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THE ACTIN CYTOSKELETON DIFFERENTIALLY REGULATES NG115-401L CELL RYANODINE RECEPTOR AND INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR INDUCED CALCIUM SIGNALING PATHWAYS

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Abstract

Regulation of bi-directional communication between intracellular Ca^{2+} pools and surface Ca^{2+} channels remains incompletely characterized. We report Ca^{2+} release mediated by inositol 1,4,5-trisphosphate receptor (IP_3R) and ryanodine receptor (RyR) pathways is diminished under actin cytoskeleton disruption in NG115-401L (401L) neuronal cells, yet despite truncated Ca^{2+} release, Ca^{2+} influx was not significantly altered in these experiments. However, disruption of cortical actin networks completely abolished IP_3R induced Ca^{2+} release, whereas RyR mediated Ca^{2+} release was preserved, albeit attenuated. Moreover, cortical actin disruption completely abolished IP_3R and RyR linked Ca^{2+} influx even though Ca^{2+} pool sensitivities were different. These findings suggest discrete Ca^{2+} store/ Ca^{2+} channel coupling mechanisms in the IP_3R and RyR pathways as revealed by the differential sensitivity to actin perturbation.

Keywords

Ca^{2+} ; actin; cytoskeleton; inositol 1,4,5-trisphosphate receptor; ryanodine receptor; Ca^{2+} release; Ca^{2+} influx

INTRODUCTION

Activation of a wide group of plasma membrane receptors by hormone, neurotransmitter and growth factor stimulants produces appropriate cellular responses in part via elevation of free cytoplasmic Ca^{2+} levels. The initial increase in Ca^{2+} is due to the release of Ca^{2+} from the inositol 1,4,5-trisphosphate (IP_3)-sensitive and ryanodine receptor (RyR)-regulated pools of Ca^{2+} thought to be contained in the endoplasmic reticulum (ER) [1]. The release of Ca^{2+} from the ER is accompanied by influx of Ca^{2+} from the extracellular milieu and this Ca^{2+} influx response is frequently mediated by plasma membrane (PM) store operated Ca^{2+} (SOC) channels [2]. The activation of some SOC pathways is dependent on the state of filling of the internal ER Ca^{2+} stores, but these pathways continue to be poorly understood. Indirect and

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direct coupling between the ER and the PM channels have both been considered as possible mechanisms activating SOC [2;3;4;5;6]. Indirect coupling involves the production of a diffusible messenger from the ER following store-depletion; but this messenger pathway has yet to be identified. Direct coupling proposes a physical interaction between the ER and the PM channels, analogous to the excitation-contraction coupling mechanism between RyRs and PM localized dihydropyridine receptors in skeletal muscle [7]. This type of conformationally-coupled mechanism between IP₃R and PM SOC channels is also regulated by ER Ca²⁺ content, and thus activated by signals that deplete ER Ca²⁺ stores. A type of conformationally coupled pathway has also recently been described wherein the ER localized stromal interacting molecule 1 (STIM 1) transmits depleted ER Ca²⁺ stores to activation of the Orai1 channel component of the Ca²⁺-Release-Activated Ca²⁺ (CRAC) channel [8;9].

We have previously reported the characterization of unique Ca²⁺ signaling pathways in the NG115-401L (401L) neuronal cell line that has contributed a better understanding of the elusive mechanisms regulating ER Ca²⁺ store coupling to PM Ca²⁺ influx responses [10]. Our most recent study described an unusual effect of the compound 2-aminoethoxydiphenyl borate (2-APB) in promoting the activation of a Ca²⁺ release pathway requiring close physical communication with PM channels/elements, given the high sensitivity to treatments interfering with actin cytoskeleton function or membrane integrity in general [11]. In this report we sought to extend these findings by examining the role of the actin cytoskeleton in regulating hormone and neurotransmitter pathways that promote Ca²⁺ release along with coupled Ca²⁺ influx responses in 401L cells. Our results suggest an intriguing pattern of differential sensitivity to actin perturbation depending on the cellular sites of actin disruption and whether Ca²⁺ release is induced by IP₃R or RyR activators.

MATERIALS AND METHODS

Materials

Ryanodine and calyculin A were purchased from LC Laboratories (Woburn, MA). Bradykinin, ATP and poly-l-lysine were from Sigma (St. Louis, MO). 2,2',3,5',6-pentachlorobiphenyl (PCB95) was purchased from Ultra Scientific (North Kingstown, RI). Cytochalasin D was purchased from Tocris (Ellisville, MO).

Cell Culture

NG115-401L neuroblastoma cells were maintained in DMEM supplemented with 10% Fetal Bovine Serum, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin. For Ca²⁺ measurements, NG115-401L cells were seeded onto poly-l-lysine coated coverslips at a cell density of $1.5-2 \times 10^6$ cells/3 ml.

Calcium Measurements

Monolayer cultures of NG115-401L cells were seeded on 9×22 mm coverslips and loaded with 1.5 µM Fura-2/AM for 30 minutes at room temperature. Changes in cytosolic Ca²⁺ were measured in cell population experiments using a fluorescence spectrophotometer (PTI). For cells loaded with Fura-2/AM, excitation of the dye was achieved by rapidly alternating monochromator settings between 340 and 380 nm with fluorescence emission measured at 510 nm. Changes in cytosolic Ca²⁺ concentrations are reported as the ratio of the fluorescence values for Fura-2 taken at 340 nm and 380 nm (Ratio F₃₄₀/F₃₈₀).

Statistical Analysis

The data are presented as the mean experimental values with statistical variation indicated by the standard error of the means ($\pm$ S.E.M.) with the number of experimental repetitions indicated in parentheses.

RESULTS

For the following results Ca^{2+} responses are presented as the changes in the fluorescence ratio values measured at 340/380 nm for Fura-2. The data are reported as either peak amplitude changes in fluorescence values or as initial rates of fluorescence changes and presented as the means $\pm$ S.E.M., with the number of experimental repetitions indicated in parentheses.

A. Global Actin Perturbation in NG115-401L Cells Diminishes $\text{IP}_3\text{R/RyR}$ - Mediated Ca^{2+} Release Without Altering Ca^{2+} Influx Pathways

The inhibition of actin polymerization leads to cytoskeletal disruption and the formation of tight bundles of actin. Pretreatment of 401L cells with 10 μM cytochalasin D for 30 min (at 37 $^{\circ}\text{C}$) induced considerable alteration in the appearance of the stress fibers. Cytochalasin D treatment triggers the formation of tightly condensed bundles of actin fibers in discrete pockets or regions of the 401L cytoplasm (not shown). The appearance of actin bundle accumulations in the cytoplasm of cytochalasin D treated 401L cells reflects global damage and disassembly of actin fibers.

In our experiments, we used the pro-inflammatory cytokines bradykinin and ATP as physiological activators of the phospholipase C/ IP_3 pathway coupled to IP_3R -mediated Ca^{2+} release from the ER. As shown in Figure 1A the addition of 1 μM bradykinin in a Ca^{2+} -free medium mobilized a rapid IP_3 -mediated Ca^{2+} release in the 401L cells (1.22 ± 0.18 fluorescence units, $n=6$). Following the decay of the signal to baseline, restoration of extracellular Ca^{2+} (2 mM) produced a Ca^{2+} influx response (0.57 ± 0.17 fluorescence ratio units/minute, $n=6$) suggesting the activation of a SOC pathway in the 401L cells (Figure 1A). Preincubation of 401L cells with cytochalasin D (10 μM , 30 min at 37 $^{\circ}\text{C}$) decreased the IP_3 -mediated Ca^{2+} release response by 51% (0.63 ± 0.18 fluorescence units, $n=6$, $p<0.05$, Figure 1A). In order to test the activity of SOC in the cytochalasin D treated cells we added 2 mM Ca^{2+} following the decay of the transient bradykinin-induced Ca^{2+} release response. The Ca^{2+} influx response in cytochalasin D treated 401L cells was not significantly different from untreated cells stimulated with bradykinin (0.55 ± 0.11 fluorescence ratio units/minute, $n=6$, Figure 1A). The damage to the actin filament network attenuated the bradykinin-mediated release of Ca^{2+} but did not affect coupled Ca^{2+} influx responses. We also tested the ability of other activators of the IP_3 pathway, such as ATP, to release ER Ca^{2+} and activate SOC following cytochalasin D-mediated inhibition of actin polymerization. Figure 1B shows that the addition of 100 μM ATP in a Ca^{2+} -free medium triggered a rapid increase of cytoplasmic Ca^{2+} from intracellular stores (1.04 ± 0.24 fluorescence units, $n=8$). The addition of 2 mM Ca^{2+} to the extracellular medium in the ATP-stimulated cells activated Ca^{2+} influx responses (0.43 ± 0.08 ratio units/minute, $n=6$), presumably mediated by SOC pathways in 401L cells (Figure 1B). However, like bradykinin responses, pretreatment with cytochalasin D caused a significant attenuation of the ATP-mediated Ca^{2+} release response to 55% of its control value (0.58 ± 0.22 fluorescence units, $n=6$, $p<0.05$). As shown in Figure 1B, the induction of SOC-mediated Ca^{2+} influx activation upon addition of 2 mM Ca^{2+} remained unchanged in the cytochalasin D treated 401L cells stimulated with ATP (0.41 ± 0.11 ratio units/minute, $n=6$).

We have previously reported that activators of ryanodine receptors couple robustly to Ca^{2+} influx pathways in the 401L cell line [10]. In this study we also examined the sensitivity of RyR-mediated Ca^{2+} responses to actin perturbation. To test whether RyR-mediated Ca^{2+}

responses are regulated by the actin cytoskeleton, we stimulated 401L cells with the pharmacological RyR activators ryanodine and pentachlorobiphenyl 95 (PCB95). In our previous study, we documented the activation of Ca^{2+} signaling pathways by various RyR agonists (ryanodine, 4-chloro-*m*-cresol, caffeine and PCB95) [10]. In the present study we used ryanodine and PCB95 as robust activators of RyRs that target different sites on the receptor/channel complex [12].

Figure 1C shows that stimulation of 401L cells with 1 μM ryanodine in Ca^{2+} -free media induced Ca^{2+} release responses from the RyR-sensitive ER Ca^{2+} pool (1.12 ± 0.18 fluorescence units, $n=6$). The RyR-mediated discharge response is accompanied by an influx of Ca^{2+} upon addition of 2 mM Ca^{2+} to the extracellular medium (0.65 ± 0.10 ratio units/minute, $n=6$, Figure 1C). In the cytochalasin D treated 401L cells, the addition of 1 μM ryanodine produced a Ca^{2+} release response that was decreased to 32% of the response compared to cells not exposed to actin disruption (0.35 ± 0.10 fluorescence units, $n=6$, $p < 0.05$). After 350 seconds, 2 mM Ca^{2+} was added back to the bathing medium to measure Ca^{2+} influx. We observed that the activation of a Ca^{2+} influx response in the ryanodine-stimulated cytochalasin D treated cells was not significantly different from the control untreated 401L cells (0.64 ± 0.14 ratio units/minute, $n=6$, Figure 1C).

PCB95 has been identified as an agent that alters the association between the FKBP/RyR complex and mediates RyR-sensitive Ca^{2+} release [13]. As shown in Figure 1D, the addition of 10 μM PCB95 increased $[\text{Ca}^{2+}]_i$ due to the release of Ca^{2+} from the RyR-sensitive ER Ca^{2+} pools (1.02 ± 0.22 fluorescence units, $n=8$). However, in the cytochalasin D treated 401L cells the addition of PCB95 produced a significantly attenuated increase in Ca^{2+} (0.39 ± 0.15 fluorescence units, $n=6$, $p < 0.05$). The treatment with cytochalasin D reduced the PCB95-induced $[\text{Ca}^{2+}]_i$ increase from the ER stores to 38% of its control value (Figure 1D). Calcium influx activated by the addition of 2 mM Ca^{2+} to PCB95 stimulated cells was not significantly different between the cytochalasin D-treated and untreated 401L cells (cytochalasin D treated response 0.55 ± 0.22 vs untreated response 0.58 ± 0.19 ratio units/minute, $n=6$, Figure 1D).

B. Condensation of cortical actin results in pronounced inhibition of 401L cell release and influx responses

To further clarify the role of the cytoskeleton in the release and activation of Ca^{2+} influx in 401L cells, we chose to use calyculin A, an agent that interferes with the communication between the ER and the PM channels [14;15]. Treatment of 401L cells with calyculin A (100 nM, 37°C) for 1 hr induced a redistribution of actin filaments, with the disappearance of the uniformly distributed stress fibers leading to a tight condensation of actin filaments in the vicinity and subjacent to the PM (not shown). The addition of 1 μM bradykinin to calyculin A treated cells in Ca^{2+} -free media failed to trigger the mobilization of Ca^{2+} stored in the IP_3 -sensitive Ca^{2+} pools (Figure 2A, $n=6$). After 350 seconds, 2 mM Ca^{2+} was added back to the medium in the bradykinin stimulated cells, but this treatment failed to activate Ca^{2+} influx in 401L cells exposed to calyculin A in contrast to responses observed when actin was disrupted with cytochalasin D or latrunculin A (Figure 2A, $n=6$). We observed a similar pronounced inhibition of Ca^{2+} release and influx responses when cells were treated with 100 μM ATP. As shown in Figure 2B, stimulation of the calyculin A treated 401L cells with 100 μM ATP in Ca^{2+} -free media failed to induce an IP_3 -mediated increase in $[\text{Ca}^{2+}]_i$ ($n=6$). Calyculin A-induced redistribution of actin filaments to the PM also abolished ATP-activated Ca^{2+} influx responses, detectable with the addition of 2 mM Ca^{2+} to the extracellular medium (Figure 2B, $n=6$).

We also investigated the effects of cortical actin condensation on RyR-mediated Ca^{2+} responses. In contrast to results observed for bradykinin and ATP stimulation, pretreatment of 401L cells with calyculin A did not abolish Ca^{2+} release responses induced by 1 μM ryanodine,

although they were significantly attenuated compared to cells not exposed to calyculin A (calyculin A treated: 0.41 ± 0.14 vs untreated cells: 1.04 ± 0.12 fluorescence units, $n=6$, $p<0.05$, Figure 2C). Despite the ability of ryanodine to stimulate an attenuated increase in $[Ca^{2+}]_i$ in calyculin A treated cells, restoration of extracellular Ca^{2+} (2 mM) failed to activate measurable Ca^{2+} influx responses (Figure 2C, $n=6$). The addition of 10 μ M PCB95 in a Ca^{2+} free medium induced Ca^{2+} release responses that were, like ryanodine, attenuated but not abolished following treatment of 401L cells with calyculin A (calyculin A treated: 0.43 ± 0.18 vs untreated cells: 0.93 ± 0.15 fluorescence units, $n=6$, $p<0.05$, Figure 2D). In contrast to untreated cells, restoration of 2 mM Ca^{2+} to the bathing medium failed to activate Ca^{2+} influx responses in calyculin A treated 401L cells following PCB95-mediated Ca^{2+} discharge from RyR-sensitive pools (Figure 2D, $n=6$).

C. Restoration of Ca^{2+} responses via cytochalasin D-mediated reversal of calyculin A induced actin condensation

Our results revealed that treatment of 401L cells with calyculin A abolished the IP_3 -mediated release and influx of Ca^{2+} , suggesting that these processes depend upon dynamic actin rearrangement. Calyculin A-induced cortical actin condensation acts as a physical barrier restricting the interaction between the ER and the PM channels, eradicating both channel-mediated Ca^{2+} release and influx pathways. Given that stabilization of cortical actin prevented Ca^{2+} release and influx, we sought to test whether cytochalasin D treatment could restore either Ca^{2+} release or influx or perhaps both responses [16].

As mentioned previously, treatment of 401L cells with calyculin A resulted in the formation of tight cortical actin layers near the PM. However, when calyculin A treated cells were subjected to 30 min cytochalasin D treatment the cortical actin condensation bundles were disbanded and a more general redistribution of actin was observed (not shown). The dense band of cortical actin disappeared and bundles of actin, typical of cytochalasin D treatment, appeared especially near the PM of the 401L cells.

As shown in Figure 2, treatment of 401L cells with 100 nM calyculin A for 1 hr prevented any stimulus-mediated changes in $[Ca^{2+}]_i$, abolishing IP_3 -sensitive release and the subsequent influx of Ca^{2+} . However, in 401L cells treated with cytochalasin D to reverse condensation of actin induced by calyculin A, the addition of 1 μ M bradykinin in Ca^{2+} -free media, resulted in the recovery of an IP_3 -mediated Ca^{2+} release response (0.85 ± 0.19 fluorescence units, $n=5$, Figure 3A). Following the decay of the bradykinin-induced signal to baseline, we tested Ca^{2+} influx responses by increasing extracellular Ca^{2+} to 2 mM. Adding back extracellular Ca^{2+} resulted in the reappearance of a Ca^{2+} entry pathway (0.44 ± 0.13 ratio units/minute, $n=5$, Figure 3A). We have also shown that IP_3 -sensitive Ca^{2+} release inducible by 100 μ M ATP addition was abolished in 401L cells following calyculin A treatment. In contrast, ATP (100 μ M) exposure was able to mobilize stored Ca^{2+} when cytochalasin D was added subsequent to the actin damage induced by calyculin A. Moreover, in contrast to cells treated with calyculin A only, the addition of 2 mM Ca^{2+} to both calyculin A and cytochalasin D treated 401L cells was able to restore Ca^{2+} influx responses (not shown).

Since the disassembly of cortical actin by cytochalasin D was able to restore IP_3 -mediated Ca^{2+} release and subsequent SOC activity in 401L cells, we next investigated whether actin disassembly could also restore RyR-mediated Ca^{2+} release and influx activity. Condensation of actin filaments at the PM by calyculin A treatment resulted in attenuated RyR-mediated Ca^{2+} release responses but also led to complete abolition of SOC activity in 401L cells. As shown in Figure 3B, the addition of 1 μ M ryanodine to 401L cells treated with calyculin A and cytochalasin D stimulated an increase in $[Ca^{2+}]_i$ (0.53 ± 0.23 fluorescence units, $n=4$) similar to cells treated with calyculin A alone. Following RyR-mediated Ca^{2+} discharge with ryanodine, Ca^{2+} influx activity was tested by adding back 2 mM Ca^{2+} to the bathing medium.

Unlike cells treated with calyculin A alone, the addition of 2 mM Ca^{2+} to 401L cells co-treated with calyculin A and cytochalasin D resulted in the reappearance of Ca^{2+} influx responses similar to those observed in the control cells (0.43 ± 0.18 ratio units/minute, $n=4$, Figure 3B). We also observed a similar recovery of Ca^{2+} influx in the calyculin A and cytochalasin D co-treated cells when the RyR activator PCB95 (10 μM) was used (not shown). As previously shown in Figure 2F, stimulation with 10 μM PCB95 failed to activate SOC activity in calyculin A treated cells. However, disassembly of the tight actin filaments by cytochalasin D-mediated reversal of actin damage induced by calyculin treatment was able to restore Ca^{2+} influx activity in the 401L cells stimulated with either ryanodine or PCB95.

DISCUSSION

We have previously reported that the NG115-401L neuronal cell line reveals an unusual Ca^{2+} signaling phenotype, that has yielded additional insight into how Ca^{2+} release pathways couple to the activation of more protracted and sustained Ca^{2+} influx responses. We recently reported, for example, that the compound 2-APB mimics a hormone stimulant in 401L cells via its actions to induce Ca^{2+} release and promote Ca^{2+} influx in a manner indistinguishable from the pro-inflammatory cytokines bradykinin and ATP [11]. Moreover, 2-APB's effects on 401L cells suggest that the compound targets a Ca^{2+} release site that physically interacts with PM components of Ca^{2+} influx pathways, given the high sensitivity of this target to actin disruption or other perturbants of ER/PM interaction. In this study we have examined whether IP_3R and RyR-mediated Ca^{2+} release pathways also display a similar strict dependence on an intact actin cytoskeleton for their role in regulating both Ca^{2+} release and also as robust activators of Ca^{2+} influx. Here we show that depending on how the actin cytoskeleton is perturbed we can discriminate between Ca^{2+} release and Ca^{2+} influx responses. We find that in 401L cells the initial Ca^{2+} release pathways (both for IP_3R and RyR-induced Ca^{2+} release) appear more sensitive to general actin perturbation than Ca^{2+} influx responses, as Ca^{2+} release signals are significantly attenuated although not completely eradicated when cells are treated with cytochalasin D. However, activation of both IP_3Rs and RyRs induce sufficient Ca^{2+} release, albeit diminished, to promote the formation of a fully functional Ca^{2+} influx pathway, even though the cell demonstrates clear disruption of dynamic actin function. These findings are again consistent with our earlier observations that the 401L cell appears to have a unique and strict dependence on the initial activation of intracellular Ca^{2+} -release channels, even though Ca^{2+} release itself may be significantly truncated. And moreover, the activation of Ca^{2+} release units (whether activated by hormones, RyR activators or 2-APB) appear highly sensitive to ER/PM integrity, suggesting that a functional Ca^{2+} -release structure requires coupling between ER and PM components, a process more commonly proposed to occur after Ca^{2+} release in the activation of SOC-based Ca^{2+} influx pathways.

Our data using calyculin A reveals critical actin-dependent changes occur in peripheral cortical regions that are likely to be necessary for forming functional Ca^{2+} influx pathways, as both IP_3R or RyR-activated Ca^{2+} influx is abolished by calyculin A treatment. Unexpectedly, we find that there is a differential sensitivity of the IP_3R and RyR intracellular Ca^{2+} release channels to cortical actin disruption with RyR-mediated Ca^{2+} release still preserved while IP_3R -mediated release is entirely abolished. This result is initial data to suggest that perhaps RyRs communicate with PM SOC elements that combine to form a Ca^{2+} release unit less dependent on cortical actin rearrangements than the corresponding $\text{IP}_3\text{R}/\text{SOC}$ functional unit. These observations are in keeping with an emerging pattern that explains diversity in SOC channel properties depending on which channel isoforms are recruited by ER localized Ca^{2+} release channels. The further characterization of the 401L cell signaling phenotype promises to add additional insight into the complex regulation and relationship of intracellular Ca^{2+} release pathways to coupled Ca^{2+} influx responses.

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Figure 1. Effect of cytochalasin D on receptor activated Ca^{2+} release and Ca^{2+} entry in 401L cells
Cells were treated with cytochalasin D (10 μM , 30 min) in HBSS. The cytochalasin D treated (solid trace) and control 401L cells (dotted trace) were stimulated with bradykinin (1 μM) (A), ATP (100 μM) (B), ryanodine (5 μM) (C) and PCB95 (10 μM) (D). Ca^{2+} influx was initiated by the addition of 2 mM Ca^{2+} to the medium. The traces are representative of six individual experiments.

Figure 2. Calyculin A induced actin redistribution prevents activation of store-operated Ca^{2+} entry in 401L cells

Cells were treated with calyculin A (100 nM, 1 hr) in HBSS at 37°C. The calyculin A treated (solid trace) and control 401L cells (dotted trace) were stimulated with bradykinin (1 μM) (A), ATP (100 μM) (B), ryanodine (1 μM) (C) and PCB95 (10 μM) (D). Ca^{2+} (2mM) was added to the medium to initiate Ca^{2+} influx in the latrunculin A treated and control 401L cells. The traces are representative of six individual experiments.

Figure 3. Restoration of Ca^{2+} release and influx by disrupting the calyculin A induced cortical actin layer in 401L cells

Cells were treated with calyculin A (100 nM, 1 hr) in HBSS at 37°C. The calyculin A treated cells were further treated with cytochalasin D (10 μM , 30 min) at 37°C. Cells treated with both calyculin A and cytochalasin D (dotted trace) and calyculin A treated 401L cells (solid trace) were stimulated with bradykinin (1 μM) (A) and ryanodine (1 μM) (B). Ca^{2+} (2mM) was added to the medium to initiate Ca^{2+} influx. The traces are representative of six individual experiments.