Surface Accessibility and Conformational Changes in the N-terminal Domain of Type I Inositol Trisphosphate Receptors

STUDIES USING CYSTEINE SUBSTITUTION MUTAGENESIS

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To identify surface-accessible residues and monitor conformational changes of the type I inositol 1,4,5-trisphosphate receptor protein in membranes, we have introduced 10 cysteine substitutions into the N-terminal ligand-binding domain. The reactivity of these mutants with progressively larger maleimide-polyethylene glycol derivatives (MPEG) was measured using a gel shift assay of tryptic fragments. The results indicate that the mutations fall into four categories as follows: sites that are highly accessible based on reactivity with the largest 20-kDa MPEG (S2C); sites that are moderately accessible based on reactivity only with 5-kDa MPEG (S6C, S7C, A189C, and S277C); sites whose accessibility is markedly enhanced by Ca2+ (S171C, S277C, and A575C); and sites that are inaccessible irrespective of incubation conditions (S217C, A245C, and S436C). The stimulation of accessibility induced by Ca2+ at the S277C site occurred with an EC50 of 0.8 μM and was mimicked by Sr2+ but not Ba2+. Inositol 1,4,5-trisphosphate alone did not affect reactivity of any of the mutants in the presence or absence of Ca2+. The data are interpreted using crystal structures and EM reconstructions of the receptor. Our data identify N-terminal regions of the protein that become exposed upon Ca2+ binding and suggest possible orientations of the suppressor and ligand-binding domains that have implications for the mechanism of gating of the channel.

Inositol 1,4,5-trisphosphate receptors (IP3R)2 are ligand-gated channels important in Ca2+ signaling triggered by diverse cellular stimuli (1). Three different isoforms are present that share 60–70% sequence homology (2–4). The type I isoform is subject to alternative splicing at three sites (5). IP3R channel activity only with 5-kDa MPEG (S6C, S7C, A189C, and S277C); sites that are moderately accessible based on reactivity with the largest 20-kDa MPEG (S2C); sites that are inaccessible irrespective of incubation conditions (S217C, A245C, and S436C). The stimulation of accessibility induced by Ca2+ at the S277C site occurred with an EC50 of 0.8 μM and was mimicked by Sr2+ but not Ba2+. Inositol 1,4,5-trisphosphate alone did not affect reactivity of any of the mutants in the presence or absence of Ca2+. The data are interpreted using crystal structures and EM reconstructions of the receptor. Our data identify N-terminal regions of the protein that become exposed upon Ca2+ binding and suggest possible orientations of the suppressor and ligand-binding domains that have implications for the mechanism of gating of the channel.

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2 The abbreviations used are: IP3R, myo-inositol 1,4,5-trisphosphate receptor; IP3R, myo-inositol 1,4,5-trisphosphate; LBD, ligand-binding domain; Ab, antibody; MPEG, methoxy-polyethylene glycol maleimide; DTT, dithiothreitol; HEDTA, N-(2-hydroxyethyl)ethylenediaminetriacetic acid; TM, transmembrane; PEG, polyethylene glycol.
study the receptor had adopted the more extended structure even in the absence of Ca\(^{2+}\) (20). Ca\(^{2+}\) has also been shown to have effects on an isolated N-terminal fusion protein (amino acids 1–604) that is consistent with enhanced rotational flexibility of the three independently folded domains around two flexible linkers (21).

Almost all studies on the conformation of IP\(_3\)Rs have utilized fusion proteins or isolated detergent-purified receptor. Our knowledge on IP\(_3\)Rs would benefit from the application of methods that might provide information on structure and conformational dynamics of the tetrameric, full-length receptor in its native membrane environment. In this study, we have utilized the thiol-reacting agent, maleimide polyethylene glycol (MPEG), to gain information on the accessibility of endogenous and mutant thiol groups in the protein. Previously, we showed that only tryptic fragments I and III of the type I IP\(_3\)R contained highly reactive cysteines capable of reacting with large MPEGs (22). Furthermore, the reactivity of trypsin fragment I was observed only in the SI(−) splice variant (22). The absence of MPEG reactivity in the SI(−) splice form provided a null background into which cysteine substitution mutants could be introduced. This was carried out in this study, and the relative accessibility of these mutant cysteines was determined using MPEG molecules of various sizes (MPEG-2, -5, and -20 kDa). We have focused our study to fragments I and II that encompass the suppressor domain and LBD because crystal structures for these domains are available (7, 23). The MPEG reactivity experiments were performed in the absence and presence of Ca\(^{2+}\) and/or IP\(_3\) to determine whether accessibility of MPEG is altered by modulation of the receptor. Changes in accessibility are interpreted as reflecting conformational changes of the protein in native membranes. The data are discussed in relation to the available structural models of the IP\(_3\)R.

**EXPERIMENTAL PROCEDURES**

*Materials*—MPEGs of 2, 5, and 20 kDa were obtained from Nektar Therapeutics (San Carlos, CA). Dithiothreitol (DTT) was purchased from Sigma. \(^{45}\)Ca\(^{2+}\) was purchased from Amer-
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between amino acids 420 and 606 was amplified using PCR primers containing flanking KpnI and XhoI sites. This fragment was introduced into a full-length type I IP$_3$R containing a “silent” XhoI site (26).

Cell Culture and Transient Transfection—COS-7 cells were grown on 150-mm plates (Sarstedt) in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 0.1 mg/ml streptomycin (Invitrogen), and 100 IU/ml penicillin (Invitrogen) until 70–80% confluent. 15 μg of IP$_3$R DNA was transfected into COS cells containing Dulbecco’s modified Eagle’s medium without serum using LT-1 (Mirus) and NovoFECT (VennNova, Inc.) at a lipid to DNA ratio of 1:1. After 24 h, serum containing Dulbecco’s modified Eagle’s medium was added, and cells were allowed to grow for 48–72 h.

Preparation of Microsomes—Cells were washed twice with ice-cold phosphate-buffered saline and scraped into a sucrose-based buffer containing 0.5 M sucrose, 20 mM Tris-HCl, pH 7.8, 50 μM EDTA and protease inhibitor mixture tablet (Roche Applied Science). Cells were lysed by passage through a 26.5-gauge needle 10 times. The lysates were spun at 2,500 rpm for 5 min to remove debris, and the supernatants were spun at 40,000 rpm for 50 min. The vesicle pellets were resuspended in storage buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 7.8, and 50 μM EGTA). The vesicles were used fresh or stored at −80 °C for further use.

Reaction with MPEG and Trypsin Digestion—MPEG reactions were carried out as described previously (22) with few modifications. Briefly, microsome preparations were incubated at a final protein concentration of 0.5 mg/ml in a buffer containing 120 mM NaCl, 20 mM Tris-HCl, pH 7.2, and 0.5 mM EDTA in the presence or absence of MPEG-2, MPEG-5, or MPEG-20. Incubations were carried out at room temperature for 5 min. Analysis of the kinetics of reactivity of selected mutants (S2C and S6C) showed that the reaction was complete within 15–20 min (data not shown). A time point of 5 min was selected as suitable to observe any effects of Ca$^{2+}$ or IP$_3$ on the reaction process. The reaction was stopped by the addition of 20 mM DTT. Trypsin digestion was carried out subsequent to MPEG reaction at a final concentration of 6 μg/ml for 5 min at 80°C. Incubations were carried out by addition of 10 μg/ml trypsin to the MPEG reaction, and shifted bands are marked by “open triangle.”

| ATrypsin Fragment I | BTrypsin Fragment II |
|---------------------|---------------------|
| IP$_3$ | Ca$^{2+}$ | MPEG-5 kDa |
| 54 | | |
| 38 | | |

**Figure 2.** MPEG-5 kDa reactivity of fragments I and II. A, microsome containing type I IP$_3$R S1(−) or the cysteine substitution mutants were incubated with 500 μM MPEG-5 kDa for 5 min in the presence or absence of 2.2 μM Ca$^{2+}$, 10 μM IP$_3$, or both. The reaction was stopped by addition of 20 mM DTT, and samples were subsequently digested with 6 μg/ml trypsin for 5 min to generate IP$_3$R fragmentic tryptic fragments. Protein samples were run on 10% SDS-PAGE, and tryptic fragments were immunoblotted with NT-1 antibody. Tryptic fragment I (−40 kDa) is indicated by the open triangle, and shifted bands are marked by closed triangles. B, reactivity of MPEG-5 kDa with fragment II cysteine substitution mutants (S436C and A575C). Experiments were performed as indicated above. The fragment II band (open triangle) appears at −67 kDa as detected by immunoblotting with KEEK and or/N3 antibody. The results shown are representative of three or more independent experiments.

sham Biosciences. All other chemicals and reagents were purchased from Fisher.

Antibodies—Previously characterized Abs against rat type I IP$_3$R were used in this study were as follows: tryptic fragment I Ab to amino acids 326–341 designated as NT-1 (22) and tryptic fragment II Ab to amino acids 401–414 designated as KEEK (22). An additional Ab against trypsin fragment II was raised against amino acids 326–341 designated as NT-1 (22) and tryptic fragment II Ab to amino acids 401–414 designated as KEEK (22). An additional Ab against trypsin fragment I was raised against amino acids 501–517 and was designated N3 (13).

Expression Constructs—cDNA encoding rat type I IP$_3$R S1(−)/SII(+)/SIII(−) splice variant in pCMV3 was the gift of Dr. Thomas Sudhof (24). The cDNA encoding the rat type I IP$_3$R S1(−)/SII(+) splice variant was the gift of Dr. Gregory Mignery (Department of Physiology, Loyola University, Chicago, IL) (25). Cysteine substitutions at positions Ser-2, Ser-6, Ser-7, Ser-171, Ala-189, Ser-217, Ala-245, and Ser-277 were generated using the QuikChange mutagenesis system (Stratagene, La Jolla, CA) using a template of IP$_3$R S1(−) in pBluescript. The mutations were transferred to the full-length receptor using KpnI restriction sites. The cysteine substitutions at positions Ser-436 and Ala-575 were made with the QuikChange kit using the pGEX-LBD plasmid encoding amino acids 1–601 of the S1(−) splice variant of the LBD. The segment containing the mutations was amplified using PCR primers containing flanking KpnI and XhoI sites. This fragment was introduced into a full-length type I IP$_3$R containing a “silent” XhoI site (26).

Cells were washed twice with ice-cold phosphate-buffered saline and scraped into a sucrose-based buffer containing 0.5 M sucrose, 20 mM Tris-HCl, pH 7.8, 50 μM EDTA and protease inhibitor mixture tablet (Roche Applied Science). Cells were lysed by passage through a 26.5-gauge needle 10 times. The lysates were spun at 2,500 rpm for 5 min to remove debris, and the supernatants were spun at 40,000 rpm for 50 min. The vesicle pellets were resuspended in storage buffer (0.5 M sucrose, 20 mM Tris-HCl, pH 7.8, and 50 μM EGTA). The vesicles were used fresh or stored at −80 °C for further use.

**Figure 2.** MPEG-5 kDa reactivity of fragments I and II. A, microsome containing type I IP$_3$R S1(−) or the cysteine substitution mutants were incubated with 500 μM MPEG-5 kDa for 5 min in the presence or absence of 2.2 μM Ca$^{2+}$, 10 μM IP$_3$, or both. The reaction was stopped by addition of 20 mM DTT, and samples were subsequently digested with 6 μg/ml trypsin for 5 min to generate IP$_3$R fragmentic tryptic fragments. Protein samples were run on 10% SDS-PAGE, and tryptic fragments were immunoblotted with NT-1 antibody. Tryptic fragment I (−40 kDa) is indicated by the open triangle, and shifted bands are marked by closed triangles. B, reactivity of MPEG-5 kDa with fragment II cysteine substitution mutants (S436C and A575C). Experiments were performed as indicated above. The fragment II band (open triangle) appears at −67 kDa as detected by immunoblotting with KEEK and or/N3 antibody. The results shown are representative of three or more independent experiments.
Hepes, pH 7.5, 0.5 mM HEDTA, and different concentrations of CaCl₂ to achieve the indicated concentrations of free Ca²⁺ as determined by calibration with the fluorescent dyes Calcium Orange and Calcium Green-5N (Invitrogen). The Kₐ values used in these calculations were 0.22 and 14 μM, respectively. Where Sr²⁺ and Ba²⁺ were used, the free divalent cation concentrations were calculated using the program MAXC, available on line. All experiments were repeated three times from three separate microsome preparations.

Electrophoresis and Immunoblotting—For protein expression, microsomes were run on 5% SDS-polyacrylamide gels. For MPEG experiments, microsomes were run on either 10% (MPEG-5 and -20) or 12% 16-cm gels (MPEG-2). Gels were transferred to nitrocellulose membranes and blocked in a 10% milk solution in Tris-buffered containing 0.1% Tween 20. Membranes were incubated with affinity-purified primary antibodies NT-1 (1:5000) or KEEK (1:1000) for 2 h and anti-rabbit secondary horseradish peroxidase antibody for 1 h. Membranes were developed with chemiluminescent substrates (Pierce). In cases where a blot was probed sequentially with more than one antibody, the nitrocellulose was stripped at 65 °C for 30 min in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8) before probing with the next antibody. All immunoblots shown are representative of three or more experiments.

**RESULTS**

Reactivity of Cysteine Substitution Mutants with MPEG-5—Fig. 1 shows the location of the 10 cysteine substitution mutants used in this study. Their positions within the primary sequence (Fig. 1, A and B) and within the published crystal structure of the suppressor and core LBD (Fig. 1, C and D) are indicated. Because the crystal structures do not provide information on the accessibility of residues in the native tetrameric receptor, the choice of residues to mutate was largely empirical. With the exception of Ala-575, all the selected residues were in loops located between secondary structures. In addition, the Ser-436 residue is the known site of phosphorylation by ERK2 mitogen-activated protein kinase (MAPK) (28, 29), and the Ser-217 residue, when mutated to phenylalanine in *Drosophila* IP₃R, enhances the IP₃ sensitivity of channel function (30). All the...
mutants expressed normally when transiently transfected into COS-7 cells (see individual figures). The functional responsiveness of the mutants was measured with saturating doses of IP₃ using ^{45}Ca²⁺ flux assays performed on microsomes prepared from transfected cells (supplemental Fig. S1). Only the A189C mutant was found to be functionally inactive.

Accessibility of the various cysteine mutants was determined in microsomes prepared in the absence of thiol reductants and incubated with 0.5 mM MPEG-5 for 5 min in the presence or absence of Ca²⁺ (2.2 μM) and/or IP₃ (10 μM). Reaction of sites located within the 40-kDa trypsin fragment I band was observed by gel shifts detected

**FIGURE 4. MPEG-2 kDa reactivity of fragments I and II.** A, microsomes containing IP₃R1 SI(−) or fragment I cysteine substitution mutants were incubated with 500 μM MPEG-2 kDa, and experiments were performed as stated in Fig. 2A with a slight modification. The protein samples were run on 12% SDS-PAGE 16-cm gels to obtain proper separation of MPEG reactive bands. An interpretation of the banding pattern for the S171C mutant is shown as a schematic assuming occupation of two endogenous and one mutant site. B, same experiment repeated with fragment II mutants, but immunoblotted with the KEEK and/or N3 antibody. The results shown in the figure are representative of three or more independent experiments.

**FIGURE 5. Reactivity of MPEG-5 and -20 kDa in DTT-treated microsomes.** Microsomes were prepared in the presence of 1 mM DTT and treated with MPEG-5 (A) or MPEG-20 (B) as indicated above. The wild-type IP₃R1 SI(−) was used as the control. Fragment (Frag) I was detected with NT-1 Ab, and fragment II was detected with N3 Ab.
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A

Suppressor domain

β-trefoil domain

α-helical domain

Ca²⁺

B

MPEG-20
~130 Å

MPEG-5
~50 Å

Ca²⁺

C

SIDE

TOP
by immunoblotting with NT-1 Ab (Fig. 2A). Similarly, reactivity of sites within the 64-kDa trypsin fragment II was detected by immunoblotting with KEEK Ab (Fig. 2B). As observed previously, the eight endogenous cysteine residues in fragment I of the wild-type SI(–) type I IP₃R did not react with MPEG-5 (22) (Fig. 2A, top panel, compare lanes 1 and 2). Similarly, we observed no reactivity for the S2C, S171C, S217C, and A245C mutants indicating that these sites are also inaccessible to MPEG-5. By contrast, the S6C, S7C, A189C, and S277C sites were reactive indicating that these locations are exposed in the IP₃R structure. Ca²⁺, which is known to induce large conformational changes in the detergent-solubilized purified protein (31), would be expected to induce changes in accessibility at some sites. The inclusion of Ca²⁺ did not enhance the reactivity of endogenous cysteines in fragment I of the wild-type SI(–) receptor (Fig. 2A, top panel, compare lanes 2 and 3). A Ca²⁺-induced enhancement of reactivity was seen for the S6C, S7C, and A189C mutants. Analysis of the time course of reactivity of the S6C mutant indicated that the effect of Ca²⁺ was primarily to accelerate the rate of the reaction with MPEG-5 (data not shown). The most dramatic change was observed for the S171C mutant that showed substantial reactivity with MPEG-5 only in the presence of Ca²⁺. The pattern of MPEG-5 reactivity for wild-type and all mutant receptors was not altered by 10 μM IP₃, which is known to induce large conformational changes in the receptor (Fig. 2B, compare lanes 1 and 2). As observed previously, the two endogenous sites were observed at 0.8 μM IP₃, and the behavior of the S171C and S277C sites with MPEG-2 was similar to that observed with MPEG-5. Notably, the S171C site reacted only in the presence of Ca²⁺, whereas the S277C was constitutively reactive. In both cases, the presence of Ca²⁺ generated a prominent doublet of markedly shifted bands (Fig. 4A, lower two panels, lane 3). The number of shifted bands is consistent with the presence of at least three reactive sites in the presence of Ca²⁺ encompassing two endogenous sites and a third contributed by the mutant cysteine (see schematic in Fig. 4A). Occupation of the mutant cysteine in combination with one or both endogenous sites would produce a doublet of shifted bands. The data suggest that the use of sufficiently small MPEG derivatives may allow visualization of the exposure of endogenous cysteines during Ca²⁺-associated conformational changes. Although these endogenous sites were not located in the present study, the apparently additive nature of the shifts suggests that the reactive sites are sufficiently separated to avoid steric interference when multiple sites are occupied.

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**Reactivity of Endogenous and Mutant Cysteines with MPEG-2**—The analysis of the IP₃R reactivity with the smaller 2-kDa MPEG derivative was complicated by two factors. First, resolution of the smaller shifts in reactivity required analysis on larger (16 cm) SDS-polyacrylamide gels. Second, the smaller MPEG-2 formed adducts with endogenous as well as cysteine substitution mutants and therefore gave a more complex pattern of bands. This is illustrated for selected IP₃R constructs in Fig. 4. The wild-type SI(–) showed reactivity at one endogenous site that was enhanced in the presence of Ca²⁺ (Fig. 4A, top panel, lanes 2 and 3). A weaker additional reactivity at a second endogenous site was observed in the presence of Ca²⁺ at higher exposures of the blot (data not shown). The S2C, S217C, and A245C mutants, which were nonreactive with MPEG-5, were also nonreactive with MPEG-2 as judged by the presence of the same background bands observed with wild-type SI(–) (Fig. 4A, 2nd panel, and data not shown for S217C and A245C). The data on MPEG-2 reactivity of the S436C and A575C also confirmed a lack of reactivity above the wild-type background in the absence of Ca²⁺, although the A575C site reacted with MPEG-2 in the presence of Ca²⁺ (Fig. 4B, 3rd panel, lanes 3 and 5). The behavior of the S171C and S277C sites with MPEG-2 was similar to that observed with MPEG-5. Notably, the S171C site reacted only in the presence of Ca²⁺, whereas the S277C was constitutively reactive. In both cases, the presence of Ca²⁺ generated a prominent doublet of markedly shifted bands (Fig. 4A, lower two panels, lane 3). The number of shifted bands is consistent with the presence of at least three reactive sites in the presence of Ca²⁺ encompassing two endogenous sites and a third contributed by the mutant cysteine (see schematic in Fig. 4A). Occupation of the mutant cysteine in combination with one or both endogenous sites would produce a doublet of shifted bands. The data suggest that the use of sufficiently small MPEG derivatives may allow visualization of the exposure of endogenous cysteines during Ca²⁺-associated conformational changes. Although these endogenous sites were not located in the present study, the apparently additive nature of the shifts suggests that the reactive sites are sufficiently separated to avoid steric interference when multiple sites are occupied.

**FIGURE 6. Summary of data and model of proposed location of reactive cysteines in the three-dimensional structure of the receptor.** A shows the three independently folded domains that constitute the N-terminal segment of the receptor and the location of the 10 cysteine substitution mutants used in this study. The accessibility to each site in the presence or absence of Ca²⁺ is indicated by the colored spheres of different sizes. The data with MPEG-2 have been omitted for clarity, but it was verified that all sites accessible to MPEG-5 are also accessible to MPEG-2. Reactivity at the S2C site was observed only when membranes are prepared in the presence of DTT, G. cross-section through two subunits of the receptor according to the EM reconstruction of da Fonseca et al. (43) has been used to illustrate a model of the localization of MPEG-reactive sites. For additional details see text. C, crystal structure of the suppressor domain (Protein Data Bank code 1XZZ) and core LBD (Protein Data Bank code 1N4K) were docked using the program GRAMM. The docked structure was oriented with the MPEG-reactive sites in the suppressor domain facing the surface of the central density. The figure is for illustration only and does not attempt to rigorously fit the crystal structures into the EM density. A side view and a view from the cytosol are shown.
Thiol Reactivity of IP$_3$ Receptors

Reactivity of Cysteine Substitution Mutants with MPEG Derivatives in DTT-treated Membranes—A lack of reactivity of the S2C mutant with MPEG-5 (Fig. 2) and MPEG-2 (Fig. 4A) could indicate that the N-terminal region is buried within the native receptor and is therefore occluded. This appears inconsistent with previous observations that showed that N-terminally tagged green fluorescent protein constructs were functionally active (29) and that N-terminally epitope-tagged IP$_3$Rs can be readily immunoprecipitated (33). Similarly, the lack of reactivity of the S436C position is surprising because this site lies within a flexible linker region between two domains (23) and is the site phosphorylated by ERK2, a 42-kDa enzyme (29). Our routine preparation method for membranes avoids the use of thiolic reductants in buffers so as to avoid compromising subsequent reaction with MPEGs. It is therefore possible that highly reactive cysteines may already have been modified and are therefore unavailable for MPEG reaction. To test this hypothesis, membranes were prepared in the presence of 1 mM DTT. The wild-type SI(-) DTT-treated membranes did not react with MPEG-5 or -20 as observed for untreated membranes (Fig. 5, A and B, top panels, lane 2). However, a shifted band was observed with MPEG-5 in the presence of Ca$^{2+}$ (Fig. 5A, top panel, lanes 3 and 5) indicating that an endogenous thiol is available for limited reaction in DTT-treated membranes. In contrast to the data observed in Fig. 2 with untreated membranes, the S2C mutant reacted with MPEG-5 and MPEG-20 in DTT-treated membranes (Fig. 5, A and B, 2nd panel). Reactivity of the S2C position with MPEG-20 was enhanced in the presence of Ca$^{2+}$ (Fig. 5B, 2nd panel, lanes 3 and 5). In contrast to the S2C mutant, the S436C mutant did not react with MPEG-5 (Fig. 5A, compare with wild-type SI(-)) or MPEG-20 (Fig. 5B) in DTT-treated membranes. The behavior of other mutants that were unreactive with MPEG-5 (S217C and A245C) was also not altered by DTT treatment (data not shown).

DISCUSSION

Fig. 6A summarizes the experimental observations made in this study regarding accessibility of the indicated 10 cysteine substitution mutants to MPEG derivatives of various sizes. The schematic in Fig. 6A locates these sites within the main structural domains identified in the N-terminal region of the receptor, namely, the suppressor domain and the core LBD made up of the $\beta$-trefoil and $\alpha$-helical domains. The results indicate that the mutants fall into four categories as follows: sites that are highly accessible, sites that are moderately accessible, sites whose accessibility is enhanced by Ca$^{2+}$, and sites that are inaccessible irrespective of incubation conditions.

The S2C position was the most highly accessible of the sites tested based on partial reactivity with MPEG-20 even in the absence of Ca$^{2+}$. Indeed, when microsomal membranes were prepared from this mutant in the absence of DTT, this site was found to be refractory to MPEG reaction. This behavior was unique to the S2C position, and the nature of the blocking modification was not explored in this study. The possibility that the result arises because only the S2C site is sufficiently accessible to a large oxidoreductase enzyme seems unlikely because other highly accessible endogenous cysteines in trypsin fragment III (22) and the A2749C mutant at the C terminus (data not shown) remain accessible to MPEG-20 even in membranes prepared in the absence of DTT.

The distinction between sites accessible to MPEG-5 and MPEG-20 is presumably related to the relative sizes of the MPEG molecules and the exact location of the cysteine substitution mutants in the three-dimensional structure of the receptor. MPEGs behave approximately like spherical molecules in aqueous solutions (34, 35), and MPEGs of different sizes have been widely used to estimate the diameter of artificial and natural ion channels (36–38). Estimates of the hydrated diameter of MPEG-5 and MPEG-20 are 41–60 Å (39) and ~130 Å (40), respectively. However, based on previous studies, MPEGs would not be expected to behave like rigid spheres because inherent flexibility may permit the molecules to penetrate into some spaces to a limited extent (41). Several groups have published EM reconstructions of detergent-solubilized purified IP$_3$R (42–44). Although there are many differences in these studies, a basic structure with 4-fold symmetry and dimensions of ~200 Å$^2$ has been visualized. In the model published by da Fonseca et al. (43), which has been described as resembling a flower, the central density (stigma) has been proposed to contain the N-terminal portion of the receptor (Fig. 6B) (43). The reactivity of the S2C site in DTT-treated membranes with MPEG-5 and with the larger MPEG-20 suggests that this residue is highly exposed and is therefore more likely to be situated at the exposed cytosolic face of the central density (Fig. 6B). In the crystal structure the position of serine 2 is not resolved, and the first N-terminal residue is serine 7. The latter residue, together with positions 171 and 189, are all on the same side of the suppressor domain. The S7C and A189C mutants are accessible to MPEG-5, and the S171C mutant becomes accessible in the presence of Ca$^{2+}$. We suggest that the suppressor domain is oriented with the MPEG-5 reactive residues at the exposed surface of the central density (Fig. 6C). Deletion of the suppressor domain eliminates channel gating (45, 46). A proposed gating mechanism has suggested that there is direct contact of the suppressor domain with the TM4-TM5 loop in the channel domain (15). However, there is no direct evidence for such contacts and a surface disposition of the suppressor domain, at some distance from the channel domain, makes such contacts unlikely. Conformational changes in the suppressor domain may be transmitted to the channel indirectly, or direct interactions with the channel domain may involve other regions of the LBD that are in closer proximity. We have selected to interpret our data using the structure of da Fonseca et al. (43) because the assignments in this model accommodate the experimental observation of a close association of N- and C-terminal segments and that the ligand-binding sites in tetramers are closely spaced (47). However, others have proposed different locations of the ligand-binding sites in the EM structures (42, 44). It should be noted that the basic arguments favoring a location of the suppressor domain at the surface of the protein are independent of the EM model selected.

IP$_3$ alone had no discernible effects on MPEG reactivity for any of the cysteine substitution mutants examined in this study. This is in line with previous observations indicating that IP$_3$ elicits more subtle changes in the protein than those induced by
Ca²⁺ (48). We cannot formally exclude the possibility that the inability to detect IP₃-mediated conformational changes may be due to the requirement for some additional factor lacking in our specific experimental conditions. By contrast, Ca²⁺ had large effects on the MPEG reactivity of specific IP₃R cysteine mutants. In the presence of Ca²⁺ the A575C position reacted with MPEG-2 (but not larger MPEGs), the S171C site reacted with MPEG-5 (but not MPEG-20), and the S277C site became accessible to MPEG-20 (Fig. 6A). Ca²⁺ also enhanced the reactivity of certain sites, e.g. S6C, S7C, A189C with MPEG-5 and S2C with MPEG-20 (Fig. 2A and Fig. 5B). EM studies on purified IP₃Rs have revealed a large transition from a compact to a windmill conformation induced by low concentrations of Ca²⁺ (31). In the model of da Fonseca et al. (43), we suggest that the structural change may involve a wider separation of the side arms from the central density (Fig. 6B, right panel). In the crystal structure of the LBD, the Ser-277 position is located within the binding crevice occupied by IP₃. Given this location, it is somewhat surprising that the S277C is so reactive with the larger MPEGs. We hypothesize that the IP₃ binding pocket may be located at the narrowest constriction in the central density. This would be compatible with the close spacing of IP₃-dimer-binding sites observed with IP₃ dimers (19, 47) and would allow a molecule of the size of MPEG-5 to be accommodated in the cavity (Fig. 6B). The dramatic increase in reactivity of the S277C mutant with MPEG-20 in the presence of Ca²⁺ may be caused by the widening of the cavity resulting from the movement of the side arm densities. It is not immediately obvious from the model in Fig. 6B why residues located at the surface of the central density (e.g. S2C and S171C) should show an increase in accessibility in the presence of Ca²⁺. The S171C position is close to the N-terminal region in the crystal structure, so the effects of Ca²⁺ on these residues may have a common mechanism. These specific locations could also become less hindered because of the outward movement of the side arms. Alternatively, conformational changes in the N-terminal region itself could contribute to the results. Biophysical measurements on purified fusion proteins indicate that Ca²⁺ may promote the movement of the suppressor domain away from the binding core (21).

Although reaction of a particular site with MPEG is unequivocal, the failure to react under any conditions (e.g. S217C and A245C) could be due to many factors. The most obvious is that the site is located in the interior of the protein or at highly restricted positions in the central density. Even residues that are on the surface could fail to react if the side chain orientation of the cysteine is not appropriate. In addition, a lack of access could also be due to an associated protein that obscures a particular site. The S436C mutant, which is located in a small flexible linker between β-trefoil and α-helical domains (Fig. 6A), was not accessible to any of the MPEGs. This is surprising because Ser-436 is a site that is proposed to be phosphorylated by ERK2 (a 42-kDa protein) both in vitro and in vivo (28, 29). We were unable to observe phosphorylation of IP₃R in COS cell microsomes incubated with purified ERK2 using a phosphospecific Ab (MPM-2) that has previously been used to recognize the consensus phosphorylation site at Ser-436 (29) (data not shown). Further work is required to resolve the issue of relative accessibility of Ser-436 in intact membranes.

This study is the first to use cysteine substitution mutagenesis to examine surface accessibility in IP₃Rs. The use of MPEG as a means of tagging accessible cysteines was originally introduced in studies of potassium channels (49) and has subsequently been employed in other experimental systems (50–52). A particular advantage of this methodology, as applied to IP₃Rs, is that it yields structural information on the tetrameric, full-length receptor in its native membrane environment. The fortuitous lack of reactivity of large MPEGs with the endogenous cysteines in the N-terminal segments of the SI(−) type I IP₃R circumvents the need to make cysteine-less versions of the receptor. No attempt was made in this study to rigorously fit the high resolution (1.8–2.2 Å) crystal structures of the suppressor and LBD domains (7, 23) into the low resolution (15–30Å) EM structures (42–44) using the accessibility criteria obtained from the present MPEG studies as a guide. This approach awaits improved EM resolution, a greater certainty of the location of the N-terminal segment, and better consensus between different EM models. Nevertheless, the methodology has the potential to provide independent information on structure and conformational dynamics that will be valuable in building a more complete and accurate picture of this complex ion channel.

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