Unique Regulatory Properties of Heterotetrameric Inositol 1,4,5-Trisphosphate Receptors Revealed by Studying Concatenated Receptor Constructs*

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The ability of inositol 1,4,5-trisphosphate receptors (IP₃Rs) to precisely initiate and generate a diverse variety of intracellular Ca²⁺ signals is in part mediated by the differential regulation of the three subtypes (R1, R2, and R3) by key functional modulators (IP₃, Ca²⁺, and ATP). However, the contribution of IP₃R heterotetramerization to Ca²⁺ signal diversity has largely been unexplored. In this report, we provide the first definitive biochemical evidence of endogenous heterotetramer formation. Additionally, we examine the contribution of individual subtypes within defined concatenated heterotetramers to the shaping of Ca²⁺ signals. Under conditions where key regulators of IP₃R function are optimal for Ca²⁺ release, we demonstrate that individual monomers within heteromeric IP₃Rs contributed equally toward generating a distinct 'blended' sensitivity to IP₃ that is likely dictated by the unique IP₃ binding affinity of the heteromers. However, under suboptimal conditions where [ATP] were varied, we found that one subtype dictated the ATP regulatory properties of heteromers. We show that R2 monomers within a heterotetramer were both necessary and sufficient to dictate the ATP regulatory properties. Finally, the ATP-binding site B in R2 critical for ATP regulation was mutated and rendered non-functional to address questions relating to the ATP regulatory properties of heteromers. We show that R2 monomers within a heterotetramer were both necessary and sufficient to dictate the ATP regulatory properties. Finally, the ATP-binding site B in R2 critical for ATP regulation was mutated and rendered non-functional to address questions relating to the ATP regulatory properties of heteromers. We show that R2 monomers within a heterotetramer were both necessary and sufficient to dictate the ATP regulatory properties. Finally, the ATP-binding site B in R2 critical for ATP regulation was mutated and rendered non-functional to address questions relating to the ATP regulatory properties of heteromers. We show that R2 monomers within a heterotetramer were both necessary and sufficient to dictate the ATP regulatory properties.

The importance of intracellular Ca²⁺ as a second messenger is underscored by its distinctive ability to regulate a multitude of diverse cellular processes, including transcription, translation, secretion of fluids, muscle contraction, motility, fertilization, memory, apoptosis, and autophagy (1–8). This remarkable capacity to precisely and often simultaneously regulate cellular events is thought to be due, at least in part, to the highly sophisticated spatial and temporal control of intracellular [Ca²⁺] by a complement of specialized proteins, collectively termed the “Ca²⁺ signaling toolkit” (7). Simplistically, this “toolkit” includes several Ca²⁺ influx and release channels whose activation brings about a rise in basal intracellular Ca²⁺ concentration. In addition, cytosolic Ca²⁺ buffers, pumps, and transporters function to reduce the intracellular Ca²⁺ concentration by extrusion into stores or the extracellular space (9). Essential components of this toolkit are the members of the endoplasmic reticulum-localized inositol 1,4,5-trisphosphate receptor (IP₃R)² family.

Stimulation of cell surface receptors by growth factors, hormones, and neurotransmitters results in Ca²⁺ mobilization as a result of the generation of IP₃, which subsequently binds to and activates IP₃Rs (10, 11). There are three major subtypes of IP₃Rs (R1, R2, and R3), encoded by a distinct gene (Itpr1, Itpr2, and Itpr3) (12, 13). These ∼300-kDa monomeric proteins co-translationally oligomerize into ∼1200-kDa tetrameric Ca²⁺ release channels (14). The three subtypes share ∼60–70% sequence homology and are conventionally divided into three functional domains. At the extreme N terminus lies the conserved ligand-binding domain, which is composed of a suppressor domain (SD) and a ligand-binding core (15). This is followed by a large, less conserved intermediary regulatory domain that contains several putative sites for regulation by different molecules, including Ca²⁺ (serving as a co-agonist), adenosine triphosphate (ATP), protein binding partners, and post-translational modifications (16). Finally, at the C terminus lies a six-transmembrane domain that, in addition to being critical for receptor oligomerization (17) and ER localization (18, 19), contains the ion-conducting pore between transmembrane helices 5 and 6 (20–22).

The variation in primary amino acid sequence between the three subtypes results in each isoform exhibiting distinct IP₃ binding affinities and modulatory properties. For instance, IP₃ binding assays performed on the N-terminal 604 amino acids revealed that R2 has an ∼3- and ∼12-fold greater IP₃ binding affinity than R1 and R3, respectively (23). Utilizing permeabilized cell Ca²⁺ release assays, our group has shown that subtype sensitivity to ATP modulation follows a similar rank order, with R2 shown to have ∼3- and ∼10-fold higher affinity for ATP than R1 and R3, respectively. Furthermore, although Ca²⁺ release of R1 and R3 is potentiated at all [IP₃] by ATP, the activity of R2 is uniquely augmented only at submaximal [IP₃]².

The abbreviations used are: IP₃R, inositol 1,4,5-trisphosphate receptor; ICR, IP₃-induced Ca²⁺ release; IP₃, inositol 1,4,5-trisphosphate; SERCA, sarcoplasmic-endoplasmic reticulum Ca²⁺-ATPase; ER, endoplasmic reticulum; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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(24, 25). IP₃Rs are also differentially regulated by kinases (PKA, PKG, and Akt) (26–33), proteolytic enzymes (34), post-translational modifications (17), and a whole host of other functional modulators (12). Importantly, all known regulatory motifs are present in each monomer, although the stoichiometry necessary for modulation is unknown.

Further complexity potentially arises from the idea that IP₃R subtypes assemble into heterotetramers. Although IP₃R expression is ubiquitous, individual cell types express distinct complements of isoforms at varying proportions. Notably, most cell types express at least two subtypes (35–37). For instance, although the majority of neuronal IP₃Rs are R1, most other peripheral cell types express R2 and R3 (38, 39). Furthermore, numerous cross-linking (40, 41), co-immunoprecipitation (39, 42, 43), and co-localization and immunostaining assays (44–47) have supported the existence of heterotetrameric IP₃R. Additionally, we demonstrated that homotetramers account for only a small proportion of IP₃Rs in mouse pancreas (39).

Given that IP₃Rs are regulated in a subtype-specific manner and that these subtypes likely oligomerize into heterotetramers, the obvious question relates to the contribution of each isoform within a heterotetramer to overall receptor function. Studies in cultured cells or isolated tissues fail to account for the exact proportions of each subtype within a tetramer. To address this, we recently described a strategy whereby concatenated IP₃R cDNA constructs were engineered to result in expression of tetrameric channels with defined composition (39). As proof of principle, we showed that concatenated homo- or heterodimers encoding R1 or R2 were stably expressed in DT40 triple IP₃R knock-out (3KO) cells, dimerized to form tetrameric channels localized to the ER membrane, were functionally responsive to Gₛ-coupled, G-protein-coupled receptor stimulation, and exhibited identical single channel activity to those expressed from monomeric constructs (39).

In this report, we examine the contribution of individual isoforms within an IP₃R heterotetramer toward shaping the Ca²⁺ signals that are generated. Under conditions where key regulators of IP₃R function are optimal for Ca²⁺ release (5 mM ATP, 200 nM free Ca²⁺⁺) (24, 25, 48, 49), individual monomers within a heterotetrameric IP₃R were found to contribute equally toward dictating the channels’ “blended” sensitivity to IP₃. IP₃ binding assays further revealed that this distinct IP₃ sensitivity is likely dictated by the unique apparent IP₃ binding affinity of heterotetrameric IP₃Rs. In contrast, under suboptimal conditions for Ca²⁺ release through the IP₃Rs, where [ATP] was varied, a single isoform was found to dictate the ATP regulatory properties of heteromeric IP₃Rs. Finally, using concatenated receptors with mutations in defined numbers of subunits, we address for the first time fundamental questions relating to the stoichiometry of IP₃R regulation by ATP.

Materials and Methods

Reagents Used—All restriction enzymes and DNA T4 ligase were obtained from New England Biolabs. Fetal bovine serum, RPMI 1640 media, chicken serum, penicillin/streptomycin, and β-mercaptoethanol were obtained from Gibco®/Life Technologies, Inc. G418 sulfate (Geneticin) was obtained from Invitrogen. Fura-2 AM and MgFluo4 AM were obtained from Molecular Probes by Life Technologies, Inc. Enhanced chemiluminescent substrate and DyLight™ 800CW secondary antibodies were from Thermo Scientific. The D₄ protein assay kit, Tris base, glycine, horseradish peroxidase-conjugated secondary antibodies, and all reagents used for SDS-PAGE were from Bio-Rad. All materials for native-PAGE, including native-PAGE 3–12% BisTris gels, were obtained from Novex® by Life Technologies, Inc. CHAPS was obtained from G Biosciences. d-Myoinositol 1,4,5-trisphosphate hexapotassium salt and protein A/G PLUS-agarose beads were obtained from Santa Cruz Biotechnology. [³H]IP₃ was obtained from PerkinElmer Life Sciences. KCl and NaCl were obtained from Amresco. Glucose was obtained from Calbiochem EMD Millipore. Mouse anti-Chicken IgM was obtained from Southern Biotech. All other materials were obtained from Sigma.

Antibodies—The antibody against the C-terminal 19 amino acids of R1 (α-R1) was generated by Pocono Rabbit Farms and Laboratories (Canadensis, PA), as were the antibodies against the N terminus (amino acids 320–338) (α-R2NT) and C terminus (α-R2CT) of R2 (amino acids 2686–2702). Mouse monoclonal antibody against residues 22–230 of human R3 (α-R3) was from BD Transduction Laboratories (San Jose, CA).

Generation of Concatenated IP₃R Constructs—R1 and R2 dimeric constructs were generated by designating IP₃R cDNA encoding the corresponding isoforms as “head” and “tail” and modifying them appropriately using a QuikChange mutagenesis strategy as described before (39). R3 head subunits were generated by silently mutating Ncol sites in the rat R3 coding sequence (primers 1–6). After the start codon, an alanine codon was added to enable insertion of a new Ncol site, in addition to a Kozak sequence (primers 7 and 8). The head subunit was further modified by deleting a stop codon and introducing a nucleotide sequence encoding the first half of the linker followed by an Agel site (primers 9 and 10), immediately after the R3 coding sequence. The R3 tail subunit was generated by introducing an Agel site followed by a nucleotide sequence encoding the second half of the linker immediately before the start codon (primers 11 and 12). Additionally, a blunt end restriction site (HpaI) was inserted immediately after the stop codon (primers 13 and 14). The coding sequence for all constructs was confirmed by sequencing. To generate IP₃R dimers encoding homo- and heterodimeric receptors, the appropriate fragments were directly ligated between the two arms of pJAZZ Mamm linear vector to generate a construct encoding two IP₃R subunits connected with a 14-amino acid linker within a single open reading frame. The tetrameric R1R1R1R2 construct was engineered by ligating C to N terminus, subunits 1–3 of the R1R1R1 homotetramer and the R2 tail subunit, generated as described previously (39).

Generation of ATPB-binding Site Mutants—A QuikChange mutagenesis strategy was employed to modify the Walker A-like motif ATPB site in cDNAs encoding the mouse R2 monomer, the R2 dimer head and tail subunits. Briefly, forward and reverse mutagenic primers (primers 15 and 16) were generated by Integrated DNA Technologies to encode mutations at G5940, G5946, and G5955, in addition to silently introducing a PspOMI restriction site. These mutations code for amino acid substitutions G1969A, G1971A, and G1974A, respectively. The
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coding sequence for all constructs was confirmed by sequencing. The R2R₂ and R₂²R₂ dimers were generated using the same approach used to generate the wild type R2 homodimer, as described above.

Cell Culture and Generation of DT40 Cells Stably Expressing IP₃R—DT40-3KO cells and DT40-3KO cells stably expressing IP₃R constructs were maintained at 39 °C and 5% CO₂ in RPMI 1640 media supplemented with 10% FBS, 1% chicken serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μM β-mercaptoethanol. Cells were maintained by subculturing every 3 days. Selection was carried out using G418 sulfate (geneticin). DT40-3KO cell transfection and stable line generation were performed as described previously (34). Cells stably expressing monomeric or concatenated IP₃R constructs were lysed using Triton X-100 lysis buffer as described before.

Western Blotting—DT40-3KO cells or DT40 cells stably expressing monomeric or concatenated IP₃R constructs were lysed using Triton X-100 lysis buffer (50 mM Tris base, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, pH 8), supplemented with protease inhibitors. All lysates were incubated on ice for 30 min, interspersed with occasional vortexing and sonication to ensure adequate disruption of membranes. Lysates were cleared by centrifugation (16,000 × g, 10 min, 4 °C) and resolved on SDS-PAGE. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) and immunoblotted using isofrom-specific primary antibodies and corresponding secondary antibodies. Membranes were visualized using an Odyssey infrared imaging system (LICOR Biosciences).

Native-PAGE—DT40-3KO cells expressing various constructs were harvested by centrifugation and lysed in CHAPS lysis buffer (40 mM NaCl, 25 mM HEPES, 10 mM CHAPS, 1 mM EDTA, pH 7.4) supplemented with protease inhibitors. After 30 min on ice at 4 °C, lysates were cleared by centrifugation at 16,000 × g for 10 min at 4 °C. Cleared lysates were mixed with of 2× sample buffer, 5% G-250 sample additive, and fractionated 3–12% native-PAGE™ Novex gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed using the indicated primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected using enhanced chemiluminescent substrate.

Size Exclusion Chromatography—A small section of frozen bovine salivary gland (Pel-Freez Biologicals Inc., Rogers, AR) was excised, washed in ice-cold homogenization buffer (10 mM Tris, 150 mM sucrose, 150 mM KCl, 1.5 mM MgCl₂, pH 7.5) containing protease inhibitors, and flash-frozen in liquid N₂. In the presence of liquid N₂, the salivary section was ground to a fine powder and lysed on ice for 1 h using CHAPS lysis buffer (10 mM CHAPS, 25 mM HEPES, 120 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8) containing protease inhibitors. During the hour, the lysate was subject to repetitive vortexing, needle homogenization using an RZR 2021 homogenizer (Heidolph Instruments, Germany) with 50 strokes at 1200 rpm. The lysates were then centrifuged twice at 1000 × g for 15 min at 4 °C. The supernatants were transferred to polyallomer Beckman centrifuge tubes and centrifuged at 130,000 × g twice for 30 min at 4 °C. A small sample of the lysate was mixed with SDS loading buffer and saved as the input control. 1100 μl of the clarified extract was injected onto the Sephacryl-400 column. 36 × 2-ml fractions were collected post-void at a 1 ml/ml flow rate. Equivalent amounts of each fraction were fractionated on 5% SDS-PAGE, transferred to nitrocellulose, and probed with the indicated antibodies. Co-immunoprecipitation was performed on pooled fractions corresponding to ~1.1 MDa by incubation with protein A/G plus-agarose beads and the described isoform-specific antibodies overnight at 4 °C, tumbled end over end. Beads were washed with Triton X-100 lysis buffer, resuspended in SDS loading buffer, and boiled at 75 °C for 5 min. The samples were resolved on 5% SDS-PAGE, transferred to nitrocellulose membranes, and probed using the indicated antibodies.

Fluorescence Imaging—DT40 cells expressing defined IP₃R constructs were loaded with 2 μM Fura-2 AM on a glass coverslip mounted onto a Warner chamber at room temperature for 20–30 min. Loaded cells were perfused with HEPES imaging buffer (137 mM NaCl, 4.7 mM KCl, 1.26 mM CaCl₂, 1 mM Na₂HPO₄, 0.56 mM MgCl₂, 10 mM HEPES, 5.5 mM glucose, pH 7.4) and stimulated with the desired agonist. Ca²⁺ imaging was performed using an inverted epifluorescence Nikon microscope with a ×40 oil immersion objective. Cells were alternately excited at 340 and 380 nm, and emission was monitored at 505 nm. Images were captured every second with an exposure of 10 ms and 4 × 4 binning using a digital camera driven by TILL Photonics software.

96-Well Permeabilized Cell IP₃-induced Ca²⁺ Release (IICR) Assays—This protocol was modified from one described previously (50). DT40-3KO cells stably expressing defined IP₃R constructs were grown up to near confluency, harvested, and washed twice with HEPES imaging buffer. Cells were incubated in darkness with 20 μM MgFluo4 AM at 1 h at room temperature on a rocker. After loading, cells were washed, resuspended in Ca²⁺-free media (140 mM KCl, 20 mM NaCl, 20 mM PIPES, 1 mM EGTA, and 2 mM MgCl₂, pH 7.0), and permeabilized using 10 μg/ml saponin. Permeabilization was confirmed by visualizing trypan blue accumulation. Permeabilized cells were subsequently washed, resuspended in Mg²⁺-free media (140 mM KCl, 20 mM NaCl, 20 mM PIPES, 1 mM EGTA, 375 μM CaCl₂, pH 7.0), and dispensed into a black-walled flat bottom 96-well plate (~500,000 cells/well). The plate was spun at 200 × g for 2 min to plate cells to the bottom of each well. The plate was allowed to rest for 30 min prior to commencing the assay. Fluorescence imaging was carried out using FlexStation 3 from Molecular Devices (excitation 490 nm and emission 525 nm) and analyzed by using SoftMax® Pro Microplate Data Acquisition and Analysis software. Stores were loaded by adding 1.5 mM Mg-ATP to activate SERCA. Upon loading, SERCA was disabled using cyclopiazonic acid, and carbonyl cyanide p-trifluoromethoxyphenyldrazo was added to uncouple mitochondria. IP₃Rs were then activated by the addition of varying [IP₃] in the presence of 5 mM ATP. The initial rates of Ca²⁺ release were determined by fitting the curves to a single exponential function (OriginPro 6.1). Dose-response curves were plotted using the determined rates.

Single Cell Permeabilized DT40 Cell IICR Assays—DT40-3KO cells stably expressing defined IP₃R constructs were
loaded with 20 μM MgFura-2 AM for 50–60 min on a glass coverslip mounted onto a Warner chamber at room temperature in darkness. Cells were subsequently permeabilized by superfusion of 40 μM β-escin in intracellular medium (ICM) (125 mM KCl, 19 mM NaCl, 10 mM HEPES, 1 mM EGTA, pH 7.3). The duration of permeabilization was dependent on the flow rate, and care was taken to prevent excessive (internal membrane) permeabilization by careful monitoring of the fluorescence. Permeabilized cells were then washed in ICM without β-escin for 15 min to allow for removal of cytosolic dye. The internal stores of permeabilized cells were loaded by activating SERCA through superfusion with ICM containing 1.4 mM MgCl₂, 3 mM Na-ATP, and 0.65 mM CaCl₂ (free [Ca²⁺] of 200 nM (MaxChelator freeware)). Upon stabilization of fluorescence, MgCl₂ was removed from the superfused solution to equally aliquoted into Eppendorf tubes. The binding reaction rates of Ca²⁺ view and was performed a minimum of three times. The initial release of Ca²⁺ was determined rates. All imaging was performed on an inverted epifluorescence Nikon microscope with a ×40 oil immersion objective. Cells were alternately excited at 340 and 380 nm, and emission was monitored at 505 nm. Images were captured every 5 s during permeabilization, loading, and disabling and every second during release. This was done with an exposure of 10 ms and 4 × 4 binning using a digital camera driven by TILL Photonics software.

Homologous Competitive IP₃ Binding Assay—DT40-3KO cells stably expressing IP₃R constructs were lysed using Triton X-100 lysis buffer supplemented with protease inhibitors. Lysates were incubated on ice for 30 min, interspersed with occasional vortexing, and subsequently cleared by centrifugation twice at 16,000 × g for 20 min at 4 °C. An overnight immunoprecipitation was performed on the cleared lysate by adding protein A/G PLUS-agarose beads and antibody against the specific isoform at 4 °C. Post-immunoprecipitation, the beads were washed with binding buffer (50 mM Tris base, 1 mM EDTA, pH 8, plus 1 mM β-mercaptoethanol added freshly upon use) and equally aliquoted into Eppendorf tubes. The binding reaction was performed in a 100-μl volume containing the immunoprecipitated protein, 2.5 nM [³H]IP₃, and varying concentrations of unlabeled IP₃ for 1 h at 4 °C, vortexed every 10 min. After incubation, beads were pelleted by centrifugation (16,000 × g for 2 min), and the supernatants were carefully aspirated. The beads were incubated with 500 μl of 1% SDS overnight at room temperature. The next day, the tube contents were transferred to scintillation vials, mixed with scintillation liquid, and bound radioactivity measured using a liquid scintillation counter. Nonspecific binding was defined as the radioactivity measured in the presence of 50 μM cold IP₃. Specific binding was determined by subtracting the counts/min values obtained in the presence of 50 μM cold from the counts/min values obtained with other conditions. Total specific binding was determined as the binding observed in the absence of cold IP₃. All values were normalized to total specific binding. Normalized specific binding from three experiments was averaged, and curves were fit using a logistic dose–response equation using the OriginPro 6.1 software.

Preparation of DT40 Nuclei—Isolated DT40 nuclei were prepared by homogenization as described previously (49). Cells were washed and resuspended in homogenization buffer (250 mM sucrose, 150 mM KCl, 3 μM β-mercaptoethanol, 10 mM Tris, 1 mM PMSF, pH 7.5, supplemented with protease inhibitor mixture (Roche Applied Science)), and nuclei were isolated using an RZR 2021 homogenizer (Heidolph Instruments, Germany) with 25 strokes at 1200 rpm. 3 μl of nuclear suspension were placed in 3 ml of bath solution (140 mM KCl, 10 mM HEPES, 500 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 246 nM free Ca²⁺, pH 7.1). Nuclei were allowed to adhere to a plastic culture dish for 10 min.

Patch Clamp Experiments—Single IP₃R channel potassium currents (Iₛ) were measured in the on-nucleus voltage clamp configuration of the patch clamp technique using PClamp9 and an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), as described previously (49). Pipette solutions contained 140 mM KCl, 10 mM HEPES, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, 200 nM free Ca²⁺, and the indicated concentrations of IP₃ and ATP. Free [Ca²⁺] was calculated using Max Chelator freeware and verified fluorometrically. Traces were consecutive 3-s sweeps recorded at −100 mV, sampled at 20 kHz, and filtered at 5 kHz. A minimum of 15 s of recordings was considered for data analyses. Current/voltage relationships were generated by obtaining multiple sweeps at the indicated holding potential. Pipette resistances were typically 20 megohms and seal resistances were >5 gigaohms.

Data Analysis—Single channel openings were detected by half-threshold crossing criteria using the event detection protocol in Clampfit. We assumed that the number of channels in any particular nucleus was represented by the maximum number of discrete stacked events observed during the experiment. Even at low Pₛ, stacking events were evident (data not shown). Only patches with one apparent channel were considered for analyses of mean open and closed times and open probability. The slope conductances were determined from the linear fits of the current-voltage relationships where \( g = I/V − V_k \). Equation parameters were estimated using a non-linear least squares algorithm.

Results and Discussion

IP₃R Isoforms Oligomerize Forming Heterotetramers in Native Tissues—The ubiquitous expression of IP₃Rs is well established, with numerous studies unequivocally demonstrating that individual cell types express at least two if not all three isoforms (38, 39). Co-immunoprecipitation experiments performed on lysates isolated from individual cell types or tissues have shown that these isoforms do associate (39, 42, 43, 51). However, they fail to exclude the possibility that these observed interactions are the result of larger intermolecular complexes of homotetrameric channels. To address this issue, lysates from bovine salivary glands, which express all three isoforms (Fig.
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FIGURE 1. IP₃R subtypes oligomerize to form heterotetramers in native tissues. Cleared lysates from bovine salivary gland were prepared using CHAPS lysis buffer. A, lysates were resolved on 5% SDS-PAGE and immunoblotted with α-R1, α-R2CT, or α-R3. Lysates from DT40 3KO cells stably expressing R1, R2, or R3 were used as controls. B, bovine salivary gland lysates cleared through ultracentrifugation were subject to size exclusion chromatography. Alternate fractions were resolved on 5% SDS-PAGE and probed using α-R1 or α-R2CT. IP₃R immunoreactivity was quantified using ImageJ and normalized to peak immunoreactivity. C, fractions 9-11 corresponding to native IP₃R (~1000–1200 kDa) were pooled at subject to co-immunoprecipitation using α-R1 and protein A/G beads. Immunoprecipitates (IP) were probed using α-R1 or α-R2CT. Lysates from DT40-3KO cells stably expressing R1, R2, or R3 were used as controls. Representative immunoblots are shown.

Establishing Stable Cell Lines Expressing Homo- or Heterodimer IP₃R Constructs—Having confirmed that different isoforms oligomerize to form heterotetrameric IP₃Rs natively, an obvious question is as follows: What is the contribution of each isoform within a heterotetramer to overall receptor function? To address this, cDNA constructs encoding concatenated IP₃R homo- and heterodimers were engineered and stably expressed in DT40-3KO cells, an IP₃R null background cell line (39). Fig. 2A demonstrates that concatenated homo- or heterodimers encoding R2 or R3 can be stably expressed in DT40-3KO cells. Immunoblots probed with α-R2NT show that monomeric R2 migrated at ~250 kDa, whereas dimers containing R2 migrated at ~500 kDa, as expected. No bands were observed for 3KO or R3 monomers or homodimers (R3R3). Similarly, immunoblots probed with α-R3 demonstrated expression of monomeric (~250 kDa) and dimeric (~500 kDa) R3 migrating as expected, with no immunoreactivity seen in lanes containing 3KO or R2 monomers or homodimer (R2R2) lysates. Additionally, no lower order degradation products were detected in cells expressing dimers, firmly establishing that the composition of concatenated IP₃Rs being studied was defined. These dimeric proteins are also capable of oligomerizing to form tetramers (Fig. 2B). Under non-denaturing blue native-PAGE conditions, IP₃R monomers or dimers migrated at ~1100–1200 kDa when probed with either α-R2NT or α-R3, corresponding to the established molecular mass of assembled tetrameric IP₃Rs.

A recent cryo-EM structure indicates that the C and N termini of adjacent IP₃R monomers are situated in close proximity to the assembled tetrameric channel (52). Nevertheless, a major concern when designing and utilizing the concatenated constructs was whether linking the N terminus, containing the IP₃ binding domain, to the C terminus of the adjacent subunit, containing the pore domain, would constrain the tetrameric channel and thereby affect the conformational change required for receptor function. Therefore, homologous competitive IP₃ binding assays were carried out to ensure that the monomers and concatenated dimers bound IP₃ with similar affinities. Monomers of the three isoforms exhibited similar apparent affinities to those previously reported (23), with R2 having the highest apparent affinity (14 ± 4 nM) followed by R1 (50 ± 5 nM) and finally R3 (171 ± 19 nM) (Fig. 2C). Furthermore, the data clearly show that the apparent IP₃ binding affinities of the monomers and homodimers are indistinguishable, with R2R2 having the highest affinity at 18 ± 4 nM, followed by R1R1 (44 nM ± 7 nM), and finally R3R3 (186 ± 17 nM). Analysis of the slopes also revealed the binding for R1 and R1R1 is non-cooperative, consistent with previous work by Iwai et al. (53). However, contrary to their findings, we show R3 and R3R3 also exhibit non-cooperative binding, whereas R2 and R2R2 display negative cooperative binding (Hill coefficients of 0.7 and 0.8, respectively).

To ensure that the monomers and homodimers were functionally indistinguishable, a direct analysis of IP₃R activity was...
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Blended IP₃ Sensitivity Dictated by Apparent IP₃ Binding Affinity—The three major essential regulators of IP₃R function are IP₃, Ca²⁺, and ATP (12). As [ATP] and [Ca²⁺] were tightly clamped in the permeabilized cell assays to achieve maximal release, we speculated that the apparent IP₃ binding affinity was potentially the major determinant of the intermediate IP₃ sensitivity observed with the heterodimers under these conditions. Using homologous competitive binding IP₃ assays, we focused on the R3R2 heterodimer as R2 and R3 have the most distinct IP₃ sensitivities observed with the heterodimers under these conditions. Using homologous competitive binding IP₃ assays, we focused on the R3R2 heterodimer as R2 and R3 have the most distinct IP₃ sensitivities observed with the heterodimers under these conditions. Using homologous competitive binding IP₃ assays, we focused on the R3R2 heterodimer as R2 and R3 have the most distinct IP₃ sensitivities observed with the heterodimers under these conditions. Using homologous competitive binding IP₃ assays, we focused on the R3R2 heterodimer as R2 and R3 have the most distinct IP₃ sensitivities observed with the heterodimers under these conditions.
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FIGURE 3. IP₃,R heterodimers exhibit blended IP₃ sensitivities at optimal [ATP] and [Ca²⁺]. IP₃-response relationships of R2R2 and R3R2 were compared with R2R3 and R3R3 (A) and R1R2 was compared with R1R1 and R2R2 (B). High throughput permeabilized cell IICR assays were carried out using FlexStation 3. IP₃-mediated Ca²⁺ release was induced through addition of varying [IP₃] in the presence of 5 mM ATP and 200 nM free Ca²⁺. All values were normalized to the maximal release rate. Each point is the mean ± S.E. of eight wells from at least three experiments.

FIGURE 4. IP₃ binding of R3R2 heterodimer. [³H]IP₃ binding curves of R2R2, R3R3, and R3R2 homo- and heterodimers were generated using homologous competitive binding assays. All values were normalized to total specific binding. Each point is mean ± S.E. from three experiments.

natively cooperative binding exhibited by R2R2 (Fig. 2C), binding in R3R2 was non-cooperative resembling R3 and R3R3.

The data presented in Fig. 4 suggest that the four individual IP₃ ligand-binding domains within a tetramer, rather than functioning as four independent sites, interact and contribute to determine the overall binding affinity of the assembled channel. Previous studies add support to the notion that adjacent subunits may interact to influence function. For example, Boehning and Joseph (40) demonstrate through a series of biochemical cross-linking and mutagenesis assays that the C terminus of one monomer interacts with the N terminus of another monomer, regardless of subtype, to influence tetramer functionality. This idea is also reinforced by a recent cryo-EM structure, which suggests that the N-terminal ligand-binding domain interacts with multiple contact sites in the C terminus of adjacent subunits to initiate the conformational changes required for channel gating (52). In total, our observations raise the possibility that IP₃,R heterotetramers with different subunit combinations could exhibit unique IP₃ binding affinities, thus extending the repertoire of IP₃ regulation of IP₃,R and in turn the sensitivity of Ca²⁺ release.

R2 Determines the ATP Regulatory Properties of R3R2 Heterodimers—The previous data suggest that the IP₃ binding affinity of heterodimers is dictated by a contribution from both constituent isoforms. We next investigated whether a similar situation occurs when heterotetramers are regulated by ATP. Fig. 5, A–C, illustrates by using single cell IICR assays that the presence of saturating ATP (5 mM) markedly potentiated Ca²⁺ release from cells expressing R2R2 only at submaximal [IP₃], consistent with previously reported findings using R2 monomers (24, 49). Moreover, the presence of ATP appeared to induce a shift in IP₃ sensitivity, reducing the EC₅₀ from 2.45 ± 0.93 μM (0 ATP) to 0.62 ± 0.18 μM (5 mM ATP). In contrast, R3R3 requires ATP to become maximally activated with 5 mM ATP enhancing the rate of release even at 100 μM IP₃ (from 0.16 ± 0.02 s⁻¹ to 0.29 ± 0.02 s⁻¹), again consistent with earlier reports (Fig. 5, D–F) (24). Furthermore, unlike R2R2, ATP does not appear to markedly alter IP₃ sensitivity (0 ATP, 6.69 ± 1.06 μM, versus 5 mM ATP, 7.26 ± 1.16 μM). It should also be noted that the EC₅₀ values observed using the permeabilized single cell IICR assays are comparable with those observed using the Flexstation 3-based IICR assays. Next, we investigated the sensitivity of Ca²⁺ release to ATP in cells expressing these constructs (Fig. 6, A and B). Single cell IICR assays were performed where ATP levels were varied between 0 and 5 mM ATP at fixed [IP₃] and [Ca²⁺]. Release was induced at 1 μM for R2R2 and 10 μM for R3R3-expressing cells because the differences in raw Ca²⁺ release rates at 0 and 5 mM ATP were high at these [IP₃]. Fig. 6 shows that R2R2 clearly exhibited a considerably higher sensitivity to ATP (34 ± 15 μM) than R3R3 (447 ± 175 μM). These values are again comparable with those observed for R2 and R3 monomer constructs (~41 μM versus ~400 μM, respectively). Having defined the properties of the homomeric R2R2 and R3R3 constructs, we then investigated the characteristics of Ca²⁺ release in cells expressing R3R2 heterodimers. Strikingly, the presence of 5 mM ATP potentiated Ca²⁺ release only at submaximal [IP₃] (Fig. 5, G–I) while reducing the EC₅₀ from 4.17 ± 0.84 μM to 2.07 ± 0.31 μM. Additionally, R3R2 heterodimers exhibited an ATP sensitivity of 47 ± 19 μM (Fig. 6C). Remarkably, these data demonstrate that both the mode of regulation of heterotetrameric R2R3 channels and the ATP sensitivity are identical to that observed for R2 channels. These data strongly suggest that R2 dictates the mode of ATP regulation in heteromers containing equal proportions of R2 and R3.

Previous investigations into IP₃,R regulation by ATP in native tissues or cultured cells corroborate our findings by demonstrating that the regulatory properties appear to be dictated by a specific isoform. For instance, Ca²⁺ release assays performed on mouse pancreatic acini revealed that native IP₃,Rs exhibited an ATP sensitivity of 38 μM and were only modulated at submaximal [IP₃], comparable with the properties of R2 (60). This is despite the fact that mouse pancreatic acini have roughly equal expression of R2 and R3, with the vast majority of receptors likely heterotetrameric in nature (39). Interestingly, pancreatic acini from R2 knock-out mice exhibited an ATP sensitivity of 450 μM and were regulated by ATP at all [IP₃], identical to R3. Similar findings were observed in AR42J (R2 predominant) and RinM5F (R3 predominant) cells, which exhibited ATP sensitivities of 10 and 430 μM, respectively. Interestingly, when RinM5F cells were transiently transfected with cDNA encoding R2, a 10-fold increase in ATP sensitivity was observed. Furthermore, Ca²⁺ release was no longer potentiated at all [IP₃], rather only at submaximal [IP₃] (60). More recently,
FIGURE 5. ATP regulatory properties of R2R2, R3R3, and R3R2 dimers. Ca^{2+} release through R2R2 (A–C), R3R3 (D–F), and R3R2 (G–I) was measured using single cell permeabilized IICR assays. A, [IP_3]-response curve shows 5 mM ATP potentiates IICR only at submaximal [IP_3], not at maximal [IP_3], in R2R2. D, [IP_3]-response curve shows ATP is required to maximally potentiate IICR at all [IP_3] for R3R3. G, [IP_3]-response curve for R3R2 shows 5 mM ATP potentiates IICR only at submaximal [IP_3], not at maximal [IP_3], comparable with R2R2. Each point is mean ± S.E. from at least three experiments. Traces show Ca^{2+} release events at 3 and 30 μM IP_3 for R2R2 (B and C), R3R3 (E and F), and R3R2 (H and I) in the presence (solid) or absence (dotted) of 5 mM ATP. Each trace is the average of ~40–60 cells and was normalized to the average Δ340/380 of the 10 s prior to IP_3 application. *, p ≤ 0.05, Student's unpaired t test.

FIGURE 6. ATP sensitivities of R2R2, R3R3, and R3R2 dimers. Ca^{2+} release through R2R2 (A), R3R3 (B), and R3R2 (C) was measured using single cell permeabilized IICR assays. Ca^{2+} release was stimulated using 1 μM IP_3 for R2R2, 10 μM IP_3 for R3R3, and 1 μM IP_3 for R3R2 at various [ATP]. Release rates were plotted to a [ATP]-response curve, and the sensitivity to ATP was determined using a unimodal logistic equation. Each point is mean ± S.E. from at least three experiments.
using the on-nucleus patch clamp technique, our group showed that the open probability \( P_o \) of R1R2 heterodimers was only enhanced by ATP at low \([\text{IP}_3]\), comparable with the properties exhibited by R2R2 (39). Taken together, these data suggest that a general property of R2 is to dictate the mode of ATP regulation of heteromers containing equal proportions of R2, irrespective of the additional subtypes in the tetramer. These data are in stark contrast to IP3 binding, where a “blending” of the characteristics of the two constituent isoforms was observed. Indeed, the high “R2-like” sensitivity to ATP exhibited by R3R2 may suggest that the presence of two R2 subunits prevents ATP from binding to and regulating R3 altogether.

**R2 Dictates the Temporal Pattern of Ca\(^{2+}\) Release of R3R2 Heterodimers**—As a consequence of the distinctive binding and regulatory properties of each IP3R isoform, the submaximal stimulation of B cell receptors in DT40 cells expressing a single isoform results in each subtype generating distinctive intracellular Ca\(^{2+}\) release “signatures.” This temporal pattern may be considered as the integrated response of the particular IP3R subtype to the multiple regulatory inputs experienced after B cell receptor stimulation. For instance, our group and others have shown that R2-expressing cells produce robust long lasting Ca\(^{2+}\) oscillations, in stark contrast to the monophasic Ca\(^{2+}\) spikes produced by R1 (24, 39, 61). A previous study from our laboratory has reported that modulation of R2 activity by ATP is important for maintaining Ca\(^{2+}\) oscillations (24). \(\alpha\)-IgM stimulation of cells expressing R3 monomers and homodimers reproducibly produced robust yet short lasting Ca\(^{2+}\) oscillations (Fig. 7B), qualitatively and quantitatively distinct from the oscillatory pattern generated by R2 expressing cells (Fig. 7A). Indeed, quantification of the number of transients elicited by cells (Fig. 7C) shows a larger percentage of responding cells expressing R3 monomers, or homodimers produced few oscillations (1–3 transients), compared with R2R2. Remarkably, R2R3 and R3R2 heterodimers (Fig. 7, D and E) both generated robust long lasting oscillatory Ca\(^{2+}\) signals, similar to those generated by R2 monomers and homodimers. This is quantitatively represented in Fig. 7F, which shows that a smaller percentage of cells produced 1–3 transients, comparable with R2R2. We had also previously reported that R1R2 heterodimers produce long lasting Ca\(^{2+}\) oscillations, similar to R2 (39). Together, these data further support the notion that two R2 subunits within a heterotetramer are sufficient to dictate the overall modulation of Ca\(^{2+}\) release, including regulation by ATP binding, necessary to initiate an oscillatory profile of Ca\(^{2+}\) signals generated by heteromeric IP3Rs.

**Stoichiometry of ATP Regulation**—By virtue of being able to define the exact composition of tetrers being expressed, this approach using concatenated IP3R can also be used to answer questions related to the stoichiometry required for channel function and regulation. The primary sequence of the three subtypes contains several putative ATP-binding Walker A-type motifs (GXGXXG) (24, 25). However, mutagenic analysis has revealed that only a single such motif, the ATPB site in R2, is critical for ATP regulation of Ca\(^{2+}\) release (16). This has led to the suggestion that sites other than the Walker A-type motifs or alternate accessory proteins are responsible for regulation by ATP in R1 and R3. Briefly, triple glycine-alanine substitutions
at G1969A, G1971A, and G1974A in R2 monomers (R2/H9004) were shown to result in 5 mM ATP being completely unable to potentiate Ca\(^{2+}\)/H11001 release at any [IP3]. Additionally, R2/H9004 lost the ability to produce the IgM-stimulated robust Ca\(^{2+}\)/H11001 oscillations characteristic of R2 (24).

Accordingly, we sought to determine how many ATPB sites are required for regulation by ATP. R2R2 homodimers with either one (R2R2/H9004) or both subunits (R2/H9004R2/H9004) containing ATPB mutations were engineered and stably expressed in DT40-3KO cells (Fig. 8A). These constructs encode proteins that would oligomerize to form tetramers with either two or all four ATPB motifs mutated. Fig. 8B confirms that R2 homotetramers with all four ATPB motifs rendered non-functional (R2\(^{2}\)/H9004) resulted in a significant reduction in oscillatory activity when stimulated with α-IgM. Remarkably, the R2\(^{2}\)/H9004 mutant dimer retained robust oscillatory activity (Fig. 8C); ~70% of R2\(^{2}\)/R2\(^{2}\) cells produced 1–2 transients, in stark contrast to the ~5–10% displayed by R2R2\(^{2}\) or R2R2 (pooled data in Fig. 8D).

We next examined the ATP regulatory properties of ATPB constructs by conducting permeabilized single cell IICR assays. Fig. 8, E–J, shows that Ca\(^{2+}\)/H11001 release was not potentiated by 5 mM ATP in R2R2\(^{2}\)/H9004, exhibiting an IP3 sensitivity of 2.57 ± 0.72 μM. This EC\(_{50}\) was comparable with that observed in Fig. 5A. Interestingly, the ability of 5 mM ATP to potentiate Ca\(^{2+}\)/H11001 release at submaximal [IP3] was retained by R2R2\(^{2}\)/H9004. The presence of ATP visibly induced a shift in IP3 sensitivity, reducing the EC\(_{50}\) from 2.37 ± 0.97 μM (0 ATP) to 0.71 ± 0.16 μM (5 mM ATP). These values are also comparable with those seen in Fig. 5A. These observations clearly suggest that two functional ATPB motifs within an R2 homotetramer are sufficient to both facilitate ATP regulation and induce Ca\(^{2+}\)/H11001 oscillations. The inability of R2\(^{2}\)/R2\(^{2}\) to maintain the characteristic Ca\(^{2+}\)/H11001 oscillations may be explained by the reduced sensitivity to IP3 observed in Fig. 8D.

FIGURE 8. Stoichiometry of ATP regulation in R2R2. A, lysates from DT40-3KO cells stably expressing R2, R2\(^{2}\), R2R2, R2R2\(^{2}\), and R2\(^{2}\)/R2\(^{2}\) were resolved on 4% SDS-PAGE. Immunoblots were probed with α-R2NT. B and C, representative Fura-2 AM recordings of four individual DT40 3KO cells stably expressing R2\(^{2}\)/R2\(^{2}\) (B) and R2\(^{2}\) (C), when stimulated with 1 μg/ml α-IgM. D, quantification of oscillatory patterns of R2R2 (red), R2\(^{2}\)/R2\(^{2}\) (green), and R2R2\(^{2}\) (blue). E–J, Ca\(^{2+}\)/H11001 release through R2\(^{2}\)/R2\(^{2}\) (E–G) and R2\(^{2}\) (H–J) measured using single cell permeabilized IICR assays. E, [IP3]-response curves show 5 mM ATP does not potentiate Ca\(^{2+}\)/H11001 release in R2\(^{2}\)/R2\(^{2}\) at any [IP3]. H, 5 mM ATP potentiates Ca\(^{2+}\)/H11001 release in R2R2 at submaximal [IP3]. Each point is mean ± S.E. from at least three experiments.
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We also sought to examine the contribution of the R2 ATPB motif to ATP regulation in cells expressing heterodimers. R2 dictates the mode of ATP regulation in R3R2 heterodimers. Characteristically, ATP potentiates Ca²⁺ release only at submaximal [IP₃] (Fig. 5, G–I). Although the sites critical for regulation in R3 are unknown, a ΔATPB mutation in the R2 subunit of the heterodimer would still be predicted to leave the ATP regulatory sites in R3 intact and capable of binding ATP. Accordingly, we hypothesized that incorporation of an R2 ΔATPB mutation into R3R2 (R3R2^Δ) would disable R2’s ability to dictate ATP regulation and induce a shift to an R3 mode of regulation. Remarkably, however, permeabilized IICR assays (Fig. 9, A and B) demonstrated that R3R2^Δ is not regulated by ATP. For example, 5 mM ATP fails to augment Ca²⁺ release at any [IP₃]. Similar observations were made with R1R2^Δ (Fig. 9, C and D). This lack of sensitivity to ATP suggests that the two R2^Δ subunits within the heterotetramer were still likely determining the mode of ATP regulation, despite the R3/R1 subunits being unmodified and fully functional. It also suggests that the higher “R2-like” sensitivity to ATP exhibited by R3R2 (Fig. 6C) is unlikely to be the only cause of the dominant R2 activity with regard to regulation of Ca²⁺ release. One possible explanation is that the presence of two R2 subunits within the heterotetramer is sufficient to prevent ATP binding to R3 or R1, thereby abrogating modulation by ATP.

Two R2 Subunits Are Necessary to Dictate ATP Regulation of Heterotetramers—Two R2 subunits within a heterotetramer are sufficient for R2 to dictate both the ATP regulatory properties and the pattern of α-IgM-stimulated intracellular Ca²⁺ signals. We next asked the question whether one R2 subunit was sufficient to dictate the function of heterotetramers. A concatenated tetramer containing three R1 subunits and one R2 subunit was successfully generated (R1R1R1R2) and stably expressed in DT40-3KO cells (Fig. 10A). Immunoblots probed with α-R1 show that monomeric R1 migrate at ~250 kDa followed by the R1R1 dimer, R1R1R1 trimer, and R1R1R1R2 heterotetramer at increasing molecular masses. The R1R1R1R2 concatamer was also functionally responsive to stimulation with submaximal [α-IgM] (Fig. 10B), producing largely single Ca²⁺ spikes that are qualitatively similar to the patterns exhibited by R1R1. Furthermore, quantitative analysis of the Ca²⁺ signals revealed that over ~50% of responding cells produced 1–2 transients, generating a histogram profile similar to that of the R1R1 (~70% producing 1–2 transients) and distinct from the R2R2 (~5%) (Fig. 10C). These data, together with the previous report showing that R1R2 heterodimers produce long lasting Ca²⁺ oscillations characteristic of R2 (39), suggest that one R2 subunit within a heterotetramer is not sufficient to dictate the patterns of Ca²⁺ signals generated.

We next carried out an examination of the tetramer’s regulation by ATP. We have previously reported that R1R2 heterodimers, forming tetramers containing two R1 and two R2 subunits, exhibited regulatory properties and single channel characteristics similar to R2R2 dimers (39). Specifically, on-nucleus patch clamp electrophysiology showed that the presence of 5 mM ATP in the patch pipette only enhanced channel activity at low (1 μM) [IP₃] and not at maximal (10 μM) [IP₃]. This was also confirmed using permeabilized single cell IICR assays (histogram, Fig. 10D). Furthermore, although ATP regulates the Pₒ of both subtypes by increasing their total activity, it does so in biophysically distinct manners (49). At the single channel level, IP₃Rs enter a “bursting” state in the presence of IP₃ regardless of subtype, with each burst characterized by relatively constant open and closed times. Accordingly, any rise in Pₒ is the result of an increase in the duration spent bursting. Uniquely, the presence of saturating [ATP] augmented the Pₒ of R1 and R1R1 homodimers by increasing the duration of bursts. In contrast, the Pₒ of R2 and R2R2 homodimers was augmented by an increase in the number of bursts over a given period. An examination of R1R2 single channel activity revealed that ATP enhanced channel Pₒ by increasing the number of bursts, indicating that R2 also dictated the single channel kinetics of heterotetramers containing equal numbers of R1 and R2 subunits (49). Strikingly, however, single channel analysis of R1R1R1R2 activity revealed that 5 mM ATP augmented the Pₒ at both submaximal (1 μM) and maximal (10 μM) [IP₃] (Fig. 10, E and F), following a distinctly R1 mode of ATP regulation. Furthermore, it did so in a biophysical manner characteristic of R1: by increasing duration spent bursting rather than the number of bursts over a given period, a characteristic typical of R1 (49). Permeabilized single cell IICR assays confirm these data (Fig. 10, H–J), showing that ATP potentiates Ca²⁺ release at both submaximal and maximal [IP₃], similar to the pattern displayed by R1R1 homodimers (Fig. 10G). These data are consistent with the requirement of at least two R2 subunits being necessary to dictate the mode of regulation of Ca²⁺ release by ATP.
Conclusion—Heterotetrameric IP₃Rs likely play major roles in the generation of diverse intracellular Ca²⁺ concentration signals in cells expressing multiple IP₃R subtypes, which is a situation that is the norm in the majority of cells. However, elucidating the specific regulatory properties of heterotetrameric IP₃R has been challenging to date, largely because of the difficulty in defining the complement of constituent isoforms expressed in a given situation. This problem is compounded by the difficulty in predicting how subtype-specific regulation of individual monomers, within the tetramer, might contribute to the channel's overall activity. To circumvent this issue, we utilized our previously described concatemer approach to produce pre-assembled IP₃Rs with defined subunit composition, and we investigated fundamental characteristics of Ca²⁺ release through heterotetrameric IP₃Rs (39). This experimental paradigm has provided unique and non-intuitive insight into how important subtype-specific regulatory events contribute to channel activity, together with the stoichiometry of input required. Specifically, we show that IP₃ binding to individual ligand-binding domain monomers, although not markedly cooperative, is nevertheless influenced by binding to neighboring subunits to establish the overall binding affinity (Fig. 11A). This unique IP₃ binding affinity likely plays a role in dictating the intermediate IP₃ sensitivity exhibited by heterotetramers containing equal proportions of R2 and either R1 or R3 (Fig. 11B). The recent IP₃R cryo-EM structure, which shows the N terminus of one subunit interacting with multiple contact points in the C terminus of adjacent subunits, supports this notion of inter-subunit cross-talk within a heterotetramer. Furthermore, it raises the tantalizing prospect that cells expressing different IP₃R heterotetramer populations may exhibit distinct IP₃ binding affinities and sensitivities that suit the physiological needs of the cell.

Our data have also shown that, in terms of regulation of Ca²⁺ release by ATP, the presence of two R2 subunits within a tetramer are necessary and sufficient for R2 to dictate the regulatory characteristics and ATP sensitivity of the heterote-
Unique Properties of Heterotetrameric IP$_3$R

![Diagram](image)

**FIGURE 11.** Novel insights into the function and regulation of IP$_3$R heterotetramers. Individual subtypes are represented as distinct colors as follows: R1, red; R2, blue; and R3, green. A, R3R2 heterodimers, which generate heterotetramers containing an equal proportion of R2 and R3, exhibit unique IP$_3$ binding properties (affinity, cooperativity) that are distinct to that of the two constituent subtypes, B, at optimal conditions for Ca$^{2+}$ release (200 nm Ca$^{2+}$, 5 mm ATP), heterodimers containing R2 and either R1 or R3 exhibit an IP$_3$ sensitivity intermediate to that of the two constituent subtypes that is potentially reflective of the heterodimers’ unique IP$_3$ binding affinity. C, when ATP levels are reduced to submaximal concentrations, two R2 subunits are sufficient to dictate the regulatory properties of heterotetramers containing equal proportions of R2 and either R1 or R3. D, one R2 monomer is not sufficient to maintain R2 regulatory dominance in a heterotetramer containing three R1 subunits.

In all, these distinct properties likely add further diversity to the spatial and temporal properties of Ca$^{2+}$ signals that can be evoked through an individual heterotetrameric IP$_3$R. Furthermore, we have extended these studies to begin to understand the stoichiometry of modulatory input of IP$_3$R by mutagenesis of defined numbers of regulatory motifs within the tetramer, specifically demonstrating that two ATPB sites within R2 homotetramers are required for ATP to potentiate Ca$^{2+}$ release. We envision that this powerful experimental platform can be utilized to define the stoichiometry of other regulatory inputs, as well as illuminate the consequences of incorporating subunits encoding splice variants or mutations associated with human diseases within tetrameric IP$_3$Rs on Ca$^{2+}$ release.

**Author Contributions**—R. C. designed and stably expressed constructs, collected and analyzed the data, drafted the manuscript, and prepared the figures. K. J. A. was responsible for designing and establishing the concatermy strategy. L. E. W. collected and analyzed data obtained through single channel electrophysiology. D. I. Y. was responsible for the conception and design of concatenated strategy, as well as data analysis, generation of figures, and editing of the manuscript. All authors approved the final version.

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