Reactivity of free thiol groups in type-I inositol trisphosphate receptors

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The IP₃R (inositol 1,4,5-trisphosphate receptor) Ca²⁺-release channel is known to be sensitive to thiol redox state. The present study was undertaken to characterize the number and location of reactive thiol groups in the type-I IP₃R. Using the fluorescent thiol-reactive compound monobromobimane we found that approx. 70 % of the 60 cysteine residues in the type-I IP₃R are maintained in the reduced state. The accessibility of these residues was assessed by covalently tagging the IP₃R in membranes with a 5 kDa or 20 kDa MPEG [methoxypoly(ethylene glycol) maleimide]. MPEG reaction caused a shift in the mobility of IP₃R on SDS/PAGE that was blocked by pretreatment of the membranes with dithiothreitol, *N*-ethylmaleimide, mersalyl or thimerosal, indicating that MPEG reactivity was specific to thiol groups on the IP₃R. Trypsin cleavage of the type-I IP₃R generates five defined domains. In cerebellum membranes, MPEG reacted over a 5 min interval with tryptic fragment I and fragment III, but not fragments II, IV or V. Fragment I appears as a doublet

in cerebellum membranes, corresponding to the presence and absence of the SI splice site in this region (SI is a spliced domain corresponding to amino acids 318–332). Only the fragment I band corresponding to the SI(+) splice form shifted after reaction with MPEG. Expression of SI(+) and SI(–) spliced forms in COS cell microsomes confirmed this result. The MPEG-induced shift was not prevented when the cysteine residue present in the SI splice domain (C326A) or the remaining seven cysteine residues in fragment I were individually mutated. Of the combination mutations screened, only the mutation of C206/214/326A blocked MPEG reactivity in fragment I. We conclude that a set of highly reactive cysteine residues in fragment I are differentially accessible in the SI(+) and SI(–) splice variants of the type-I IP₃R.

Key words: Alternative splicing, calcium, endoplasmic reticulum, IP₃ receptor, thiol groups.

INTRODUCTION

Changes in cytosolic Ca²⁺ regulate a wide array of cellular processes [1]. RyRs (ryanodine receptors) and IP₃Rs (inositol 1,4,5-trisphosphate receptors) are related families of intracellular Ca²⁺-release channels that play key roles in mediating changes in cytosolic Ca²⁺ [1–3]. RyR channels are known to be highly sensitive to changes in thiol redox state. Thus, of the 100 cysteine residues in each RyR1 subunit, approx. 50 are free for modification by oxidation, nitrosylation or alkylation [4,5]. The functional effects of these modifications are complex and are determined by the exact modifying agent, as well as the dose and time of treatment. Overall, it has been concluded that RyRs contain multiple classes of reactive thiol groups which can either activate or inhibit channel activity [4,6].

In contrast with RyRs, there is relatively little known regarding the regulation of IP₃Rs by changes in thiol redox status. Previous studies have shown that the thiol-reactive agents *t*-butylhydroperoxide and TMS (thimerosal) can promote repetitive Ca²⁺ spiking in many cell systems [7–9]. It has also recently been reported that NO may directly modify the IP₃R in brain, particularly under hypoxic conditions [10]. The effects of *t*-butylhydroperoxide are thought to be mediated by increased levels of oxidized glutathione, which has been shown to enhance IP₃ (inositol 1,4,5-trisphosphate) binding and channel function in permeabilized hepatocytes [11]. TMS has been shown to directly modify functionally reconstituted IP₃Rs [12], and its effects on Ca²⁺ signalling are believed to arise from a sensitization of IP₃Rs to ambient levels of IP₃ [12,13]. Previously, we have shown that even structurally related thiol agents, such as TMS and mersalyl, can have quite different effects on IP₃R channel function [11].

These data suggest that IP₃Rs, like RyRs, may also have multiple classes of reactive thiol groups and that modification of these sites may be important under physiological or pathophysiological conditions.

A first step to understanding regulation of thiol groups on IP₃Rs is to determine how many of the total cysteine residues in the molecule are available for modification and to identify the location of the most reactive of these groups. The present study was undertaken with these objectives as its goal. We focused on the type-I IP₃R isoform using cerebellum membranes or heterologous expression of type-I IP₃R constructs as experimental systems. The studies indicate that approx. 70 % of the thiol groups on the receptor are available for modification and that the thiol groups that are most easily accessed by maleimide-PEG [poly(ethylene glycol)] derivatives are located in the ligand binding domain and the central coupling domain of the receptor. Three different sites of alternative splicing [SI (spliced domain corresponding to amino acids 318–332), SII and SIII] have been identified in the type-I IP₃R [2]. The present study shows that the SI(+) and SI(–) splice variants, which differ by 15 amino acids, have different accessibilities of the reactive thiol groups in the ligand binding domain.

EXPERIMENTAL

Materials

MPEG [methoxypoly(ethylene glycol) maleimide] (MPEG-20, molecular-mass 20 kDa, catalogue number 2F2MOP01; MPEG-5, molecular-mass 5 kDa, catalogue number 2F2MOHO1) were from Nektar Therapeutics. Rat brains were obtained

Abbreviations used: Ab, antibody; DTT, dithiothreitol; ER, endoplasmic reticulum; IP₃(R), *myo*-inositol 1,4,5-trisphosphate (receptor); mBB, monobromobimane; 2ME, 2-mercaptoethanol; MPEG, methoxypoly(ethylene glycol) maleimide; NEM, *N*-ethylmaleimide; PEG, poly(ethylene glycol); RyR, ryanodine receptor; SB, solubilization buffer; TBS, Tris-buffered saline; TMS, thimerosal.

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Table 1 Primers used for cysteine mutagenesis

The mutated codon is underlined.

Mutant	Primers	
	Sense (5' → 3')	Antisense (3' → 5')
C15S	5' catatcggagacatt <u>ttc</u> ctctctgtatgcagag	3' gtatagcctctgtaaaggagagacatagcttc
C37S	5' ttggtgatgaccgt <u>tcc</u> gtgtacagccagaa	3' aaccaactactggcaaggcaacatgtcggctt
C57/61S	5' ttcagagac <u>tcc</u> ctcttaagctatccctatgaat	3' aagtccttgaggagaaatcagatgggtagtagta
C206/214S	5' cgggc <u>tcca</u> atgaggtcaactccgcaact <u>tcca</u> acaca	3' gcccgaggttactccagttgagcgagttgtgt
C253S	5' gagaagtttctcag <u>tcc</u> gatgagcacaggaag	3' gtgttcaagagtgacggtactctgttccttc
C292S	5' gtccagcatgaccat <u>tcc</u> cggggtggagctggg	3' caggctgtactgggtaggggcccccactcgaccc
C326S	5' gactttgaggaagaat <u>tcc</u> ctggagtttcagccg	3' ctgaaactcctcttagggagcctcaagtcggc

from Pel-Freez Biologicals. Mersalyl, TMS, NEM (*N*-ethylmaleimide), DTT (dithiothreitol), and mBB (monobromobimane) were purchased from Sigma-Aldrich and [³H]IP₃ from PerkinElmer.

Abs (antibodies)

Peptides were synthesized with an N-terminal cysteine residue by Research Genetics and coupled with keyhole-limpet haemocyanin. Abs were raised in rabbits by Cocalico Biologicals and were affinity-purified using a peptide, coupled with Ultralink beads, as described in the manufacturer's (Pierce) protocol. The new or previously characterized Abs against rat type-I IP₃R used in the present study were as follows: tryptic fragment I (Ab to amino acids 139–155; designated NT-1, produced as described above), tryptic fragment II (Ab to amino acids 401–414; designated KEEK [14]), tryptic fragment III (Ab to amino acids 1554–1569; designated FragIII) and tryptic fragment V (Ab to amino acids 2733–2749; designated CT-1 [15]). The Ab raised against amino acids 1829–1848 of rat type-I IP₃R tryptic fragment IV (designated DEVD) was purchased from Affinity Bioreagents. The locations of the Ab epitopes are indicated in Figure 3 (below).

Expression constructs

The cDNA encoding rat type-I IP₃R SI(–)/SII(+)/SIII(+) splice variant in pCMV3 was the gift of Dr Thomas Sudhof (Center for Basic Neuroscience, Department of Molecular Genetics and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) [16]. The cDNA encoding the rat type-I IP₃R SI(+) variant was the gift of Dr Gregory Mignery (Department of Physiology, Loyola University, Chicago, IL, U.S.A.) [17]. The generation of the SI(+) type-I IP₃R variant encoding point mutations that converted cysteine residues into serine was accomplished using the QuikChange mutagenesis system (Stratagene). A cassette containing an EcoRI/KpnI segment of the ligand binding domain in pBluescript SK(+) was used as the PCR template. The sequences of the forward and reverse primers used are described in Table 1. The sequence-verified mutant was then subcloned into EcoRI/KpnI-restriction-enzyme-digested type-I SI(+) plasmid, propagated in JM109 cells (Invitrogen), and purified using a plasmid purification kit (Promega).

Cell culture and transfection

COS7 cells were grown to approx. 70% confluence in Dulbecco's modified Eagle's medium supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin and 10% fetal bovine serum in a 37°C humidified incubator in an atmosphere of 5% CO₂. TransIT LT-1 (Mirus) was used as the trans-

fection reagent and transfection carried out according to the manufacturer's protocol.

Preparation of microsomes

At 48 h post-transfection, confluent COS7 cells were washed twice with ice-cold PBS and scraped into isolation buffer containing 320 mM sucrose, 0.5 mM EGTA and 10 mM Tris (pH 7.8) supplemented with protease-inhibitor cocktail (Roche). 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 centrifuged 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 unstripped rat brains. The tissue was homogenized ten times in isolation buffer using a Dounce homogenizer with a tight-fitting pestle. The homogenate was centrifuged at 500 *g* for 15 min and the supernatant was removed and centrifuged for an additional 50 min at 100 000 *g*. The microsome pellet was resuspended in isolation buffer. All microsomes were either used fresh or stored frozen in liquid nitrogen.

Reaction with mBB

Cerebellum microsomes, prepared in the absence of any thiol reductants, were lysed in SB (solubilization buffer) containing 150 mM NaCl, 50 mM Tris/HCl (pH 7.8), 1% (w/v) Triton X-100, 1 mM EGTA and supplemented with protease-inhibitor cocktail. After removal of insoluble material by centrifugation (12 000 *g*, 10 min), 300 µg of lysate protein was immunoprecipitated overnight with CT-1 Ab and Protein A-Sepharose beads. The beads were washed twice in SB and three aliquots of the beads (50 µl) were then incubated for 10 min at 25°C with 2ME (2-mercaptoethanol; 5 mM), NEM (1 mM) or no further additions respectively. The beads were washed twice in SB and then resuspended in 50 µl of buffer containing 200 mM Tris/HCl (pH 7.8), 1% (w/v) SDS and 1 mM EDTA. The beads were heated to 95°C for 20 min to completely denature the IP₃R, cooled to 25°C and then incubated with 2 mM mBB in the dark for 20 min. The reaction was terminated by the addition of SDS/PAGE sample buffer [50 mM Tris/HCl (pH 6.8), 1% (w/v) SDS, 0.1% (w/v) Bromophenol Blue, 1% (v/v) 2ME and 5% (v/v) glycerol]. After electrophoresis (SDS/5% PAGE gels) the gels were fixed in two changes of 50% methanol for 5 min each, followed by 50% (v/v) trichloroacetic acid for 30 min. Fluorescent bands were detected with a Kodak image station 440CF (excitation/emission wavelengths of 365/450 nm) and quantified using ImageMaster™ 1D Image Analysis densitometric software (Amersham Pharmacia Biotech). The gel was subsequently stained with Coomassie Blue.

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 1 mM EGTA, in the presence or absence of MPEG-5 or MPEG-20. Routinely incubations were carried out at 25°C for 5 min. 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 12.5 µg/ml for 5 min at 25°C. The digestion was stopped by addition of 0.4 mg/ml soybean trypsin inhibitor (Sigma–Aldrich). In some experiments (e.g. Figures 4–6 and 9 below) the sample was centrifuged at 100 000 *g* in a Beckman TLA100 rotor. The supernatant was discarded and the membrane pellets were solubilized in SDS/PAGE sample buffer. This procedure improved the appearance of the gels, presumably by removing free MPEG.

Electrophoresis and immunoblotting

Gels (5 % or 10 %) were transferred to nitrocellulose membranes (Bio-Rad Laboratories) and blocked in a 10 % (w/v) dried milk solution in TBS (Tris-buffered saline) containing 0.1 % (v/v) Tween-20. Blots were developed with chemiluminescent substrates (Pierce). In cases where a blot was probed sequentially with more than one Ab, the nitrocellulose was stripped at 60°C for 30 min in stripping buffer [2 % (w/v) SDS, 100 mM 2ME, 62.5 mM Tris/HCl (pH 6.8)] before probing with the next Ab.

[³H]IP₃ binding assays

Isolated microsomes (from cerebellum or COS7 cells) were allowed to react with MPEG or TMS for various times as described above. Then 175 µl of the initial incubation was removed and added to 175 µl of labelling mixture containing 120 mM KCl, 20 mM Tris/Hepes (pH 8.3), 1 mM EDTA and 10 nM [³H]IP₃ for 5 min on ice. Triplicate samples (75 µl) were removed and vacuum-filtered through glass-fibre filters (Gelman A/E; Fisher Scientific). The filters were washed with 15 ml of 50 mM Tris/HCl (pH 8.3), 1 mM EDTA, and 0.5 mg/ml BSA. Non-specific binding was determined by incubation with 8 µM IP₃ (Calbiochem). The filters were counted for radioactivity in scintillation fluid (Budget Solve; RPI Corp.) and the amount of IP₃ bound was calculated from the specific radioactivity of the labelled IP₃ after correction for non-specific binding.

RESULTS

Basic characterization of IP₃R thiol groups

There are 60 cysteine residues in the 2749 amino acids of the rat type-I IP₃R [16]. In order to determine how many of these were present as free thiol groups we used mBB as a membrane-permeant, thiol-group-specific, fluorescent probe, which has previously been used for this purpose in studies on RyRs [4,5]. The type-I IP₃R was immunoprecipitated from a rat cerebellum microsome lysate and the immunoprecipitates were either treated with 2ME (to fully reduce all cysteine residues) or NEM (to covalently react with all available free thiol groups). The immunoprecipitates were washed to remove the thiol reagents and then allowed to react with an excess of mBB under denaturing conditions. The amount of fluorescently tagged IP₃R visualized on SDS/5 % PAGE was then compared after treatment under control, 2ME and NEM conditions. As expected, NEM pretreatment completely prevented reaction with mBB and the maximal signal was obtained when all the thiol groups were fully reduced with 2ME (Figure 1). Assuming that the treatment with 2ME led to reduction of 100 % of the available cysteine residues, it can be quantified

Figure 1 mBB reactivity of IP₃R in cerebellum microsomes

IP₃R immunoprecipitated from cerebellum microsomes were allowed to react for 10 min in the presence or absence of 5 mM 2ME and 1 mM NEM. The samples were then denatured and incubated with 2 mM mBB for 20 min as described in detail in the Experimental section. After electrophoresis the gels were fixed and visualized for fluorescently labelled IP₃R and for IP₃R protein by Coomassie Blue staining.

from the mBB fluorescence that the control, untreated sample had 72.2 ± 1.9 % (mean $\pm$ S.E.M.; *n* = 3) of available thiol groups in the reduced state.

The accessibility of these thiol groups in native membranes was investigated using a protein-tagging approach utilizing a maleimide attached to a 5 kDa (MPEG-5) or 20 kDa (MPEG-20) PEG. The covalent attachment of MPEG derivatives to the IP₃R would be expected to shift the size of the protein on SDS/PAGE. We used an Ab directed at the C-terminus to detect the type-I IP₃R in cerebellar membranes by immunoblotting. The observed molecular-mass shift, as a function of dose and time of incubation with MPEG-20, is shown in Figures 2(A) and 2(B). A large shift was observed at low concentrations of MPEG-20 (0.1 mM for 5 min) and at short times of incubation (0.5 mM for 15 s), although some unchanged IP₃R was still present. At higher concentrations and longer incubation times, the shift was more complete, but there was also evidence of loss of IP₃R signal from the blots. This may be a consequence of the large number of IP₃R-bound MPEG-20 molecules interfering with the reactivity of the CT-1 Ab or the ability of the protein to enter or transfer from the SDS/PAGE gel. A concentration of 0.5 mM MPEG-20 and a 5 min incubation time were considered optimal to investigate the most highly reactive thiol groups on the IP₃R and were used for further experiments. To confirm that the observed molecular-mass shift was due to covalent reaction with thiol groups, we examined the effect of 5 min preincubation with a number of thiol-group-reacting reagents, including NEM, DTT, TMS and mersalyl (Figure 2C). All these agents completely blocked the shift induced by MPEG-20, suggesting that the gel shift is due to covalent reaction with exposed thiol groups.

Highly reactive thiol group(s) are present in the N-terminal fragment I, but only in the SI(+) splice variant

To determine the locations of MPEG reaction sites, we utilized the fact that the IP₃R in cerebellum membranes is cleaved by trypsin into five distinct shorter fragments [18]. The boundaries of these fragments and the Abs used to detect them are shown in Figure 3. Initially, we focused our attention on the N-terminal fragment I as detected by immunoblotting with NT-1 Ab (Ab to amino acids 326–341). This fragment appears as a doublet of

Figure 2 Molecular-mass shift induced in cerebellum type-I IP₃R upon reaction with MPEG-20

(A) Cerebellum microsomes, prepared in the absence of DTT, were incubated with the indicated concentrations of MPEG-20 for 5 min at 25 °C. The samples were electrophoresed on a 5% gel and immunoblotted with CT-1 Ab. (B) shows the time course of the molecular-mass shift upon reaction with 0.5 mM MPEG-20. (C) Microsomal membranes were pre-treated with the indicated thiol reagents for 5 min at 25 °C. The concentrations used were as follows: NEM (10 mM), DTT (10 mM), TMS (1 mM) and mersalyl (1 mM). The samples were then incubated with 0.5 mM MPEG-20 for 5 min and processed for IP₃R detection as described above.

Figure 3 Diagram illustrating the intact and trypsin-fragmented type-I IP₃R

The upper diagram shows the intact receptor. The approximate locations of the three known splice sites (SI, SII, SIII), the ligand binding domain (LBD) and the six transmembrane helices are indicated (vertical bars). The lower diagram shows the approximate molecular-masses of the five tryptic fragments. Their amino-acid boundaries are indicated at the beginning of each fragment. Both fragment I and fragment IV have two alternative molecular-masses depending on the presence or absence of the spliced sequences present within these fragments. Also shown are the epitopes and names of the Abs used to detect the individual fragments.

bands with molecular-masses of 38 kDa and 43 kDa (Figure 4A, lane 1). These bands are thought to correspond to the fragment I from the SI(–) and SI(+) splice variants of the type-I IP₃R [18]. In agreement with this suggestion, only single NT-1-reactive bands were observed upon trypsin cleavage of membranes from COS cells transfected with SI(+) or SI(–) type-I IP₃R, and the

Figure 4 Reactivity of trypsin fragment I with MPEG in cerebellum, SI(+) and SI(–) type-I IP₃Rs

(A) Cerebellum microsomes (Cb, lanes 1 and 2) or microsomes from COS cells transfected with the SI(+) (lanes 3 and 4) or SI(–) (lanes 5 and 6) splice variants of the type-I IP₃R were incubated with 0.5 mM MPEG-20 for 5 min. The reaction was terminated with DTT and the samples were then trypsin-digested as described in the Experimental section. The samples were then electrophoresed on SDS/10% PAGE gels and immunoblotted with NT-1 Ab. Shifted bands are labelled with open circles. (B) Samples were as in (A) but processed on the same gel at the same exposure to illustrate the relative position of the doublet of bands seen in the cerebellum microsomes. (C) The same experiment as in (A) but using a 5 kDa MPEG derivative. (D) The indicated membranes were incubated with MPEG-20 (0.5 mM for 5 min). The molecular-mass shift in the full-length receptor was monitored by immunoblotting with CT-1 Ab.

mobility of these bands aligned with the cerebellum upper and lower doublet bands respectively (Figure 4B). The addition of MPEG-20 to cerebellar membranes caused the appearance of a single shifted NT-1-reactive band at 89.1 ± 1.4 kDa ($n = 19$) and diminished the intensity of the 43 kDa upper band in the doublet (Figures 4A and 4B, lane 2). These results suggest that only the SI(+) spliced trypsin fragment I in cerebellum has thiol groups that are accessible to MPEG-20. This was confirmed by the observation that only the 43 kDa band in SI(+) COS cell membranes gave a shift to 89 kDa (Figure 4A, lane 4), whereas the 38 kDa fragment I band in SI(–) membranes did not react with MPEG-20 (Figure 4A, lane 6). A similar pattern of results was obtained when the membranes were incubated with a smaller 5 kDa derivative of MPEG. Notably, the upper band of the cerebellum and the 43 kDa band in SI(+) membranes shifted to 57.4 ± 0.6 kDa ($n = 3$), but the 38 kDa band in the SI(–) membranes did not react with MPEG-5. It should be pointed out that the appearance of shifted bands was not the result of an altered pattern of trypsin digestion in the presence of MPEG, since the

Figure 5 Reactivity of tryptic fragments II, III, IV and V in cerebellum, SI(+) and SI(-) type-I IP₃Rs

Microsomal membranes from cerebellum (Cb) or COS cells transfected with the SI(+) or SI(-) type-I IP₃R construct were incubated with 0.5 mM MPEG-20 for 5 min and then cleaved with trypsin for 5 min as described in the Experimental section. The presence of each of the indicated tryptic fragments was monitored by immunoblotting with the Abs directed at the epitopes depicted in Figure 3. The expected position of the primary tryptic fragment is indicated by the arrowhead and shifted bands are indicated by open circles.

same results were obtained if the MPEG reaction was conducted after the membranes had been cleaved with trypsin (results not shown).

Reaction of MPEG with tryptic fragments II–V of the type-I IP₃R

Although the trypsin fragment I in SI(-) membranes does not show a shift with MPEGs, it is apparent that the intact full-length SI(-) IP₃R is reactive with MPEG-20 in a similar manner to that observed with SI(+) membranes (Figure 4D). This implies that there are other sites that are reactive with MPEG-20 outside of the fragment I domain. To determine the locations of additional MPEG-reactive sites, we analysed trypsin fragments II, III, IV and V using epitope-specific Abs (Figure 5). Our affinity-purified Ab to trypsin fragment II recognized multiple bands in addition to the expected primary fragment II at 67 kDa. However, none of the bands were altered by a 5 min incubation with 0.5 mM MPEG-20 (Figure 5). The predicted size of fragment IV is 40 kDa, although, as noted by others [18], this fragment often appeared as a doublet. Fragment IV contains the SII splice site, but this is unlikely to be the reason for the doublet, since all the membranes used in the present study are exclusively SII(+). In any case, there was no reactivity of the fragment IV bands with MPEG-20. The 91 kDa C-terminal fragment V was also not reactive.

The predicted size of trypsin fragment III is 76 kDa [18], but in our hands the fragment consistently appeared at the higher molecular-mass of 87.2 ± 1.9 kDa ($n = 11$). This band showed reactivity with MPEG-20 and appeared to shift to two distinct slower migrating bands (100.2 ± 3.9 kDa and 136.9 ± 2.9 kDa; $n = 11$). Unlike the observations with fragment I, the pattern of

Figure 6 Relative kinetics of the binding of MPEG to fragment I and fragment III bands

(A) COS cell microsomes from cells transfected with SI(+) (lanes 1–3) or SI(-) (lanes 4–6) type-I IP₃R were incubated with 0.5 mM MPEG-20 for 15 s (lanes 2 and 5) and 5 min (lanes 3 and 6) before being trypsin-digested for 5 min. The digested membranes were electrophoresed on SDS/10% PAGE gels and immunoblotted with NT-1 Ab for detection of fragment I and with Frag3 Ab for fragment III. Shifted bands are indicated by open circles. (B) SI(+) microsomes were allowed to react with MPEG-20 for 15 s or 5 min and then treated with trypsin for 5 min as described in the Experimental section. The shifts in fragment I were detected with NT-1 Ab. In lane 4 the membranes were treated with trypsin for 5 min and then treated with a 10-fold excess of soybean trypsin inhibitor before reaction with MPEG-20 for 5 min. (C) The experiment was performed as in (B) but using MPEG-5.

shifts in fragment III were identical in cerebellum, SI(+) and SI(-) membranes. Thus the overall conclusion from the present study is that the most highly reactive surface-exposed thiol groups are located exclusively in fragment I and fragment III of the type-I IP₃R.

Figure 6(A) shows the relative kinetics of the MPEG-20 interaction with fragment I and fragment III. It was apparent that the full-length receptor had significantly shifted its molecular-mass within 15 s of reaction with 0.5 mM MPEG-20 (Figure 4D). However, analysis of the tryptic fragment I in SI(+) membranes indicated that only a small percentage of the 43 kDa band had shifted by this time (Figure 6A). In contrast, fragment III showed a significant accumulation of the two shifted MPEG-20 bands even after 15 s. This indicated that the thiol groups in fragment III react more quickly than those in fragment I. It should also be noted that we were unable to see an intermediate band accumulating when examining the shift of the fragment I band, which is consistent with only a single reactive thiol group in fragment I participating in the MPEG-20 reaction. Two shifted bands were also observed in fragment III when MPEG-20 was added after trypsin digestion (Figure 6B, lane 4), excluding the possibility that the bands

Figure 7 Effect of MPEG on [³H]IP₃ binding to membranes

Membranes from cerebellum or COS cells transfected with SI splice variants of type-I IP₃R (last two columns) were incubated with the indicated thiol compounds and assayed for [³H]IP₃ binding as described in the Experimental section. Binding was expressed as a percentage of the binding observed in the absence of thiol compound. All assays were performed in triplicate and each bar represents the mean $\pm$ S.E.M. for three to nine independent experiments. * Statistically significant $P > 0.05$.

reflect altered tryptic digestion products. If the intermediate band represented a singly PEGylated species and the upper band a doubly PEGylated product we would expect that the singly PEGylated form would accumulate transiently and diminish as it was converted into the doubly PEGylated form. This was not observed in our time-course experiments, suggesting that both PEGylated bands represent independent products with distinct mobilities on SDS/PAGE. In contrast with the two bands observed with MPEG-20 only a single shifted band was seen in fragment III after reaction with MPEG-5 (Figure 6C) and no intermediate band was observed during the reaction. If it is assumed that both MPEG-5 and MPEG-20 react with the same number of thiol groups in fragment III, this would suggest that multiple thiol groups react simultaneously with MPEG-5 but not with MPEG-20.

Functional effects of the MPEG reaction

We examined the effect of MPEG-5 and MPEG-20 on [³H]IP₃ binding to microsomal membranes (Figure 7). Reaction of cerebellum microsomes for 5 min with MPEG-5 did not affect [³H]IP₃ binding. By contrast, reaction with MPEG-20 produced an approx. 50% inhibition of binding which was evident within 15 s. TMS is a thiol agent which markedly potentiates IP₃-mediated Ca²⁺ release [13,19]. In agreement with previous studies, we found an approx. 2-fold enhancement of [³H]IP₃ binding to cerebellum membranes [20]. The addition of MPEG-20, either before or after TMS, did not prevent the stimulatory effect. The results suggest that the TMS effect is dominant and occurs by reaction with thiol(s) that are different from those accessed by MPEG-20. Although both SI(+) and SI(−) type-I IP₃R splice variants react with MPEG agents, it is clear that only the SI(+) form shows reactions within the N-terminal ligand binding domain (Figure 4). The effects of MPEG-20 on [³H]IP₃ binding to SI(+) and SI(−) membranes are also shown in Figure 7. MPEG-20 reaction inhibited [³H]IP₃ binding to SI(+) membranes although the inhibition was smaller than observed for cerebellum microsomes. MPEG-20 did not affect binding to SI(−) membranes. Thus the results from the [³H]IP₃ binding measurements are in general agreement with the observations from gel-shift assays.

Location of reactive thiol(s) in fragment I of the IP₃R

Figure 8 highlights the nine cysteine residues in the primary sequence of tryptic fragment I and also indicates the position of the

Figure 8 MPEG reactivity of a cysteine residue present within the SI(+) splice site of the type-I IP₃R

(A) The Figure shows the rat SI(+) IP₃R sequence terminating at the boundary of fragment I established by N-terminal sequencing of proteolytic fragments [18]. The nine cysteine residues in the tryptic fragment I are shaded and the SI splice site is boxed. (B) COS7 cells were transfected with the C326S mutant of type-I IP₃R and the membranes were treated with 0.5 mM MPEG-20 or MPEG-5 for 5 min before being digested with trypsin for a further 5 min. The fragment I bands were monitored by immunoblotting with NT-1 Ab. Shifted bands are shown by open circles.

15 amino acids encompassing the SI splice site. It can be noted that a cysteine residue is present within the SI splice site (Cys³²⁶). Since only the SI splice variant appears to react with MPEG (Figure 4), this cysteine residue is an obvious candidate for the MPEG-reactive thiol group. We therefore mutated Cys³²⁶ to a serine residue and tested the reactivity of this mutant with MPEG-20 and MPEG-5 (Figure 8B). Surprisingly, the Cys³²⁶ mutant continued to show MPEG-induced shifts that were indistinguishable from that observed for wild-type receptors (see Figure 4). One possible explanation is that although the SI splice site may not be directly involved, its presence may modify the accessibility of another cysteine residue in fragment I. We therefore individually mutated each of the eight cysteine residues present in tryptic fragment I. Where two cysteine residues were relatively close in the primary sequence they were mutated simultaneously (e.g. C56/61S and C206/214S). The results shown in Figure 9 (upper panel) indicate that all of the cysteine mutants continued to react with MPEG-5. This result would be consistent with the presence of multiple, independently-reactive thiol groups. We therefore tested the effects of mutating Cys³²⁶ in the SI splice site in combination with each of the other eight cysteine residues. The results from this analysis indicate that C206/214/326S was the only combination mutant that failed to react with MPEG-5 (Figure 9).

DISCUSSION

The skeletal isoform of RyR1 contains 100 cysteine residues per subunit and, of these, approx. 20–50 are available for modification by thiol-reactive agents [4–6]. Recently, seven hyper-reactive

Figure 9 Mutation of cysteine residues singly or in combination and effects on MPEG-5 reactivity

Microsomal membranes prepared from COS cells transiently transfected with the indicated point mutations were screened for reactivity with MPEG-5 (0.5 mM) for 5 min as described in the Experimental section. The results shown are representative of two or three independent experiments.

cysteines have been identified in RyR1 using an MS approach [21]. Our measurements on SDS-denatured type-I IP₃R indicate that almost 70 % of the available 60 thiol groups can be modified by the small lipophilic thiol-specific probe mBB. We conclude that IP₃R shares with RyRs the property that a large number of the available cysteine residues are present in the reduced state. Although our measurements were made in the absence of redox buffers, the basic conclusion is also likely to be valid under the highly reducing conditions found in the cytoplasm.

The accessibility of these thiol groups has been addressed in the present study with a covalent mass-tagging approach using a maleimide conjugated to PEGs of different sizes. It is assumed that the bulky size of these compounds would limit attachment to only the most reactive, surface-accessible thiol groups. The ability to easily detect the molecular-mass shift by immunoblotting and to perform the experiment with receptors in their native environment are some of the advantages of this approach. A complex pattern of PEGylation may have been anticipated when dealing with a protein that has approx. 40 cysteine residues available for modification. Surprisingly, only a few available cysteine residues seem to be accessible in the native receptor when reaction with MPEG derivatives is monitored over a short time interval (< 5 min). These are confined to tryptic fragments I and III. Potentially, the magnitude of the gel shifts could be used to predict the number of reactive thiol groups. It is known that the hydrodynamic properties of MPEG cause target proteins to migrate more slowly than expected on gels [22,23]. For example MPEG-5 reaction with a test protein having only one available cysteine residue caused a molecular-mass shift of 10–15 kDa [22]. Although this precludes use of the MPEG gel-shift assay as a quantitative probe, the presence of multiple bands and elimination of gel shifts by selective mutagenesis can still provide information on the number and identity of accessible reactive thiol groups.

A surprising observation was the finding that MPEG reactivity within tryptic fragment I was restricted to the SI(+) splice variant of the type-I IP₃R. Mutation of the single cysteine residue (Cys³²⁶) present within the 15-amino-acid sequence encoded by SI did not eliminate MPEG reactivity. Nor was reactivity ablated by individual mutation of all nine cysteine residues within fragment I. This result suggests that more than one cysteine residue is reactive with MPEG. Crystal structures are available for the N-terminal segment of the type-I SI(+) IP₃R [24,25]. The N-terminal 223 amino acids, referred to as the 'suppressor domain', contain six of the cysteine residues mutated in the present study. Of these only Cys²¹⁴ is present in a highly exposed loop. Although we did not individually mutate Cys²⁰⁶, its side chain appears occluded like that of the other cysteine residues in the suppressor domain. The remaining three cysteine residues are present in the core ligand

binding domain [24]. Cys³²⁶ in the SI splice site is not visible in the crystal structure, but the orientation of the adjacent β -strands and analogy with the β -trefoil structure in the suppressor domain suggest that this residue would be highly accessible. Cys²⁵³ appears to be in an occluded location and Cys²⁹² is not visible in the structure. Thus, on the basis of mutagenesis and examination of the crystal structures, Cys²¹⁴ and Cys³²⁶ appear to be the most likely candidates for highly reactive surface-exposed thiol groups in tryptic fragment I. A hypothesis that is consistent with the experimental results is that Cys²¹⁴ in the suppressor domain and Cys³²⁶ in the ligand binding domain are in close proximity. Reaction of the bulky MPEG molecule with one of the thiol groups would sterically hinder access to the other reactive group. This would explain why mutation of either residue singly does not block MPEG reactivity. When the SI splice site is absent, we suggest that the structure is altered such that the remaining reactive Cys²¹⁴ thiol group is occluded. The model is consistent with recent data showing that suppressor and ligand binding domains of the type-I IP₃R interact *in vitro* [26].

The implication of the results obtained in our experiments is that the presence or absence of the 15-amino-acid sequence in the SI splice site may impact on the structure of the N-terminal segment of the receptor and/or the interactions between different domains. Functional studies comparing the type-I SI(+) and SI(-) splice variants have not revealed any significant differences in the affinity of IP₃ binding or the biophysical properties of the Ca²⁺ channel [17,27]. Recently, Regan et al. [28] have shown that there are differences in the Ca²⁺ sensitivity of [³H]IP₃ binding between SI(+) and SI(-) variants stably expressed in HEK-293 cells. These cells also showed differences in the pattern of Ca²⁺ transients elicited by angiotensin II [28]. Several studies have shown that the SI splice variants are differentially expressed in various tissues and during neuronal development [28,29]. Ca²⁺ influx stimulated by 25 mM KCl or *N*-methyl-D-aspartate in cultured granule cells selectively induces the SI(+) variant of the type-I IP₃R [30]. The possibility that the exposure of reactive thiol groups in the SI(+) variant may alter redox regulation of IP₃R function or the selective association with other regulatory proteins remains to be explored.

Cysteine-substitution mutagenesis has been extensively employed to study the structure and function of ion channels and receptors. The specific use of MPEG derivatives to probe the accessibility of endogenous and engineered thiol groups in voltage-gated K⁺ channels has been pioneered by Lu and Deutsch [22]. Normally, these studies involve making a cysteine null mutant - a daunting task in IP₃R which contain 60 cysteine residues. The absence of reactivity to the bulky MPEG molecule in the SI(-) tryptic fragments I, II, IV and V offers an experimentally null

background in which the topological accessibility of cysteine-substitution mutants can be investigated. Large conformational changes in the IP₃R induced by IP₃ or Ca²⁺ would also be expected to alter the MPEG reactivity of endogenous or engineered thiol groups and this can be assayed with the receptor in its native membrane environment. The potential for using MPEG to study the surface topology and conformational changes in IP₃Rs is currently being investigated.

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