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

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Contributed by Solomon H. Snyder, December 30, 2003

It has been considered that Ca\(^{2+}\) release is the causal trigger for Ca\(^{2+}\) entry after receptor activation. In DT40 B cells devoid of inositol 1,4,5-trisphosphate receptors (IP\(_R\)R), the lack of Ca\(^{2+}\) entry in response to receptor activation is attributed to the absence of Ca\(^{2+}\) release. We reveal in this article that IP\(_R\)R recognition of IP\(_3\) determines agonist-induced Ca\(^{2+}\) entry (ACE), independent of its Ca\(^{2+}\) release activity. In DT40 IP\(_R\)R\(^{-/-}\) cells, endogenous ACE can be rescued with type 1 IP\(_R\)R mutants (both a \(\Delta C\)-terminal truncation mutant and a D2550A pore mutant), which are defective in Ca\(^{2+}\) release channel activity. Thus, in response to B cell receptor activation, ACE is restored in an IP\(_R\)R-dependent manner without Ca\(^{2+}\) store release. Conversely, ACE cannot be rescued with mutant IP\(_R\)Rs lacking IP\(_3\) binding (both the \(\Delta 90–110\) and R265Q IP\(_3\)-binding site mutants). We conclude that an IP\(_3\)R-dependent conformational change in the IP\(_R\)R, not endoplasmic reticulum Ca\(^{2+}\) pool release, triggers ACE.

C\(^{a+}\) 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\(_2\)) into the second-messenger molecules: inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). IP\(_3\) mediates rapid Ca\(^{2+}\) store release by activating IP\(_3\) receptors (IP\(_3\)Rs) in the endoplasmic reticulum (ER), whereas DAG activates protein kinase C (PKC) (2). After this initial Ca\(^{2+}\) release phase, external Ca\(^{2+}\) enters through plasma membrane channels, providing a secondary and more prolonged Ca\(^{2+}\) signal (1), a phenomenon designated here as agonist-induced Ca\(^{2+}\) entry (ACE) (3).

To date, the molecular identity of these Ca\(^{2+}\) entry channels as well as their coupling mechanism remain unknown, although several mechanisms have been proposed. Intracellular Ca\(^{2+}\) release through the IP\(_R\)R could trigger ACE by means of capacitative Ca\(^{2+}\) entry (CCE) (4), which can be activated in a PLC-independent manner (3) by the ER Ca\(^{2+}\) pump blocker thapsigargin or the Ca\(^{2+}\) ionophore ionomycin (4, 5). In this scheme, the luminal drop in ER Ca\(^{2+}\) activates “store-operated” Ca\(^{2+}\) channels in the plasma membrane, although the basis for this coupling mechanism is entirely unknown. Although the release activity of IP\(_3\)Rs may mediate Ca\(^{2+}\) entry, others have suggested that IP\(_3\)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\(^{2+}\) entry channels (TRPC) (8).

We recently demonstrated a functional distinction between ACE and CCE based on the requirement of the former for PLC-\(\gamma\), 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\(_R\)R-mediated Ca\(^{2+}\) release triggers ACE and/or whether a conformational alteration of the IP\(_R\)R is sufficient for ACE. Evidence for the activation by Ca\(^{2+}\) pool emptying alone (and the dispensability of the IP\(_R\)R for Ca\(^{2+}\) entry) includes the ability of thapsigargin or ionomycin, but not PLC activation, to stimulate Ca\(^{2+}\) entry in the DT40 triple IP\(_R\)R knockout cell line, a form of B lymphocytes devoid of any IP\(_R\)R (9). These interpretations assume that ACE and CCE are functionally overlapping mechanisms. In the present study, we demonstrate that IP\(_R\)R recognition by the IP\(_R\)R but not the receptor’s Ca\(^{2+}\) 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\(_R\)R\(^{-/-}\) 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 \(\mu\)g of yellow fluorescent protein (YFP) cDNA \(\pm 20\ \mu\)g of IP\(_R\)R cDNA was used for transfection.

Ca\(^{2+}\) Imaging. Ca\(^{2+}\) measurements were as described (11). Fura-2/acetoxymethyl 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\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) ATPase (SERCA)-2b. Functional rescue of Ca\(^{2+}\) responses in the DT40 IP\(_R\)R\(^{-/-}\) 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\(_R\)R, 49 out of 261 cells (18.7% rescue, 0.36 SEM); (iii) AC IP\(_R\)R, 58 out of 230 cells (25% rescue, 1.62 SEM); (iv) \(\Delta 90–110\) IP\(_R\)R, 1 out of 239 cells (0.4% rescue, 0.19 SEM); (v) D2550A IP\(_R\)R, 67 out of 258 cells (25.9% rescue, 0.47 SEM).

Abbreviations: IP\(_3\), inositol 1,4,5-trisphosphate; IP\(_R\)R, IP\(_3\) receptor; ACE, agonist-induced Ca\(^{2+}\) entry; PLC, phospholipase C; DAG, diacylglycerol; ER, endoplasmic reticulum; CCE, capacitative Ca\(^{2+}\) entry; TRPC, transient receptor potential Ca\(^{2+}\) entry channel; YFP, yellow fluorescent protein; SERCA, sarco/endo-plasmic ER Ca\(^{2+}\) ATPase; RE, Ca\(^{2+}\) release and Ca\(^{2+}\) entry; RNE, Ca\(^{2+}\) release with little to no Ca\(^{2+}\) entry; NRE, little to no Ca\(^{2+}\) release yet substantial Ca\(^{2+}\) entry; CCH, carbachol; HEK, human embryonic kidney.

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Immunohistochemistry. Immunohistochemistry was as described in ref. 3.

IP$_3$R Mutagenesis. Δ90–110 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); carbethol (CCH), bradykinin, and vasopressin were from Sigma; Fura-2/acetoxyethyl 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 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).

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.
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 IRP (R₁R₁⁻/⁻) (17). Stimulation of DT40 cells with the B cell receptor agonist anti-IgM (IgM) leads to a nonreceptor tyrosine kinase-linked activation of PLC-γ 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 WT DT40 and mutant IP₃R cells. In rescue experiments, transfection of DT40 IP₃R−/− cells with YFP and WT type 1 IP₃R (WT) (Fig. 3 A–C) restores PLC-γ-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-γ, these subpopulations occur with comparable frequency as the PLC-β-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 IRPs on ACE. We transfected the cells with two different IP₃R mutants: (i) C-terminal truncation mutant (ΔC), 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 (Δ90–110), producing a channel with a ~100-fold decrease in IP₃ binding (19). Compared with WT IP₃R rescue (Fig. 4A), expression of ΔC 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 Ca²⁺ entry is evident in cells transfected with Δ90–110 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 ΔC deletion involves removal of ~300 amino acids, other receptor functions may have been altered. Additionally, the Δ90–110 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 ΔC 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 Δ90–110 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 IP₃R 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 IP₁R constructs in transfected DT40 IP₃R−/− cells (Fig. 4 A–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 requires recognition of Ca²⁺ by the IP₃R.
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,
changes in the movement of IP3Rs into or out of functional ER stores, as suggested by overexpression experiments of SERCA-2b in HEK293 cells that decrease RE and NRE but augment the RNE phenotypically in an activation deactivation loop. Recently, Delmas et al. (27) demonstrated that G protein-coupled receptors signal differently to specific microdomains of IP3Rs in rat sympathetic neurons. By using overexpressed TRPC1 and TRPC6, these authors correlated Ca2+ release with TRPC1 activation and lack of Ca2+ 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 IP3R, with the activation of TRPC1 merely being coincident with Ca2+ release.

Our findings support a model whereby Ca2+ entry is elicited by an IP3R pool in which no release occurs but not by an IP3R 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 model is presumably a functional IP3-bound IP3R in a nonfunctional ER Ca2+ pool. Alternatively, but less likely, overactive expressed SERCA-2b might scavenge Ca2+ as it enters the cell, thereby obscuring our measurements. Compatible with the former hypothesis, expression of ΔC or D2550A IP3R, that can bind IP3 but not release, augments ACE. Our model fits with suggestions of Parekh et al. (14), that distinct IP3-sensitive ER stores function in release and entry modes, and Delmas et al. (27), who demonstrated IP3-signaling microdomains in neurons. As discrete processes, Ca2+ release and Ca2+ entry may be dynamically regulated through changes in the movement of IP3Rs into or out of functional ER pools, generating microdomains that could respond differentially to various receptor stimuli and Ca2+-signaling functions.

Expression of WT IP3Rs in the DT40 IP3R−/− rescues the NRE response, as does the pore mutant. If coupling occurs within a nonfunctional ER pool, then Ca2+ release by WT IP3Rs would need to be rendered nonfunctional, at least in zones of Ca2+ entry. Modification of ER Ca2+ store content could be accomplished by proteins such as SERCA or by ER Ca2+ buffers (e.g., calsequestrin). Alternatively, but not mutually exclusive, a soluble truncated form of the IP3R, containing the N-terminal IP3 binding domain alone could couple IP3 recognition to Ca2+ entry. Cells contain transcripts for IP3Rs lacking Ca2+ channels (28), although it is not known whether these transcripts are translated into functional proteins. We suggest that such a protein may provide physiological signals for ACE, binding IP3 and TRPCs but unable to release Ca2+. Overall, this mechanism resembles proposals of Irvine (6) and Berridge (7) over a decade ago for IP3R-mediated conformational coupling.

We thank Drs. Joseph P. Kao, Robert E. Rothe, Gabriela Caraveo, and Klick Klerpa for useful discussion, Dr. Suresh Joseph for the kind gift of mutant IP3R constructs, and Dr. Tomohiro Kurosaki (Kansai Medical University, Moriguchi, Japan) for the gift of the DT40 IP3R−/− cell line. This research was supported by U.S. Public Health Service Grants MH-18501 and DA-000260 and Research Scientist Award DA-0074 (to S.H.S.), National Institutes of Health Grant HL5426 (to D.L.G.), American Heart Association Grant 0130268N (to K.K.), and National Research Service Awards NH65090 (to R.L.P.) and NS-043580 (to D.B.).