Molecular and Functional Evidence for Multiple Ca\(^{2+}\)-binding Domains in the Type 1 Inositol 1,4,5-Trisphosphate Receptor*

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The inositol 1,4,5-trisphosphate receptor (InsP\(_3\)-R) is an intracellular Ca\(^{2+}\) release channel that is modulated by various physiological ligands such as inositol 1,4,5-trisphosphate (InsP\(_3\)), Ca\(^{2+}\), nucleotides, calmodulin (CaM), FK506BP, phosphatases, and kinases (reviewed in Refs. 1 and 2). The InsP\(_3\)-R can be divided into three functionally different domains as follows: an N-terminal ligand-binding domain, a modulatory domain, and a channel domain near the C terminus (3, 4). Among the various modulators, Ca\(^{2+}\) itself plays a pivotal role and may be considered as a co-agonist exerting both positive and negative effects on InsP\(_3\)-induced Ca\(^{2+}\) release. Cytosolic Ca\(^{2+}\) has a bell-shaped effect on the InsP\(_3\)-R, with low concentrations stimulating the release and high concentrations inhibiting the release (5–8). Both phases are time-dependent, but the inhibition develops more slowly than the stimulation (6, 9–11). Although these kinetically distinct effects are believed to be crucial for the generation of Ca\(^{2+}\) oscillations and waves (12), little is known about how Ca\(^{2+}\) interacts with the InsP\(_3\)-R. Regulation might be exerted by direct binding of Ca\(^{2+}\) to the InsP\(_3\)-R and/or via Ca\(^{2+}\)-sensitive protein(s), e.g. via Ca\(^2+\)/CaM-dependent protein kinase II and protein phosphatase 2B (13) or via a Ca\(^2+\)-sensitizing factor (14). Studies in which effects of Sr\(^{2+}\) were compared with those of Ca\(^{2+}\) revealed that at least two interaction sites must exist: a stimulatory site that is modestly sensitive to Sr\(^{2+}\) and an inhibitory site that is nearly insensitive to Sr\(^{2+}\) (15). One Ca\(^{2+}\)-binding domain has been localized on the cytosolic side of the InsP\(_3\)-R-1 (16, 17), but whether this domain represents a stimulatory or an inhibitory interaction site is still unclear. Luminal Ca\(^{2+}\) also regulates the InsP\(_3\)-induced Ca\(^{2+}\) release. Loading of the InsP\(_3\)-R with Sr\(^{2+}\) stores results in a relatively more pronounced InsP\(_3\)-induced Ca\(^{2+}\) release (18–21). A high affinity Ca\(^{2+}\)-binding site was detected on the luminal loop (17), but its functional significance is not yet clear.

The aim of the present work was two-fold. First, we wanted to investigate, at the structural level, the presence of direct Ca\(^{2+}\)-binding sites on InsP\(_3\)-R-1. Second, we wanted to functionally demonstrate multiple Ca\(^{2+}\) interactions on the InsP\(_3\)-R by a kinetic analysis of the effects of Ca\(^{2+}\) and Sr\(^{2+}\) on the InsP\(_3\)-induced Ca\(^{2+}\) release. For the first part of this study we constructed and expressed a number of GST fusion proteins that contain cytosolic fragments of the InsP\(_3\)-R sequence. The ability of these fusion proteins to bind 45Ca\(^{2+}\) and ruthenium red was measured by overlay procedures and verified by staining with Stains-all. These studies identified multiple Ca\(^{2+}\)-binding domains in the InsP\(_3\)-R sequence.

In the second part of the study we accurately determined the

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The abbreviations used are: InsP\(_3\)-R, inositol 1,4,5-trisphosphate receptor; InsP\(_3\), inositol 1,4,5-trisphosphate; InsP\(_3\)-R-1, type 1 InsP\(_3\)-R; GST, glutathione S-transferase; RyR, ryanodine receptor; CaM, calmodulin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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Primers—The following oligonucleotides were synthesized with a 5′-flanking BamHI or EcoRI restriction site (underlined) for the forward primers and with a 5′-flanking EcoRI restriction site (underlined) for the reverse primers: Cyt1F, 5′-ATCGAGTACCCGGCTTACCTCTCAGCTTGCACTTCTATTCCC-3′ (nucleotide position 393–526); Cyt1R, 5′-ATAGCGAATTCCGGCTTACCTCTCAGCTTGCACTTCTATTCC-3′ (nucleotide position 1145–1481); Cyt3F, 5′-ATCGAGTACCCGGCTTACCTCTCAGCTTGCACTTCTATTCCC-3′ (nucleotide position 1238–1265); Cyt3F, 5′-ATAGCGAATTCCGGCTTACCTCTCAGCTTGCACTTCTATTCC-3′ (nucleotide position 1238–1265); Cyt5F, 5′-ATCGAGTACCCGGCTTACCTCTCAGCTTGCACTTCTATTCCC-3′ (nucleotide position 1145–1481); Cyt4F, 5′-ATAGCGAATTCCGGCTTACCTCTCAGCTTGCACTTCTATTCC-3′ (nucleotide position 1238–1265); Cyt4R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt9R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt10R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3485–3487); Cyt10F, 5′-ATCGGCCAAGCTCCACGAGG-3′ (nucleotide position 3539–3562); Cyt1bR, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt11R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt15F, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt15R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3485–3487); Cyt16F, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt17R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt1R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt12R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt13R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt14R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt16R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt17R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt18F, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515); Cyt18R, 5′-ATGAGAATTCCAGACTCTCCAGGGTTTGG-3′ (nucleotide position 3490–3515);}

EXPERIMENTAL PROCEDURES

Materials—[56CaCl2 (2.2 mCi/ml, 134 μg of Ca2+ /ml) was obtained from Amersham Int., UK. Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer Mannheim, Belgium, or New England Biolabs, Beverly, MA. InsP3, and isopropanol. The remaining phosphoglycerate kinase was supplied by Boehringer Mannheim. Immobilon P (polyvinylidene difluoride) membrane filters were from Millipore Corp., MA. Plasmid pGEX-2T and glutathione-Sepharose 4B were from Pharmacia Biotech Inc., Sweden. New England Biolabs supplied broad range protein markers. Triton X-100, N-lauroylsarcosine (sarcosyl), ruthenium red, and Stains-all were purchased from Sigma. The Escherichia coli hosts used in this study were E. coli strain DH5α (Westminster, Great Britain) probe sonicator. The sonicated material was centrifuged (10,000 rpm for 10 min in a Sorvall SS-34 rotor). At this stage, the procedure differed for the soluble GST fusion proteins and the insoluble GST fusion proteins. The supernatant of the soluble GST fusion proteins was immediately incubated with glutathione-Sepharose 4B beads, whereas the initial supernatants of the insoluble recombinant proteins were discarded, and the pellets were

TABLE I

| Name | Length | aa position in InsP3R-1 | Primer pair |
|------|--------|-------------------------|-------------|
| Cyt1 | 495    | 6–159 | Cyt1F-Cyt1R |
| Cyt2 | 468    | 154–309 | Cyt2F-Cyt2R |
| Cyt3a | 441    | 304–450 | Cyt3F-Cyt3R |
| Cyt3b | 219    | 378–450 | Cyt3bF-Cyt3R |
| Cyt4 | 405    | 448–583 | Cyt4F-Cyt4R |
| Cyt5 | 495    | 581–745 | Cyt5F-Cyt5R |
| Cyt5a | 240    | 581–660 | Cyt5F-Cyt5R |
| Cyt5b | 258    | 660–745 | Cyt5bF-Cyt5R |
| Cyt6 | 606    | 741–902 | Cyt6F-Cyt6R |
| Cyt6a | 327    | 741–849 | Cyt6F-Cyt6R |
| Cyt6b | 300    | 849–942 | Cyt6F-Cyt6R |
| Cyt7 | 357    | 941–1015 | Cyt7F-Cyt7R |
| Cyt7a | 168    | 941–996 | Cyt7F-Cyt7R |
| Cyt7b | 198    | 994–1059 | Cyt7bF-Cyt7R |
| Cyt8 | 444    | 1060–1207 | Cyt8F-Cyt8R |
| Cyt10 | 450    | 1347–1496 | Cyt10F-Cyt10R |
| Cyt10a | 240    | 1347–1426 | Cyt10F-Cyt10R |
| Cyt10b | 225    | 1421–1496 | Cyt10bF-Cyt10R |
| Cyt11 | 453    | 1499–1669 | Cyt11F-Cyt11R |
| Cyt12 | 412    | 1649–1792 | Cyt12F-Cyt12R |
| Cyt13 | 501    | 1793–1959 | Cyt13F-Cyt13R |
| Cyt14 | 231    | 1961–2039 | Cyt14F-Cyt14R |
| Cyt16 | 165    | 2219–2273 | Cyt16F-Cyt16R |
| Cyt18 | 447    | 2592–2741 | Cyt18F-Cyt18R |

Effects of Ca2+ and Sr2+ on the cooperativity and on the EC50 of the InsP2-induced Ca2+ release in permeabilized A7r5 cells using our recently developed rapid 45Ca2+ efflux technique (22). The effects of Ca2+ on the Vmax of InsP2-induced Ca2+ release were not determined in the present experiments because our previous work on fully loaded stores already indicated that the Ca2+ release in response to a supramaximal [InsP2] (320 μM) was not affected when the cytosolic [Ca2+]i was varied between 10 nM and 10 μM (11, 23). The stimulation of the release by cytosolic Ca2+ was due to a shift in EC50 toward lower InsP2 concentrations. The inhibition by Ca2+ was due, first, to a decrease in cooperativity, and second, to a shift in EC50 toward higher InsP2 concentrations. Both types of inhibition had a different [Ca2+]i dependence. Sr2+ only mimicked the effect on the cooperativity indicating that both inhibitory effects were exerted at different sites. Luminal Ca2+ did not have an effect on the EC50 nor on the cooperativity indicating that luminal Ca2+ did not exert its effect by acting on a cytosolic Ca2+-binding site. Therefore at least four Ca2+-binding sites on Type 1 InsP3R...
The effects of the experiment on the Ca\textsuperscript{2+} remaining in the stores was released by incubation in 1 ml of 2% (w/v) SDS for 30 min.

**RESULTS**

\textit{45Ca\textsuperscript{2+}} and Ruthenium Red Binding to GST Fusion Proteins—Recombinant peptides have been widely used for the identification of the binding sites of various ligands such as Ca\textsuperscript{2+}, ATP, and CaM. In earlier studies on the InsP\textsubscript{3}R-1 (17), we already identified a cytosolic and a luminal region with Ca\textsuperscript{2+}-binding properties. To detect additional Ca\textsuperscript{2+}-binding sites on the InsP\textsubscript{3}R-1, we performed a complete screening of the remaining cytosolic InsP\textsubscript{3}R-1 sequence for Ca\textsuperscript{2+}-binding sites. We constructed and expressed a total of 26 GST fusion proteins that contain small fragments (56–202 amino acids) of the InsP\textsubscript{3}R-1 in E. coli. These short fragments of the InsP\textsubscript{3}R covered the complete cytosolic N-terminal 2275 amino acids, including the S-I and the S-II splice domain (29) and the C-terminal 150 amino acids of the mouse InsP\textsubscript{3}R-1 (Fig. 1). Ca\textsuperscript{2+} binding to these fusion proteins was examined by a \textit{45Ca\textsuperscript{2+}}-overlay procedure as described earlier (17). The cytosolic Ca\textsuperscript{2+}-binding domain labeled d and the Ca\textsuperscript{2+}-binding domain on the luminal loop labeled l (Fig. 1) were characterized previously (17) and are therefore not further considered in the present study.

Fig. 2A shows a Coomassie Blue-stained gel of parental GST (pGST) and GST fusion proteins Cyt1–14, -16, and -18. Most of the GST fusion proteins migrated as single prominent bands corresponding to the expected molecular weights. Mainly little degradation was observed after expression and purification. The only exceptions were Cyt4, -8, -12, and -18 (Fig. 2A), which were strongly degraded. Fig. 2B demonstrates \textit{45Ca\textsuperscript{2+}} binding of the different fusion proteins covering the cytosolic domains of the InsP\textsubscript{3}R. Strong \textit{45Ca\textsuperscript{2+}} binding was detected to Cyt3, -5, -6, -7, and -10 (Fig. 2B). \textit{45Ca\textsuperscript{2+}} binding to Cyt7 was much stronger than \textit{45Ca\textsuperscript{2+}} binding to the other Ca\textsuperscript{2+}-binding fusion proteins, probably reflecting a higher affinity of this site for Ca\textsuperscript{2+}. pGST, the bacterial fusion partner, which has no known Ca\textsuperscript{2+}-binding site was used as a negative control and showed no \textit{45Ca\textsuperscript{2+}} binding (Fig. 2B).

To define more precisely the sequences involved in Ca\textsuperscript{2+} binding within the positive domains Cyt3, -5, -6, -7, and -10, each domain was further divided into two parts. The subfragments of these InsP\textsubscript{3}R sequences were expressed as GST fusion proteins and designated Cyt3a, -3b, -5a, -5b, -6a, -6b, -7a, -7b, -10a, and -10b (Fig. 1). The estimated molecular mass of most of the GST fusion proteins corresponded approximately to the predicted value, except for Cyt7b (predicted value 34 kDa) which migrated more slowly, with an apparent mobility corresponding to a polypeptide of 37 kDa (Fig. 3A). Fig. 3B shows that \textit{45Ca\textsuperscript{2+}} bound to Cyt3a, -3b, -5b, -6a, -7b, and -10b but not to -5a, -6b, -7a, and -10b. It is of interest that the first degradation product of Cyt3a also bound \textit{45Ca\textsuperscript{2+}}, whereas the second degradation product that corresponds to pGST did not.
The pattern of ruthenium red binding was similar to the pattern of $^{45}\text{Ca}^{2+}$ binding in Fig. 3B, except for fusion protein Cyt5b, which failed to bind with ruthenium red (data not shown). Cyt7b stained dark blue with Stains-all, and the blue staining of the Cyt3a, -3b, -5a, -5b, -6a, -6b, -7a, -7b, -10a, and -10b fusion proteins were affinity purified. Fusion proteins were separated by SDS-PAGE as described in the legend to Fig. 2A. B, fusion proteins were solubilized, separated electrophoretically, and transferred to an Immobilon-P membrane as described in Fig. 2. SDS-PAGE resolved proteins of a similar gel were transferred to Immobilon-P membranes, and a $^{45}\text{Ca}^{2+}$ overlay was carried out. pGST was used as negative control.

We have therefore further explored the kinetics and mechanism of the interaction of $\text{Ca}^{2+}$ with InsP$_3$-induced $\text{Ca}^{2+}$ release. We used $\text{Ca}^{2+}$ as well as $\text{Sr}^{2+}$ to discriminate and characterize between different modes of interaction with divalent cations.

**Lack of InsP$_3$-induced InsP$_3$R Inactivation in A7r5 Cells**

The cooperativity and the EC$_{50}$ of InsP$_3$-induced $\text{Ca}^{2+}$ release can be determined by loading the $\text{Ca}^{2+}$ stores to steady state with $^{45}\text{Ca}^{2+}$ and then incubating them with a progressively increasing [InsP$_3$] in efflux medium (22). This protocol only allows an accurate determination of the cooperativity and the EC$_{50}$ if there is no InsP$_3$-induced inactivation. To exclude such inactivation, we challenged the stores in permeabilized A7r5 cells with a progressively increasing or a progressively decreasing [InsP$_3$]. If the InsP$_3$R in A7r5 cells would exhibit the same incremental inactivation as in hepatocytes (30), then both protocols should result in a different final extent of $\text{Ca}^{2+}$ release. The protocol starting with the highest [InsP$_3$] should inactivate all InsP$_3$R at the beginning of the challenge. In the protocol with the increasing [InsP$_3$], the first addition should only inactivate the most sensitive receptors (30), leaving the stores fully responsive to further increases in [InsP$_3$]. The protocol with the increasing [InsP$_3$] should therefore result in less inactivation and in a lower $\text{Ca}^{2+}$ content at the end of the experiment. Fig. 4A shows that, in the presence of 5 nM $\text{Ca}^{2+}$, the $\text{Ca}^{2+}$ content of the stores at the end of the experiment did not depend on whether the [InsP$_3$] was gradually increased (closed circles) or decreased (open circles).

The inactivation of the hepatocyte InsP$_3$R recorded by Dufour et al. (31) already occurred at 1 nM $\text{Ca}^{2+}$, whereas that observed by Hajnóczky and Thomas (30) required stimulatory cytosolic $\text{Ca}^{2+}$ concentrations. The experiment was therefore repeated at 1 $\mu$M free $\text{Ca}^{2+}$, which is an optimal effective [Ca$^{2+}$] for stimulating the $\text{Ca}^{2+}$ release in A7r5 cells (11). Fig. 4B shows that the final $\text{Ca}^{2+}$ content of the stores was again independent of whether the [InsP$_3$] was increasing or decreasing. We conclude that InsP$_3$-dependent incremental inactivation of the InsP$_3$R did not occur under our assay conditions. As a consequence, our protocol of adding cumulative concentrations of InsP$_3$ can be used to measure the cooperativity and the EC$_{50}$.

**Effect of Cytosolic $\text{Ca}^{2+}$ and $\text{Sr}^{2+}$** — $\text{Ca}^{2+}$-loaded stores were incubated in thapsigargin-containing efflux medium containing 5 nM, 1 or 4 $\mu$M free $\text{Ca}^{2+}$, and challenged with a progressively increasing [InsP$_3$]. Fig. 5A gives the decrease in store...
Hill coefficient was 2.7 at 5 nM free Ca\(^{2+}\) and its modulation by a progressively increasing \((\bigcirc)\) or decreasing \((\bigtriangleup)\) [InsP\(_3\)]. The [InsP\(_3\)] was changed in a logarithmic way between 10 nM and 3.2 \(\mu\)M in 50 individual steps each lasting 6 s, as shown by the full lines in the insets below the traces. The Ca\(^{2+}\) content of the stores after 5 min of efflux was set as 100%. The curves were averages of five experiments.

Ca\(^{2+}\) content as a function of the corresponding [InsP\(_3\)] increased from 5 nM to 1 \(\mu\)M in 50 individual steps each lasting 6 s, as shown by the open symbols. A less rapid decline in store Ca\(^{2+}\) content occurred at 4 \(\mu\)M free Ca\(^{2+}\). These data confirm the bell-shaped activation of the InsP\(_3\)R by cytosolic Ca\(^{2+}\).

Fig. 5B gives the rates of Ca\(^{2+}\) release as a function of the corresponding [InsP\(_3\)] for the three different Ca\(^{2+}\) concentrations of Fig. 6A. The threshold [InsP\(_3\)] for inducing Ca\(^{2+}\) release was 0.1 \(\mu\)M at 5 nM Ca\(^{2+}\). The threshold became 0.02 \(\mu\)M at 1 \(\mu\)M Ca\(^{2+}\) and increased again to 0.5 \(\mu\)M at 4 \(\mu\)M Ca\(^{2+}\). In contrast, the release rate curves became less steep as the free [Ca\(^{2+}\)] increased from 5 nM to 1 and 4 \(\mu\)M. The Hill coefficients for these traces, and also for the traces measured at intermediate Ca\(^{2+}\) concentrations (data not shown), were calculated as described (22) and plotted by the open symbols in Fig. 6A. The Hill coefficient was 2.7 at 5 nM free Ca\(^{2+}\). This value is higher than that reported before (2.0; Ref. 22) because the present efflux medium also contained 1 \(\mu\)M ATP, which increases the cooperativity of the release (32). The Hill coefficient decreased as the free [Ca\(^{2+}\)] increased. We (22) and others (33) already reported a decrease in cooperativity at very high Ca\(^{2+}\) concentrations (10 \(\mu\)M). The present work extends these observations, and we now report a gradual decrease in Hill coefficient as the cytosolic [Ca\(^{2+}\)] increased. This decrease in cooperativity occurred with an EC\(_{50}\) for Ca\(^{2+}\) of 0.21 \(\mu\)M and was already very pronounced at 1 \(\mu\)M Ca\(^{2+}\), i.e. at the optimal effective concentration for stimulating the release (11).

We have also studied the effect of a progressively increasing [InsP\(_3\)] in the presence of various free Sr\(^{2+}\) concentrations. Sr\(^{2+}\) also decreased the Hill coefficient of the InsP\(_3\)-induced Ca\(^{2+}\) release (Fig. 6A, closed symbols). The EC\(_{50}\) value for Sr\(^{2+}\) was 8.3 \(\mu\)M, indicating that Sr\(^{2+}\) was thus 40 times less potent than Ca\(^{2+}\) in this respect. The sensitivity of InsP\(_3\)-induced Ca\(^{2+}\) release with respect to InsP\(_3\) was analyzed. The EC\(_{50}\) was calculated at the various Ca\(^{2+}\) concentrations and plotted by the open symbols in Fig. 6B. Increasing the free [Ca\(^{2+}\)] from 5 nM to 0.3 \(\mu\)M lowered the EC\(_{50}\) from 0.35 to about 0.15 \(\mu\)M. Such a decrease also occurred in rat hepatocytes (10). The present work, however, extends this previous observation to inhibitory Ca\(^{2+}\) concentrations. Interestingly, the EC\(_{50}\) shifted to higher InsP\(_3\) concentrations if the free [Ca\(^{2+}\)] was raised to 1 \(\mu\)M or higher. The closed symbols

**Fig. 4. Effect of a gradually increasing or decreasing InsP\(_3\) challenge on the non-mitochondrial Ca\(^{2+}\) stores of permeabilized A7r5 cells.** The curves show the decrease in store Ca\(^{2+}\) content during incubation in efflux medium containing 5 nM (A) and 1 \(\mu\)M (B) free Ca\(^{2+}\) and its modulation by a progressively increasing \((\bigcirc)\) or decreasing \((\bigtriangleup)\) [InsP\(_3\)]. The [InsP\(_3\)] was changed in a logarithmic way between 10 nM and 3.2 \(\mu\)M in 50 individual steps each lasting 6 s, as shown by the full lines in the insets below the traces. The Ca\(^{2+}\) content of the stores after 5 min of efflux was set as 100%. The curves were averages of five experiments.

**Fig. 5. Biphasic effect of cytosolic Ca\(^{2+}\) on InsP\(_3\)-induced Ca\(^{2+}\) release.** A shows the decrease in store Ca\(^{2+}\) content during a challenge with a progressively increasing [InsP\(_3\)] in efflux medium buffered at 5 nM \((\bigcirc)\), 1 \(\mu\)M \((\bullet)\), or 4 \(\mu\)M \((\bigstar)\) free Ca\(^{2+}\). The experimental protocol was the same as in Fig. 4, but the store Ca\(^{2+}\) content was plotted as a function of the [InsP\(_3\)] instead of as a function of time. The store Ca\(^{2+}\) content at the beginning of the InsP\(_3\) challenge was set at 100%. B shows the rate of Ca\(^{2+}\) release as a function of the corresponding [InsP\(_3\)] at the indicated free Ca\(^{2+}\) concentrations. The rate of Ca\(^{2+}\) release at each [InsP\(_3\)] was normalized to the Ca\(^{2+}\) content of the stores at that time point. The number of experiments at each [Ca\(^{2+}\)] ranged between 3 and 5.
in Fig. 6 illustrate the calculated EC50 for InsP3-induced Ca2+ release in the presence of various free Sr2+ concentrations. Sr2+, in contrast to Ca2+, lowered the EC50 for InsP3-induced Ca2+ release over the entire concentration range tested (up to 100 μM).

Effect of Luminal Ca2+—The InsP3R is stimulated by Ca2+ inside the store (9, 11, 18–21, 23, 34–41). Some groups have proposed that this effect could be exerted at the cytosolic side of the InsP3R (9, 20, 42). We therefore investigated whether luminal Ca2+ would have the same effect as cytosolic Ca2+ on the cooperativity and the EC50 of the release. Fig. 7 compares the effect of a gradual increase in [InsP3] starting after 5 min (filled stores, closed symbols) or after 29 min of efflux (less filled stores, open symbols). The stores contained 3.6 times less Ca2+ after 29 min than after 5 min of efflux. The level of store loading had no effect on the threshold for InsP 3-induced Ca2+ release (Fig. 7B). The EC50 values were similar (0.38 μM InsP3 for the less filled stores and 0.34 μM InsP3 for the more filled stores), and the Hill coefficients were equal (2.7 for the less filled stores as well as for the more filled stores). The only difference was the rate of Ca2+ release at each [InsP3], which was higher for the filled stores, in accordance with our previous data (22). The stimulatory effect exerted by luminal Ca2+ (no effect on the EC50 or on the Hill coefficient) therefore completely differed from that induced by stimulatory cytosolic Ca2+ concentrations (a decrease in both parameters).

DISCUSSION

We made major progress in the identification and localization of high affinity Ca2+-binding sites on the mouse InsP3R-1. These include regions 3, 5, 6, 7, and 10 (Fig. 1), in addition to regions 15 and 17 that we described earlier (17). These five domains were further subdivided into two parts to map the Ca2+-binding sites with more precision. Region 3 was located within the InsP3-binding domain (residues 226–578 (43)). More precise mapping revealed that both subregion 3a and 3b, lying between residues 304–381 and 378–450, respectively, bound 45Ca2+. The other Ca2+-binding stretches were localized in the modulatory domain (between residues 651 and 2275). Three Ca2+-binding stretches, regions 5, 6, and 7, were located in the 500-amino acid region just after the InsP3-binding domain. 45Ca2+ binding to these regions was further localized to subregion 5b, lying between residues 660 and 745, to subregion 6a, lying between residues 741 and 849, and to subregion 7b, lying between residues 994 and 1059. Another 45Ca2+-binding domain (region 10) was detected in the proximity of the calmodulin-binding site (amino acids 1564–1585 (44)) and was further localized to subregion 10a, lying between residues 1347 and 1426 (Fig. 8). The present molecular screening data together with the previously described (17) 45Ca2+ and ruthenium red binding in region 15 mapped to the cytosolic residues 2124–
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2146 (d) and in region 17 mapped to the luminal residues 2463–2528 (l) (Fig. 1) give a total of seven cytosolic 45Ca$^{2+}$-binding regions and one luminal 45Ca$^{2+}$-binding region for InsP$_3$R-1. The short cytosolic C-terminal extension did not bind 45Ca$^{2+}$ in our assay.

One of the interesting observations in this study is the dem-onstration of two 45Ca$^{2+}$-binding domains within the InsP$_3$-binding domain. Up to now, complex effects of Ca$^{2+}$ on the binding of InsP$_3$ to its receptor have been reported. An inhibition of the binding was observed in several (14, 45, 46) but not in all (47) studies. In hepatocytes Ca$^{2+}$ converts the InsP$_3$R from a low affinity to a high affinity conformational state, thereby increasing the binding (10, 48). Moreover, recent studies on InsP$_3$Rs overexpressed in insect Sf9 cells demonstrate differences in Ca$^{2+}$ sensitivity of the InsP$_3$ binding between InsP$_3$R isomers 1 and 3 (49, 50). The demonstration in this study of multiple Ca$^{2+}$-binding sites in the direct neighborhood of the InsP$_3$-binding domain may therefore give a clue to the understanding of these complex effects of Ca$^{2+}$ on InsP$_3$ binding.

Colocalization of the Ca$^{2+}$-binding domain in region 10a and the CaM-binding domain that is located about 190 residues downstream is reminiscent of what was observed for the ryanodine receptor (RyR), another intracellular Ca$^{2+}$ release channel. The RyR contains two strong CaM-binding sites separated by less than 200 residues from high affinity Ca$^{2+}$-binding sites, and a third CaM-binding site is flanked by a high affinity Ca$^{2+}$-binding site (51). A similar arrangement is reported in the plasma membrane Ca$^{2+}$-ATPase sequence, where two or three Ca$^{2+}$-binding sites flank a CaM-binding site (52). The significance of this colocalization of Ca$^{2+}$- and CaM-binding sites is not clear. It is possible that the Ca$^{2+}$- and CaM-binding sites interact with each other and that such interactions may modulate Ca$^{2+}$- and/or CaM binding to these regions.

Different types of Ca$^{2+}$-binding domains in proteins have been elucidated by sequence analysis. On the basis of the primary structures responsible for the actual binding of these domains, intracellular Ca$^{2+}$-binding proteins can be classified in the following three categories: EF-hand proteins, Ca$^{2+}$/phospholipid-binding proteins (annexins and C2 region proteins), and Ca$^{2+}$ storage proteins (e.g. calsequestrin, calreticulin) (53).

We have compared the typical sequence motifs of these three categories of Ca$^{2+}$-binding proteins with the Ca$^{2+}$-binding regions that we have detected in the InsP$_3$R. None of the regions showed resemblance with known motifs. Since the InsP$_3$R is structurally related to the RyR, we also compared the Ca$^{2+}$-binding sequences of the InsP$_3$R-1 and the RyR-1, which also contains seven cytosolic Ca$^{2+}$-binding regions (51, 54). Again we could not detect a significant sequence homology. The different Ca$^{2+}$-binding amino acid sequences of the InsP$_3$R all contain clusters of aspartic and glutamic acid residues, which are likely to be involved in the Ca$^{2+}$ binding (Fig. 8). Other Ca$^{2+}$-binding proteins, like the RyR (51, 54), also have clusters of acidic amino acid residues that are implicated in their Ca$^{2+}$-binding behavior. Comparison of the different Ca$^{2+}$-binding amino acid sequences of the InsP$_3$R did not reveal any apparent motifs, repeats, or consensus sequences.

The functional significance of 45Ca$^{2+}$ binding to each of the particular regions 3a, 3b, 6a, 7b, 10a, d, and l remains to be investigated. Some of the detected sites may represent potentially regulatory Ca$^{2+}$-binding sites. Direct binding of Ca$^{2+}$ may induce a profound conformational change within a single domain and as such evoke an interconversion between two (or more) receptor affinity states. Other Ca$^{2+}$-binding sites may have a structural role, e.g. structural stabilization of important domains in the neighborhood or protection against proteolysis of an exposed region (52). Ca$^{2+}$ may also bridge adjacent domains and thus direct the relative membrane orientation and supramolecular structure.

Our observation that there are multiple Ca$^{2+}$-binding sites on InsP$_3$R-1 is in agreement with our detailed kinetic analysis which points to several different types of interaction of divalent cations with InsP$_3$-induced Ca$^{2+}$ release. For that purpose, we have further characterized the well known bell-shaped activation of the InsP$_3$-induced Ca$^{2+}$ release by cytosolic Ca$^{2+}$ in permeabilized A7r5 cells by studying the effect of Ca$^{2+}$ and Sr$^{2+}$ on the EC$_{50}$ and cooperativity of the release measured at different [InsP$_3$].

We first investigated whether our protocol of adding cumulative concentrations of InsP$_3$ could be used to measure cooperativity. Since the Ca$^{2+}$ content of the stores at the end of the experiment did not depend on the way of InsP$_3$ administration (progressively increasing or progressively decreasing) (Fig. 4), we may conclude that InsP$_3$-induced inactivation of the InsP$_3$R does not occur in A7r5 cells. This contrasts with findings in liver cells (30, 31) and in RBL-2H3 cells (55) which may be due to the expression of mainly InsP$_3$R-1 in A7r5 cells, whereas the main form in liver and in RBL-2H3 cells is InsP$_3$R-2 (56, 57). Alternatively, differences in assay conditions may underlie this finding.

The bell-shaped activation of the release by cytosolic Ca$^{2+}$ (Fig. 5A) represented effects on both the cooperativity and the EC$_{50}$ of the release process. Ca$^{2+}$ exerted a biphasic effect on the EC$_{50}$ with a shift toward lower InsP$_3$ concentrations in the presence of low Ca$^{2+}$ concentrations and a shift toward higher InsP$_3$ concentrations at Ca$^{2+}$ concentrations above 0.3 μM. In contrast, no such biphasic effect was observed for the cooperativity, since the Hill coefficient gradually decreased as the free [Ca$^{2+}$] increased (Fig. 6). There are therefore two different mechanisms causing inhibition, a decrease in cooperativity and a shift in EC$_{50}$ toward higher InsP$_3$ concentrations. Sr$^{2+}$ could only mimic the inhibitory effect on the cooperativity but not on the EC$_{50}$. This different dependence of both types of inhibition on the [Ca$^{2+}$] and on [Sr$^{2+}$] suggests that two different inhibi-
Ca²⁺-binding sites were involved. The increase in EC₅₀ may be related to the Ca²⁺-induced inhibition of InsP₃ binding (14, 45, 46), especially since the inhibition by Ca²⁺ in A7r5 cells (11, 23) and in cerebellum (58, 59) can be overcome by increasing the [InsP₃].

The stimulation by cytosolic Ca²⁺ and the associated shift in EC₅₀ toward lower InsP₃ concentrations probably represent the interaction with a third Ca²⁺-binding site. It is not clear as yet whether [³H]InsP₃ binding is increased under these conditions, perhaps as a consequence of isoform diversity in the response to Ca²⁺ (49, 50).

The less steep responses in the presence of stimulatory Ca²⁺ concentrations (1 µM) are not in agreement with the generally accepted view that positive feedback by cytosolic Ca²⁺ causes the very steep rising phase of the InsP₃-induced Ca²⁺ spikes in the intact cell (60). A possible explanation lies in the fact that the inhibition by Ca²⁺ is a time-dependent process (6, 9–11, 61). Our experiments were performed after 6.5 min incubation in the high [Ca²⁺], and positive feedback by Ca²⁺ in the intact cell is assumed to occur before the time-dependent inhibition occurs.

The mechanism by which luminal Ca²⁺ stimulates the release is still a matter of debate. Some groups (11, 17, 19, 62) believe that luminal Ca²⁺ binds to a luminal site. Other groups propose a model where luminal Ca²⁺ leaks out and acts through cytosolic binding sites on the same channel or on closely associated channels (9, 20, 42). We investigated whether the effect of luminal Ca²⁺ on the EC₅₀ and cooperativity of the release (Fig. 7) is similar to the effects excreted by cytosolic Ca²⁺ described above. The activation of the release by luminal Ca²⁺ did not affect the EC₅₀ nor the Hill coefficient. This finding makes it unlikely that the stimulatory effects of luminal Ca²⁺ in A7r5 cells are exerted on the cytosolic side of the receptor. We therefore continue to propose, at least for A7r5 cells, that effects of luminal Ca²⁺ do not involve the cytosolic Ca²⁺-binding sites (11). Luminal Ca²⁺ may regulate the Ca²⁺ release from within the lumen through the luminal Ca²⁺-binding site on the InsP₃R (17) or via associated proteins like calreticulin (39).

In conclusion, we have conducted experiments designed to obtain molecular as well as functional data regarding the mechanism involved in the regulation of the InsP₃R by Ca²⁺. We have provided evidence that multiple Ca²⁺-binding regions are located on the InsP₃R and that at least four different modes of interaction with Ca²⁺ are involved in the complex feedback regulation of the release by Ca²⁺. Further characterization of the regulatory (stimulatory as well as inhibitory) Ca²⁺-binding sites is necessary to elucidate the structure-function relationships in the InsP₃R.

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