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Regulation of the type III $InsP_3$ receptor by $InsP_3$ and calcium

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Abstract

It has been proposed that the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) type III acts as a trigger for $InsP_3$-mediated calcium (Ca^{2+}) signaling, because this $InsP_3$ isoform lacks feedback inhibition by cytosolic Ca^{2+}. We tested this hypothesis in RIN-m5F cells, which express predominantly the type III receptor. Extracellular ATP increases Ca^{2+} in these cells, and we found that this effect is independent of extracellular Ca^{2+} but is blocked by the $InsP_3R$ antagonist heparin. There was a dose-dependent increase in the number of cells responding to ATP and two-photon flash photolysis of caged-Ca^{2+} heightened the sensitivity of RIN-m5F cells to this increase. These findings provide evidence that Ca^{2+} increases the sensitivity of the $InsP_3R$ type III in intact cells and supports the idea that this isoform can act as a trigger for hormone-induced Ca^{2+} signaling. © 2002 Published by Elsevier Science (USA).

Keywords: Ion channel gating; Calcium channels; Calcium signaling; Lipid bilayers

Inositol 1,4,5-trisphosphate ($InsP_3$) mediates the release of stored calcium (Ca^{2+}) in cells stimulated with a range of neurotransmitters, growth factors, or hormones [1]. $InsP_3$ can be generated by either G protein-linked or tyrosine kinase-linked activation of phospholipase C [1]. Once formed, $InsP_3$ opens the $InsP_3$ receptor ($InsP_3R$) Ca^{2+} channel in the endoplasmic reticulum, which releases Ca^{2+} from the endoplasmic reticulum into the cytosol.

Three isoforms of the $InsP_3R$ have been identified [2–7]. Initial work on the $InsP_3R$ focused on the type I isoform isolated from canine cerebellum [8,9]. Recently, the single channel properties of the types II [10,11] and III [12,13] $InsP_3R$ have been determined. The three $InsP_3R$ isoforms are similar in their domain structure. Each one contains an $InsP_3$ binding domain and a pore-forming domain. Between these two domains is a large regulatory domain which contains several Ca^{2+} binding sites, specific binding sites for ATP, phosphorylation sites for tyrosine kinase and protein kinase A (PKA), and sites of interaction with accessory proteins such as FKBP12 and calmodulin [14]. The $InsP_3R$ isoforms also possess a high degree of homology. For these reasons, many of the functional properties of the $InsP_3R$ isoforms are similar such as activation by $InsP_3$ [8,10,12], the magnitude of the single channel current [9,11,12], and activation by concentrations of Ca^{2+} less than 250 nM [8,11,12].

The functional properties of the $InsP_3R$ type III are interesting because this receptor has distinctive $InsP_3$ binding properties [15,16]. Specifically, $InsP_3$ binding to the $InsP_3R$ type III is not inhibited by high free Ca^{2+} as found for the type I $InsP_3R$ [12]. Therefore, one would predict that $InsP_3$-mediated Ca^{2+} signals, in cells expressing only the type III isoform, would result in rapid, complete release of stored Ca^{2+}, rather than Ca^{2+} oscillations. Indeed, this has been observed in two separate cell lines [12,17]. The $InsP_3R$ type III also possesses the lowest relative affinity for $InsP_3$ [4,18], yet, unlike the $InsP_3R$ type I, this isoform can be fully activated by $InsP_3$ when cytosolic Ca^{2+} levels are at resting levels [12,13]. Both of these properties make the $InsP_3R$ type

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III suitable for the initiation of intracellular Ca^{2+} signals. The goal of this project was to determine whether $InsP_3$ production induces intracellular Ca^{2+} signals in cells expressing predominantly $InsP_3R$ type III and to test whether Ca^{2+} decreases the threshold for signaling via the $InsP_3R$ type III in intact cells.

Experimental

Materials. Adenosine 5′-triphosphate (ATP), low molecular weight heparin, and de-*N*-sulfated heparin were obtained from Sigma Chemical Company (St. Louis, MO). Fluo-3-AM, cell-impermeant fluo-3, and cell-permeant DMNP–EDTA were obtained from Molecular Probes (Pitchford, OR). DM-nitrophen was obtained from CalBiochem (La Jolla, CA). All other chemicals were of the highest quality commercially available.

Cytosolic Ca^{2+} measurements in single cells. Cytoplasmic Ca^{2+} was measured in RIN-m5F cells using time-lapse confocal microscopy [19]. RIN-m5F cells were used for these studies because they express $InsP_3R$ type III almost exclusively [20]. Cells were grown in DMEM, 10% fetal calf serum, and 1% penicillin/streptomycin, and maintained at 37 °C in a 95% O_2/5% CO_2 incubator. Cells were plated on glass coverslips and used 24 h after plating. The cells were loaded with fluo 3-AM (6 μM) for 20 min at 37 °C [21] in Leibovitz L-15 medium containing 10% fetal calf serum. Coverslips containing the cells were transferred to a perfusion chamber on the stage of a BioRad MRC-1024 confocal microscope and observed using a 63×, 1.4 NA objective. Fluo-3 was excited with the 488 nm line of an air-cooled argon laser. Increases in Ca^{2+} were measured at the emission wavelength 522 nm with a spatial resolution of 0.320 μm/pixel and were expressed as percentage of baseline fluorescence. In certain experiments, cells were perifused with a HEPES-buffered solution and stimulated with ATP (1–100 μM). In other experiments, cells were perifused with the HEPES solution, and then switched to a Ca^{2+}-free medium containing 1 mM EGTA 30 s before stimulation with ATP.

Microinjection with heparin or de-N-sulfated heparin. RIN-m5F cells were plated on glass coverslips and prepared as described above. The cells were microinjected, using an Eppendorf 5242 Microinjector and an Eppendorf 5171 Micromanipulator, with fluo-3 (1 mM) to detect cytosolic Ca^{2+} and as a marker for injection, plus either heparin (1 mg/ml) to block $InsP_3R$-mediated Ca^{2+} signaling or de-*N*-sulfated heparin (1 mg/ml) to act as a negative control. These substances were dissolved in an intracellular-like solution containing 150 mM KCl and 1 mM HEPES. Heparin is a high-affinity competitive agonist of the $InsP_3R$ [22] whereas its de-*N*-sulfated analog has limited interaction with the receptor [23]. Cells were examined by confocal microscopy as described above.

Photorelease of caged Ca^{2+}. RIN-m5F cells were prepared as above, then either injected with DM-nitrophen (75 mM), $CaCl_2$ (50–70 mM), and fluo-3 (1 mM) or else loaded with fluo-3 AM (6 μM), plus DMNP–EDTA (1 μM). In either case, Ca^{2+} was released in discrete subcellular regions using two-photon flash photolysis [24,25]. For these studies, a mode-locked Spectra-Physics Tsunami Titanium:Sapphire (Ti:S) laser pumped by a 10 W diode-pumped solid-state laser (Millenia X) was used. Typically, the Ti:S laser was scanned across a predetermined 20×20 pixel ($6.4 \times 6.4\,\mu m^2$) intracellular region in order to restrict photorelease of Ca^{2+} to that region. The Ti:S and argon lasers were co-aligned to ensure that Ca^{2+} was photoreleased in the focal plane. The Ti:S laser was tuned to 730 nm which is the optimal wavelength for photorelease of caged Ca^{2+} [24]. The amount of Ca^{2+} photoreleased by two-photon excitation was calculated as (based on [24]):

$$\text{number of atoms released} = \sigma_{unc} g I^2 N_v T,$$

where σ_{unc} is the effective two-photon uncaging cross-section, g is the "second-order coherence," I is the intensity in photons/(area × time), N_v is the number of caged molecules in the two-photon excitation focal volume, and T is the duration of the uncaging pulse. The two-photon cross-section for DM-nitrophen uncaging (σ_{unc}) is $10^{-52}\,cm^4\,s$ (0.01 GM). The second-order coherence can be approximated as $0.56/R\tau$, where 0.56 is the "pulse shape factor," R is the repetition rate of the laser (80 MHz), and τ is the pulsewidth (~200 fs). yielding a value of 3.5×10^4 for g. The intensity I was determined by converting from power (in milliWatts) to photons/second and dividing by the effective area of the illumination in the focal plane, which is $\pi(0.174\,\mu m)^2$. Based on direct measurements, the power at the focal point in our system is ~4 mW for a 32% neutral density (ND) filter and ~2.5 mW for a 20% ND filter. N_v is (concentration of caged compound) × (Avogadro's number) × (effective focal volume). The effective volume is calculated as the integral over all space of the three-dimensional Gaussian volume that approximates the squared illumination point spread function. Under our experimental conditions using a 1.4 NA lens at 730 nm, the effective volume is 7×10^{-16} liter, so that $N_v = (4.2 \times 10^8$ molecules) × (liter) × (concentration of caged compound). Assuming we scan across 20 pixels within a cell at a speed of 1.5 μs/pixel, then $T = 3 \times 10^{-5}$ s. Further assuming that the intracellular concentration of the caged compound is 100 μM, then we would expect to release ~200 Ca^{2+} atoms during experiments using a 32% ND filter, and ~80 atoms with a 20% ND filter. Thus we would expect to increase the free Ca^{2+} concentration by ~5 μM in the focal volume where Ca^{2+} is released when a 32% ND filter is used, and Ca^{2+} would increase by ~2 μM when a 20% ND is used. These calculated values are consistent with our observations.

Statistical analysis. Comparisons were made between groups using a two-sample t test. Results are expressed as means ± SEM unless noted otherwise.

Results

ATP-induced Ca^{2+}-signals are mediated by $InsP_3$. RIN-m5F cells mobilize intracellular Ca^{2+} in response to stimulation by extracellular ATP (Fig. 1). These cells express the P_{2Y} class of ATP receptors, where activation of the P_{2Y} cascade stimulates the production of $InsP_3$ which is linked to intracellular Ca^{2+} signaling [26–28]. Initial studies were designed to determine whether $InsP_3$-mediated Ca^{2+} signals are solely responsible for the ATP-induced Ca^{2+} signals. In cells perifused with Ca^{2+}-containing medium, ATP (100 μM) increased fluorescence in 79% ($n = 31/39$) of the cells by 198 ± 18%. Similarly, in cells perifused with Ca^{2+}-free medium, ATP (100 μM) increased fluorescence in 90% ($n = 38/40$) of the cells by 184 ± 23% ($p > 0.3$ relative to Ca^{2+}-containing medium) (Fig. 1A). Although the initial increase in cytosolic Ca^{2+} was similar regardless of the presence of extracellular Ca^{2+} (Fig. 1B), cytosolic Ca^{2+} returned to baseline more rapidly in cells in Ca^{2+}-free medium (Fig. 1A); the time required for fluo-3 fluorescence to decrease by 50% from peak to baseline was 14.0 ± 1.1 s ($n = 30$) in Ca^{2+}-containing medium, but was only 9.9 ± 0.7 s ($n = 38$) in Ca^{2+}-free medium ($p < 0.0009$ by one-tailed t test). The difference in the duration of the Ca^{2+} transient likely reflects the fact that as the internal stores are depleted the store operated channels are activated to replace intracellular Ca^{2+}. These results suggest that the initial increase in Ca^{2+} in

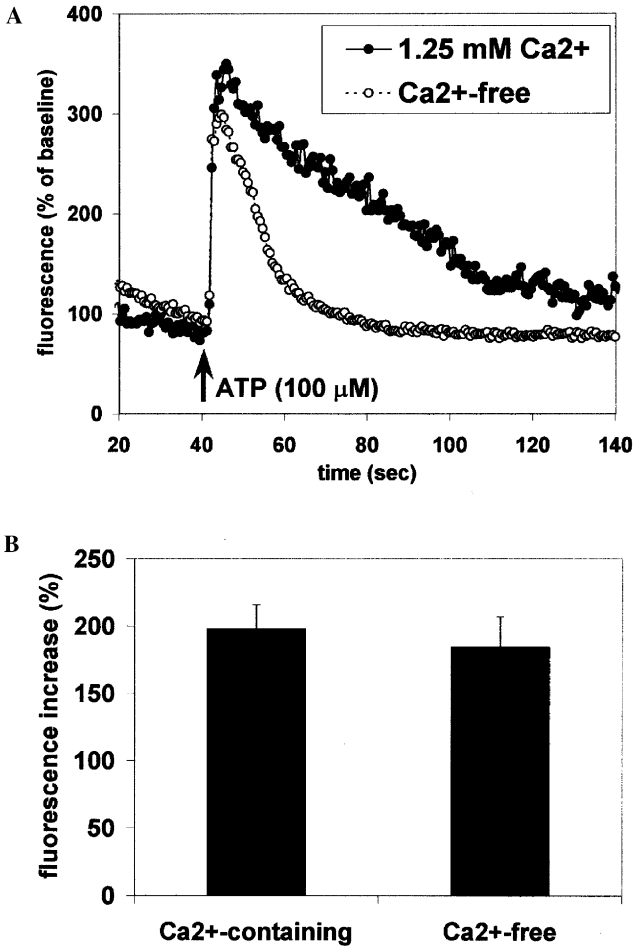

Fig. 1. Effects of extracellular Ca^{2+} on cytosolic Ca^{2+} signaling in RIN-5mF cells. Cells were perifused with 100 μM ATP and observed by time-lapse confocal microscopy. (A) Graphical representation of the increase in fluorescence detected in cells stimulated in both Ca^{2+}-containing and Ca^{2+}-free medium. The peak increase in fluo-3 fluorescence is similar under both conditions and is representative of that seen in 79 cells. (B) Summary of results. The peak increase in fluo-3 fluorescence is the same in the presence or absence of extracellular Ca^{2+}.

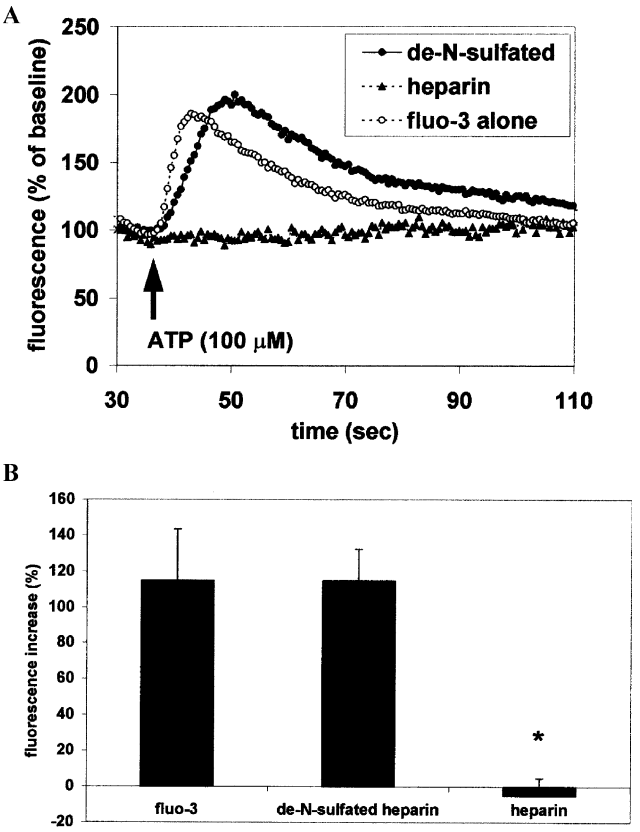

Fig. 2. Effects of heparin on cytosolic Ca^{2+} signaling in RIN-5 mF cells perifused with 100 μM ATP. Graphical representation of the increase in fluorescence detected in RIN-5mF cells microinjected with either fluo-3 (control), de-*N*-sulfated heparin, or heparin. The increase in fluorescence in cells injected with fluo-3 ($N = 10$) and de-*N*-sulfated heparin ($N = 11$) is similar, while that for heparin-injected cells ($N = 18$) is nearly absent. (B) Summary of results. The peak increase in fluo-3 fluorescence is similar in cells injected with fluo-3 with or without de-*N*-sulfated heparin, but is significantly reduced in heparin-injected cells ($*p < 0.0001$).

RIN-m5F cells stimulated with ATP is due entirely to release of Ca^{2+} from intracellular stores.

In order to determine whether ATP-induced Ca^{2+} signaling occurs exclusively via the $InsP_3R$, RIN-m5F cells were microinjected with either heparin, de-*N*-sulfated heparin, or buffer. To identify which cells were successfully injected, all cells were co-injected with fluo-3 (1 mM). When stimulated with ATP (100 μM), fluorescence increased by 115 ± 28% in 42% ($n = 10/24$) of the cells injected with fluo-3 alone (Fig. 2A and B). Similarly, fluorescence increased by 115 ± 17% in 58% ($n = 11/19$) of the cells injected with de-*N*-sulfated heparin ($p > 0.5$ relative to fluo-3 alone). In contrast, no Ca^{2+} increase was detected in all cells ($n = 18$) injected with heparin (Fig. 2A and B; mean change in fluorescence, −5 ± 10%; $p < 0.0001$ relative to de-*N*-Sulfated heparin or fluo-3 alone). These findings demonstrate that ATP-induced Ca^{2+} signaling in RIN-m5F cells depends upon activation of $InsP_3Rs$.

Ca^{2+} decreases the threshold for signaling via the $InsP_3R$ type III. Ca^{2+} has been shown to act as a coagonist for the $InsP_3R$ type III at the single channel level [12] and in permeabilized cells [29]. However, the coagonist effect of Ca^{2+} has not been demonstrated in intact cells. Since the number of RIN-m5F cells which respond to ATP increases in a concentration-dependent fashion [12] we tested whether Ca^{2+} decreases the threshold for ATP-induced signaling by uncaging Ca^{2+} in submicron-sized regions within the cytosol. In non-stimulated cells, photoreleased Ca^{2+} caused a small and highly transient increase in Ca^{2+} (Fig. 3A). Cells exposed to 0.1 μM ATP or less do not respond to ATP, as shown previously (Fig. 3C; [12]). However, in cells stimulated with a subthreshold concentration of ATP (0.1 μM), uncaging Ca^{2+} triggered a robust ATP-induced Ca^{2+} signal (Fig. 3B and C). As greater

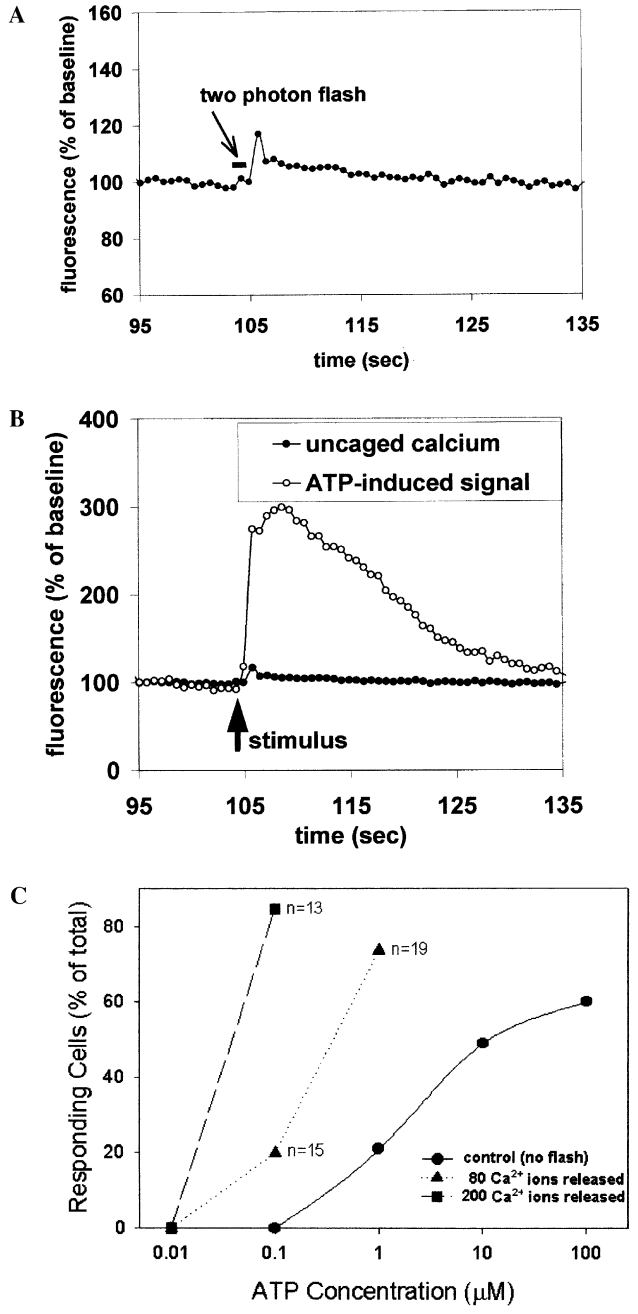

Fig. 3. Release of caged Ca^{2+} by two-photon flash photolysis in RIN-m5F cells. Cells were injected with the fluorescent Ca^{2+} dye fluo-3 and observed by time-lapse confocal microscopy. (A) Flash photolysis of caged Ca^{2+} induces a small, highly transient increase in Ca^{2+}. A 32% neutral density filter was used (80 Ca^{2+} ions released). (B) Comparison between the amount of Ca^{2+} released during flash photolysis and during stimulation with ATP. This emphasizes the increased sensitivity of the $InsP_3R$ to Ca^{2+} in the presence of subthreshold amounts of $InsP_3$. (C) Dose–response curves for RIN-5mF cells stimulated with different concentrations of ATP. More cells responded at higher ATP concentrations. Note that 0.1 μM ATP evoked no response. However, release of smaller amounts of caged-Ca^{2+} (▲, using a 32% neutral density filter; 80 Ca^{2+} ions released) enabled some cells to respond to 0.1 μM ATP and release of slightly larger amounts of Ca^{2+} (■, with a 20% neutral density filter; 200 Ca^{2+} ions released) enabled most cells to respond to 0.1 μM ATP. Values listed indicate total number of cells observed under each condition.

amounts of Ca^{2+} were uncaged, more cells responded to addition of ATP (Fig. 3C). Moreover, global cellular Ca^{2+} responses were triggered by highly localized and limited increases in free Ca^{2+}. These results show that Ca^{2+} acts locally as a coagonist for the $InsP_3R$ and decreases the threshold for signaling.

This type of Ca^{2+} signaling with the $InsP_3R$ type III suggests that a Ca^{2+} wave will spread quickly throughout the cell. To measure the speed of a Ca^{2+} wave, caged $InsP_3$ was photo-released at one end of a RIN-m5F cell and the time until the wave appeared at the other end of the cell was monitored. The Ca^{2+} wave encompasses the entire cell in less than 275 ms ($n = 7$), a time which corresponds to the shortest interval required to obtain an image of the cell during these experiments. It was not possible to collect images of the cell at a faster rate due to technical limitations. These experiments, however, show that the Ca^{2+} wave spreads in RIN-m5F as a single, global, and rapid increase in cytosolic Ca^{2+}.

Discussion

Here we report that Ca^{2+} lowers the threshold for $InsP_3$-mediated Ca^{2+} signaling in RIN-m5F cells. It was initially proposed that the $InsP_3R$ type III, due to its relatively low affinity for $InsP_3$ [15,16], is poorly activated by $InsP_3$ and inhibited by cytoplasmic levels of Ca^{2+}. Our results instead show that the low affinity for $InsP_3$ does not necessarily dictate the concentration at which the $InsP_3R$ type III opens. Rather, the $InsP_3$ concentration needed to initiate Ca^{2+} release from intracellular stores is determined by the concentrations of both Ca^{2+} and $InsP_3$. In addition, we show that the $InsP_3R$ type III supports an increase in Ca^{2+} that can spread rapidly.

The type I $InsP_3R$ was the first isoform to be identified and cloned, and has been characterized most extensively. Like the type III receptor, $InsP_3R$ type I has been characterized at the single channel level. It has been shown that Ca^{2+} and $InsP_3$ serve as co-agonists for both isoforms of the $InsP_3R$. However, the type I isoform has a bell-shaped dependence on cytoplasmic Ca^{2+} and closes in the presence of high concentrations of cytosolic free Ca^{2+} [8,30,31]. This feedback inhibition of the type I isoform appears to be mediated by calmodulin [32]. Moreover, this property of the receptor supports the generation of Ca^{2+} oscillations [33]. In addition, type I receptors initiate discrete Ca^{2+} signals called "puffs" at focal sites within the cell, and these puffs add spatially and temporally to create Ca^{2+} waves [34]. These waves can remain spatially restricted due to the type I receptor's negative feedback mechanism. The type III receptor generates "puffs" similar in amplitude to those generated by the type I receptor [35], but the lack of Ca^{2+}-dependent negative feedback of the type III

$InsP_3R$ [12] suggests that this receptor isoform is important for triggering global release of intracellular Ca^{2+} in intact cells. Indeed, in two cell types expressing predominantly $InsP_3R$ type III, RIN cells and DT40 cells genetically engineered to contain only one receptor isoform, single large Ca^{2+} transients were produced after addition of extracellular agonists [12,17] rather than oscillations or localized responses.

The properties of the type III receptor have important implications in terms of cell function. Many cells contain multiple isoforms of the $InsP_3R$, each of which may serve different functions. For example, the $InsP_3R$ type III is localized to the apical pole of salivary gland cells [36], nonpigmented ciliary epithelia [37], and pancreatic acinar cells [37–40]. This region has been called the trigger zone, because Ca^{2+} signals originate in this region in each of these cell types [36,37,41,42]. Since $InsP_3R$ type I is localized to the basolateral pole in nonpigmented ciliary epithelia, the observation that Ca^{2+} waves begin apically in those cells suggests that the type III isoform is more prone to initiate Ca^{2+} signals when the two isoforms are co-expressed in intact cells [37]. Ca^{2+} signaling in pancreatic acinar cells has been studied in more detail, but that cell system is complicated by the fact that all three $InsP_3R$ isoforms are present at the apical pole [36,39]. It had been proposed that Ca^{2+} signaling in acinar cells could be explained by a two-pool model, with $InsP_3R$ type I gating the low-threshold pool to initiate the Ca^{2+} signal and $InsP_3R$ type III gating the high threshold pool to amplify or enhance the signal. However, recent evidence based upon preferential phosphorylation of the type III isoform by PKA [42] suggests that this isoform instead is responsible for localized signal initiation in pancreatic acinar cells. It appears contradictory that the isoform with the lowest affinity for $InsP_3$ in binding [7,18], single channel experiments [11,13], and Ca^{2+} release from permeabilized cells [43] would be responsible for triggering Ca^{2+} release in epithelial cells. However, plasma membrane receptors are concentrated along the lateral membrane in pancreatic acinar cells, whereas $InsP_3Rs$ are concentrated at the apical pole, and this juxtaposition may abrogate the need for the generation of large amounts of $InsP_3$ throughout the cell in order to activate Ca^{2+} release [44]. In addition, the lack of Ca^{2+}-dependent inhibition in the type III isoform allows for autocatalytic release of Ca^{2+} from localized intracellular stores [45]. $InsP_3R$ type I located nearby can then support Ca^{2+} oscillations to exert distinct effects on the cell. Although a biphasic Ca^{2+}-dependence has been observed in some lines of RIN cells [46], those cells contain a significant amount of $InsP_3R$ type I (17%, see [46]) which may dominate the response to changes in cytoplasmic Ca^{2+}. The properties of the $InsP_3R$ type III allows this isoform to be activated in situations where other $InsP_3R$ isoforms are less likely to open.

There are circumstances under which the type III $InsP_3R$ may not be the isoform responsible for preferential triggering of Ca^{2+} signals. In neuronal cells activation of RyRs and voltage-dependent Ca^{2+} channels on the plasma membrane play major roles in Ca^{2+} signaling [1]. The ubiquitous localization of RyR and voltage-dependent Ca^{2+} channels in neurons suggests that the $InsP_3$ concentration may become a more critical factor in determining the site of signal initiation in such cells. In this case, the $InsP_3R$ type I would be expected to become the prime target for signal initiation due to the higher $InsP_3$ affinity of this isoform relative to the $InsP_3R$ type III, and this indeed has been observed [47]. An additional factor in neurons is that the $InsP_3R$ type III is concentrated in the soma and lacking at the neurites whereas $InsP_3R$ type I are found in both the soma and neurites [47]. The observation that intracellular Ca^{2+} signals begin at the neurites in these cells [47], even though the $InsP_3R$ type I is found throughout the cell suggests that heterotetrameric complexes of the $InsP_3R$ may exhibit complex behavior, or else additional cellular processes are needed to explain the basis of signal initiation in neurons.

In conclusion, we show that ATP-stimulated Ca^{2+} release from intracellular stores occurs exclusively through $InsP_3$-gated channels. Moreover, we show that the response curve to ATP can be shifted to a higher sensitivity by the co-agonist properties of Ca^{2+} and $InsP_3$ on the $InsP_3R$ type III. That the $InsP_3R$ type III could initiate Ca^{2+} signals in secretory cells has been proposed by several lines of evidence [12,42,45]. In this paper additional important support is provided for the role of the type III receptor isoform in secretory cells by showing that focal release of Ca^{2+} facilitates a global Ca^{2+} signal. Thus, the properties of the $InsP_3R$ type III show that this isoform is well suited to function as a trigger for $InsP_3$-mediated Ca^{2+} signaling in secretory cells.

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