Mapping of the ATP-binding Sites on Inositol 1,4,5-Trisphosphate Receptor Type 1 and Type 3 Homotetramers by Controlled Proteolysis and Photoaffinity Labeling*

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Inositol 1,4,5-trisphosphate (IP$_3$)$^3$ is an intracellular second messenger that mediates the release of Ca$^{2+}$ from internal stores. ATP binds to the IP$_3$ receptor (IP$_3$R), an intracellular Ca$^{2+}$-release channel (1). The IP$_3$R is composed of three functionally different domains: an N-terminal IP$_3$-binding region, a large transducing domain, and a C-terminal channel region (2). The transducing domain contains interaction sites for several modulators of IP$_3$-induced Ca$^{2+}$ release such as Ca$^{2+}$, calmodulin, kinases, phosphatases, ATP, and FKBP12 (reviewed in Refs. 1 and 3). IP$_3$Rs are encoded by three different genes, IP$_3$R1 and IP$_3$R3, both proteins were expressed in Sf9 insect cells and covalently labeled with 8-azido-[α-32P]ATP. ATP regulates the IP$_3$R in a concentration-dependent manner: Submillimolar concentrations enhance IP$_3$-induced Ca$^{2+}$ release (16–20), whereas millimolar levels of ATP inhibit IP$_3$-induced Ca$^{2+}$ release by competing with IP$_3$ for the IP$_3$-binding site (18–23). The stimulatory effect of ATP is to occur via binding to one or more sites on the IP$_3$R, because purified IP$_3$Rs bind [α-32P]ATP in a specific manner (17, 20, 24). The number and the localization of these sites have, however, not yet been determined. Based on the glycine-rich amino acid sequence (25), two ATP-binding sites were postulated on the neuronal form of IP$_3$R1 (aa 1773–1780 and 2016–2021, respectively). In IP$_3$R3, only one fragment was labeled. This fragment contained the GXGXXG sequence (amino acids 1920–1925), which is conserved in the three IP$_3$R isoforms. The presence of multiple interaction sites for ATP was also evident from the IP$_3$-induced Ca$^{2+}$ release in permeabilized A7r5 cells, which depended on ATP over a very broad concentration range from micromolar to millimolar.

Submillimolar ATP concentrations strongly enhance the inositol 1,4,5-trisphosphate (IP$_3$)-induced Ca$^{2+}$ release, by binding specifically to ATP-binding sites on the IP$_3$ receptor (IP$_3$R). To locate those ATP-binding sites on IP$_3$R1 and IP$_3$R3, both proteins were expressed in Sf9 insect cells and covalently labeled with 8-azido-[α-32P]ATP. IP$_3$R1 and IP$_3$R3 were then purified and subjected to a controlled proteolysis, and the labeled proteolytic fragments were identified by site-specific antibodies. Two fragments of IP$_3$R1 were labeled, each containing one of the previously proposed ATP-binding sites with amino acid sequence GXGXXG (amino acids 1773–1780 and 2016–2021, respectively). In IP$_3$R3, only one fragment was labeled. This fragment contained the GXGXXG sequence (amino acids 1920–1925), which is conserved in the three IP$_3$R isoforms. The presence of multiple interaction sites for ATP was also evident from the IP$_3$-induced Ca$^{2+}$ release in permeabilized A7r5 cells, which depended on ATP over a very broad concentration range from micromolar to millimolar.

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† The abbreviations used are: IP$_3$, inositol 1,4,5-trisphosphate; IP$_3$R, IP$_3$ receptor; aa, amino acids; GST, glutathione S-transferase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

EXPERIMENTAL PROCEDURES

Materials—CHAPS was obtained from Pierce (Rockford, IL). Chymotrypsin, N-tosyl-l-phenylalanine chloromethyl ketone, heparin-agar-

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ose, and N-acetyl-D-glucosamine were from Sigma Chemical Co. (St. Louis, MO). 8-Azido-α-[32P]ATP (2 µCi/ml, 12 Ci/mmol) was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). 45CaCl2 (2.2 mM/ml, 134 µCi of Ca2+/ml), wheat germ agglutinin-Sepharose 6MB, Rainbow molecular mass markers, the anti-mouse and anti-rabbit alkaline phosphatase-conjugated secondary antibodies, and the Vistra ECF substrate were from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

Expression of IPr1 and IP3R in Insect Sf9 Cells—The full-length mouse IPr1 and the full-length rat IP3R were expressed in insect Sf9 cells as described by Sipma et al. (30) and by Maes et al. (24), respectively.

Photoaffinity Labeling with 8-Azido-α-[32P]ATP of Recombinant IPr1—Microsomes of Sf9 insect cells were prepared as described (31). Photoaffinity labeling of microsomes containing either IPR1 or IP3R with 8-azido-α-[32P]ATP was performed exactly as described in Maes et al. (24).

Purification of Recombinant IPr1 and IP3R from Sf9 Microsomes—The purification of IPr1 was based on the method described by Parys et al. (32). Microsomes of Sf9 cells expressing IPr1 and IP3R in a concentration of 10 mg of protein/ml were centrifuged, and the pellet was solubilized (at 5 mg/ml) for 1.5 h at 4 °C in buffer A (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.53 mM benzamidine, and 10 mM 2-mercaptoethanol) with addition of 200 mM NaCl, 77 mM aprotonin, 1.1 µM leupeptin, 0.7 µM pepstatin A, 2.5% (w/v) β-mercaptoethanol and 0.1% (v/v) chymotrypsin. After centrifugation, the supernatant was diluted with an equal volume of buffer A with addition of 400 mM NaCl. The diluted supernatant was incubated for 30 min with heparin-agarose beads (112.5 µg of protein). The eluate obtained in buffer A with 600 mM NaCl, 0.75% CHAPS, and 0.3% L-α-phosphatidylycholine, was incubated for 2 h with wheat germ agglutinin-Sepharose (75 µg of protein). After wash steps in high (600 mM) and low (100 mM) salt conditions, the specifically bound proteins were eluted in low salt conditions with 300 mM N-acetyl-D-glucosamine. All centrifugation steps were for 17 min at 35,700 × g at 4 °C.

Controlled Proteolysis—Purified IPr1 was partially digested with chymotrypsin (0.05 µg/ml) for 2, 5, 10, or 30 min on ice as described previously (32). The digestion was stopped by the addition of 100 µg/ml N-tosylphenylalanine chloromethyl ketone and by boiling the samples for 5 min in sample buffer for SDS-PAGE.

Antibodies and Western Blotting—The monoclonal antibody against the C terminus of mouse IPr1 (Rbt03), the mouse monoclonal antibody against an N-terminal epitope of human IP3R3 (MMAType3) (Transduction Laboratories, Lexington, KY) and the polyclonal antibody against the Ca2+-binding domain cytI3b (amino acids 378–450) in the IP3-binding domain of mouse IPr1 (32) were characterized earlier (4, 30, 34). A novel antibody was raised against the luminal Ca2+-binding domain of mouse IPr1 (Rbt03), the mouse monoclonal antibody against residues 1829–1848 of human IP3R1 was purchased from Alexis Corp. (Läufelfingen, Switzerland). A polyclonal antibody against the C terminus of human IP3R3 was from Santa Cruz Biotechnology (Santa Cruz, CA). The various microsomal preparations were analyzed on 3–12% Laemmli-type gels and transferred to Immobilon-P (Millipore Corp., Bedford, MA). Immunodetection of the proteins on the transfers was performed as described previously (30, 36).

Ca2+ Fluxes—IP3-induced Ca2+ release from permeabilized A5T monolayers was described elsewhere (16). The added ATP concentrations are indicated in the legend to Fig. 4. The medium for the challenge with IPr1 contained 120 mM KCl, 30 mM imidazole-HCl, pH 6.8, and 1 mM EGTA.

RESULTS AND DISCUSSION

Purification of IPR1s from Sf9 Insect Cells—To allow an accurate analysis involving controlled proteolysis and immunostaining, purification of the IPR1s is needed. IPr1 has been purified from cerebellum (37, 38), smooth muscle (39, 40) and oocytes (32). Until now, no IP3R has been purified due to the lack of a known cell type that abundantly expresses this isoform. We therefore expressed IPR1 or IP3R in Sf9 insect cells, resulting in a 2.5 times higher expression of IPR1 and a >50 times higher expression of IP3R as compared with rabbit cerebellar and 16HBE140-microsomes, respectively (24, 30). In the purification procedures described by Chadwick et al. (39) and Parys et al. (32), microsomes were first solubilized by a detergent followed by chromatography on heparin- and lectin-based matrices. This method was based on the ability of hep- airin to bind to the IP1R with high affinity (41–43) and on the presence of N-glycosylation sites on 2 asparagine residues present in IPR1 (44). It has been suggested that IP3R is also a glycoprotein (45), although only one N-glycosylation site is predicted based on the primary sequence (46).

In this study, we have used an identical approach to purify recombinant IPR1 and IP3R overexpressed in Sf9 insect cells. It had to be verified whether the glycosylation patterns of these proteins were the same as in mammalian cells. Briefly, microsomes from Sf9 cells overexpressing either IPR1 or IP3R were solubilized with 2.5% CHAPS. Subsequently, the solubilized microsomes were incubated with heparin-agarose. After elution of the bound fraction, the latter was incubated with wheat germ agglutinin-Sepharose. Both receptors could be purified with high efficiency and were recognized by isoform-specific antibodies (Fig. 1, A and B, first lane of each blot), confirming that they are both glycoproteins and that the post-translational glycosylation of the IP3Rs in insect cells is similar to that in mammalian cells. The purified IP1R migrated on SDS-PAGE with a molecular mass of 273 kDa, which deviated from the molecular mass of 313 kDa predicted from the primary structure (Fig. 1A, first lane of each blot). The purified IP3R also migrated with a lower apparent molecular mass (248 kDa) than predicted (304 kDa) (Fig. 1B, first lane of each blot). Because a similar behavior is also found for endogenous IP3Rs from, e.g., cerebellar or 16HBE140-cells (20, 47), this discrepancy is likely due to aberrant mobility of higher molecular mass proteins on SDS-PAGE.

Controlled Proteolysis and Identification of Proteolytic Fragments—The purified IP3Rs were subjected to a controlled proteolysis with chymotrypsin (0.05 µg/ml, up to 30 min on ice), and the digestion fragments were detected by a panel of different site-specific antibodies (Fig. 1, A and B, and Table I). No degradation of the intact IP3R was observed during incubation without chymotrypsin (Fig. 1, first lane of each blot).

For IP1R, four site-specific antibodies were used, of which the epitopes were spread over the whole sequence. The anti cytI3b-2 antibody (30) is directed against a Ca2+-binding site in the IP3-binding domain (33). The anti-(1829–1848) antibody (Alexis Corp.) recognized an amino acid stretch (residues 1829–1848) located in the regulatory domain between the two putative ATP-binding sites (residues 1773–1780 and 2016–2021) (2, 26–28). A third antibody, anti-loop117a-2, was raised against the luminal Ca2+-binding fragment (35). Finally, Rbt03 (30, 34) recognized the C terminus of IP3R. The proteolytic pattern, resulting from up to 30 min of incubation with chymotrypsin, as well as detected by the four antibodies against IP1R, is shown in Fig. 1A. We determined the length of the fragments using Rainbow molecular mass markers. Based on these data, we were able to localize the chymotrypsin-sensitive sites on IP3R (Fig. 2A). The sum of the molecular mass of the five major proteolytic fragments (40, 65, 80, 40, and 90 kDa) was close to the molecular mass of the intact IP3R (313 kDa). This result was in complete agreement with the study of Yoshikawa et al. (48), where trypsin was used to digest cerebellum-purified IP3R and where five similar major proteolytic-insensitive fragments were found. Although we were able to

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2 J. B. Parys, unpublished data.
recognition of the intermediate digestion products with site-specific antibodies, two proteolytic fragments, which were predicted based on Fig. 2A, could not be detected when the digestion was performed for 30 min. Particularly, a 145-kDa fragment, precursor of the 65- and 80-kDa fragments should be recognized by the anti-cytI3b antibody and a 185-kDa fragment, precursor of the successive fragments of 65, 80, and 40 kDa should be recognized by the anti-(1829–1848) antibody. Because it is possible that these intermediate fragments have a short life time, we decreased the time of proteolysis to 2, 5, and 10 min, respectively (Fig. 1A, insets of blots 1 and 2). Upon staining with the anti-cytI3b-2 antibody (blot 1 and inset), we detected a proteolytic band corresponding to a molecular mass of 145 kDa, which is most intense at 2 and 5 min of incubation with chymotrypsin. This fragment is rapidly degraded into smaller fragments, because it is poorly or not visible in the proteolytic patterns representing 10 and 30 min of incubation of IP3R1 with chymotrypsin. Upon staining with the anti-(1829–1848) antibody, no clear fragment with a mass of 185 kDa was visible, even at shorter time points (blot 2 and inset). Because the corresponding predicted fragment was also not detected in the study of Yoshikawa et al. (48), it is conceivable that the latter intermediate fragment is rapidly degraded into smaller subfragments during proteolysis and therefore a steady-state level below the detection limit for the antibodies. All identified fragments, with indication of their molecular mass and the recognizing antibodies, are represented in Table II.

The same type of experiment was performed for IP3R3. Only two site-specific antibodies are available for this isoform: the MMAtype3 antibody (Transduction Laboratories) (4) directed against the N terminus, and the anti-CIII antibody (Santa Cruz Biotechnologies) against the C terminus. However, the anti-loopI17a-2 antibody could also recognize IP3R3, although with lower sensitivity. The proteolytic pattern as stained by the three antibodies against IP3R3 is shown in Fig. 1B. The time dependence of the occurrence of the proteolytic fragments was also investigated for IP3R3, but incubation with chymotrypsin for shorter times (2–10 min) revealed the same pattern of proteolytic fragments (data not shown). All identified fragments, with their molecular mass and the recognizing antibodies, are represented in Table III. In addition we have verified the N-terminal boundaries of some of the major proteolytic fragments by N-terminal amino acid microsequencing (data not shown). A schematic presentation of IP3R3 with the major proteolytic fragments (105, 70, 35, and 95 kDa) is shown in Fig. 2B. The sum of the molecular mass of the fragments was close to the molecular mass of the intact receptor (304 kDa) as calculated from the cloned rat IP3R3. The general structure of IP3R3 resembled that of IP3R1: Both receptor isoforms were sensitive to proteolysis at similar sites. Only the chymotrypsin-sensitive site that is present in the IP3-binding domain of IP3R1, could not be detected in IP3R3. This could however be due to the lack of an antibody that recognized the relevant part of the IP3-binding domain. Alternatively, it is also possible that IP3R3 lacks the chymotrypsin-sensitive site in the IP3-binding domain. It is conceivable that the proteolysis-sensitive sites represent regions that are exposed on the surface of the protein and thereby accessible to the proteolytic enzymes as well as to different modulators of IP3-induced Ca2+ release. Because functional IP3Rs are mostly organized in heterotetramers (9–12), it can be expected that corresponding regions of the different IP3R isoforms are exposed at the surface of the receptor protein so that they can be properly regulated.

Identification of Photoaffinity-labeled Proteolytic Fragments—In a previous study, we showed that two GST fusion proteins, each containing a putative ATP-binding domain of IP3R1, could bind ATP (29). Both predicted ATP-binding domains were situated near chymotrypsin-sensitive sites (Fig.

### Table I

| Antibody          | IP3R subtype | Epitope | References/source |
|-------------------|--------------|---------|-------------------|
| Anti-cytI3b-2     | 1            | m378–450 | (30)              |
| Anti-(1829–1848)  | 1            | h1829–1848 | Alexis Corp.     |
| Anti-loopI17a-2   | 1            | m2463–2528 | This study       |
| Rbt03             | 3            | r2391–2456 |                |
| MMAtype3          | 3            | m2735–2749 | (30, 34)         |
| Anti-CIII         | 3            | h22–230  | (4)               |
| Anti-CIII         | 3            | h2652–2671 | Santa Cruz Biotechnologies |

*m, mouse; h, human; r, rabbit.*
ATP-binding Sites on IP\textsubscript{3}R

It is therefore likely that they are both accessible to ATP in the intact protein. To prove this, we incubated microsomes from Sf9 cells expressing recombinant IP\textsubscript{3}R1 with the photoaffinity label 8-azido-[\textalpha;\textsuperscript{32}P]ATP. Covalent labeling of the ATP-binding sites by UV irradiation was followed by purification and controlled proteolysis of IP\textsubscript{3}R1 and identification of the labeled proteolytic fragments by site-specific antibodies. The two smallest labeled proteolytic fragments of IP\textsubscript{3}R1 (90 and 40 kDa, Fig. 2A) were recognized by the Rbt03 antibody and the anti-(1829–1848) antibody, respectively (Table II), indicating that they represented the proteolytic fragments containing the previously proposed ATP-binding sites (Fig. 2A).

IP\textsubscript{3}R3 contained only one of these proposed ATP-binding sites, which is conserved in all IP\textsubscript{3}R isoforms and which is also located near a chymotrypsin-sensitive site (Fig. 2B). To confirm this, we performed the same photoaffinity labeling experiment for the IP\textsubscript{3}R3 isoform. The smallest labeled band of 95 kDa was recognized by the MMAtype3 antibody and the anti-loopI17a-2 antibody, respectively (Table III). The details of the photoaffinity labeling, purification, and controlled proteolysis are described under "Experimental Procedures."
After SDS-PAGE and blotting, labeled IP3Rs were visualized using the lower EC50 (40 μM) than IP3R1 (24 μM), because it bound equally well ATP and GTP. More recently, Hagar and Ehrlich (49) demonstrated that IP3R3 incorporated in lipid bilayers was shown to be dependent on the IP3R isoform composition in the particular cell type.

**Fig. 3.** Photoaffinity labeling of IP3R1 (A) and IP3R3 (B) followed by controlled proteolysis. Microsomes from Sf9 insect cells were incubated with 20 μM 8-azido-[α-32P]ATP and subsequently irradiated with UV light for 2.5 min. IP3R1 and IP3R3 were purified from those microsomes after solubilization of the microsomes with CHAPS and binding to heparin-agarose and subsequently wheat germ agglutinin-Sepharose. IP3R3 were digested with 0.05 μg/ml chymotrypsin for 30 min on ice (lanes 2) or were not treated with chymotrypsin (lanes 1). After SDS-PAGE and blotting, labeled IP3R3 were visualized using the Storm 840 PhosphorImager (Molecular Dynamics). Positions of the molecular mass markers (in kDa) are indicated. The details of the photoaffinity labeling and the proteolysis are described under “Experimental Procedures.”

(Fig. 3B) was recognized by the anti-CIII antibody (Table III), indicating that this band represented the proteolytic fragment containing the putative ATP-binding site of IP3R3 (Fig. 2B). In summary, covalent labeling with 8-azido-[α-32P]ATP occurred at two different proteolytic fragments of IP3R1 and only at one proteolytic fragment of IP3R3. The labeled fragments contained the two previously proposed ATP-binding sites, one of which is conserved in all IP3R isoforms.

The unequal number of ATP-binding sites found in IP3R1 and IP3R3 may explain the differential modulation of these isoforms by ATP. IP3R1 showed a higher affinity for ATP than IP3R3 (13, 14, 24), suggesting that the upstream ATP-binding site, which is only present in IP3R1, is a high-affinity binding site. Moreover, IP3R3 displayed a broader nucleotide specificity than IP3R1 (24), because it bound equally well ATP and GTP. The latter property can be assigned to the ATP-binding site present in IP3R3 and conserved in all IP3R isoforms. The ATP-binding site that is only present in IP3R1 was more specific for adenine nucleotides like ATP and ADP (24).

**ATP Dependence of IP3-induced Ca2+ Release**—In permeabilized A7r5 cells, which express IP3R1 and IP3R3 in a 3 to 1 ratio (5), ATP dependence of IP3-induced Ca2+ release was found over a very broad concentration range (Fig. 4). ATP stimulated IP3-induced Ca2+ release from the low micromolar range up to 1 mM. At still higher ATP concentrations, the release was inhibited probably due to competition of ATP for the IP3-binding site (18–23). The broad concentration dependence in A7r5 cells is in very good agreement with the different ATP affinities described previously for recombinant IP3R1 and IP3R3 (EC50 values of 1.6 μM and 177 μM, respectively) (24). This difference in ATP affinities between IP3R1 and IP3R3 was also observed by other groups: recent findings of Hagar and Ehrlich (49) demonstrated that IP3R3 incorporated in lipid bilayers was activated by ATP with an EC50 of about 3 μM, whereas a much lower EC50 (40 μM) was observed for IP3R1 (19). Moreover, IP3- induced Ca2+ release in genetically engineered DT40 B cells that express a single IP3R subtype was also found to respond differently to ATP. In IP3R1-expressing cells, the rate of Ca2+ release was enhanced by ATP with an EC50 of 0.39 mM, whereas IP3R3-expressing cells were much less sensitive to ATP (14).

Because it was not possible to resolve the ATP concentration dependence in A7r5 cells by curve-fitting procedures, the presence of two IP3R isoforms in A7r5 is reflected in two separate stimulatory ATP-binding sites. However, for preparations from rat cerebellum containing nearly exclusively IP3R1, the maximum stimulation by ATP was found at 50 μM. At 1 mM ATP, IP3-induced Ca2+ release in cerebellar preparations was close to control values (50), whereas 1 mM ATP was the maximum stimulatory concentration in A7r5 cells. The much higher maximum for ATP stimulation found for A7r5 cells is therefore very probably a reflection of the presence of IP3R3. It was also not possible to decide whether these properties of IP3R3 are inferred in A7r5 cells by homo- or heterotetramers. Immunoprecipitation experiments indicated that a significant fraction of IP3R1 and IP3R3 expressed in A7r5 cells is present as heterotetramers. Our data clearly showed that the presence of different ATP-binding sites on IP3R1 and IP3R3 resulted in a nucleotide sensitivity of IP3-induced Ca2+ release that extended over a broad concentration range. The ATP concentration that yielded maximum stimulation seems very variable and to be dependent on the IP3R isoform composition in the particular cell type.

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