We have developed a novel recombinant hyperaffinity inositol 1,4,5-trisphosphate (IP₃) absorbent, called the “IP₃ sponge,” which we constructed on the basis of the ligand-binding site of the mouse type 1 IP₃ receptor (IP₃R1). The IP₃ sponge exhibited -1000-fold higher affinity for IP₃ than the parental IP₃R1 and specifically competed with the endogenous IP₃R for binding to IP₃. Trapping IP₃ with the IP₃ sponge inhibited IP₃-induced Ca²⁺ release (IICR) from cerebellar microsomes in a dose-dependent manner. The IP₃ sponge expressed in HEK293 cells also inhibited IICR in response to stimulation with carbachol or ATP. Its inhibitory effects were dependent upon the level of its expression over the increased IP₃ contents. Moreover, the IP₃ sponge significantly reduced the carbachol-induced phosphorylation of cAMP-response element-binding protein element-binding protein in HEK293 cells, indicating that the activation of cAMP-response element-binding protein by Ca²⁺-dependent phosphorylation may be partly attributable to IICR.

Many cellular responses to diverse biological stimuli, such as neurotransmitters, hormones, and growth factors, are mediated by the intracellular second messenger inositol 1,4,5-trisphosphate (IP₃). IP₃ subsequently induces Ca²⁺ mobilization from intracellular stores by activating its receptor (IP₃R) (1, 2). IP₃R channels form homo- or heterotetramers via the co-assembly of distinct types of IP₃R subunits (types 1–3) (3) and bind IP₃ in a stoichiometric manner (4). The IP₃R-induced Ca²⁺ release (IICR) not only results in a transient increase in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) but also evokes complex spatiotemporal dynamics of [Ca²⁺]ᵢ, called Ca²⁺ waves and Ca²⁺ oscillations (5). Thus, IP₃R functions as a signal converter from IP₃ to Ca²⁺, which exhibits more complex dynamics in time and space inside cells and acts on thousands of downstream targets that play key roles in many aspects of cell physiology (1, 2).

Type 1 IP₃R (IP₃R1) is composed of 2749 amino acids (molecular mass about 313 kDa) and is structurally divided into three parts as follows: a large N-terminal cytoplasmic arm region (83% of the receptor molecule); a putative six membrane-spanning region clustered near the C terminus, which is thought to constitute an ion channel by forming a tetramer; and a short C-terminal cytoplasmic tail region (6). In our previous studies (7, 8) on the ligand-binding site of mouse type 1 IP₃R (mIP₃R1), we found that the core region, essential for the high affinity of ligand binding, was localized in amino acid residues 226–576. We also found that the affinity of residues 1–604 (T604) of mIP₃R1 for IP₃ (Kᵦ = 45 nM) was comparable with that of the native cerebellar IP₃R (83 nM) (4), indicating that T604 could form a well folded or compact conformation for the functional IP₃-binding pocket (7, 9). The N-terminal residues 1–223, which we assigned as a suppresser region, instead of being directly responsible for IP₃ binding, suppressed the binding activity of the core region (7, 9). Without the putative suppresser region, residues 224–604 showed markedly higher affinity for IP₃ than did the parental T604 (7). We therefore inferred that such a high affinity IP₃-binding protein could function as an IP₃ absorbent in living cells and compete with the native IP₃R for binding to IP₃.

In this paper, we report the development of a novel recombinant IP₃ absorbent, the “IP₃ sponge,” which has hyperaffinity for IP₃ (Kᵦ = 0.092 nM). The IP₃ sponge was constructed on the basis of the ligand-binding site of mIP₃R1, and we assumed that this hyperaffinity IP₃ sponge could compete with IP₃R for the IP₃ signal. We showed that its application actually caused specific inhibition of in vitro IICR in a dose-dependent manner. Moreover, the IP₃ sponge exogenously expressed in HEK293 cells could trap IP₃ produced in response to stimulation with either carbachol (Chc) or ATP, resulting in inhibition of IICR. The IP₃ sponge appeared to be effective in competing for IP₃ with multiple types of tetrameric IP₃R channels, because HEK293 cells expressed all three IP₃R subunits. The effectiveness of the IP₃ sponge on IP₃-Ca²⁺ signaling-dependent cell physiology was verified by providing an invaluable insight into the functional role of IICR in the phosphorylation of cAMP-response element-binding protein (CREB), which was previously thought to be activated mainly by Ca²⁺ influx.

**EXPERIMENTAL PROCEDURES**

**Construction and Expression of IP₃ Sponge—PCR cloning, and sequencing were carried out as described elsewhere** (8, 9). Mouse IP₃R1 was inserted into the expression vector pCEP4 (Life Technologies) in the BamHI and EcoRI sites. Insertion was confirmed by restriction analysis. The expressed protein was purified in the presence of 0.5 M guanidine hydrochloride. The amino acid composition of the purified IP₃ sponge was determined using a model 975A amino acid analyzer (Hitachi) at 418 nm. The purification of the IP₃ sponge was not attempted because of the instability of the highly purified protein in solution.
cDNA was used for all constructs used in this study (13). pET-T604 consisted of the Ip3-binding sequence encoding residues 1–604 cloned in pET-3a (7, 8). pET-T224–604 containing the residue 224–604 coding sequence was produced by PCR-based mutagenesis, as described elsewhere (8-9). To express an N-terminal fused protein of residues 224–604 with the GST S-transferase domain, pGEX-G224 was obtained by cloning the BamH1-EcoRI fragment containing the residue 224–604 coding sequence isolated from pET-224–604 into pGEX-2T (Amersham Biosciences). Site-directed mutagenesis of K508A and R441Q was introduced into pGEX by two-step PCR, as described elsewhere (8). The sequences and junctions of all constructs were verified by DNA sequencing. Expression of recombinant proteins in Escherichia coli cells was carried out by the low temperature method (7, 8) with minor modifications, followed by glutathione-Sepharose 4B column chromatography (Amersham Biosciences) performed according to the manufacturer’s protocol. The glutathione S-transferase (GST)-fused proteins were then applied to a PD-10 column containing Sephadex G-25M (Amersham Biosciences) pre-equilibrated with elution buffer (10 mM HEPES-KOH, pH 7.2, 88 mM NaCl, 1 mM KCl). Protein concentrations were determined by the Bradford modified protocol. The glutathione S-transferase gene (GST) fused to the cDNA from pET-(224–604) into pGEX-2T; G224, GST alone (pGEX-2T). Protein expression induced by the removal of Dox was verified by Western blot analysis (7). We then examined specific Ip3/IP3 binding of K508A and GST (2 μg) was carried out as described elsewhere (7, 8). For analysis of G224 and R441Q (0.05 μg) specific binding was determined by subtracting nonspecific binding, measured in the presence of 1 μM unlabeled IP3 (Dejindo) from total binding obtained with 0.15–9.6 μM [3H]IP3 (PerkinElmer Life Sciences). Specific IP3 binding of G224 and R441Q (0.05 μg) was assessed as described elsewhere (7, 8). The competitive effect of G224 and R441Q (0.781–50 μg/ml) was assayed by directly adding them to binding mixtures containing 0.96 or 9.6 nM [3H]IP3 and cerebellar microsomes (40 μg) prepared from ddY mice (8–10 weeks old; Nippon SLC), as described elsewhere (9, 14).

**Measuring Ca2+ Release from Microsomes**—ICR from cerebellar microsomes was measured with fura2 and a fluorospectrometer, CAF110 (Nihon Bunko), as described elsewhere (14). Ca2+ was loaded into microsomes by activating Ca2+-ATPase with 1 mM ATP (Sigma), and Ca2+ release was triggered by activating IP3, with 100 nM, 500 nM, and 1 μM IP3. Various amounts of the GST-fused proteins were added 1 min before the addition of IP3. Before completing each set of experiments, Ca2+ release via the ryanodine receptor was determined by adding 40 μM caffeine (Sigma). Maximum and minimum values were obtained in the presence of an excess amount of CaC12 and EGTA, respectively, to calculate Ca2+ concentration as described elsewhere (15).

**Tetracycline-controllable Expression of IP3 Sponge in HEK293 Cells**—The Ip3 sponge K508A, and GST coding one with the 5’-Kozak initiation consensus sequence were cloned into the tetracycline (Tet)-negative inducible vector pETRE (CLONTECH). We used a HEK293 Tet-off cell line (CLONTECH), as described elsewhere (16, 17). Cells were maintained at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% Tet system-approved fetal bovine serum (CLONTECH), penicillin, streptomycin (50 units/ml), and 100 μg/ml kanamycin B (CLONTECH), penicillin, streptomycin (50 units/ml), and 100 μg/ml kanamycin B (CLONTECH). Ip3/IP3 sponge, K508A, and GST (3 μg) were co-transfected with pTK-Hyg by using LipofectAMINE (Invitro-gene). We obtained stable transformants resistant to 200 μg/ml hygromycin B (CLONTECH). Expression of exogenous proteins was suppressed by culturing in medium containing a Tet derivative, 20 ng/ml doxycycline (Dox). Protein expression induced by the removal of Dox was verified by Western blot analysis (7). We then examined specific [3H]IP3 binding activity by using sonicated nuclei-free S1 fractions.

**Phospholipase C (PLC) Assay**—PLC activity was assayed by the methods described previously (18). In brief, a reaction mixture containing 50 mM Mes buffer, pH 6.0, 400 μM CaCl2, 1 mg/ml bovine serum albumin, 1 pmol of PiP2, 22,000 pmol of [3H]PiP2, and the homogenate of established stable HEK293 cells or native HEK293 cells (130 μg) in the presence of the bacterially expressed GST-fused IP3-binding proteins (1, 10, and 100 pmol) was incubated at 37 °C for 10 min. The reaction was terminated by the addition of 2 ml of chloroform/methanol (2:1), and radioactive inositol trisphosphate was extracted with 1 N HCl. We measured the radioactivity with a scintillation counter.

**Cellular Ca2+ Imaging**—HEK293 cells were grown for 48 h on 35-mm glass-bottom dishes (coated with 50 μg/ml poly-lysine Sigma) in the presence or absence of 20 ng/ml Dox. The cells were then incubated in Dulbecco’s modified Eagle’s medium containing 5 μM fura2-AM (Dejindo) and 10% fetal bovine serum at 37 °C for 30 min, washed once with Hanks’ balanced salt solution, and then kept in Hanks’ balanced salt solution in the dark at 30 °C for 30 min. Before measurements, cells were washed twice in Ca2+-free Hanks’ balanced salt solution supplemented with 1 mM MgCl2 and 15 mM EGTA. Fura2 fluorescence images were analyzed using a video image analysis system (Argus-50/CA, Hamamatsu Photonics), as described elsewhere (19). Small volumes of stock solutions containing carbamol (CCh) (Sigma), ATP, or thapsigargin (Wako) were added to the bath medium to achieve appropriate concentrations. At the end of each recording, intracellular Ca2+ concentration ([Ca2+]i) was estimated from the maximal and minimal ratio (15) determined by adding 20 mM CaCl2 and 1 μM ionophore 4-Bromo A23187, followed by the addition of 30 mM EGTA.
cells, judged by their impermeability to trypan blue, were used throughout all the experiments. After every experiment, the cells were fixed for 20 min by adding formaldehyde solution (38% formalin; Wako) to a final concentration of 4%, and cells were then permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 5 min. After being washed and blocked with 2% normal rabbit serum in PBS-T, the cells were incubated with 1:500 anti-GST antibody (Amersham Biosciences) and then with 1:200 FITC-conjugated anti-goat antibody (Vector). Immunofluorescence was analyzed with a Zeiss LSM 410 inverted laser scan microscope and a ×40 objective lens.

**CREB Phosphorylation**—HEK293 cells (4 × 10^5 cells per 6-cm dish) were cultured for 48 h in the presence or absence of 20 ng/ml Dox. Cells were stimulated with CCh or forskolin for 15 min under Ca^{2+}-free conditions, as described above. Stimulated cells were fixed for 30 min by adding 10% trichloroacetic acid solution and lysed in 100 μl of SDS-PAGE sampling buffer supplemented with 1 mM Na3VO4, 1 mM NaF, and protease inhibitors. After sonication, the lysates were neutralized with 2 μl of 1 M Tris and boiled for 10 min. A 25-μl volume of cell lysate was then resolved by 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-phospho-CREB or anti-CREB antibody (Upstate Biotechnology, Inc.). The anti-phospho-CREB blots were quantitatively analyzed with a Molecular Dynamics FluorImager after staining with an Olympus laser scan confocal microscope and a ×60 objective lens.

**RESULTS**

**Construction of a Novel Recombinant IP3 Absorbent Protein**—The IP3-binding protein of amino acid residues 224–604 of mouse type 1 IP3R (mIP3R1), with markedly high affinity for IP3a, was not as efficiently expressed as a soluble active form in *E. coli* as residues 1–604 (T604) (7, 9). To solve this problem, we fused residues 224–604 of mIP3R1 with the GST gene instead of the putative suppressor region (Fig. 1A). Expression of the GST-fused protein, named G224, improved to ~30 mg/liter of *E. coli* culture using a low temperature method (versus 19 mg/liter for T604). Fig. 1B shows a specific immunoreactive band of G224 (66 kDa) after partial purification with column chromatography for glutathione affinity and gel filtration. G224 showed strikingly greater binding affinity, 500–1000-fold higher (Kd = 34 ± 3.9 nM) (Fig. 1C). Site-directed mutational analyses revealed that three basic amino acid residues (Arg-265, Lys-508, and Arg-511) are critical for specific IP3 binding and that substitution of Glu for Arg-441 enhances binding activity (7, 8). To determine the effect of these mutations on G224, we produced two mutants, K508A and R441Q, each having a single amino acid substitution on parental G224. Mutant R441Q had double the IP3 binding activity (Kd = 45 ± 2.1 μM) (Fig. 1C), to 3700-fold less than that of parental G224. Mutant R441Q had double the IP3 binding activity (Kd = 45 ± 2.1 μM) (Fig. 1C) of G224 and 1900-fold the binding activity of cerebellar IP3R.

We analyzed the ligand specificity of G224 and R441Q by competing 1.45×10^{-5}[^{3}H]IP3 binding to these proteins by various inositol phosphates (Fig. 1D). Both G224 and R441Q had ligand-binding specificity comparable with that of native cerebellar IP3R (4) in the following order: 1.45×10^{-5}[^{3}H]IP3 > 1.34×10^{-5}[^{3}H]IP3 > 10^{-5}[^{3}H]IP6.

G224 and R441Q Compete with IP3R for Binding to IP3—To determine whether G224 and R441Q behave as IP3R absorbents, we initially assessed the inhibitory effect of these high affinity IP3-binding proteins on[^{3}H]IP3 binding to cerebellar microsomes, an enriched source of IP3R protein (4) (Fig. 1E). In assays with 0.96 nM[^{3}H]IP3, IP3 binding to microsomes de-

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**FIG. 2.** Specific inhibition by the IP3 sponge of in vitro IICR in cerebellar microsomes. IICR in mouse cerebellar microsomes (200 μg of protein) was measured with Tris (2 μM). The GST-fused IP3-binding proteins were added when the extramicrosomal basal Ca^{2+} concentration was below 200 nM after ADP-dependent loading of microsomal vesicles with Ca^{2+}, and 1 min later Ca^{2+} release was induced by the addition of IP3. A, IICR with 100 nM IP3 in the absence of IP3-absorbent proteins (a, control) and in the presence of GST (b, at 200 μg/ml), K508A (c, at 200 μg/ml), or G224 (d, at 6.25–100 μg/ml). Insert show Ca^{2+} release traces induced by 40 μM caffeine after each measurement with IICR. Vertical bar, 100 nM [Ca^{2+}]; horizontal bar, 1 min. B, the dose-response curves for inhibition of IICR by G224 and R441Q (6.25–200 μg/ml). Peak heights of IICR were normalised to those seen in the absence of IP3-absorbent proteins. G224 is represented by the open symbols, and R441Q by the filled symbols. IICR was evoked by the addition of 100 nM IP3 (circles), 500 nM (triangles), and 1 μM (squares) IP3. For K508A (open inverse triangles) and GST (open rhombuses), IICR was evoked with 100 nM IP3. Values are the means ± S.D. of three experiments.

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G224 and R441Q Compete with IP3R for Binding to IP3—To determine whether G224 and R441Q behave as IP3R absorbents, we initially assessed the inhibitory effect of these high affinity IP3-binding proteins on[^{3}H]IP3 binding to cerebellar microsomes, an enriched source of IP3R protein (4) (Fig. 1E). In assays with 0.96 nM[^{3}H]IP3, IP3 binding to microsomes de-
increased to about 20% of the control in the presence of either G224 or R441Q at 6.25 μg/ml. By contrast, even at a 8-fold higher concentration (50 μg/ml), K508A reduced binding to only 70%, and GST had no effect. Moreover, in assays with 9.6 nM [3H]IP3, G224 and R441Q at 12.5 μg/ml reduced IP3 binding to 30%. Therefore, both G224 and R441Q appeared to function as effective IP3 absorbents, and they dose-dependently competed with microsomal IP3R for binding to IP3.

G224 and R441Q Trap IP3, Resulting in Inhibition of in Vitro IICR—We next examined the inhibitory effect of G224 and R441Q on IICR from mouse cerebellar microsomes. In the absence of IP3-absorbent proteins, half-maximal Ca2+ release occurred in response to stimulation with 100 nM IP3 (data not shown). In the presence of either G224 or R441Q at 12.5 μg/ml, the peak height of IICR with 100 nM IP3 decreased to about 50% of the control, and almost maximal inhibition was recorded at 100 μg/ml (Figs. 2A, a and d, and B). IICR stimulated with 500 nM IP3 and 1 μM IP3 was inhibited in the presence of 150 μg/ml G224 and R441Q to as low as 15 and 30% of the control, respectively (Fig. 2B). By contrast, at 200 μg/ml K508A and GST had no effect on IICR (Figs. 2A, b and c, and B) (even at 500 μg/ml; data not shown). Ca2+ release induced with 40 mM caffeine, which activates the ryanodine receptor (21), was unaffected by the addition of G224 (Fig. 2A, insets). Therefore, both G224 and R441Q specifically and dose-dependently inhibited in vitro IICR from microsomes in a competitive manner.

Thus, we named G224 an “IP3 sponge.”

Carbachol- and ATP-evoked Ca2+ Increases Are Inhibited by IP3 Sponge Expressed in HEK293 Cells—To analyze the inhibitory effect of the IP3 sponge on IICR in HEK293 cells, we obtained tetracycline (Tet)-controllable stable transfectants (16, 17), in which expressions of the IP3 sponge, K508A, or GST could be induced only by removing doxycycline (Dox) from the culture medium (Fig. 3A). More specifically, we established two IP3 sponge-expressing cell lines (clone 2 (C2) and clone 17 (C17)). Immunoblotting analysis using total cell lysates demonstrated that the level of IP3 sponge expression in C2 cells was higher than that in C17 cells. This difference of the level of induced expression was in agreement with immunostaining analysis with anti-GST antibody (Fig. 3B). Exogenous protein expression in this Tet-controllable expression system reached its maximal level within 48 h (data not shown). The IP3 sponge, K508A, and GST expression-induced HEK293 cells were viable 48 h after induction, as judged by their impermeability to trypan blue (data not shown), and they showed no remarkable changes of cell morphology at a light microscopic level when compared with non-induced cells. The levels of expression of the endogenous IP3Rs were unaltered in cells that expressed IP3 sponge, K508A, or GST (Fig. 3A). The IP3 sponge and K508A expressed were homogeneously distributed throughout the cytoplasm (Fig. 3B). After removing Dox, significant IP3 binding was present in the homogenate of the IP3 sponge expression-induced C2 cells but not in the K508A, GST expression-induced cells, or the native HEK293 cells (Fig. 3C).

To exclude the possibility that the IP3 sponge affected the production of IP3, we examined the activity of PLC in the IP3 sponge expression-induced C2 cells. As shown in Fig. 3D, the expression of neither the IP3 sponge nor K508A inhibited the PLC activity in the homogenate of HEK293 cells. Additionally, the presence of the bacterially expressed IP3 sponge as well as K508A and GST had no effect on the PLC activity of native HEK293 cells (Fig. 3E). These results indicated that the IP3 sponge expressed in this system was a functional IP3-binding protein that did not affect the production of IP3 and that K508A could be used as a negative control.

We found that there were variations in the level of expression of exogenous proteins from cell to cell in each stable cell line generated by selection with G418 and hygromycin (Figs. 3B, 4A, and 5A), and a similar variety of levels of expression in the Tet-controllable expression system has been described for other proteins (22, 23), although the reason was unknown. We therefore identified the expressing cells immunocytochemically.
The increase in [Ca\textsuperscript{2+}] was determined by fluorescence imaging with fura-2-AM. Representative traces in each paradigm are shown for non-induced C2 cells (Fig. 4, A, Rest). The mean amplitudes of the increase in [Ca\textsuperscript{2+}] were obtained in GST-expressing cells (K508A-expressing cells), and IP\textsubscript{3} sponge-expressing cells (K508A-expressing cells), and the other cells displayed a smaller and often delayed (29 ± 18, mean ± S.D., seconds) rise in [Ca\textsuperscript{2+}].

In marked contrast, such an increase in [Ca\textsuperscript{2+}] was observed by 200 μM CCh in C2 cells, and 200 μM CCh triggered a larger increase in [Ca\textsuperscript{2+}] with a slowly decreasing phase. The same results were obtained in GST-expressing cells and non-induced K508A or GST cells in which expression of K508A or GST was suppressed, respectively (data not shown).

We used these stable transfectants to analyze IICR in response to stimulation with the muscarinic agonist CCh (24) in the absence of extracellular Ca\textsuperscript{2+} (Fig. 4). There was no significant difference in resting levels of [Ca\textsuperscript{2+}], regardless of expression of the exogenous proteins (Fig. 4, A, Rest). In the non-induced C2 cells in which expression of the IP\textsubscript{3} sponge was suppressed in the presence of Dox (Fig. 4, A, Rest), K508A-expressing cells (Fig. 4, A, Rest), and K508A-expressing cells (K508A-expressing cells, and IP\textsubscript{3} sponge-expressing cells in 5–6 experiments); *, p < 0.001 compared with K508A-expressing cells (Student’s t-test). The mean amplitudes of the increase in [Ca\textsuperscript{2+}], in response to CCh in non-induced cells were 210 ± 22 nM with 20 μM and 410 ± 43 nM with 200 μM in K508A cells, 190 ± 17 nM with 20 μM CCh and 400 ± 22 nM with 200 μM CCh in C2 cells, and 200 ± 37 nM with 20 μM and 390 ± 10 nM with 200 μM in C17 cells.
FIG. 5. The IP₃ sponge inhibits ATP-evoked Ca²⁺ increases in HEK293 cells. A, fluorescence Ca²⁺ imaging of IP₃ sponge-expressing HEK293 cells before and after 100 μM ATP stimulation; open arrowheads, induced C2 cells that did not show any immunoreactivity; filled arrowheads, cells that showed a delayed increase in [Ca²⁺]; Scale bar represents 10 μM. The details of the legend for Fig. 5 are described in Fig. 4. B and C, effect of IP₃ sponge expression on ATP-evoked IICR. Before and after induction, [Ca²⁺] changes in response to the addition of 100 μM (open bars) or 10 μM ATP (hatched bars) followed by 0.2 μM TG (filled bars) were monitored in HEK293 cells. Typical traces in each paradigm are shown for non-induced C2 cells (a), K508A-expressing cells (b), IP₃ sponge-expressing C2 cells (c and d), and IP₃ sponge-expressing C17 cells (e). Vertical bar, 1.0 fluorescence ratio (340:380); horizontal bar, 1 min. D, inhibition ratios of 10 μM (filled) or 100 μM ATP (open)-evoked increases in [Ca²⁺] in IP₃, sponge-expressing C2 or C17 cells (n = 70–98 expressing cells in 5–6 experiments); *, p < 0.001 compared with K508A-expressing cells (Student’s t test). In non-induced cells, the mean amplitudes of the increase in [Ca²⁺], in response to ATP were 200 ± 26 nM with 10 μM and 290 ± 45 nM with 100 μM in K508A cells, 190 ± 10 nM with 10 μM ATP and 290 ± 28 nM with 100 μM ATP in C2 cells, and 290 ± 18 nM with 10 μM and 270 ± 45 nM with 100 μM in C17 cells.

middle column, and B, d). Thus, the exogenously expressed IP₃ sponge was able to inhibit significantly CCh-evoked IICR (Fig. 4, A, and C, p < 0.001).

We next examined whether the inhibitory effect of IP₃ sponge would be influenced by the expression levels. As in the result with C2 cells, we observed a significant small and often delayed Ca²⁺ response in sponge-expressing C17 cells after CCh stimulation (Fig. 4, A, bottom column, B, e, C, d, and D, C17; p < 0.001). However, no Ca²⁺ increase in response to stimulation with 20 μM CCh was seen in only 26% of the sponge-expressing C17 cells (Fig. 4C, e), indicating that the inhibitory effect in a high level sponge-expressing cell line, C2 cells, could be significantly more potent than that in a low level sponge-expressing cell line, C17 cells (Fig. 4D; p < 0.001, Student’s t test). These results indicate that the exogenously expressed IP₃ sponge reached a concentration sufficient to trap IP₃, resulting in the complete inhibition of IICR.

The subsequent Ca²⁺ oscillation induced by stimulation with 20 μM CCh was not detected in most of the sponge-expressing C2 and C17 cells (Fig. 4C, d). Interestingly, some IP₃ sponge-expressing cells displayed a small rise in [Ca²⁺] in response to 200 μM CCh followed by oscillation (Fig. 4B, e) that resembled the response elicited by 20 μM CaCl₂ in the non-induced cells.

We also investigated whether IP₃ sponge would inhibit the Ca²⁺ response to the purinergic agonist ATP (25) under the same conditions we used to measure the response to CCh. Application of ATP to K508A-expressing cells (Fig. 5, A, top column, B, h, and C, b) and GST-expressing cells (data not shown) as well as all non-induced cells (Fig. 5, B, a, and C, a) caused a steep rise in [Ca²⁺], that depended on the ATP concentration. In contrast, a smaller and delayed Ca²⁺ increase was typically seen in sponge-expressing C2 cells (filled arrowheads in Fig. 5, A, middle column, B, c, C, c, and D, C2; p < 0.001), whereas induced C2 cells that did not show any immunoreactivity (open arrowheads in Fig. 5A, middle column) displayed marked Ca²⁺ release. Moreover, in the same manner as CCh-evoked IICR, complete inhibition of the increase in [Ca²⁺] was observed in 20–30% of sponge-expressing C2 cells stimulated with both 10 and 100 μM ATP (Fig. 5, A, middle column, B, d, and C, d). Most Ca²⁺ increases evoked by ATP in sponge-expressing C17 cells were significantly reduced (Fig. 5, B, e, C, e, and D, C17; p < 0.001) and often delayed (filled arrowheads in Fig. 5A, bottom column). However, no rise in Ca²⁺ was detected in only 13% of cells stimulated with 10 μM ATP (data not shown). These findings show that IP₃ sponge could also efficiently trap IP₃ produced in response to ATP and significantly inhibited ATP-evoked IICR. All IP₃ sponge-expressing cells examined after CCh or ATP stimulation showed obvious 0.2 μM thapsigargin (TG)-induced Ca²⁺ release as well as K508A- and GST-expressing cells and all non-induced cells (Fig. 4, B and C, and Fig. 5, B and C), indicating that the inhibition of IICR by IP₃ sponge did not result from a lack of Ca²⁺ storage.

IP₃ Trapping by the IP₃ Sponge Reduces CCh-induced CREB Phosphorylation in HEK293 Cells—We next analyzed whether trapping of IP₃ by the IP₃ sponge would influence cell physiology downstream. It has been well established that Ca²⁺ signaling patterns in the form of single transients and sustained plateaux, including repetitive oscillations generated by cell permeant caged IP₃ ester (26), play a critical role in gene transcription (27). One of the responses to an increase in [Ca²⁺], is activation of the transcription factor CREB (28, 29).
Encended the level of CREB phosphorylation in cells treated with 20 μM CCh (Fig. 6). In contrast, the level of CREB phosphorylation in IP₃ sponge expression-induced C2 cells was less than half that in non-induced C2 cells (Fig. 6, A and B, n = 8; p < 0.001, Student’s t test), indicating that the IP₃ sponge had a significant inhibitory effect on the CCh-induced CREB phosphorylation. Immunocytochemical analysis of CREB phosphorylation using anti-phospho-CREB antibody (Fig. 6C) showed that the nuclear immunoreactivity of K508A-expressing cells treated with 20 μM CCh (Fig. 6C, d) was up-regulated in comparison with that of unstimulated cells (Fig. 6C, a and b). In IP₃ sponge-expressing C2 cells (Fig. 6C, e), CREB phosphorylation triggered by 20 μM CCh was almost completely inhibited, whereas the increase of nuclear immunoreactivity to anti-phospho-CREB antibody was clearly observed in both non-induced C2 cells (Fig. 6C, e) and induced C2 cells that did not show the expression of IP₃ sponge (arrowhead in Fig. 6C, e). On the other hand, trapping IP₃ did not influence 20 μM forskolin-induced CREB phosphorylation (Fig. 6, A and B, n = 3–4), suggesting that the IP₃ sponge specifically inhibited IICR-dependent CREB phosphorylation.

**DISCUSSION**

We have developed a novel recombinant IP₃-absorbent protein, called the IP₃ sponge, that has >1000-fold higher affinity for IP₃ than the endogenous IP₃-R, and we found that it specifically and dose-dependently inhibited in vitro IICR in a competitive manner. Exogenously expressed IP₃ sponge was also found to trap efficiently IP₃ and inhibit IICR in living cells, depending on the level of expression. Trapping IP₃ with the IP₃ sponge significantly reduced CCh-induced CREB phosphorylation at Ser-133.

All of the inhibitors of IICR reported previously have targeted IP₃-R, and there have been problems with specificity, isomeric selectivity, and/or permeability. Application of heparin, a competitive inhibitor for IP₃ binding (31), is limited, because it performs actions on multiple targets in cells and is not membrane-permeable. Microinjection of the monoclonal antibody 18A10 specifically inhibits IICR via type 1 IP₃-R by binding to its C terminus (32), but this approach cannot be applied to phenomena likely to involve type 2 or 3 IP₃-R, and it is difficult to deliver efficiently this antibody inside cells (33). There are two potent membrane-permeable IICR inhibitors that do not block IP₃ binding, the xestospongins, a group of macrocyclic bis-1-oxaquinolizidines isolated from the Australian sponge (34), and 2-aminoethoxy diphenylborate, which we developed previously (35). However, Missiaen and colleagues recently reported that xestospongins C (36) and 2-aminoethoxy diphenylborate (37) are not specific for IICR, because both equally block the endoplasmic reticulum Ca²⁺ pump. In the present study, we have demonstrated that the IP₃ sponge could overcome these problems.

One of the unique properties of the IP₃ sponge is that it directly traps IP₃ signals. The specificity of the IP₃ sponge for various inositol phosphates was similar to that of native cerebellar IP₃-R (Fig. 1D), indicating that the IP₃ sponge retained specific IP₃ binding properties that were the same as those of endogenous IP₃-R. The inhibitory effect of IP₃ sponge on in vitro IICR from microsomes (Fig. 2) resulted from trapping extravesicular IP₃ in a dose-dependent manner rather than affecting the de novo production of IP₃. In addition, the caffeine-induced Ca²⁺ response was unaffected by adding the IP₃ sponge (Fig. 2A, insets), indicating that the IP₃ sponge had no effect on Ca²⁺ release from ryanodine-sensitive stores. We also identified that the exogenously expressed IP₃ sponge in HEK293 cells was distributed homogeneously in the cytoplasm (Figs. 3B, 4A, and 5A) and had no effect on the activity of PLC (Fig. 3, D and E).
Thus, IP$_3$ sponge could trap the increased intracellular IP$_3$ without affecting the production of IP$_3$. Because the inhibition of ICR by the IP$_3$ sponge was due to the superiority in the affinity and specificity for IP$_3$ over the parental IP$_3$R, the inhibitory effect could be enhanced by using mutant R441Q, the substitution of Gln for Arg-441 on the IP$_3$ sponge (G224), which had double the IP$_3$ binding activity of the IP$_3$ sponge.

Another unique feature of the IP$_3$ sponge is the product encoded in its cDNAs, which allows trapping of intracellular IP$_3$ by exogenously expressing the IP$_3$ sponge. Moreover, HEK293 cells express all three subunits of the IP$_3$R family (10), and thus the IP$_3$ sponge appears to be effective in competing for IP$_3$ with multiple types of tetrameric IP$_3$R channels, consisting of various subunit compositions. Therefore, spatiotemporally well-controlled expression of the IP$_3$ sponge will enable it to trap intracellularly produced IP$_3$ and to approach IP$_3$ signaling-dependent Ca$^{2+}$ physiology, irrespective of cell or tissue type. Because the levels of IP$_3$ trapping seem to influence Ca$^{2+}$ oscillation, the IP$_3$ sponge might be useful for the analysis of temporal Ca$^{2+}$ dynamics. Moreover, targeting the expression of this recombinant IP$_3$ sponge might be useful for the analysis of cell type- and cell stage-specific ICR events or intracellular compartment-specific miniature ICR events.

We also used the IP$_3$ sponge to measure whether inhibition of ICR would affect activation of the transcription factor CREB, which was crucially dependent on phosphorylation of Ser-133 (28, 29). Although it was well known that an increase in [Ca$^{2+}$]$_i$, via L-type Ca$^{2+}$ channels or NMDA receptors acts on CREB phosphorylation (11, 12), the involvement of IP$_3$-Ca$^{2+}$ signaling in CREB phosphorylation had never been directly investigated. Finkbeiner et al. (38) reported that the brain-derived neurotrophic factor-induced activation of PLC$_{y}$ stimulated CREB phosphorylation via ICR, and Hardingham et al. (39) recently showed that ICR was critical for propagation of Ca$^{2+}$ waves from synaptic NMDA receptors to the nucleus, which consequently induced Ca$^{2+}$-dependent phosphorylation of CREB. The present study showed that the up-regulation of CREB phosphorylation in response to 20 μM CCH was inhibited in the IP$_3$ sponge-expressing C2 cells, in which the rise in Ca$^{2+}$ evoked by 20 μM CCH was almost completely blunted. Thus, the application of the IP$_3$ sponge provided the first direct evidence that ICR enhanced phosphorylation at Ser 133 of CREB. These data suggest a link between ICR and gene transcription via CREB phosphorylation, involved in many neuronal activities, such as development, plasticity, and survival (40–42). Trapping of IP$_3$ by the IP$_3$ sponge actually influenced cell physiology including a downstream cascade and would be effective for exploring cellular events mediated by IP$_3$-Ca$^{2+}$ signaling.

In conclusion, we have characterized the recombinant hyper-affinity IP$_3$ sponge protein, which is useful for exploring IP$_3$-Ca$^{2+}$ signal conversion. This novel inhibitory system should provide a powerful means of analyzing not only the signaling effects of different intracellular IP$_3$ levels and dynamics but also a variety of aspects of IP$_3$-Ca$^{2+}$ physiology in vivo by expressing cell-, tissue-, and organelle-specific IP$_3$ sponge.
A Novel Recombinant Hyperaffinity Inositol 1,4,5-Trisphosphate (IP$_3$) Absorbent Traps IP$_3$, Resulting in Specific Inhibition of IP$_3$-mediated Calcium Signaling
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