Modulation of Ca^{2+} entry by polypeptides of the inositol 1,4,5-trisphosphate receptor (IP3R) that bind transient receptor potential (TRP): Evidence for roles of TRP and IP3R in store depletion-activated Ca^{2+} entry

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Homologues of *Drosophilia* transient receptor potential (TRP) have been proposed to be unitary subunits of plasma membrane ion channels that are activated as a consequence of active or passive depletion of Ca^{2+} stores. In agreement with this hypothesis, cells expressing TRPs display novel Ca^{2+}-permeable cation channels that can be activated by the inositol 1,4,5-trisphosphate receptor (IP3R) protein. Expression of TRPs alters cells in many ways, including up-regulation of IP3Rs not coded for by TRP genes, and proof that TRP forms channels of these and other cells is still missing. Here, we document physical interaction of TRP and IP3R by coimmunoprecipitation and glutathione *S*-transferase-pulldown experiments and identify two regions of IP3R, F2q and F2g, that interact with one region of TRP, C7. These interacting regions were expressed in cells with an unmodified complement of TRPs and IP3Rs to study their effect on agonist- as well as store depletion-induced Ca^{2+} entry and to test for a role of their respective binding partners in Ca^{2+} entry. C7 and an F2q-containing fragment of IP3R decreased both forms of Ca^{2+} entry. In contrast, F2g enhanced the two forms of Ca^{2+} entry. We conclude that store depletion-activated Ca^{2+} entry occurs through channels that have TRPs as one of their normal structural components, and that these channels are directly activated by IP3Rs. IP3Rs, therefore, have the dual role of releasing Ca^{2+} from stores and activating Ca^{2+} influx in response to either increasing IP3 or decreasing luminal Ca^{2+}.

Stimulation of cells that elevates inositol 1,4,5-trisphosphate (IP3) causes the release of Ca^{2+} from internal stores and its entry from the external milieu (for reviews see refs. 1–3). The release from internal stores occurs through channels formed by IP3 receptors (IP3Rs), and entry is mediated by a set of functionally heterogeneous but ubiquitous channels that are activated by the store depletion event *per se*. Transient receptor potential (TRP) proteins have been hypothesized to be structural components of Ca^{2+} entry channels (4, 5) and to be activated by IP3R in response to IP3 or store depletion. However, neither has the presence of TRP in Ca^{2+} entry channels been proven nor has the mechanism(s) by which the channels are activated been clearly elucidated. Indeed, the mechanism by which Ca^{2+} entry channels are activated has received considerable attention, and arguments have been set forth (*i*) for activation by second messengers or mediators that include cGMP, IP3, diacylglycerol, a G protein, arachidonic acid derivatives, and a complex termed CIF (6–14), (*ii*) for translocation from internal pools with involvement of an exocytotic event (15, 16), and (*iii*) for short-range physical coupling between the membrane delimiting the store and the plasma membrane (17, 18). The short-range physical-coupling model proposed that membrane Ca^{2+} entry channels may be activated by the same protein that is responsible for store depletion, i.e., the IP3R (for details see ref. 1).

The first functional evidence for a direct role of IP3R in Ca^{2+} entry was obtained by Kiselyov *et al.* (19), who showed that Ca^{2+} entry channels found in HEK cells expressing transfected TRP3 in stable form can be activated in inside-out membrane patches by addition of either IP3R-rich cerebellar microsomes or liposomes carrying recombinant IP3R protein truncated at its C terminus to inactivate its channel-forming capacity. However, this study did not determine whether the protein with which IP3R interacted was TRP3. Indeed, TRP was shown to cause changes in protein expression other than TRP, e.g., up-regulation of IP3Rs, and attempts to coimmunoprecipitate IP3R and TRP3 failed (19). We now show that IP3R and TRP can be coimmunoprecipitated. We thus sought to identify interacting domains using *in vitro* protein:protein interaction tests and, if we found them, to test for their function. Such domains were identified and, upon expression in cells whose TRP and IP3R complement had not been manipulated, were found to modulate natural Ca^{2+} entry stimulated by either a G protein-coupled pathway or store depletion. The data support a model in which store depletion-activated Ca^{2+} entry is mediated by TRP-based channels that are activated by the IP3R.

Materials and Methods

Molecular Biology. Standard molecular biology techniques were used throughout (20). IP3R and TRP cDNAs have been described (21–23) and are identified in Fig. 3. When expressed in cells, TRP3 and TRP6 were epitope-tagged at their C termini with the hemagglutinin (HA) or the c-*myc* epitope to give $TRP3^{HA}$, $TRP6^{HA}$, or $TRP3^{myc}$. C-terminal extension of TRPs with epitopes does not interfere with their ability to enhance Ca^{2+} entry into cells (24, 25). For expression in cells, cDNAs were cloned into pcDNA3 (Stratagene) or pEBFP (enhanced blue fluorescent protein, CLONTECH) downstream of the cytomegalovirus promoter. For protein:protein interaction stud-

Abbreviations: AVP, arginine vasopressin; GST, glutathione *S*-transferase; IP, immunoprecipitate; IP3, inositol 1,4,5-trisphosphate; IP3R, IP3 receptor; TG, thapsigargin; TRP, transient receptor potential, V2R, type-2 vasopressin receptor; HA, hemagglutinin; EBFP, enhanced blue fluorescent protein.

See commentary on page 14669.

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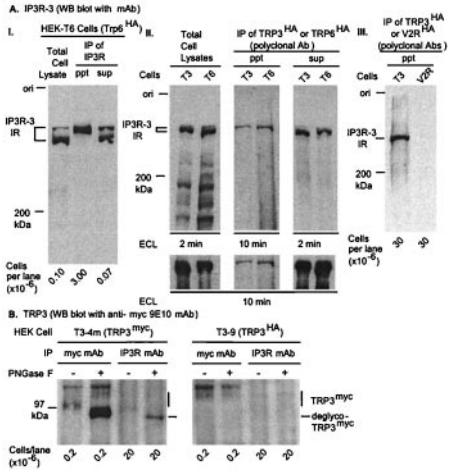

Fig. 1. Reciprocal coimmunoprecipitation of IP3R and TRP. T3–9 or T6–12 cells, stably expressing HA epitope-tagged TRP3 or TRP6, were lysed, and immunoreactive IP3R was analyzed by Western blotting. (*A*) IPs of TRP3 and TRP6 contain type-3 IP3R. (*I*) IP3R-3 in cell lysates, immunoprecipitates (IP, ppt) and IP supernatant (IP, sup). (*II*) IP3R-3 in detergent extracts and in the respective IP ppt and IP sup. Lower panels of *A.II* provide an idea of the relative proportion of total IP3R coimmunoprecipitated with TRP3 or TRP6. (*III*) Lack of significant nonspecific coimmunoprecipitation of IP3R3 immunoreactive material in an IP of V2R, an unrelated integral membrane protein expressed stably in HEK cells (32). Western blot with anti-human IP3R-3 mAb. (*B*) IPs of IP3R from T3 cell extracts contain TRP3. Detergent extracts of HEK-T3 cells stably expressing TRP6^{myc} or TRP3^{HA} were treated or not with PNGase-F and incubated overnight either with anti-type-1/2/3 IP3R mAb or with 9E10 anti-*myc* mAb, both preadsorbed to protein-A Sepharose. IPs were washed, dissolved, and electrophoresed as described in *Materials and Methods*. TRP3^{myc} in IP3R and TRP3 IPs was visualized with 9E10 mAb and detected by enhanced chemiluminescence (ECL).

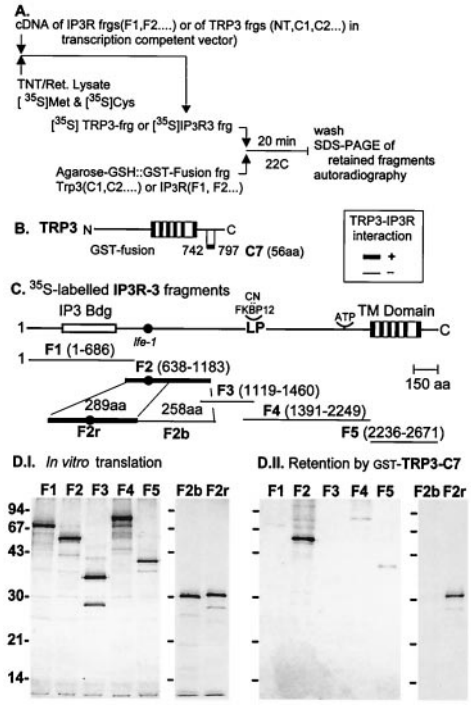

Fig. 2. Binding of IP3 receptor fragments to human TRP3. *In vitro*-translated IP3R3 F1–F5 fragments, labeled with [^{35}S]Met and [^{35}S]Cys, were incubated with a TRP segment fused to GST and adsorbed to glutathione-Sepharose. After 30 min at room temperature, Sepharose beads were washed, and the adsorbed complexes were resolved by SDS/PAGE, stained, and autoradiographed. In other experiments, the inverse strategy was used: the *in vitro*-translated fragment was from hTRP3, and the protein fused to GST was from the human IP3R3. (*A*) Design of the GST-pulldown experiments. (*B*) Linear diagram of the IP3R3 and TRP3, represented with the same scale and location of some of the molecular landmarks of IP3R3. (*C*) Representative results showing binding of F2, but not the other IP3R fragments, to TRP-C7.

ies, the indicated sequences were cloned into pGEX-4T-1 (Amersham Pharmacia) to produce C-terminal glutathione *S*-transferase (GST) fusion proteins in bacteria or into pAGA2 (26) for *in vitro* translation. Mutant GST-TRP3-C7^{mut3} was made with the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with wild-type pGST-TRP3-C7 as donor DNA. The nucleotide composition of the final constructs was confirmed by sequencing of double-stranded DNA in both directions (27).

Antibodies. Type-3 IP3R (IP3R3) and type-1, -2 and -3 (IP3R-123) mAbs were from Transduction Laboratories (Lexington, KY) and Chemicom, respectively. Peptide-directed polyclonal rabbit Abs nos. 1092, 1110, and 2 were custom-made by Quality Control Biochemicals (Hopkinton, MA). No. 1092 is anti-TRP3/TRP6 (epitope: NIEKEFKNDYRKLSC), no. 1110 is anti-TRP6 (epitope: CYPSFRGNENRLTHRRQ), and no. 2 is anti-type 2 vasopressin receptor (V2R) (epitope: VPGPSERPGGRRRGR). 12CA5 mAb (anti-HA) or 9E10 mAb (anti-*myc*) was obtained in ascites fluids and used without further treatment.

Immunoprecipitation and Western Blots. *Lysates and detergent extracts.* Whole-cell lysates (Fig. 1): Cells were harvested by trypsinization, resuspended in Dulbecco's PBS with 1 mM EDTA and 100 μM PMSF (PBSEP), pelleted, and lysed by addition of 1 vol of 2× Laemmli's sample buffer (28) at a ratio of 150 μl per 10^6 cells. The mixtures were sonicated in the cold, cleared by centrifugation, and subjected to SDS/PAGE for further analysis. Lysates for immunoprecipitation: Cells were rinsed, scraped into 2 ml of PBSEP per 10-cm dish, pelleted, and detergent-extracted by addition of ice-cold detergent-extraction buffer [5–30 μl per 10^6 cells; detergent-extraction buffer: 1% (vol/vol) Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 5 mM EDTA, 1 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 100 μM PMSF, and 50 mM Tris·HCl (pH 8.0)], followed 10 passages each through 20- and 25-gauge needles. Cell extracts were cleared by centrifugation.

Deglycosylation. When indicated, prior to immunoprecipitation, detergent extracts were incubated for 3 hr at 4°C with 2.5–15 units/ml of PNGase F (Roche Molecular Biochemicals).

Immunoprecipitation. TRPs and IP3R3 or IP3R-1, -2, and -3 were immunoprecipitated from detergent extracts by incubation for 3–15 hr at 4°C with the appropriate Ab, followed by addition of protein A-Sepharose in detergent-extraction buffer and incubation overnight at 4°C on a rocking platform. The Sepharose beads were collected by centrifugation. When needed, supernatants were mixed with 1 vol of 2.5× Laemmli's sample buffer for analysis. The protein A-Sepharose with immunoprecipitates (IPs) bound to them were washed, and the IPs were solubilized in 30–120 μl of 2× Laemmli buffer.

SDS/PAGE. Electrophoresis of IP3R was through standard SDS/PAGE gels (6 mA for 12 hr). Heavy–heavy Ig chain dimer and TRP3 were separated at 11 mA for 4.75 hr on 7.5% acrylamide 4–8 M urea gradient gels with a 5% acrylamide 4 M urea stacking gel. Standard 10% or 15% SDS/PAGE (20) was used to resolve GST-fusion proteins and the radioactive polypeptides bound to them.

Western blots. Proteins were transferred to nitrocellulose (0.2 μm, Trans-Blot; Bio-Rad) in buffer containing 150 mM glycine, 20 mM Tris-base, and 20% (vol/vol) methanol. Transfer of proteins for the *myc* Western blot was performed at 275 mA, 4°C,

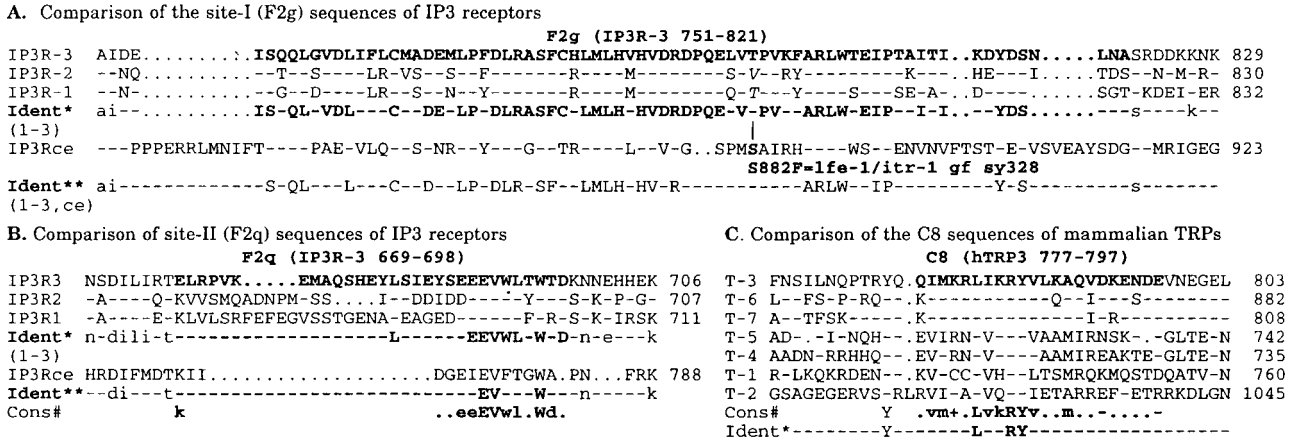

Fig. 3. F2g (*A*) and F2q (*B*) IP3R-3 sequences and the C8 TRP sequences (*C*) are in bold. (*A* and *B*) IP3R1, IP3R2, and ceIP3R sequences are compared to that of the IP3R3 sequence (*Top*). (*C*) TRP1–TRP7 sequences are compared to that of TRP3 (*Top*). For these comparisons: - denotes identity, otherwise the amino acid is given in single-letter code. Ident and Cons, identical and consensus amino acids. ''Ident,'' Identities in upper case. *, Includes the three human IP3Rs. **, Includes three human and one *Caenorhabditis elegans* IP3R. #, Consensus lists dominant amino acid or amino acid type: +, K or R; –, D or E; ·, no consensus. GenBank accession numbers: IP3R1, L38019; IP3R2, D26350; IP3R3, D26351; T-1 (hTRP1), U31110; T-2 (mTRP2), AF111108; T-3 (hTRP3), U47050; T-4 (mTRP4), AF011543; T-5 (mTRP5), AF060107; T-6 (mTRP6), U49069; T-7 (mTRP7), AF139923.

overnight; for the IP3R3 Western blot, 0.05% SDS was added to the buffer, and transfer was at 400 mA, 4°C, overnight. Blots were stained with Ponceau S to visualize marker proteins, destained with TBS [20 mM Tris·HCl, 137 mM NaCl (pH 7.6)], blocked with TBS plus 0.05% Tween 20 and 5% nonfat dry milk for either 3 hr at room temperature or overnight at 4°C. For detection of IP3R3, the IP3R3 mAb was diluted 1:1,000 in blocking buffer. For detection of the *myc* epitope, the 9E10 ascites fluid was diluted 1:250 in blocking buffer. Incubations with Abs were for either 3 hr at room temperature or overnight at 4°C under constant agitation. Blots were washed with blocking buffer containing 2% milk at room temperature. mAbs retained on the blots were visualized with NEN′s enhanced chemiluminescence Renaissance detection system.

Other. Cell cultures, transient transfection conditions, preparation of GST-fusion proteins in *E. coli*, *in vitro* translation of cDNA fragments, binding of GST-fusion proteins to *in vitro*-translated fragments, and measurement of agonist and thapsigargin (TG)-stimulated Ca^{2+} entries into fura 2-loaded cells by videomicroscopy have been described (21, 22, 29, 30). Efficiencies of transfection of EBFPs into HEK-293T cells were between 80% and 90%.

Results

Coimmunoprecipitation of IP3R and TRP. The activation of a cation-permeable channel in inside-out membrane patches of T3 cells by recombinant IP3R suggested that IP3R and TRP3 have interacting domains, provided that the effect was direct and that the channels in question indeed had TRP3 as a structural component, either forming the channel proper or attached to the channels as regulatory subunits. Formation of stable TRP and IP3R complexes was tested in coimmunoprecipitation and GST-pulldown experiments.

Fig. 1*A* shows that IP3R is present in TRP IPs from HEK cells stably expressing epitope-tagged TRPs. In *A*, we immunoprecipitated TRP3 or TRP6 with either an Ab to a peptide that is conserved in TRP3 and TRP6 (Ab no. 1092) or an Ab raised against a peptide unique to TRP6 (Ab no. 1110) and tested for presence of IP3R in the IPs by classical Western blotting with an anti-IP3R mAb. This mAb recognizes two bands in total cell lysates, of which it can immunoprecipitate only the less-abundant, upper band. This band was found in immunoprecipitated TRPs. The same IP3R-reactive band was precipitated from cells stably expressing TRP3 or TRP6, clones T3–9 and T6–12, respectively. Because the proportion of IP3R present in TRP3 or TRP6 IPs was very small, we tested whether IP3R would also be found in IPs of an unrelated membrane protein, the Gs-coupled V2R, using cells and a peptide-directed polyclonal Ab (no. 2) described in Innamorati *et al.* (31). Only a minor amount of immunoreactive IP3R was found in the V2R IP when compared with that found in IPs of TRP3 derived from an equal number of T3 cells. This indicated that the immunoreactive IP3R in the TRP IP was not the result of nonspecific trapping.(Fig. 1 *A.III*).

We also immunoprecipitated IP3R with a mAb that recognizes all three IP3Rs and tested for presence of TRP (tagged with the *myc* epitope) in the IP. Fig. 1*B* (*Left*) shows that the IP3R IP was positive for *myc*-tagged TRP3. Visualization of TRP3 in the IP required deglycosylation, and the amount of TRP that coimmunoprecipitated with IP3R was again very small. We tested whether the 9E10-reactive band might be nonspecific by preparing an IP3R IP with the same mAb from the same amount of detergent extract, but from cells expressing $TRP3^{HA}$ rather than $TRP3^{myc}$ (Fig. 1*B Right*). The absence of a 9E10-reactive band in the right-most lane of Fig. 1*B* indicates that the reactive band in the fourth lane of the left panel corresponds to the *myc* epitope and represents TRP^{myc} coimmunoprecipitated with the IP3Rs.

It should be noted that, while TRP3-expressing cells undoubtedly are expressing high levels of TRP compared with the natural complement of TRP, this amount does not appear to overwhelm the biochemical carbohydrate-maturing machinery of the cells. This is shown by the fact that essentially all of the *myc*-TRP3 in the cells is fully glycosylated, needing deglycosylation to be visible by Western blotting (Fig. 1*B Left*). Other experiments showed that deglyco-TRP could not be obtained by digestion with endoglycosidase H, indicating that IP3R only associates with mature TRP.

***In Vitro* Interactions of IP3R with TRP.** Using cDNA fragments derived from the human IP3R3 and the human TRP3, we set up GST-pulldown experiments following the strategy shown in Fig. 2*A* (29). Fig. 2*B* presents linear diagrams of IP3R3 and TRP3, identifying the location of notable landmarks (32). Fig. 2*C* shows that the region denoted as F2 has the ability to bind to a C-terminal fragment of TRP3. In similar GST-pulldown exper-

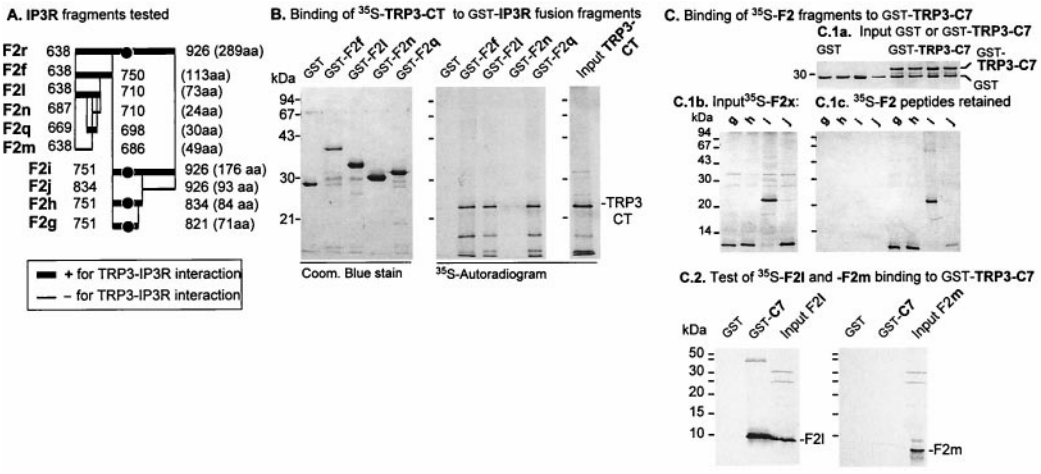

Fig. 4. Two regions of IP3R interact with the C-terminus of TRP3. Design and analysis of results is as in Fig. 2. The amino acid compositions of hIP3R3 and hTRP3 correspond to those found in GenBank accession numbers D26351 and U47050, respectively. Beginning and ending amino acid numbers are shown of the fragments translated *in vitro* or fused to GST.

iments, we studied the converse interaction of TRP-fragments with the IP3R3 F2 fragment. This led to the identification of TRP-C8 (hTRP3[777–797]; Fig. 3) as a 21-aa sequence of TRP3 able to associate with IP3R (data not shown). Using the whole C terminus of TRP3 as a probe, we then proceeded to narrow down the TRP3-interacting domain of IP3R. To our surprise, IP3R has not one but two TRP-interacting sequences, F2q (Fig. 4*B*) and F2g (Fig. 4*C*). Of these, a subfragment of F2g, F2t (IP3R3[768–805]), was also positive for interaction with the C terminus of TRP3 (data not shown).

Functional Relevance of *in Vitro*-Interacting Sequences. The IP3R-interacting TRP fragment C7 and TRP-interacting IP3R fragments were expressed transiently in HEK-293T cells, and their effect was tested on Ca^{2+} entry stimulated either by a pathway involving a Gq-coupled receptor, the arginine vasopressin (AVP)-responsive V1a receptor, or by TG-induced store depletion. For expression in HEK-293T cells, the fragments to be tested were fused to EBFP (see *Materials and Methods*). As shown in Fig. 5 for TRP-C7, presence of this IP3R-interacting sequence at the end of EBFP inhibited AVP-stimulated Ca^{2+} entry between 25% and 30%. Expression of a control fusion protein, formed of EBFP and TRP-C11, which we found did not interact with IP3R (data not shown), did not affect agonist-stimulated Ca^{2+} entry, nor did a mutated C7 that had lost its affinity for IP3R-F2 (Fig. 5 *A* and *C*). C7 not only interfered with full development of Ca^{2+} entry stimulated by the Gq-PLC-IP3 pathway (Fig. 5 *C1*), but also with Ca^{2+} entry activated by store depletion (Fig. 5 *C2*).

Both TRP-interacting regions of IP3R3, also expressed as EBFP-fusion proteins, altered Ca^{2+} entry activated by the Gq-PLC-IP3 pathway or by store depletion, but did so in opposing ways. The IP3R region containing the F2q fragment, F2l, had an inhibitory effect (Fig. 6*A*); F2g, on the other hand, enhanced Ca^{2+} entry by prolonging its active phase (Fig. 6*C*). In all cases, the differences between the Ca^{2+} accumulation curves in cells expressing control constructs and those expressing the interacting sequences fused to EBFP were statistically significant at probability levels that ranged from $P < 0.025$ for the effect of F2l on AVP-stimulated Ca^{2+} entry to better than $P < 0.01$ for all others.

Discussion

Although only a very small proportion of cellular TRP was found to associate with a small proportion of the cell's IP3R complement, this interaction does not appear to be nonspecific. We obtained similar results with two IP3R Abs and with two

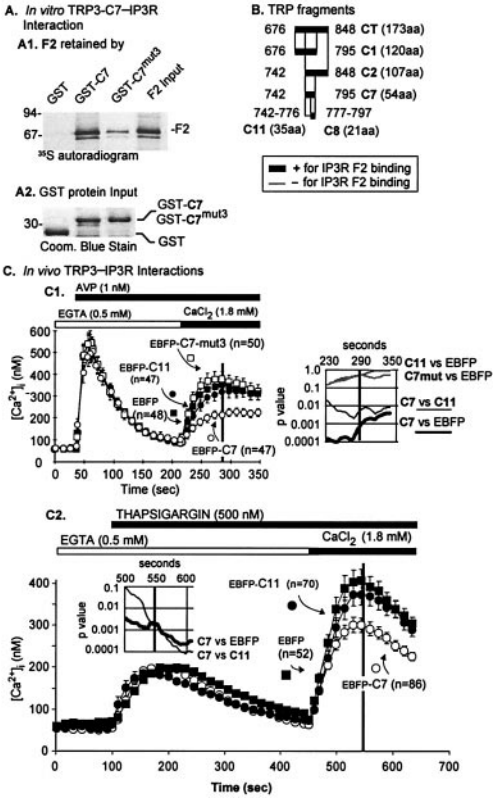

Fig. 5. Binding of wild-type and mutant TRP-C7 to IP3R-F2 and partial interference of agonist and TG-induced Ca^{2+} entry by TRP3-C7 but not by fragments that do not interact with IP3R. (*A*) Binding of TRP3 fragments to IP3R-F2. (*B*) Summary of IP3R-F2 binding to C-terminal fragments of hTRP3. (*C*) Effect of EBFP and EBFP-TRP fusions on Ca^{2+} entry stimulated by TG (*C2*) or by AVP in cells cotransfected with the Gq-coupled V1a receptor (*C1*). C7, hTRP3[742–795], $C7^{mut3}$, hTRP3[742–795] with K780D/R781E/K784D/R785E. The strategy and methods described in ref. 19 were used to determine Ca^{2+} transients. Points are means ± SEM of the number of cells shown analyzed in three to four independent transfections. *Insets*, Significance of differences between $[Ca^{2+}]_i$ curves; *P* value, Student's *t* test of the null hypothesis.

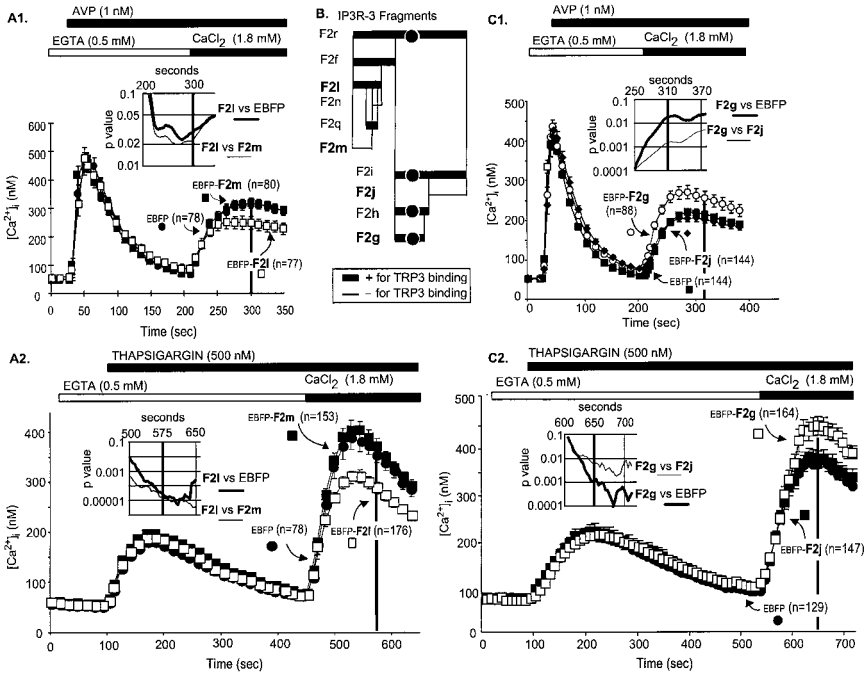

Fig. 6. Effects of expressing F2q-containing F2l (*A*) or F2g (*C*), fused to EBFP, in HEK-293T cells on Ca^{2+} entry activated either by the Gq-coupled V1a receptor or by TG-induced store depletion. Presentation of data and experimental designs and analyses were the same as in Fig. 5. Points are means ± SEM of the number of cells analyzed in three independent transfections for each of the experiments shown.

TRP-expressing cells lines immunoprecipitating TRP with either monoclonal anti-epitope or two polyclonal Abs. Further, IP3R did not associate with an unrelated membrane protein, V2R. The fact that only minor amounts of these molecules are found associated should not be surprising because not many channels need to be activated to account for the type of Ca^{2+} entry monitored in fura 2 or electrophysiological studies, and TRP3 is rather homogeneously distributed on the cell surface of T3 cells (33). Also, when analyzed by immunoelectronmicroscopy, IP3R is essentially absent from plasma membranes (34). Points of contact are therefore expected to be few, consistent with our observation. The fact that TRP and IP3R coimmunoprecipitate may signify a certain degree of precoupling, and, in turn, may be a reason for not obtaining larger effects of interacting peptides in Ca^{2+} entry studies.

The findings reported here bear on three aspects of regulated Ca^{2+} entry into cells by mechanisms that do not involve either voltage- or ligand-gated ion channels: (*i*) the participation of a TRP in Ca^{2+} entry, be it activated by stimuli that increase IP3 or by mere store depletion without overt phospholipase C stimulation, (*ii*) the participation of IP3R in store depletion-activated Ca^{2+} entry, and (*iii*) the mechanism by which Ca^{2+} entry channels are activated.

The identity, even partial, of the molecule(s) that make up store depletion-activated Ca^{2+}-entry channels has been a matter of speculation. Depending on the TRP subtype, the channels detected in TRP-expressing cells do (35–37) or do not (5, 19, 22) exhibit Ca^{2+} selectivity, but the degree of Ca^{2+} selectivity exhibited by macrophage and lymphocyte store depletion-activated Ca^{2+} entry (I_{CRAC}) has not been recapitulated with TRPs. This raised doubts about their role in store depletion-activated Ca^{2+} entry. TRP-binding peptides are potential TRP probes and pharmacological tools with which to test for involvement of a TRP in Ca^{2+} entry in cells whose TRP and IP3R compositions have not been manipulated. Our findings (Fig. 6) are strong evidence for the involvement of a TRP in the normal response of a cell to stimuli that increase IP3 as well as in store depletion-activated Ca^{2+} entry. The two peptides that bind to TRP3 affected TG-activated Ca^{2+} entry—the classical mode of eliciting store depletion-stimulated Ca^{2+} entry without activation of phospholipase C or elevation of IP3. This suggests that Ca^{2+}-entry channels, activated either by agonists that stimulate PLC activity or by mere depletion of the internal Ca^{2+} store, are either made of a combination of TRPs or, at a minimum, have a TRP as one of their components.

In analogy to F2 fragments, TRP3-C7 is an IP3R probe and, as such, functionally identified this protein as participating not only in agonist-activated Ca^{2+} entry (which was known) but also in store depletion-activated Ca^{2+} entry (Fig. 5). IP3R must therefore act not only as a link between the Ca^{2+} store and plasma membrane but also as a monitor of the degree or state of filling of the store, i.e., a Ca^{2+} sensor. Modulation of the Ca^{2+} release channel activity of the IP3R by luminal Ca^{2+} is a well described, yet complex phenomenon (*cf.* ref. 38), and a Ca^{2+}-binding site has been described for the IP3R that is located in the lumen, close to the pore (39). It is tempting to speculate that this may be the Ca^{2+}-sensing domain of the IP3R, which, when Ca^{2+} dissociates from it, triggers exposure of a cytosolic signal-transfer domain that allows the IP3R to activate TRP-based Ca^{2+} entry channels. IP3R regulation of Ca^{2+} entry, independent of its function as a Ca^{2+} release channel, may be an explanation for finding that TG-induced Ca^{2+} entry is preserved in a chicken cell line in which the Ca^{2+} release function of all three IP3Rs had been inactivated but the luminal Ca^{2+}-binding site of IP3R-1 had been left unaltered (40).

One of the two TRP-interacting IP3R segments, F2g (and F2t), encompasses an activating point mutation in the orthologous IP3R of the nematode *Caenorhabditis elegans*. This mutation, *Ce*IP3R[S882F] (Fig. 3), is responsible for the gain of function *sy328* allele that suppresses the sterility of a hypomorphic LET-23/EGF receptor mutant (41), presumably by increasing Ca^{2+} availability to the egg-laying apparatus. We envision F2g to be part of the signal transfer region of the IP3R-to-TRP signaling pathway and that IP3 binding and/or luminal sensing

of Ca^{2+} by the IP3R would cause a conformational change that uncovers the signal transfer domain. This domain would then be free to activate nearby (precoupled?) plasma membrane TRP-based or TRP-containing ion channels and trigger Ca^{2+} entry. Not investigated so far is whether other portions of IP3R may also interact with Ca^{2+} entry channels. The kinetics of the F2g effect suggest that it prolongs, rather than mimics, Ca^{2+} entry. This raises the possibility that activation of entry channels involves a multipronged attachment of the IP3R to the entry channel. Physiologic activation of Ca^{2+}-entry channels by IP3R may involve cooperative interactions that have not been tested.

In conclusion, the results presented above indicate that TRP proteins are integral parts of agonist and store depletion-activated Ca^{2+} entry channels and that these channels are regulated directly by IP3Rs. The signal that activates the Ca^{2+} entry-stimulating activity of the IP3R is IP3 or a decrease of luminal Ca^{2+} or both. Our studies do not rule out other mechanisms that enhance Ca^{2+} entry or the existence of Ca^{2+} entry channels not made up of TRPs, be they affected by IP3R or not. Notably Yao *et al.* (16) showed that a dominant-negative mutant of SNAP25, a component of vesicle fusion machineries, as well as maneuvers that inhibit exocytosis, prevent development of store depletion-induced Ca^{2+} entry in *Xenopus* oocytes. Kanzaki *et al.* (15) recently found that activation of a distant relative of TRPs, the growth factor receptor-activated channel, involves its translocation to the plasma membrane by an exocytotic event. It remains for future studies to test whether Ca^{2+}-entry channels are of two main categories: those formed of TRPs and activated by IP3R and those formed by TRP-related proteins that have the ability to shuttle between the plasma membrane and an internal compartment.

While this manuscript was in preparation, a report appeared showing IP3-dependent activation of T3 cell channels by a soluble 787-aa IP3-binding fragment of IP3R1 (BD, ref. 42). The C terminus of IP3R1 BD corresponds to amino acid 782 of the IP3R3 used here. This suggests that the signal-transfer domain of IP3R3, exposed/activated in response to either cytosolic IP3 or luminal Ca^{2+} depletion, resides within IP3R3[751–782], some 200 amino acids downstream of the IP3-binding domain located approximately between amino acids 225 and 575 (32).

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