M23 and M1 in transfected glial cell cultures from AQ4P knock-out mice. As expected for SPT data, the populations obey a gamma distribution (42) (Fig. 6C). The distribution of ranges for native AQ4P in wild-type glial cells fits well to a sum of two gamma distributions (Fig. 6D, left), one of which was fixed to the canonical M1 distribution corresponding to free AQ4P tetramers (see “Experimental Procedures”). The second gamma distribution in the sum represents the diffusion of AQ4P molecules in OAPs versus non-OAPs and the apparent size distribution of OAPs. This method is an extension of the threshold range analysis, as done in Fig. 4 and in prior studies (35, 41), which provides a good description of one-component systems. However, a simple threshold analysis provides limited biological insight in complex systems where M1 and M23 co-expression produce broad distributions of AQ4P tetramers in OAPs.

Fig. 6A, left, shows SPT of native (non-epitope-tagged) AQ4P isoforms in transfected COS-7 cells, in which Qdot labeling was done with rAb-53. The expected distributions of diffusion range were found for M23 and M1. As an additional control, we found that the diffusion of native mouse M23 and M1, when transiently transfected into primary glial cell cultures from AQ4P knock-out mice, was similar to that seen in COS-7 cells (Fig. 6A, right). Notably, AQ4P diffusion in (non-transfected) glial cell cultures from wild-type mice was similar to that seen in COS-7 cells that were transfected with a 1:1 M23-to-M1 ratio (Fig. 6, A and B), supporting the conclusion that native M23 and M1 interact strongly in live glial cells at 37 °C.

We developed a numerical method to resolve in native glial cells the number of AQ4P molecules in OAPs versus non-OAPs and the apparent size distribution of OAPs. This method is an extension of the threshold range analysis, as done in Fig. 4 and in prior studies (35, 41), which provides a good description of one-component systems. However, a simple threshold analysis provides limited biological insight in complex systems where M1 and M23 co-expression produce broad distributions of AQ4P diffusion range as in Figs. 2 and 6A. We began by examining the distributions of pure M23 and M1 in transfected glial cell cultures from AQ4P knock-out mice. As expected for SPT data, the populations obey a gamma distribution (42) (Fig. 6C). The distribution of ranges for native AQ4P in wild-type glial cells fits well to a sum of two gamma distributions (Fig. 6D, left), one of which was fixed to the canonical M1 distribution corresponding to free AQ4P tetramers (see “Experimental Procedures”). The second gamma distribution in the sum represents the diffusion of AQ4P in OAPs. As summarized in Fig. 6D, right, there is a significant shift (p < 0.01) in the distribution of AQ4P in OAPs in wild-type glial cell cultures when compared with...
pure M23 OAPs, likely corresponding to a shift to smaller OAP size. Integration of the two distributions indicated that 51/11006 4% of AQP4 molecules were confined within OAPs in glial cell cultures from wild-type mice.

Regulated OAP Assembly in Primary Glial Cell Cultures—We tested in glial cell cultures the key prediction from M1/M23-co-transfected COS-7 cells that post-translational palmitoylation of M1 affects OAP assembly. Incubation of glial cell cultures overnight with BrPA produced a marked shift of the entire distribution of AQP4 diffusion to a lower range (Fig. 6E, top). Mathematical deconvolution of the OAP and non-OAP populations as described above indicated an increase to 81 ± 7% of AQP4 molecules within OAPs after treatment with BrPA and a small although significant (p < 0.01) increase in the distribution of OAP size, with the median range of the OAP fraction decreasing from 51 to 40 nm (Fig. 6, F and G). Palmitoylation, thus, affects OAP assembly in native glial cells.

We further tested whether OAP organization in primary glial cell cultures can be modified by the major cellular signaling pathways. A wide range of inter- and intracellular events in glial cells are regulated by Ca2+ oscillations and propagation of Ca2+ waves (43). Thapsigargin is a cell-permeable inhibitor of the sarco-endoplasmic reticulum Ca2+-ATPase that increases cytosolic Ca2+ concentration in all cell types including glial cells. Incubation of Qdot-labeled glial cell cultures with thapsigargin produced a modest increase in AQP4 diffusion (Fig. 6E, bottom), with the median range at 1 s increasing by 44% from 109 to 157 nm. We also tested activators of two major phosphorylation pathways; forskolin, which increases cyclic adenosine monophosphate and thereby activates protein kinase A, and phorbol 12-myristate-13-acetate (PMA), which activates protein kinase C. AQP4 contains one consensus protein kinase A phosphorylation site at Ser-111 and two protein kinase C sites at Thr-107 and Ser-180 (Fig. 1A). Although forskolin did not affect AQP4 diffusion, PMA produced a comparable effect to that of thapsigargin, with an increase in median range to 154 nm at 1 s (Fig. 6E, bottom). Interestingly, mathematical deconvolution of the distributions did not show significant changes (p > 0.05) in the apparent OAP size distribution with thapsigargin or PMA (Fig. 6F, bottom) but instead showed a 20% decrease in the overall fraction of OAP-assembled AQP4 tetramers (Fig. 6G). Thapsigargin, forskolin, or PMA had no effect on AQP4 diffusion in cells transfected with M1 or M23 alone (data not shown). Possible mechanisms of Ca2+- and protein kinase C-mediated regulation of AQP4 OAPs are discussed below.

DISCUSSION

Strong M1/M23 Interactions Are Responsible for Regulation of OAP Size—As mentioned above, co-expression of M1 with M23 reduces the size of AQP4 OAPs when compared with those formed by M23 alone, and large OAPs of > 100 particles, which are routinely seen in M23-transfected cells, are rare in glial cell membranes (33). The nature of the putative interactions between M1 and M23 has been largely unknown. Using a dual-labeling approach, we conclude here that M1 interacts strongly with M23, probably forming M1/M23 heterotetramers, and that M1 effectively partitions into OAPs. We found here that the diffusion of Myc-tagged M1 and HA-tagged M23 became nearly identical upon co-expression over a range of M23-to-M1 molar ratios and that M1 partially migrated in OAP bands on native gels (Fig. 2).
fined oscillations by AQP4 tetramers and diffusion of whole AQP4 arrays of various sizes, with smaller OAPs likely diffusing faster than larger OAPs. We and others have found by freeze-fracture electron microscopy that M23-only OAPs can vary in size from $10^2$ to $10^3$ intramembrane particles (33, 34). The distribution of ranges for pure M23 (Figs. 1 and 6A), thus, includes contributions from M23 diffusion in OAPs of different size. In Fig. 4 and in previous studies we used the range at which $P$ (range) = 0.95 for pure M23 as a threshold to estimate the fraction of AQP4 isoforms or mutants residing in OAPs under various conditions (35, 41). Such analysis is useful in simple one-component systems having relatively narrow OAP size distributions, as found for various AQP4 mutants, but has limited utility in describing more complex systems where AQP4 diffuses as free tetramer as well as OAP-only fractions. This approach provided useful information about whether selected maneuvers alter OAP size distribution and/or the fraction of AQP4 molecules within OAPs, as discussed further below.

**Regulation of OAPs by S-Palmitoylation**—A role for post-translational lipid modification of AQP4-M1 in OAP formation was first proposed by Fujiyoshi and co-workers (40), who showed formation of OAPs in cells transfected with cysteine mutants of M1. We reported conflicting data indicating that M1 cysteine mutants diffuse identically to native M1 in live cells at 37 °C (35). Recently, we were able to resolve these apparently contradictory results by showing that OAP formation by M1 cysteine mutants in live cells was temperature-dependent, with OAPs seen only at reduced temperature (41). By studying co-expressed M23 and M1 cysteine mutants here, we found that M1 palmitoylation does significantly alter AQP4 assembly at physiological temperature, not by altering the ability of M1 alone to form OAPs but, instead, by regulating the formation of mixed M1/M23 arrays. The physiological relevance of M1 palmitoylation on OAP regulation was shown here directly in primary glial cell cultures.

OAP formation is driven by hydrophobic interactions near the M23 N terminus, likely occurring at or near the plasma membrane. Prevention of OAP formation by M1 tetramers requires seven residues upstream from Met-23, although the specific sequence or palmitoylation state of these residues is not important (35). In a mixed AQP4 heterotetramer, the number of “active” N termini involved in OAP formation is dependent on the M1/M23 ratio, but in the absence of palmitoylatable cysteines M1 cannot modulate OAP formation, allowing growth into large immobile OAPs similar to those formed by M23 alone (Fig. 3C). We propose that in mixed heterotetramers the extra residues comprising the M1 N terminus actively block tetramer-tetramer contacts between adjacent M23 N termini, but to do so they must be anchored in the cytoplasmic face of
Aquaporin-4 Orthogonal Arrays

The plasma membrane near the site of interaction. Lipid modification provides the anchor, thereby allowing M1 to modulate OAP formation and growth.

The extent to which M1-C13A/C17A associated with OAPs when co-expressed with M23 was initially surprising. One might expect a significant fraction of AQP4 tetramers in the membrane to be M1-C13A/C17A homotetramers, which do not form OAPs on their own, and yet no population of freely diffusing M1-C13A/C17A was observed (Fig. 3C). However, we recently showed that M1-C13A/C17A homotetramers can...
form OAPs at reduced temperatures (41). Together, these data suggest that the additional residues in the M1 N terminus inhibit nucleation of M1 OAPs at 37 °C. Meanwhile, lipid anchoring of the N terminus in the plasma membrane further prevents M1 homotetramers from associating with prenucleated OAPs. Removing the barrier to nucleation either by lowering the temperature or by co-expression of M23 allows non-palmitoylated M1 homotetramers to assemble in OAPs.

S-Palmitoylation is a reversible modification that is highly regulated and involved in cellular signaling, but little is known about the mechanisms controlling the process (44). It remains to be determined whether and how glial cells actively regulate AQP4 assembly by altering the palmitoylation of M1.

Regulation of OAPs by Cellular Signaling—Fig. 6E shows that Ca²⁺- and protein kinase C-mediated signaling affect AQP4 assembly in glial cells. OAP assembly in live cells at 37 °C is controlled by at least three factors: M1/M23 ratio, M1 palmitoylation, and the amount of AQP4 in the membrane. Signaling events could potentially affect each of these processes. Earlier work showed increased endocytosis of AQP4 after exposure to phorbol esters (45) and apparent rearrangements of OAPs after exposure to histamine, which elevates Ca²⁺ (46). However, these previous experiments were performed in cells expressing M23 only, in which essentially all AQP4 is locked in large immobile arrays. It is unclear how phosphorylation of M23 alone could lead to OAP rearrangement. Indeed, we found no effect of PMA or thapsigargin in cells expressing only M23. In glial cells AQP4 exists in a dynamic state, with M1 appearing to be the major active modulator of OAP assembly. In mixed M1/M23 systems, preferential endocytosis of OAP-associated AQP4 could explain our observation of increased diffusion after treatment with thapsigargin or PMA. Non-specific depletion of AQP4 from the membrane could have a similar effect because OAP assembly is driven by thermodynamic processes of mass action (41).

Biological Relevance of Regulated OAP Assembly—The potential biological consequences of regulated AQP4 assembly are largely unknown. M1 expression in glia is higher than in other cell types where AQP4 is found, suggesting that AQP4 assembly is more dynamically regulated in the central nervous system than in peripheral tissues, where M23 is the predominant isoform. Assembly in small-to-medium-sized OAPs that can diffuse in the membrane may allow glial cells to more efficiently sort and traffic large numbers of water channels to specific locations, such as endfoot processes or lamellipodia. Upon arrival, further assembly into large, immobile arrays could prevent diffusion away from these sites where high water permeability is required, without the need for continuous energy-dependent regulation.

Recently, much AQP4 research has focused attention on the inflammatory demyelinating disease NMO, which produces optic nerve and spinal cord lesions (47). The majority of NMO patients contain in their serum autoantibodies (NMO-IgG) recognizing AQP4 on the surface of glial cells (12). A recent study suggested that NMO-IgG specifically targets AQP4 OAPs, based on imaging data showing preferential staining of M23-transfected cells but not M1-transfected cells (26). This finding suggests that OAPs are the site of NMO pathogenesis. We show here, however, that whole serum from an NMO patient as well as a monoclonal AQP4-specific recombinant antibody derived from an NMO patient (rAb-53) stains M1-transfected cells quite well (Fig. 5A). In general, serum from a single NMO patient contains many variants of NMO-IgG with differential abilities to stain AQP4 isoforms. The incorrect conclusion of the prior study that NMO-IgG stains only OAPs may be related to biased serum sampling or to inadequate sensitivity in detection of NMO-IgG staining of M1, perhaps because cellular M1 expression is uniform rather than punctate. The role, therefore, of AQP4 OAPs in the pathogenesis of NMO remains to be determined.

Conclusion—Our data from two-color SPT in transfected cells and SPT of native AQP4 in primary glial cell cultures have revealed new information about the nature of interactions between M1 and M23 isoforms and how these interactions modulate the assembly of AQP4 into OAPs. We found that M1 and M23 isoforms of AQP4 interact strongly, so much so that they are nearly indistinguishable when co-expressed in the same cell membrane. We confirmed suggestions based on direct evidence (48) that M1 co-associates with M23 in small OAPs. We found that AQP4 assembly is strongly dependent on the M1-to-M23 molar ratio. We also found that OAPs can be actively regulated in primary glial cell cultures by altering reversible S-palmitoylation of N-terminal cysteines in M1 or by Ca²⁺- and phosphorylation-mediated signaling. However, these active processes of regulation require co-expression of M1 and M23 isoforms, as no effect was found on the assembly of either isoform alone at 37 °C. Our findings, thus, mandate a shift in thinking about AQP4 assembly in the central nervous system away from models concerning the behaviors of distinct M1 and M23 isoforms but, instead, to models representative of real glial cells in which M1 and M23 are co-expressed, and OAP formation is regulated through signaling processes that control AQP4 M1/M23 ratio, S-palmitoylation, and phosphorylation state.

Acknowledgments—we thank Dr. Hua Zhang, Dr. Courtney Crane, Dr. Jianhua Ran, and Wali Danish for providing brain tissues and primary glial cell cultures.

REFERENCES

1. Nielsen, S., Nagelhus, E. A., Amiry-Moghaddam, M., Bourque, C., Agre, P., and Ottersen, O. P. (1997) J. Neurosci. 17, 171–180
2. Rash, J. E., Yasumura, T., Hudson, C. S., Agre, P., and Nielsen, S. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 11981–11986
3. Manley, G. T., Fujimura, M., Ma, T., Noshita, N., Filiz, F., Bollen, A. W., Chan, P., and Verkman, A. S. (2000) Nat. Med. 6, 159–163
4. Saadoun, S., Bell, B. A., Verkman, A. S., and Papadopoulos, M. C. (2008) Brain 131, 1087–1098
5. Yang, B., Zador, Z., and Verkman, A. S. (2008) J. Biol. Chem. 283, 15280–15286
6. Papadopoulos, M. C., Manley, G. T., Krishna, S., and Verkman, A. S. (2004) FASEB J. 18, 1291–1293
7. Bloch, O., Auguste, K. I., Manley, G. T., and Verkman, A. S. (2006) J. Cereb. Blood Flow Metab. 26, 1527–1537
8. Padmawar, P., Yao, X., Bloch, O., Manley, G. T., and Verkman, A. S. (2005) Nat. Methods 2, 825–827
9. Binder, D. K., Yao, X., Zador, Z., Sick, T. J., Verkman, A. S., and Manley, G. T. (2006) Glia 53, 631–636
Aquaporin-4 Orthogonal Arrays

10. Saadoun, S., Papadopoulos, M. C., Watanabe, H., Yan, D., Manley, G. T., and Verkman, A. S. (2005) *J. Cell Sci.* **118**, 5691–5698

11. Auguste, K. I., Jin, S., Uchida, K., Yan, D., Manley, G. T., Papadopoulos, M. C., and Verkman, A. S. (2007) *FASEB J.* **21**, 108–116

12. Lennon, V. A., Kryzer, T. J., Pittock, S. J., Verkman, A. S., and Hinson, S. R. (2005) *Exp. Med.* **202**, 473–477

13. Hinson, S. R., Pittock, S. J., Lucchetti, C. F., Roemer, S. F., Fryer, J. P., Kryzer, T. J., and Lennon, V. A. (2007) *Neurology* **69**, 2221–2231

14. Landis, D. M., and Reese, T. S. (1974) *J. Cell Biol.* **60**, 316–320

15. Wolburg, H. (1995) *J. Hirnforsch.* 36, 239–258

16. Frigeri, A., Gropper, M. A., Umenishi, F., Kawashima, M., Brown, D., and Verkman, A. S. (1995) *J. Cell Sci.* **108**, 2993–3002

17. Yang, B., Brown, D., and Verkman, A. S. (1996) *J. Biol. Chem.* **271**, 4577–4580

18. Verbavatz, J. M., Ma, T., Gobin, R., and Verkman, A. S. (1997) *J. Cell Sci.* **110**, 2855–2860

19. Yang, B., van Hoek, A. N., and Verkman, A. S. (1997) *Biochemistry* **36**, 7625–7632

20. van Hoek, A. N., Ma, T., Yang, B., Verkman, A. S., and Brown, D. (2000) *Am. J. Physiol. Renal Physiol.* **278**, F310–F316

21. Silberstein, C., Bouley, R., Huang, Y., Fang, P., Pastor-Soler, N., Brown, D., and Van Hoek, A. N. (2004) *Am. J. Physiol. Renal Physiol.* **287**, F501–F511

22. Amyr-Moghadam, M., Frydenlund, D. S., and Ottersen, O. P. (2004) *Neuroscience* **129**, 999–1010

23. Hiroaki, Y., Tani, K., Kamegawa, A., Gyobu, N., Nishikawa, K., Suzuki, H., Walz, T., Sasaki, S., Mitsuoka, K., Kimura, K., Mizoguchi, A., and Fujiyoshi, Y. (2006) *J. Mol. Biol.* **355**, 628–639

24. Schotland, D. L., Bonilla, E., and Wakayama, Y. (1981) *Acta Neuropathol.* **54**, 189–197

25. Hatton, J. D., and Ellisman, M. H. (1984) *Epilepsia* **25**, 145–151

26. Nicchia, G. P., Mastrototaro, M., Rossi, A., Pisani, F., Tortorella, C., Ruggieri, M., Lia, A., Trojano, M., Frigeri, A., and Svelto, M. (2009) *Glia* **57**, 1363–1373

27. Hasegawa, H., Ma, T., Skach, W., Matthyay, M. A., and Verkman, A. S. (1994) *J. Biol. Chem.* **269**, 5497–5500

28. Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B., Baraban, J. M., and Agre, P. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 13052–13056

29. Yang, B., Ma, T., and Verkman, A. S. (1995) *J. Biol. Chem.* **270**, 22907–22913

30. Lu, M., Lee, M. D., Smith, B. L., Jung, J. S., Agre, P., Verdijk, M. A., Merkx, G., Rijs, J. P., and Deen, P. M. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10908–10912

31. Neely, J. D., Christensen, B. M., Nielsen, S., and Agre, P. (1999) *Biochemistry* **38**, 11156–11163

32. Moe, S. E., Sorbo, J. G., Sogaard, R., Petter Ottersen, O., and Holen, T. (2008) *Genomics* **91**, 367–377

33. Furman, C. S., Gorelick-Feldman, D. A., Davidson, K. G., Yasumura, T., Neely, J. D., Agre, P., and Rash, J. E. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 13609–13614

34. Crane, J. M., Van Hoek, A. N., Skach, W. R., and Verkman, A. S. (2008) *Mol. Biol. Cell* **19**, 3369–3378

35. Crane, J. M., and Verkman, A. S. (2009) *J. Cell Sci.* **122**, 813–821

36. Ma, T., Yang, B., Gillespie, A., Carlson, E. J., Epstein, C. J., and Verkman, A. S. (1997) *J. Clin. Invest.* **100**, 957–962

37. Wingerchuk, D. M., Lenon, V. A., Pittock, S. J., Lucchini, C. F., and Weinshenker, B. G. (2006) *Neurology* **66**, 1485–1489

38. Bennett, J. L., Lam, C., Kalluri, S. R., Saikali, P., Bautista, K., Dupree, C., Glogowska, M., Case, D., Antel, J. P., Owens, G. P., Gilden, D., Nessler, S., Stadelmann, C., and Hemmer, B. (2009) *Ann. Neurol.*, in press

39. Crane, J. M., and Verkman, A. S. (2008) *Biophys. J.* **94**, 702–713

40. Suzuki, H., Nishikawa, K., Hiroaki, Y., and Fujiyoshi, Y. (2008) *Biochim. Biophys. Acta* **1778**, 1181–1189

41. Crane, J. M., and Verkman, A. S. (2009) *Biophys. J.*, in press

42. Sonnleitner, A., Schutz, G. J., and Schmidt, T. (1999) *Biophys. J.* **77**, 2638–2642

43. Simard, M., and Nedergraad, M. (2004) *Neuroscience* **129**, 877–896

44. Dietrich, L. E., and Ungermann, C. (2004) *EMBO Rep.* **5**, 1053–1057

45. Han, Z., Wax, M. B., and Patil, R. V. (1998) *J. Biol. Chem.* **273**, 6001–6004

46. Carmosino, M., Procino, G., Nicchia, G. P., Mannucci, R., Verbavatz, J. M., Gobin, R., Svelto, M., and Valenti, G. (2001) *Epilepsia* **42**, 1235–1243

47. Wingerchuk, D. M., Lennon, V. A., Lucchini, C. F., Pittock, S. J., and Weinshenker, B. G. (2007) *Lancet Neurol.* **6**, 805–815

48. Sorbo, J. G., Moe, S. E., Ottersen, O. P., and Holen, T. (2008) *Biochemistry* **47**, 2631–2637