Agonist-induced Ca\textsuperscript{2+} entry determined by inositol 1,4,5-trisphosphate recognition

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 It has been considered that Ca\textsuperscript{2+} release is the causal trigger for Ca\textsuperscript{2+} entry after receptor activation. In DT40 B cells devoid of inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}R), the lack of Ca\textsuperscript{2+} entry in response to receptor activation is attributed to the absence of Ca\textsuperscript{2+} release. We reveal in this article that IP\textsubscript{3}R recognition of IP\textsubscript{3} determines agonist-induced Ca\textsuperscript{2+} entry (ACE), independent of its Ca\textsuperscript{2+} release activity. In DT40 IP\textsubscript{3}R\textsuperscript{-/-} cells, endogenous ACE can be rescued with type 1 IP\textsubscript{3}R mutants (both a ΔC-terminal truncation mutant and a D2550A pore mutant), which are defective in Ca\textsuperscript{2+} release channel activity. Thus, in response to B cell receptor activation, ACE is restored in an IP\textsubscript{3}R-dependent manner without Ca\textsuperscript{2+} store release. Conversely, ACE cannot be rescued with mutant IP\textsubscript{3}Rs lacking IP\textsubscript{3} binding (both the Δ90–110 and R265Q IP\textsubscript{3}R-binding site mutants). We conclude that an IP\textsubscript{3}-dependent conformational change in the IP\textsubscript{3}R, not endoplasmic reticulum Ca\textsuperscript{2+} pool release, triggers ACE.

 Ca\textsuperscript{2+} transients elicited in response to cell surface receptor activation by neurotransmitters, hormones, and other molecular messengers are major messengers of intracellular communication (1). Stimulation of G protein-coupled receptors, tyrosine kinase receptors, and nonreceptor tyrosine kinases activate phospholipase C (PLC), catalyzing the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) into the second-messenger molecules: inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG). IP\textsubscript{3} mediates rapid Ca\textsuperscript{2+} store release by activating IP\textsubscript{3} receptors (IP\textsubscript{3}Rs) in the endoplasmic reticulum (ER), whereas DAG activates protein kinase C (PKC) (2). After this initial Ca\textsuperscript{2+} release phase, external Ca\textsuperscript{2+} enters through plasma membrane channels, providing a secondary and more prolonged Ca\textsuperscript{2+} signal (1), a phenomenon designated here as agonist-induced Ca\textsuperscript{2+} entry (ACE) (3).

 To date, the molecular identity of these Ca\textsuperscript{2+} entry channels as well as their coupling mechanism remain unknown, although several mechanisms have been proposed. Intracellular Ca\textsuperscript{2+} release through the IP\textsubscript{3}R could trigger ACE by means of capacitative Ca\textsuperscript{2+} entry (CCE) (4), which can be activated in a PLC-independent manner (3) by the ER Ca\textsuperscript{2+} pump blocker thapsigargin or the Ca\textsuperscript{2+} ionophore ionomycin (4, 5). In this scheme, the luminal drop in ER Ca\textsuperscript{2+} activates “store-operated” Ca\textsuperscript{2+} channels in the plasma membrane, although the basis for this coupling mechanism is entirely unknown. Although the release activity of IP\textsubscript{3}Rs may mediate Ca\textsuperscript{2+} entry, others have suggested that IP\textsubscript{3}Rs play a conformational role in the coupling process (6, 7). Also, DAG may directly initiate ACE, because DAG can activate overexpressed “canonical” transient receptor potential Ca\textsuperscript{2+} entry channels (TRPC) (8).

 We recently demonstrated a functional distinction between ACE and CCE based on the requirement of the former for PLC-γ, in a lipase-independent manner (3). However, it is unclear whether endogenous ACE is an integrated process accounting for both receptor and store-operated channel activation. At the molecular level, it is unclear whether IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release triggers ACE and/or whether a conformational alteration of the IP\textsubscript{3}R is sufficient for ACE. Evidence for the activation by Ca\textsuperscript{2+} pool emptying alone (and the dispensability of the IP\textsubscript{3}R for Ca\textsuperscript{2+} entry) includes the ability of thapsigargin or ionomycin, but not PLC activation, to stimulate Ca\textsuperscript{2+} entry in the DT40 triple IP\textsubscript{3}R knockout cell line, a form of B lymphocytes devoid of any IP\textsubscript{3}R (9). These interpretations assume that ACE and CCE are functionally overlapping mechanisms. In the present study, we demonstrate that IP\textsubscript{3}R recognition by the IP\textsubscript{3}R but not the receptor’s Ca\textsuperscript{2+} channel activity is required for activation of endogenous ACE.

 Materials and Methods

 Culture of Cells. Rat PC12 cells (passage numbers 6–15), human embryonic kidney (HEK)293 cells, rat aortic smooth muscle A7r5 cells (passage numbers 10–25), and DT40 chicken B lymphocyte IP\textsubscript{3}R\textsuperscript{-/-} cells were cultured as described (3, 9, 10).

 Expression Protocols. DT40, HEK293, PC12, and A7r5 were transfected as described (3). For experiments shown in Figs. 3 and 4, 5 μg of yellow fluorescent protein (YFP) cDNA ± 20 μg of IP\textsubscript{3}R cDNA was used for transfection.

 Ca\textsuperscript{2+} Imaging. Ca\textsuperscript{2+} measurements were as described (11). Fura-2/acetoxyethyl ester loading was for 25 min at 20°C for DT40 cells, 1 h at 20°C for PC12 cells, 25 min at 20°C for HEK293 cells, and 30 min at 20°C for A7r5 cells. Transfected enhanced YFP served as the transfection marker and was detected at excitation wavelength 485 nm. Resting Ca\textsuperscript{2+} levels in cell lines were similar, 100–200 nM, and cells with higher basal levels were excluded from data collection because these cells tend to have constitutive Ca\textsuperscript{2+} entry. All measurements shown are representative of a minimum of three and, in most cases, a larger number of independent experiments. For the population studies in Fig. 1, YFP-transfected cells were used to control for experiments in HEK293 cells transfected with YFP plus the sarcoplasmic ER Ca\textsuperscript{2+} ATPase (SERCA)-2b. Functional rescue of Ca\textsuperscript{2+} responses in the DT40 IP\textsubscript{3}R\textsuperscript{-/-} cells was totaled from five separate experiments with six conditions each: (i) YFP alone, 0 out of 241 cells (0% rescue, 0.0 SEM); (ii) WT IP\textsubscript{3}R, 49 out of 261 cells (18.7% rescue, 0.36 SEM); (iii) ΔC IP\textsubscript{3}R, 58 out of 230 cells (25% rescue, 1.62 SEM); (iv) Δ90–110 IP\textsubscript{3}R, 1 out of 239 cells (0.4% rescue, 0.19 SEM); (v) D2550A IP\textsubscript{3}R, 67 out of 258 cells (25.9% rescue, 0.36 SEM).

 Abbreviations: IP\textsubscript{3}, inositol 1,4,5-trisphosphate; IP\textsubscript{3}R, IP\textsubscript{3} receptor; ACE, agonist-induced Ca\textsuperscript{2+} entry; PLC, phospholipase C; DAG, diacylglycerol; ER, endoplasmic reticulum; CCE, capacitative Ca\textsuperscript{2+} entry; TRPC, transient receptor potential Ca\textsuperscript{2+} entry channels; YFP, yellow fluorescent protein; SERCA, sarcoplasmic ER Ca\textsuperscript{2+} ATPase; R, Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry; N, Ca\textsuperscript{2+} entry; N, little to no Ca\textsuperscript{2+} entry; N, little to no Ca\textsuperscript{2+} entry yet substantial Ca\textsuperscript{2+} entry; LHC, carbachol; HEK, human embryonic kidney.

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studies are almost exclusively based on average responses within large numbers of cells, despite the risks of interpreting Ca^{2+} signals from populations (14). In the present study, we have compared Ca^{2+} release and Ca^{2+} entry in individual cells. We measured single-cell receptor-mediated Ca^{2+} responses to bradykinin receptors in PC12 cells (neuronal origin), muscarinic receptors in HEK293 cells (kidney origin), and vasopressin receptors in A7r5 cells (vascular smooth muscle-derived). Ca^{2+} release was induced by the addition of agonist in nominally Ca^{2+}-free media (Fig. 1, arrows). Subsequent addition of agonist with 1 mM extracellular Ca^{2+} induces an increase in cytosolic Ca^{2+}, resulting from ACE (Fig. 1, bars). In averaged responses from multiple (30–50) cells, Ca^{2+} release coincides with ACE in each of the three cell types examined (Fig. 1, leftmost traces). However, analysis of the single-cell responses comprising these averages reveals three subpopulations of Ca^{2+} signals. Overall, among the three cell types, 62–72% of individual cells display both Ca^{2+} release and Ca^{2+} entry (RE), 17–20% of cells exhibit Ca^{2+} release with little to no Ca^{2+} entry (RNE), and 10–17% of cells manifest little to no Ca^{2+} release yet substantial Ca^{2+} entry (NRE) (Fig. 1 and Table 1). In all three cell types, constitutive Ca^{2+} entry is a rare event (<1% of cells), and a similarly small population of cells do not show any Ca^{2+} response (these cells were excluded from the population analyzed).

We characterized the relationship between Ca^{2+} release and entry in HEK293 cells by performing scatter analysis of the two parameters in response to the muscarinic receptor agonist CCH (100 μM) (Fig. 2). There is no correlation between extent of peak Ca^{2+} pool release and peak Ca^{2+} entry (n = 40; R = –0.05258). Thus, ACE in cells is not graded with respect to the magnitude of IP_{3}-mediated Ca^{2+} release. This result agrees with the nonlinear activation of the store-operated Ca^{2+} current (I_{cra}) by IP_{3} (14).

Because our imaging assay reflects only whole-cell Ca^{2+} transients, we cannot exclude the possibility of subthreshold/local IP_{3}-mediated Ca^{2+} release within the subpopulations of cells exhibiting NRE. Undetectable release from possibly subthreshold/agonist-specific coupling pools could trigger ACE and subsequent Ca^{2+}-induced Ca^{2+} release through Ca^{2+}-release channels. Under this paradigm, NRE (Fig. 1, rightmost traces) might be a mixture of Ca^{2+} release and Ca^{2+} entry. To overcome the difficulty in

**Immunohistochemistry.** Immunohistochemistry was as described in ref. 3.

**IP_{3}R Mutagenesis.** ΔR90–110 IP_{3}R was constructed with two rounds of PCR by using overlapping 40-mers in which the bases 270–330 were omitted.

**Antibodies and Reagents.** Plasmids were obtained from the following sources: enhanced YFP vector cDNA was from Clontech; IP_{3}R WT type 1, AC terminus, D2550A, and R265Q vectors were from Suresh Joseph (Thomas Jefferson University, Philadelphia, PA); carbachol (CCH), bradykinin, and vasopressin were from Sigma; Fura-2/acetoxymethyl ester and goat-anti rabbit Alexa 568 were from Molecular Probes; anti-chicken IgM (supernatant, M-4 clone) was from Southern Biotechnology Associates; and polyclonal anti-IP_{3}R was from Affinity BioReagents (Golden, CO).

**Results**

Many studies, including our own, have shown that Ca^{2+} entry is coincident with IP_{3}R-mediated Ca^{2+} release (3, 10, 12, 13). Such

**Table 1. Frequency of various Ca^{2+} entry responses to agonist stimulation**

<table>
<thead>
<tr>
<th>Cell/agonist</th>
<th>n</th>
<th>RE, %</th>
<th>RNE, %</th>
<th>NRE, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12/1 μM bradykinin</td>
<td>427</td>
<td>72.60</td>
<td>17.56</td>
<td>9.84</td>
</tr>
<tr>
<td>A7r5/10 nM vasopressin</td>
<td>208</td>
<td>62.98</td>
<td>20.19</td>
<td>16.83</td>
</tr>
<tr>
<td>HEK293/100 μM CCH</td>
<td>411</td>
<td>72.70</td>
<td>17.03</td>
<td>10.21</td>
</tr>
</tbody>
</table>

Ca^{2+} transients were collected for YFP-transfected PC12, A7r5, and HEK293 cells over five standardized experiments. These responses were categorized into their respective groups by a semiquantitative approach in which Ca^{2+}-release events <20 nM were considered little to no release and Ca^{2+} entry events <20 nM were considered little to no entry.

**Fig. 1.** Ca^{2+} entry in the absence of IP_{3}R-mediated Ca^{2+} release. Free Ca^{2+} measurements were made in YFP-transfected PC12, HEK293, and A7r5 cells. (A) Ca^{2+} pools were released by bradykinin (BK, 1 μM; arrow) in nominally Ca^{2+}-free medium followed by replacement with bradykinin and normal 1 mM Ca^{2+} medium (bar). (B) Ca^{2+} pools were released by CCH (100 μM; arrow) in nominally Ca^{2+}-free medium followed by replacement with CCH and normal 1 mM Ca^{2+} medium (bar). (C) Ca^{2+} pools were released by vasopressin (Vaso, 1 μM; arrow) in nominally Ca^{2+}-free medium followed by replacement with vasopressin and normal 1 mM Ca^{2+} medium (bar). Average traces are representative of 30–50 cells, and the subpopulation traces are representative single cells extracted from that average.

rescue, 0.64 SEM); and (vi) R265Q IP_{3}R, 0 out of 255 cells (0% rescue, 0.0 SEM).
Analyzing microdomains of local IP3R-mediated Ca2+ release, we used the mutant DT40 chicken B lymphocyte cell line, which is deficient in all three genes for the IP3R (IP3R−/−) (17). Stimulation of DT40 cells with the B cell receptor agonist anti-IgM (IgM) leads to a nonreceptor tyrosine kinase-linked activation of PLC-γ2 and subsequent production of IP3 and DAG (3, 18). Introduction of Ca2+ reveals corresponding ACE as described (3, 9). Importantly, neither IP3/DAG production nor thapsigargin- or ionomycin-induced activation of CCE differs between the DT40 wild-type and mutant IP3R−/− cells (9, 12). In rescue experiments, transfection of DT40 IP3R−/− cells with YFP and WT type 1 IP3R (WT) (Fig. 3A–C) restores PLC-γ2-dependent activation of ACE. Similar subpopulations of Ca2+ responses are seen in these rescued cells as in WT DT40 cells (data not shown). Moreover, even though mediated by PLC-γ2, these subpopulations occur with comparable frequency as the PLC-γ2-stimulated responses in PC12, HEK293, and A7r5 cells (Fig. 1). Because YFP control DT40 IP3R−/− cells lack any Ca2+ release or ACE (Fig. 3D), we conclude that each of the three Ca2+ response subtypes requires the IP3R. Thus, even the NRE response (Fig. 3C) is restored by IP3R expression.

The DT40 IP3R−/− cells allowed us to assess directly the role of functionally modified IP3Rs on ACE. We transfected the cells with two different IP3R mutants: (i) C-terminal truncation mutant (∆C), which binds IP3, but cannot release Ca2+ because of defects in Ca2+ channel gating; or (ii) a mutant IP3R lacking N-terminal amino acids 90–110 (∆90–110), producing a channel with a ~100-fold decrease in IP3 binding (19). Compared with WT IP3R rescue (Fig. 4A), expression of ∆C restores ACE without any Ca2+ release (Fig. 4B) (see Materials and Methods for the percentage of cells rescued), because the only response seen is the NRE response. In contrast, neither agonist-induced Ca2+ release nor NRE entry is evident in cells transfected with ∆90–110 IP3R, which cannot bind IP3 (Fig. 4C). Because neither mutant channel can elicit intracellular Ca2+ release, the functional requirement for coupling to ACE appears to be IP3 recognition by the IP3R.

As the ∆C deletion involves removal of ~300 amino acids, other receptor functions may have been altered. Additionally, the ∆90–110 IP3R mutant still can bind IP3, albeit with ~100-fold lower affinity than WT. We addressed these issues in rescue experiments by using single point-mutant IP3R constructs: (i) an IP3R pore mutant (D2550A) lacking a critical aspartate-2550, mutation of which to alanine abolishes Ca2+ channel activity (20), or (ii) an IP3R ligand-binding mutant (R265Q) lacking a critical arginine-265, mutation of which to glutamine abolishes IP3 binding (21). The pore mutant (D2550A), like ∆C inactive channel preparation, restores Ca2+ entry but not release (NRE) (Fig. 4D), establishing that intracellular Ca2+ release is not required for entry. The deficient IP3 binding mutant (R265Q), like the ∆90–110 mutant that cannot bind IP3, displays neither Ca2+ release nor ACE (Fig. 4E).

In control experiments, we assessed the expression of DT40 IP3R−/− cells transfected with YFP alone, YFP plus WT IP3R, or YFP plus mutant IP3Rs by using a rabbit polyclonal antibody against an N-terminal IP3R epitope and YFP as a marker for transfected cells. Confocal immunocytochemistry with goat anti-rabbit Alexa-568 secondary antibodies confirms the expression of the IP3R constructs in transfected DT40 IP3R−/− cells (Fig. 4A–E, YFP (Left) and anti-IP3R (Right)). In summary, our IP3R mutational analysis establishes that ACE requires functionally active IP3Rs and depends on IP3 recognition but is independent of Ca2+ release activity.

**Discussion**

The present study shows that, unlike CCE, ACE activation does not require ER Ca2+ release. Rather, ACE recognition...
of IP3 by the IP3R, irrespective of any Ca2+ release activity. These conclusions are supported by several key findings. First, single-cell Ca2+ measurements reveal subpopulations of Ca2+ responses (RE, RNE, and NRE), and the magnitude of Ca2+ release does not correlate with the magnitude of ACE. Second, in DT40 IP3R−/− cells devoid of ACE, restoration of WT IP3R expression rescues ACE in the three modes described, illustrating the requirement of the IP3R for each response type. Third, ACE is rescued in the DT40 IP3R−/− cells by two distinct Ca2+ release-deficient IP3R mutants but not by two different ligand-binding mutants. Thus, the IP3R is coupled to ACE independently of its Ca2+ release activity, supporting the notion that conformational changes in the IP3R, subsequent to IP3 binding, gate endogenous Ca2+ entry channels.

Our findings on the requirement of IP3 for ACE are consistent with studies showing that the IP3R binds TRPC3 in HEK293 cells (22, 23) and that an N-terminal IP3R fragment bound to IP3 is sufficient to gate TRPC3 in reconstituted vesicles (22, 24). Moreover, the IP3-dependence on ACE accords with experiments in the DT40 PLC-γ−/− cells, in which a lipase-inactive PLC-γ mutant rescues ACE only in the presence of receptor-generated IP3 (3). Our findings also complement observations of Penner and colleagues (25) that repetitive subthreshold CCH application stimulates ACE independently of Ca2+ release in RBL-2H3-M1 cells.

DAG. The other product of phosphatidylinositol 4,5-bisphosphate (PIP2) degradation, also has been proposed as a physiological activator of ACE, given that it can stimulate overexpressed TRPC3, -6, and -7 channels, even in the absence of IP3Rs (8, 9). Conversely, recent work reveals DAG-induced PKC activation as an inhibitor of endogenous ACE as well as overexpressed TRPC channels (26). In our experiments, endogenous ACE is not demonstrable in DT40 IP3R−/− cells, even though these cells contain active PLC and can produce DAG (9,
Thus, if DAG is an activator of Ca\(^{2+}\) entry, it is unlikely to function alone. Alternatively, IP\(_3\) and DAG may function coordinately in an activation–deactivation loop. Recently, Delmas et al. (27) demonstrated that G protein-coupled receptors signal differently to specific microdomains of IP\(_3\)R in rat sympathetic neurons. By using overexpressed TRPC1 and TRPC6, these authors correlated Ca\(^{2+}\) release with TRPC1 activation and lack of Ca\(^{2+}\) release with TRPC6 (i.e., DAG activation). Our findings suggest that, although both TRPC1 and TRPC6 respond differentially to DAG, both still may be regulated by the IP\(_3\)R, with the activation of TRPC1 merely being coincident with Ca\(^{2+}\) release.

Our findings support a model whereby Ca\(^{2+}\) release is elicited by an IP\(_3\)R pool in which no release occurs but not by an IP\(_3\)R pool in which only release occurs. This interpretation is supported by overexpression experiments of SERCA-2b in HEK293 cells that decrease RE and NRE but augment the RNE phenotype. Accordingly, the preferred coupling mode is presumably a functional IP\(_3\)-bound IP\(_3\)R in a nonfunctional ER pool. Alternatively, IP\(_3\) and DAG may function coor-
dinately in an activation loop. Recently, Delmas et al. (27) demonstrated that G protein-coupled receptors signal differently to specific microdomains of IP\(_3\)R in rat sympathetic neurons. By using overexpressed TRPC1 and TRPC6, these authors correlated Ca\(^{2+}\) release with TRPC1 activation and lack of Ca\(^{2+}\) release with TRPC6 (i.e., DAG activation). Our findings suggest that, although both TRPC1 and TRPC6 respond differentially to DAG, both still may be regulated by the IP\(_3\)R, with the activation of TRPC1 merely being coincident with Ca\(^{2+}\) release.

This mechanism resembles proposals of Irvine (6) and Berridge (7) over a decade ago for IP\(_3\)-mediated conformational coupling. We thank Drs. Joseph P. Kao, Robert E. Rothe, Gabriela Caraveo, and Klick Klerpa for useful discussions. Dr. Suresh Joseph for the kind gift of mutant IP\(_3\)R constructs, and Dr. Tomohiro Kurosaki (Kansai Medical University, Moriguchi, Japan) for the gift of the DT40 IP\(_3\)R\(^{-/-}\) cell line. This research was supported by U.S. Public Health Service Grants MH-18501 and DA-000266 and Research Scientist Award DA-00074 (to S.H.S.), National Institutes of Health Grant HL55426 (to D.L.G.), American Heart Association Grant 0130285N (to K.K.), and National Research Service Awards NH65090 (to R.L.P.) and NS-043850 (to D.B.).