

# Calcium-dependent Conformational Changes in Inositol Trisphosphate Receptors\*

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We have used limited trypsin digestion and reactivity with PEG-maleimides (MPEG) to study Ca<sup>2+</sup>-induced conformational changes of IP<sub>3</sub>R in their native membrane environment. We found that Ca<sup>2+</sup> decreased the formation of the 95-kDa C-terminal tryptic fragment when detected by an Ab directed at a C-terminal epitope (CT-1) but not with an Ab recognizing a protected intraluminal epitope. This suggests that Ca<sup>2+</sup> induces a conformational change in the IP<sub>3</sub>R that allows trypsin to cleave the C-terminal epitope. Half-maximal effects of Ca<sup>2+</sup> were observed at ~0.5 μM and was sensitive to inhibition by IP<sub>3</sub>. Ca<sup>2+</sup> also stimulated the reaction of MPEG-5 with an endogenous thiol in the 95-kDa fragment. This effect was eliminated when six closely spaced cysteine residues proximal to the transmembrane domains were mutated (C2000S, C2008S, C2010S, C2043S, C2047S, and C2053S) or when the N-terminal suppressor domain (amino acids 1–225) was deleted. A cysteine substitution mutant introduced at the C-terminal residue (A2749C) was freely accessible to MPEG-5 or MPEG-20 in the absence of Ca<sup>2+</sup>. However, cysteine substitution mutants in the interior of the tail were poorly reactive with MPEG-5, although reactivity was enhanced by Ca<sup>2+</sup>. We conclude the following: a) that large conformational changes induced by Ca<sup>2+</sup> can be detected in IP<sub>3</sub>R *in situ*; b) these changes may be driven by Ca<sup>2+</sup> binding to the N-terminal suppressor domain and expose a group of closely spaced endogenous thiols in the channel domain; and c) that the C-terminal cytosol-exposed tail of the IP<sub>3</sub>R may be relatively inaccessible to regulatory proteins unless Ca<sup>2+</sup> is present.

Inositol 1,4,5-trisphosphate receptors (IP<sub>3</sub>R)<sup>3</sup> are large tetrameric intracellular Ca<sup>2+</sup>-release channels that mediate the release of Ca<sup>2+</sup> from endoplasmic reticulum stores into the cytosol in response to IP<sub>3</sub> (1–5). Three different isoforms exist, which can form homo- and heterooligomers. All three isoforms consist of an IP<sub>3</sub> binding domain in the N-terminal region, a

channel domain containing six transmembrane segments in the C-terminal region and an intervening regulatory domain. Apart from IP<sub>3</sub>, the principal regulator of the channel is Ca<sup>2+</sup>, which exerts a biphasic effect on channel function with activation at low concentrations and inhibition at high concentrations. The structural organization of this complex protein and the mechanism by which binding of IP<sub>3</sub> and Ca<sup>2+</sup> are linked to channel gating are active areas of investigation.

An important feature of the mechanism of the channel involves conformational changes in the protein. In particular, the gating mechanism is thought to involve conformational changes initiated by IP<sub>3</sub> binding that are propagated to various regions of the channel domain (6, 7). The IP<sub>3</sub> binding domain of the receptor is organized into a “core” segment that binds IP<sub>3</sub> with high affinity (amino acids 226–604) and a “suppressor domain” that inhibits IP<sub>3</sub> binding to the core (amino acids 1–225). Crystal structures for the suppressor domain and the IP<sub>3</sub>-liganded core domain have been obtained (8, 9). Biophysical measurements using fusion proteins encoding the ligand binding domain have suggested that IP<sub>3</sub> favors the formation of compact conformations, whereas Ca<sup>2+</sup> promotes the formation of more extended structures (10). Conformational changes induced by IP<sub>3</sub> also have been observed by gel-filtration analysis (11) and have been utilized to design probes for the measurement of IP<sub>3</sub> in cells (12, 13). Conformational changes promoted by Ca<sup>2+</sup> have been observed in electron microscopy analysis of detergent purified IP<sub>3</sub>R from mouse cerebellum. In this system, Ca<sup>2+</sup> transforms receptors from a square to a windmill shape (14, 15). The relationship of the conformational changes detected in fusion proteins or isolated purified receptors to the conformational dynamics of full-length receptors embedded in native membranes remains to be established.

In the present study, we have utilized two indirect biochemical approaches to measure the Ca<sup>2+</sup>-induced conformational changes in native IP<sub>3</sub>R *in situ*. Our experiments are focused on the 95kDa C-terminal trypsin fragment containing the channel domain and the cytosol-exposed C-terminal tail. The results provide insights into the structural changes of the receptor and suggest that accessibility of critical regions of the receptor to regulatory proteins also could be altered by Ca<sup>2+</sup>-induced conformational changes.

## EXPERIMENTAL PROCEDURES

**Materials**—Methoxy-polyethylene glycol maleimide (MPEG) (molecular weight of 20,000) and (molecular weight of 5,000) were from NEKTAR Therapeutics (San Carlos, CA). Rat brains

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<sup>3</sup> The abbreviations used are: IP<sub>3</sub>R, inositol 1,4,5-trisphosphate receptor; CT, C-terminal epitope; C-tail, C-terminal tail; HA, hemagglutinin; IP<sub>3</sub>, myo-inositol 1,4,5-trisphosphate; Ab, antibody; MPEG, methoxy-polyethylene glycol maleimide; HEDTA, N-(2-hydroxyethyl)ethylenediaminetriacetic acid.

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were obtained from Pel Freeze Biologicals (Rogers, AR). All other chemicals were purchased from Sigma-Aldrich.

**Antibodies**—The Ab recognizing the C-terminal epitope (amino acids 2733–2749; designated CT-1 Ab) of type I IP<sub>3</sub>R present in trypsin fragment V has been characterized previously (16). An alternative Ab (designated IL-3) recognizing an epitope within the intraluminal loop (amino acids 2499–2516) between transmembrane domains 5 and 6 also has been described previously (17). Both Abs were raised in rabbits by Cocalico Biologicals (Reamstown, PA) and were affinity-purified using the antigenic peptide coupled to Ultralink beads by procedures described by the manufacturer (Pierce, Rockford, IL).

**Expression Constructs**—The wild-type cDNA encoding the rat type I IP<sub>3</sub>R SI(-)/SII(+)/SIII(+) splice variant in pCMV3 was a kind gift of Dr. Thomas Sudhof (University of Texas, Southwestern Medical Center) (18). The point mutants introducing cysteine substitutions into the C-terminal tail (S2681C, S2703C, S2716C, and A2749C) were made using the QuikChange mutagenesis kit (Stratagene, CA) utilizing a cassette encompassing the BstBI/XbaI fragment of the type I IP<sub>3</sub>R in pBluescript (Invitrogen). Mutants were confirmed by sequencing, and the BstBI/XbaI digested inserts were subcloned into the full wild-type IP<sub>3</sub>R cDNA in pCMV3. The D442N mutant has been described previously (19). The E2100D Ca<sup>2+</sup> regulation mutant (20) was a kind gift of Dr. Kevin Foskett. The 1–225 suppressor domain deletion mutant was a kind gift of Drs. Humberdt deSmedt and Jan Parys.

**Cell Culture and Transfection**—COS-7 cells were grown on 100- or 150-mm plates (Sarstedt) in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 0.1 mg/ml streptomycin, and 100 international units/ml penicillin (all from Invitrogen) until 70–80% confluent. Transfections typically were done overnight in Dulbecco's modified Eagle's medium without serum. LT-1 (Mirus) and NovaFECTOR (VennNova, Inc.) were used together during transfections, and each was added at a cationic lipid to DNA ratio of 1:1. Transfections typically involved 5 μg and 20 μg of DNA for 100- and 150-mm plates, respectively. After 24 h, serum containing Dulbecco's modified Eagle's medium was added, and cells were used 48 h after transfection.

**Preparation of Microsomes**—At 48-h post-transfection, confluent COS-7 cells were washed twice with ice-cold phosphate-buffered saline and scraped into isolation buffer containing 320 mM sucrose, 0.5 mM EGTA, and 10 mM Tris (pH 7.8). 50 ml of this buffer was supplemented with one protease inhibitor mixture tablet (Roche Applied Science). Lysates were prepared from the cells by passing them five times through a 26.5-gauge needle. Cell debris was removed by centrifuging at 500 × *g* for 5 min, and the supernatant was spun for an additional 50 min at 100,000 × *g*. The microsome pellet was resuspended in isolation buffer. Cerebellum microsomes were prepared by first excising the cerebellum from frozen rat brains. The tissue was homogenized 10 times in isolation buffer using a Dounce homogenizer with a tight fitting pestle. The homogenate was spun at 500 × *g* for 15 min, and the supernatant was removed and spun for an additional 50 min at 100,000 × *g*. The micro-

some pellet was resuspended in isolation buffer. All microsomes were either used fresh or stored frozen in liquid nitrogen.

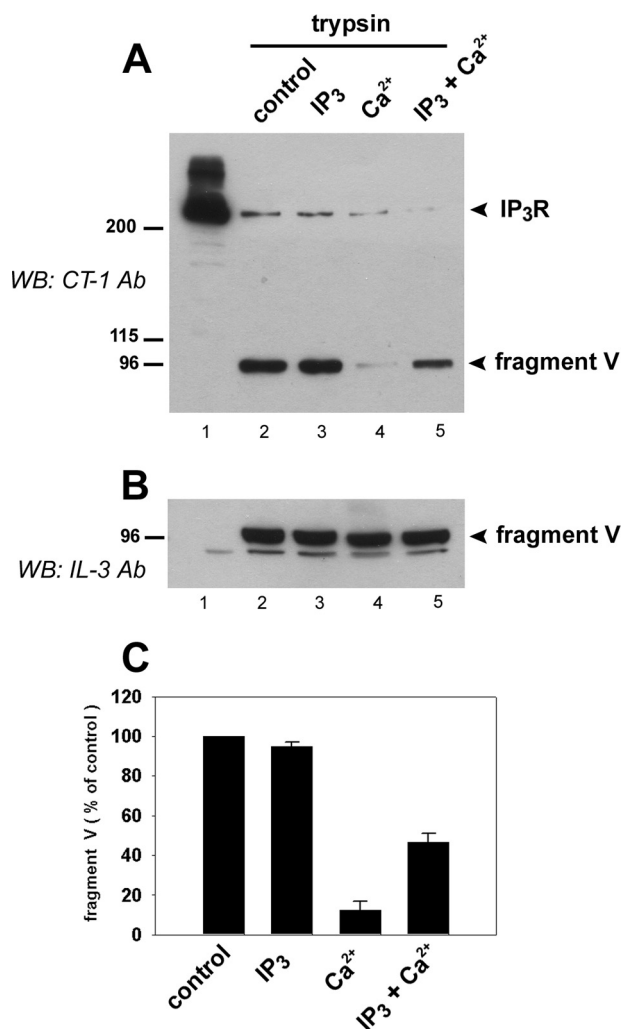
**Reaction with MPEG and Trypsin Digestion**—Microsome preparations were incubated at a final protein concentration of 0.5 mg/ml in a buffer containing 120 mM NaCl, 20 mM Tris-HCl (pH 7.2) and 0.5 mM HEDTA in the presence or absence of MPEG-5 or MPEG-20. Incubations were carried out at room temperature for the indicated times. The reaction was stopped by the addition of 20 mM dithiothreitol. Trypsin digestion was carried out subsequent to MPEG reaction at a final concentration of 4 μg/ml for 5 min at room temperature. Titrations with different concentrations of Ca<sup>2+</sup> were carried out by addition of 10× stock solutions of Ca<sup>2+</sup> buffers containing (final concentration) 5 mM TrisHepes (pH 7.5), 0.5 mM HEDTA, and different concentrations of CaCl<sub>2</sub> to achieve the indicated concentrations of free Ca<sup>2+</sup> as determined by calibration with the fluorescent dyes Calcium orange and Calcium green-5N (Invitrogen). The *K<sub>d</sub>* values used in these calculations were 0.22 and 14 μM, respectively. Where Sr<sup>2+</sup> and Ba<sup>2+</sup> were used, the free divalent cation concentrations were calculated using the program MAXC (Chris Patton, Stanford University).

**Electrophoresis and Immunoblotting**—7% gels were transferred to nitrocellulose membranes (Bio-Rad) and blocked in a 10% milk solution in Tris-buffered saline containing 0.1% Tween 20. Blots were developed with chemiluminescent substrates (Pierce). In cases where a blot was probed sequentially with more than one antibody, the nitrocellulose was stripped at 60 °C for 30 min in stripping buffer (2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris-HCl (pH 6.8)) before probing with the next antibody.

**Akt Kinase Phosphorylation of IP<sub>3</sub>Rs**—Cerebellum microsomes were first stripped of any endogenous associated kinases by treatment for 20 min in 0.5 M KCl, 50 mM Tris-HCl (pH 7.2), and 0.25 mM EDTA. The membranes were reisolated by centrifugation (30 min, 100,000 × *g*) and incubated at 1 mg protein/ml in the presence or absence of 2.2 μM free Ca<sup>2+</sup> and 0.1 mM MgATP, 10 nM okadaic acid, 20 μCi [γ-<sup>32</sup>P]ATP, and 160 ng of recombinant activated human Akt kinase-1 (Biomol). The membranes were incubated for 2 h at 30 °C and lysed in 500 μl of a buffer containing 150 mM NaCl, 50 mM TrisHepes (pH 7.8), 1% Triton X-100, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche Applied Science). IP<sub>3</sub>R was immunoprecipitated overnight with CT-1 Ab and processed on a 5% gel. The gel was transferred to nitrocellulose, and radioactive IP<sub>3</sub>R was detected and quantitated by phosphorimaging.

## RESULTS

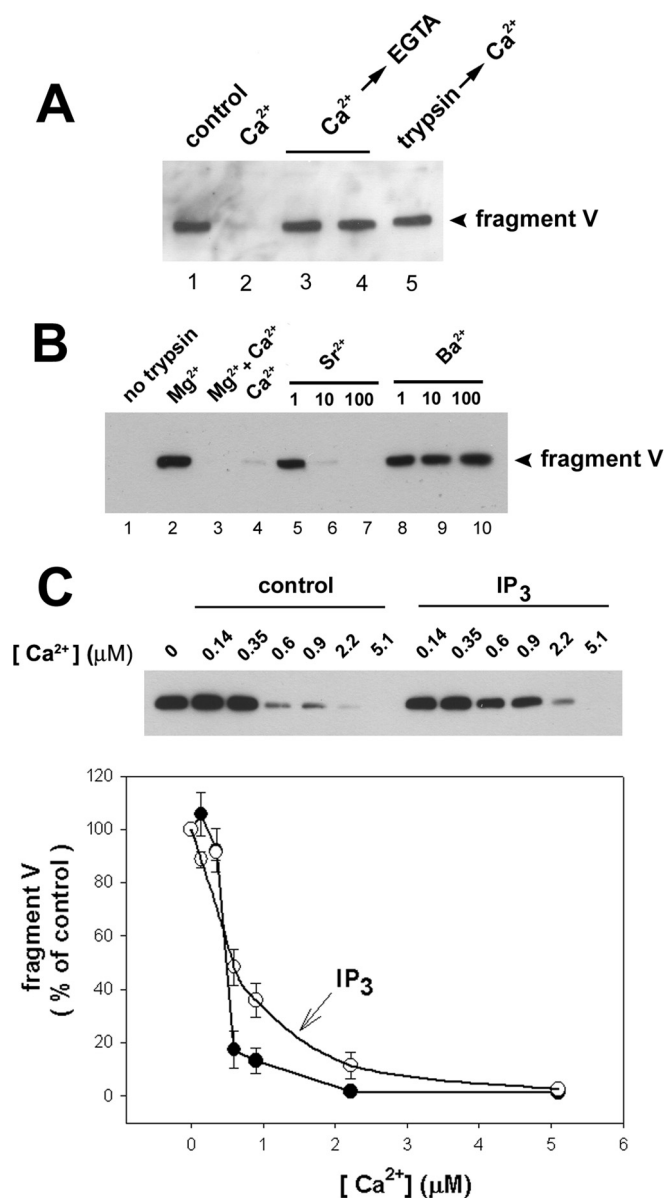
**Ca<sup>2+</sup> Alters Accessibility of the C-terminal Tail to Trypsin**—Trypsin cleaves the type I IP<sub>3</sub>R into five well defined fragments of which the ~95-kDa trypsin fragment V contains the channel domains and the cytosol-exposed C-terminal tail of 160 amino acids (21). The formation of fragment V in cerebellum microsomes after a 5-min trypsin treatment is shown in Fig. 1A, lane 2. Fragment V was detected by immunoblotting with a C-terminal Ab (CT-1) recognizing the last 18 amino acids located in the C-tail. The inclusion of 0.9 μM free Ca<sup>2+</sup> for 5 min prior to trypsin digestion led to a marked inhibition of the formation of



**FIGURE 1. The effect of Ca<sup>2+</sup> and IP<sub>3</sub> on the formation of trypsin fragment V.** Cerebellum microsomes were incubated for 5 min with 0.9 μM free Ca<sup>2+</sup> and/or 10 μM IP<sub>3</sub> as indicated (A). The membranes were then digested with trypsin (4 μg/ml) for a further 5 min, and the samples were then analyzed by SDS-PAGE and immunoblotting with an Ab directed at the terminal 17 amino acids of the C terminus (CT-1 Ab) or an Ab raised to a peptide sequence in the intraluminal loop between TM-5 and -6 (IL-3 Ab) (B). Densitometric quantitation of blots in A from three separate experiments are shown (mean ± S.E.) (C). Quantitation was carried out using NIH ImageJ software. WB, Western blot.

fragment V (Fig. 1A, lane 4). This partially was prevented by the inclusion of 10 μM IP<sub>3</sub> together with Ca<sup>2+</sup> (Fig. 1A, lane 5), although IP<sub>3</sub> added alone was without effect (lane 3). The data from multiple experiments are quantitated in Fig. 1C. The apparent loss of fragment V in the presence of Ca<sup>2+</sup> was not due to altered proteolysis because the same blots probed for fragment V with an Ab that recognized a protected endoplasmic reticulum intraluminal epitope (IL-3 Ab) showed no changes in the levels of fragment V (Fig. 1B). Our interpretation of this data is that Ca<sup>2+</sup> induces a conformational change in the receptor that allows trypsin access to the C-terminal tail, resulting in cleavage and loss of the CT-1 Ab epitope.

Additional experiments using the loss of the CT-1 epitope as a probe for the Ca<sup>2+</sup>-dependent conformational change are shown in Fig. 2. The effects of Ca<sup>2+</sup> could be reversed by the addition of EGTA 30 s prior to addition of trypsin (Fig. 2A, lane 4). The addition of Ca<sup>2+</sup> after trypsin digestion had been inac-

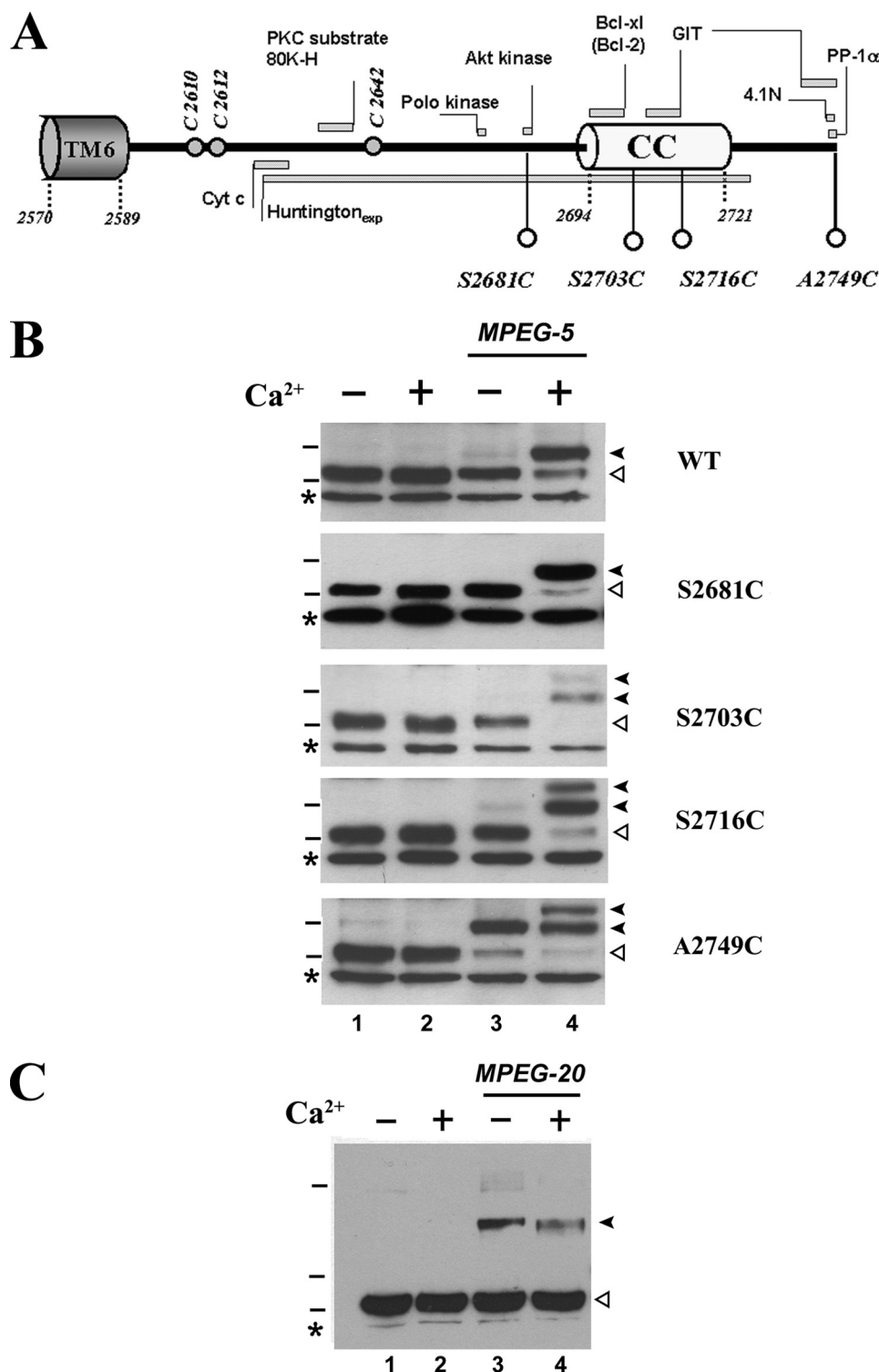


**FIGURE 2. Reversibility, divalent cation dependence and concentration dependence of the effects of Ca<sup>2+</sup>.** A, cerebellum microsomes were incubated for 5 min in the absence (lane 1) or presence (lane 2) of 2.2 μM free Ca<sup>2+</sup> for 5 min, followed by a 5-min digestion with trypsin (4 μg/ml). 1 mM EGTA was added 1 min or 30 s prior to trypsin digestion in lanes 3 and 4, respectively. In lane 5, the trypsin digestion was carried out first for 5 min in the absence of Ca<sup>2+</sup> (0.5 mM HEDTA). The digestion was stopped by the addition of 10 μg/ml soybean trypsin inhibitor, and 0.4 mM CaCl<sub>2</sub> was added to give a final free concentration 2.2 μM. B, microsomes were incubated for 5 min with 1 mM Mg<sup>2+</sup>, 2.2 μM Ca<sup>2+</sup>, or the indicated free concentrations (μM) of Sr<sup>2+</sup> or Ba<sup>2+</sup> (as described under "Experimental Procedures"). The membranes were then digested for 5 min with trypsin. C, cerebellum microsomes were incubated for 5 min with the indicated concentrations of buffered free Ca<sup>2+</sup> in the presence or absence of 10 μM IP<sub>3</sub>. The samples were digested for 5 min with trypsin and immunoblotted with CT-1 Ab. The top panel shows a representative immunoblot of the 95-kDa fragment V, and the lower panel is a compilation of the data from three separate experiments (mean ± S.E.).

tivated produced no effect on the detection of fragment V. The effects of Ca<sup>2+</sup> were not mimicked or inhibited by Mg<sup>2+</sup>, suggesting a high degree of specificity consistent with the Ca<sup>2+</sup> regulatory sites identified previously in IP<sub>3</sub>Rs (22). Sr<sup>2+</sup> mimicked the effect of Ca<sup>2+</sup>, albeit at higher concentrations, whereas Ba<sup>2+</sup> was relatively ineffective (Fig. 2B). This divalent



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**FIGURE 3. The effect of calcium on the MPEG reactivity of cysteine substitution mutants in the C-terminal tail.** *A*, schematic of the C-terminal tail of the IP<sub>3</sub>R with the location of three endogenous cysteines and four cysteine substitution mutants indicated by *open circles*. The location of the transmembrane domain 6 (S6) and the coiled-coil domain (CC) as well as several sites for interaction with key proteins are indicated (for additional details, see text). *GIT*; G-protein-coupled receptor kinase-interacting protein, *PP1-α*; protein phosphatase 1- $\alpha$ , *4.1N*; neuron-specific isoform of erythrocyte protein band 4.1; *Cyt c*, cytochrome c; *PKC*, protein kinase C. *B*, microsomal membranes were prepared from COS cells transfected with wild-type IP<sub>3</sub>R (WT) or the indicated cysteine substitution mutants and then reacted with 0.5 mM MPEG-5 for 5 min in the presence or absence of a free [Ca<sup>2+</sup>] of 2.2  $\mu$ M. The samples were then digested with trypsin. Cleaved fragments from the C-terminal domain were detected on immunoblots with affinity-purified IL-3 Ab. The *open arrow* indicates the position of the 95-kDa fragment V, and the *closed arrows* indicate its shift to higher molecular weights. The *asterisk* identifies a nonspecific IL3-reactive band that is not shifted by MPEG-5. *C*, experiments with MPEG-20 were performed with membranes expressing the A2749C mutant as described in *B*.

cation specificity is similar to that reported for an activatory modulatory site on rat liver IP<sub>3</sub>R (23). The concentration dependence on Ca<sup>2+</sup> is shown in Fig. 2C. Half-maximal inhibition of the CT-1 detectable fragment V occurred at  $\sim 0.5 \mu$ M Ca<sup>2+</sup>. The shape of the titration curve was particularly steep in the range 0.35–0.6  $\mu$ M. The presence of IP<sub>3</sub> made the titration curve shallower and decreased the sensitivity to Ca<sup>2+</sup>. The effects of IP<sub>3</sub> were not observed with the inactive isomer Ins-(1,3,4)-P<sub>3</sub> (data not shown).

*Ca<sup>2+</sup> Alters MPEG Reactivity with Endogenous and Mutant Cysteines in the C-terminal Domain*—To examine accessibility and conformational changes of domains, we used the alternative strategy of measuring reactivity of endogenous and mutant cysteines with MPEG using gel-shift assays (24, 25). There are 13 potentially MPEG-accessible endogenous cysteines in fragment V of which three are located in the C-terminal tail, which is also the site of interaction with several important regulatory proteins (Fig. 3A). To assess accessibility we introduced four additional cysteines into the C-terminal tail (S2681C, S2703C, S2716C, and A2749C). All of these substitution mutants retained channel function as measured with a <sup>45</sup>Ca<sup>2+</sup> flux assay (26) (data not shown). In agreement with our previous observations (25), none of the endogenous cysteines in the wild-type receptor fragment V was reactive with MPEG-5 in the absence of Ca<sup>2+</sup> based on the lack of a gel shift of fragment V detected with IL-3 Ab (Fig. 3B, top panel, lane 3). However, in the presence of Ca<sup>2+</sup>, a single shifted band was observed (Fig. 3B, top panel, lane 4), indicating that Ca<sup>2+</sup> led to the exposure of at least one endogenous reactive cysteine. The cysteine substitution mutant placed at the extreme C terminus of the protein (A2749C) was highly accessible, as indicated by reaction with MPEG-5 even in the absence of Ca<sup>2+</sup> (Fig. 3B, bottom panel, lane 3). However, the three cysteine substitution mutants placed

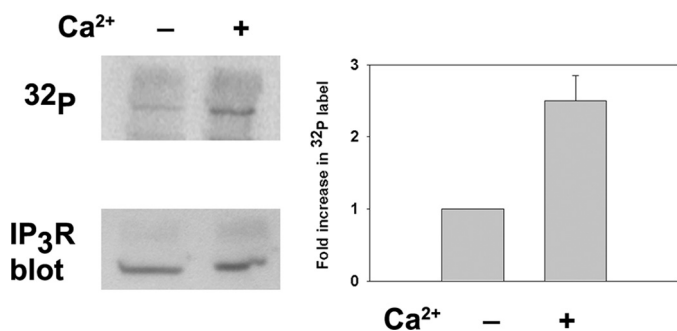


FIGURE 4. Phosphorylation of IP<sub>3</sub>Rs in cerebellum microsomes by Akt kinase in the presence and absence of Ca<sup>2+</sup>. Cerebellum microsomes were phosphorylated with [ $\gamma$ -<sup>32</sup>P]ATP and recombinant Akt kinase in the presence or absence of 2.2  $\mu$ M Ca<sup>2+</sup> as described under "Experimental Procedures." After 2 h at 30 °C, the membranes were lysed and immunoprecipitated with CT-1 Ab. The immunoprecipitates were analyzed for radioactivity by phosphorimaging and for IP<sub>3</sub>R levels by immunoblotting. The fold increase in radioactivity was quantitated and is shown as mean  $\pm$  S.E. for three experiments.

further into the tail did not react with MPEG-5 over the 5-min incubation period. When Ca<sup>2+</sup> was present the A2749C mutant producing a doublet of shifted bands, presumably corresponding to the combined reaction of the endogenous and mutant cysteine sites (Fig. 3B, bottom panel, lane 4). The same doublet also was seen for the S2716C and S2703C mutant. Because neither of these mutants reacted appreciably in the absence of Ca<sup>2+</sup>, this suggests that Ca<sup>2+</sup> may enhance the accessibility of this portion of the C-tail. Only the reaction with the endogenous cysteine was seen in the S2681C mutant incubated in the presence of Ca<sup>2+</sup>. The degree of accessibility of the C-terminal A2749C residue was assessed by examining reactivity with the larger 20-kDa MPEG derivative (Fig. 3C). This mutant reacted with MPEG-20 in the absence of Ca<sup>2+</sup>, but no additional bands were observed in the presence of Ca<sup>2+</sup>. These results are compatible with a restricted environment around the endogenous cysteine that would be unfavorable for reaction with the larger MPEG-20.

Ser<sup>2681</sup> is the site proposed to be phosphorylated by Akt kinase (27, 28). It is therefore somewhat surprising that a cysteine mutant introduced at this site was unreactive with MPEG-5. However, reactivity to MPEG was monitored over a short period, whereas incubations with kinases are carried out for longer times, and the accessibility criteria for a protein kinase and an MPEG molecule could be very different. The physiological significance of an increased access of the C-tail in the presence of Ca<sup>2+</sup> was examined by measuring the phosphorylation of cerebellar IP<sub>3</sub>Rs by Akt kinase *in vitro* in the presence or absence of Ca<sup>2+</sup> (Fig. 4). The incubation of isolated membranes for 2 h with activated Akt kinase led to the phosphorylation of the receptor, which was stimulated 2.5-fold by incubation in the presence of 2.2  $\mu$ M free Ca<sup>2+</sup>. Other substrate proteins in the membranes did not show a similar enhancement (data not shown), indicating that the effect of Ca<sup>2+</sup> is not due to a general enhancement of the catalytic activity of the recombinant Akt kinase. The data are consistent with the hypothesis that a Ca<sup>2+</sup>-induced conformation change in the IP<sub>3</sub>R causes an increased accessibility of the C-tail to interacting proteins. We attempted to test this further by examining the effects of Ca<sup>2+</sup> on the interaction of added cytochrome *c* or endogenous Bcl-2,

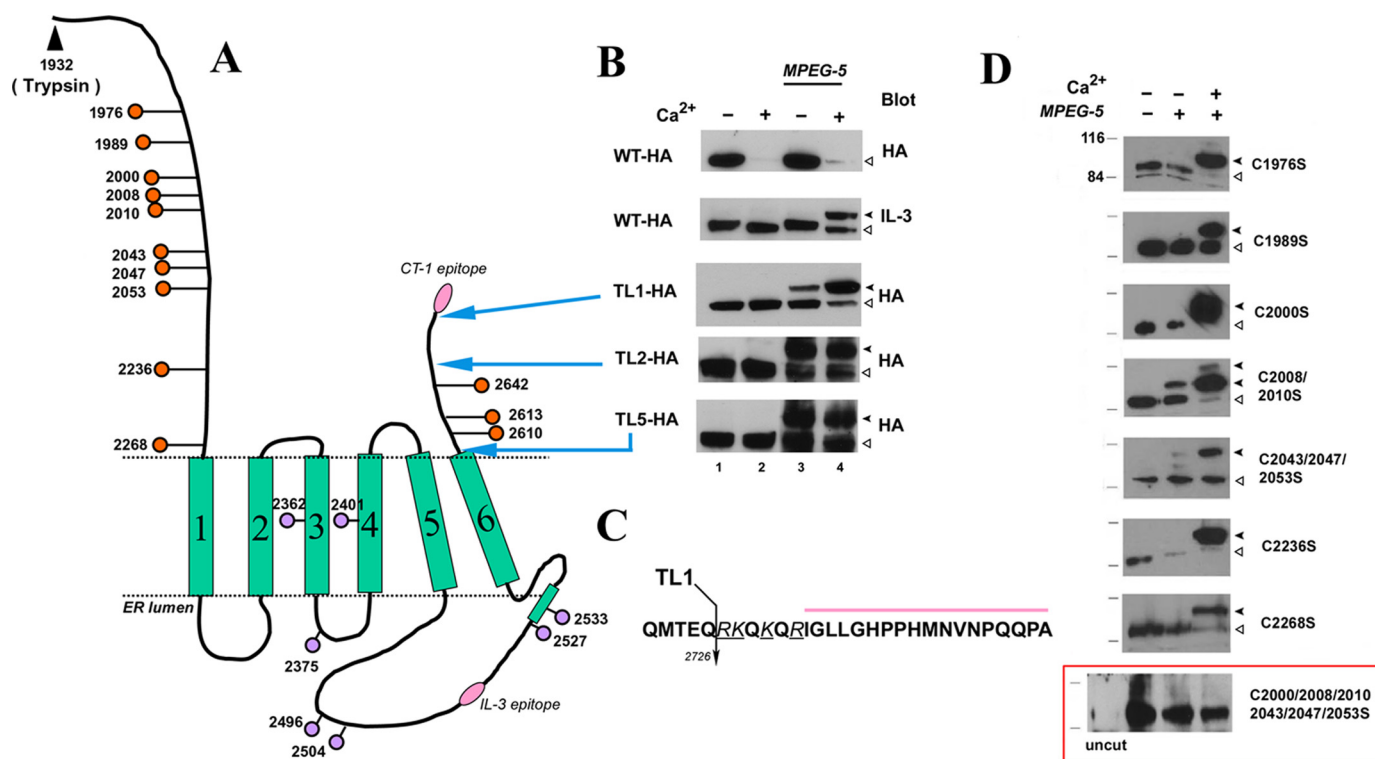
but we were not able to co-immunoprecipitate these proteins with IP<sub>3</sub>Rs under our experimental conditions (data not shown). The presence of Ca<sup>2+</sup> did not alter the co-immunoprecipitation of IP<sub>3</sub>Rs with PP1- $\alpha$  (data not shown), as anticipated for a protein that interacts with the last 12 C-terminal residues (Fig. 3A) (29).

**Mutagenesis Studies to Identify Endogenous Reactive Cysteines and Ca<sup>2+</sup> Modulatory Sites**—To locate the endogenous cysteine(s) reacting with MPEG-5 in the presence of Ca<sup>2+</sup>, we have utilized a mutagenesis approach (Fig. 5A). Initially, we examined the effect of removing the three endogenous cysteine residues in the C-tail by making progressive deletions from the C terminus (Fig. 5B). These "tail-less" mutants were tagged at the C terminus with the HA epitope, and as a control, we examined the behavior of the wild-type receptor tagged with HA. Fig. 5B shows that Ca<sup>2+</sup> treatment caused the loss of HA but not IL-3 immunoreactivity in fragment V, confirming that the native cerebellum and recombinant receptor behave similarly. Deletion of 24 amino acids from the C-tail (TL1 mutant) completely prevented the loss of HA signal induced by Ca<sup>2+</sup>. This suggests that the trypsin cleavage site must be located within the terminal 24 amino acids, and several candidate residues are underlined in the sequence shown in Fig. 5C. The cleavage at sequences in the distal portion of the C-tail also is consistent with the lack of a noticeable decrease of molecular weight of the IL-3 reactive fragment V band. Interestingly, the TL1 deletion mutant showed some reactivity with MPEG-5 even in the absence of Ca<sup>2+</sup>. The deletion of 60 (TL2 mutant) or 141 amino acids (TL5 mutant) showed maximum reactivity with MPEG-5 in the complete absence of Ca<sup>2+</sup>. These data suggest that the endogenous cysteine exposed by Ca<sup>2+</sup> in wild-type receptors already is exposed when significant portions of the C-tail are deleted. Because an MPEG-5 shift is observed with the TL-5 mutant, we conclude that the endogenous reactive cysteine probably does not involve the three residues located in the C-tail.

We addressed the role of the 10 cysteine residues located proximal to the transmembrane domains by making mutations individually or in groups (Fig. 5, A and D). The data show that each of the mutations retained a Ca<sup>2+</sup>-dependent MPEG-5 shift and, in some cases, even showed enhanced multiple shifts (e.g. C2008S/C2010S). It is possible that MPEG-5 may react with multiple cysteines in a closely spaced cluster. The reaction with any one cysteine may prevent reaction at adjacent cysteines because of steric hindrance due to the large MPEG molecule and the relatively restricted space available at the reactive site. In accord with this hypothesis, the mutation of six cysteines (C2000S, C2008S, C2010S, C2043S, C2047S, and C2053S) in two closely spaced clusters was necessary to eliminate the Ca<sup>2+</sup>-dependent MPEG-5 shift. It is possible that the absence of MPEG-5 reactivity could be secondary to a major structural defect in the six-cysteine mutant. This seems unlikely because the mutant was cut by trypsin to form the normal fragment V (Fig. 5D) and retained 43  $\pm$  7% ( $n$  = 3) of the channel activity of the wild-type receptor measured with 10  $\mu$ M IP<sub>3</sub> using <sup>45</sup>Ca<sup>2+</sup> flux assays (data not shown).

In Fig. 6, we have used mutations that are known to modify the Ca<sup>2+</sup> sensitivity of channel function. The D442N mutant

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**FIGURE 5. MPEG-5 reactivity with cysteine mutants.** *A*, a schematic of trypsin fragment V with the cytosolic-facing 13 endogenous cysteines potentially accessible to MPEG-5 (orange) and seven inaccessible endogenous cysteines (purple). The central panel of blots (*B*) shows the effect of deleting segments of the C-terminal tail corresponding to the removal of 24 (TL-1), 60 (TL-2), and 141 (TL-5) amino acids. The latter construct removes the three endogenous cysteines in the C-tail. Each of the tail-less constructs contained a C-terminal HA-tag for detection. Microsomal membranes were prepared and incubated in the presence or absence of 2.2  $\mu\text{M}$  free  $[\text{Ca}^{2+}]$  for 5 min followed by a 5-min incubation with 0.5 mM MPEG-5 (lanes 3 and 4). All samples were processed on 7% SDS-PAGE. Unshifted and shifted bands (open and closed triangles, respectively) were detected by immunoblotting with IL-3 or HA antibodies. The sequences in *C* are derived from the C-terminal region of the receptor and indicate potential trypsin cleavage sites (*underlined italic*) preceding the CT-1 Ab epitope (pink line). The blots in *D* show the effect of mutating each of the 10 endogenous cysteines in the segment prior to the transmembrane domains. The highlighted panel shows the elimination of the MPEG shift upon mutating a cluster of six closely spaced cysteines. The experimental conditions are as described for *B* except IL-3 Ab was used for detection of all blots. *ER*, endoplasmic reticulum.

previously has been shown to have a decreased sensitivity to inhibition of the channel by high concentrations of  $\text{Ca}^{2+}$  (19). The E2100D mutation has been shown to result in shifts of both the activation and inhibition of channel function to higher  $\text{Ca}^{2+}$  concentrations (20). The D442N mutation did not have any effect on the  $\text{Ca}^{2+}$ -induced MPEG shift of the 95-kDa fragment. However, the E2100D mutant did decrease the sensitivity of the  $\text{Ca}^{2+}$  effect (Fig. 6*B*). We also tested a construct in which the entire N-terminal suppressor domain was deleted ( $\Delta 1$ –225). A putative  $\text{Ca}^{2+}$  binding site is thought to be present in this domain (30). This construct was cleaved normally by trypsin to generate the C-terminal 95-kDa fragment, but no reactivity with MPEG-5 was observed in the presence of  $\text{Ca}^{2+}$ . The  $\Delta 1$ –225 mutant also failed to show a  $\text{Ca}^{2+}$ -induced trypsin cleavage of the CT-1 epitope (data not shown).

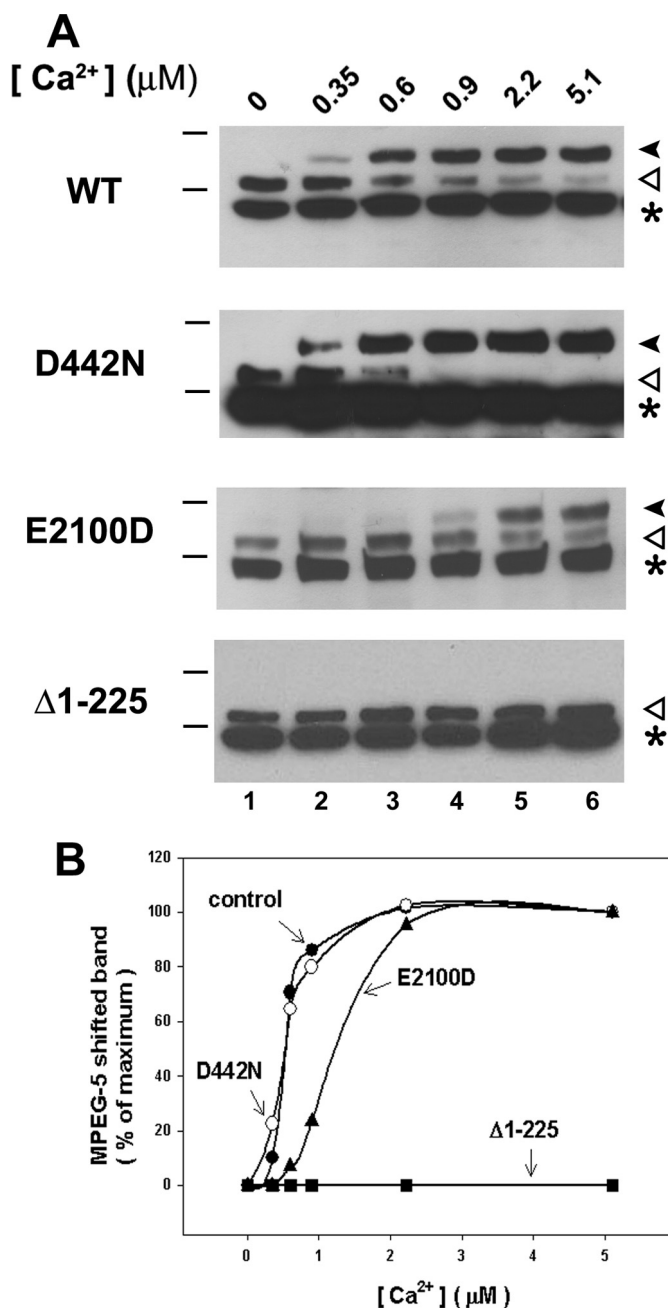
### DISCUSSION

In the present study, we have used indirect biochemical approaches to observe conformational changes in full-length IP<sub>3</sub>Rs in their native membrane environment. To do this, we measured changes in accessibility of the protein to trypsin and changes of MPEG reactivity with endogenous and mutant thiols. The experiments focused on the 95-kDa C-terminal trypsin fragment containing the channel domain and C-terminal tail. The results reveal large changes in accessibility induced by

$\text{Ca}^{2+}$  in this segment of the receptor. It is possible that this reflects direct effects of  $\text{Ca}^{2+}$  on the conformation of the 95-kDa fragment. However, large conformation transitions in the density corresponding to the channel domain were not observed previously in EM studies (15). Hence, we favor the view that the changes in accessibility arise indirectly because of  $\text{Ca}^{2+}$ -induced conformational changes occurring elsewhere in the protein. The structural and physiological implications of the results are discussed below.

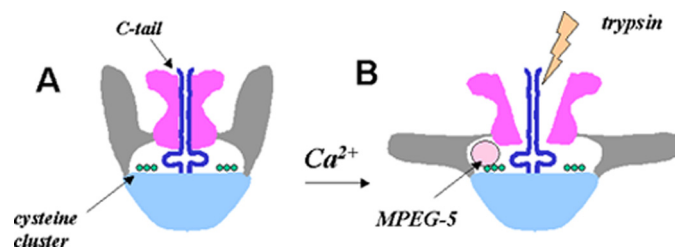
Although homology models of the channel domain have been constructed (31), a detailed molecular structure of the entire 95-kDa C-terminal segment is not available. However, several groups have obtained low resolution structures of purified IP<sub>3</sub>Rs from electron microscopy studies (32–34). Although there are considerable differences in these reports, particularly in the assignment of the density corresponding to the ligand binding domain, all the structures visualized are  $\sim 200\text{\AA}^3$  and show overall 4-fold symmetry. The receptor has been described variously as resembling a flower (32), a hot air balloon (33), or a pinwheel (34). A model consistent with our observations is shown in Fig. 7 and is based on the EM structure reported by da Fonseca *et al.* (32). The model shows the presence of a small vestibule/space on the cytosolic side between the channel domain (Fig. 7, shown in blue) and the rest of the receptor,





**FIGURE 6. Ca<sup>2+</sup> sensitivity of MPEG-5 reactivity in selected IP<sub>3</sub>R mutants.** *A*, MPEG-5 reaction of the endogenous cysteine in the 95-kDa C-terminal trypsin fragment of microsomes prepared from COS cells transfected with the indicated mutants was measured at different buffered free [Ca<sup>2+</sup>] by immunoblotting with the intraluminal IL-3 Ab. The position of the 95-kDa unshifted and shifted bands are shown by the open and closed arrows, respectively. The asterisk indicates the presence of a variable intensity, nonspecific band detected by the Ab. *B*, the magnitude of the shifted band was estimated densitometrically using low exposure blots and is expressed as a percentage of the maximum shift. The data are the mean of two experiments on independent microsome preparations. WT, wild-type.

which is narrow at the center of the receptor (~15 Å) and widens at the periphery (~30 Å) (32). A similarly located cavity also has been noted in the other EM models (33, 34). We suggest that in the presence of Ca<sup>2+</sup>, a large conformational transition of the receptor widens this space sufficiently to allow MPEG-5 (hydrated diameter of ~50 Å (35)) to access a cluster of reactive cysteines located in this compartment. The reactivity of more



**FIGURE 7. Model to account for Ca<sup>2+</sup> effects on MPEG-5 reactivity and trypsin cleavage of the C-terminal tail.** *A*, the model shows a representation of the IP<sub>3</sub>R based on the EM structure reported by da Fonseca *et al.* (32) in which the densities corresponding to the ligand binding (pink), regulatory (gray), and channel domains (blue) are as shown. A cross-sectional side view with just two subunits is shown for clarity. The C-terminal tail is shown as projecting perpendicularly from the membrane and running in a central channel formed by the ligand binding domain. The model draws attention to a small vestibule above the channel domain, which is proposed to enlarge as a result of a Ca<sup>2+</sup>-driven conformational change arising from the outward movement of ligand binding and regulatory domains. This results in increased accessibility of an endogenous cluster of cysteines located in the vestibule to MPEG-5. The opening of a crevice in the ligand binding domain also allows increased accessibility of trypsin to a cleavage site in the tail. For additional details, see text.

than one cysteine is inferred from mutagenesis experiments, which indicate that a group of six closely spaced cysteines must be mutated to remove the MPEG-5 reactivity. The available space around the reactive cysteines must be relatively restricted because no reactivity is observed with the much larger MPEG-20 (hydrated diameter of ~130 Å (36)). In Fig. 7, the Ca<sup>2+</sup>-dependent conformational transition is shown as a large outward movement of the densities that have been assigned to the regulatory domain of the receptor (Fig. 7, shown in gray) (32). This Ca<sup>2+</sup>-induced change could be the basis of the previously documented “square” to “windmill” conformational transition seen in EM studies of the purified cerebellar IP<sub>3</sub> receptor (15). This model predicts that Ca<sup>2+</sup> also should promote large changes in the MPEG accessibility of cysteines in the ligand binding domain (Fig. 7, shown in red). This has been confirmed experimentally (37). It should be noted that the Ca<sup>2+</sup>-dependent change in MPEG accessibility of fragment V also was observed when the MPEG reaction was carried out after the receptor had been first cleaved with trypsin (data not shown). This is in agreement with previous findings that the receptor retains function even when partially digested with trypsin (38).

Our experiments also provide information on the accessibility of residues in the C-terminal tail. A cysteine substitution introduced at the last amino acid of the tail (A2749C) was readily accessible to both MPEG-5 and MPEG-20 in the absence of Ca<sup>2+</sup> (Fig. 3). By contrast, none of other three cysteine substitution mutants in the C-tail (S2681C, S2703C, and S2716C) or the three endogenous cysteines (Cys<sup>2610</sup>, Cys<sup>2613</sup>, and Cys<sup>2642</sup>) were reactive with MPEG-5. The S2703C and S2716C sites are located in a coiled-coil domain present in the C-tail (Fig. 3A), and it is therefore possible that these sites are occluded because of oligomerization of this domain (7). However, in the presence of Ca<sup>2+</sup>, there was an enhanced reactivity of the S2716C site and to a lesser extent the S2703C site. We also have shown that trypsin (22 kDa) gains access to a cleavage site within the last 24 amino acids of the C-tail only in the presence of Ca<sup>2+</sup>. Interestingly, Yoshikawa *et al.* (21) also observed that the tip of the C-terminal tail was labile to trypsin,

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although in their study, this occurred only with higher concentrations of trypsin, and Ca<sup>2+</sup> was not included in the incubations. We conclude from our data that only the most terminal amino acids of the C-tail are solvent-exposed in the absence of Ca<sup>2+</sup> and that the conformational changes induced by Ca<sup>2+</sup> cause an increase in accessibility of the tail which is most marked (based on the MPEG gel-shift assay) for the ~35 C-terminal amino acids.

Our interpretation of these observations is based on the hypothesis that the 160 amino acid C-tail projects perpendicularly from the membrane and runs in a central channel formed by the ligand binding domain (Fig. 7). This arrangement is consistent with the limited access of endogenous or substituted cysteine residues in the C-tail in the absence of Ca<sup>2+</sup>. In this model, the terminal residues of the C-tail are proposed to project from the central density into the solvent space. This orientation is analogous to the C-tail of the full-length KcsA channel, whose recently published crystal structure shows a long perpendicular helix with a small bulge helix near the membrane (39). This model also is consistent with the reported ability of recombinant domains from the IP<sub>3</sub>R C-tail to form dimers (40) or tetramers (7). When Ca<sup>2+</sup> is present, we suggest that a conformational transition initiated in the ligand binding domain and transmitted to the connected side arms, results in the opening of a crevice in the central density that would account for the enhanced accessibility of the C-tail under these conditions (Fig. 7B). Deletion mutants of the C-tail appear to enhance reactivity of the endogenous cysteines in trypsin fragment V even without added Ca<sup>2+</sup> (Fig. 5B). The reason for this is not clear, but we speculate that the absence of a correctly folded C-tail may increase the MPEG-5 accessible space around the reactive cysteines.

A number of proteins of different sizes have been proposed to interact with the C-tail at various locations (Fig. 3A). These include 4.1N, 98 kDa (41); BclXL, 26 kDa (42); PP1- $\alpha$ , 37 kDa (29); cytochrome *c*, 12 kDa (43), 80K-H protein kinase C substrate, 80 kDa (44), G-protein-coupled receptor kinase-interacting protein, 95 kDa (45); and Huntington-associated protein 1A, 70 kDa (46). In addition, Ser<sup>2618</sup> is the phosphorylation site for Akt kinase (56 kDa (27, 28)), and Thr<sup>2656</sup> is the phosphorylation site for Polo kinase (68 kDa (47)). Because of these interactions, it has generally been assumed that the C-tail is freely accessible along its entire length in the native receptor. The present data using MPEG reactivity and trypsin cleavage suggest that this may not be the case. In those instances where the protein interacts with the C-terminal 14 amino acids (e.g. PP1- $\alpha$  and 4.1N) accessibility should not be an issue. However, proteins interacting further into the tail would be expected to require Ca<sup>2+</sup>-induced conformational changes or other structural alterations of the receptor to facilitate accessibility to their binding sites. We have shown that the phosphorylation of the receptor by Akt kinase is indeed enhanced in the presence of Ca<sup>2+</sup> (Fig. 4). Interestingly, the functional effect of cytochrome *c* is observed only in the presence of Ca<sup>2+</sup> (48), and the interaction with G-protein-coupled receptor kinase-interacting protein also is stimulated by cytosolic Ca<sup>2+</sup> elevation (45). Based on the available data, we would suggest that Ca<sup>2+</sup> could be an important factor regulating protein-protein interactions with

the IP<sub>3</sub>R and that even a transient elevation of Ca<sup>2+</sup> might be sufficient to allow association of interacting proteins with the C-tail.

Ca<sup>2+</sup> activates channel function at low concentrations and has an inhibitory effect at higher concentrations. What, then, is the functional significance of the Ca<sup>2+</sup>-induced conformational changes observed in the present study? Whether these changes accompany activation or inhibition of the channel is difficult to assess from the present data. The half-maximal concentration over which the Ca<sup>2+</sup> effects are observed are ~0.5  $\mu$ M. These concentrations are larger than required to activate the channel in <sup>45</sup>Ca<sup>2+</sup> flux assays on COS cell microsomes (26, 31). A key feature of the inhibitory site is that the affinity for Ca<sup>2+</sup> is decreased by IP<sub>3</sub> (5). The sensitivity of the Ca<sup>2+</sup> effect on trypsin cleavage was decreased by IP<sub>3</sub>, although IP<sub>3</sub> had no effects on the Ca<sup>2+</sup> stimulation of MPEG reactivity (data not shown). This does not necessarily mean that Ca<sup>2+</sup> mediates two different effects at two distinct sites. In the model shown in Fig. 7, conformational changes induced by IP<sub>3</sub> in the central domain could interfere with the access of trypsin without necessarily affecting MPEG access. In our experiments Sr<sup>2+</sup>, rather than Ba<sup>2+</sup>, facilitated the conformational changes in the receptor. This is the specificity reported for the activatory binding site in the liver (23). The location and function of divalent cation binding sites in the IP<sub>3</sub>R has not been established firmly, and multiple Ca<sup>2+</sup> binding sites could function to both activate and inhibit the channel (5). A "Ca<sup>2+</sup> sensor" mutation (E2100D) has been described in which the Ca<sup>2+</sup> sensitivity for activation and inhibition of the channel are decreased (20). This mutant also showed a decreased sensitivity for the Ca<sup>2+</sup> effect on MPEG reactivity. In addition, the removal of the N-terminal suppressor domain was sufficient to prevent the Ca<sup>2+</sup> effect entirely. These data support the view that the suppressor domain may be critically important for driving Ca<sup>2+</sup>-dependent conformational changes in the receptor. Further mutagenesis studies are required to identify distinct Ca<sup>2+</sup> binding sites in the receptor and to understand the relationship of conformational changes in the protein to channel function.

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