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The Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) Activator CDN1163 Exerts Complex Time-Dependent and SERCA Isoform-Specific Effects on T Lymphocyte Ca^{2+} Store Functions

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Abstract: The allosteric SERCA (Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase) activator CDN1163 has been recently added to the group of pharmacological tools for probing SERCA function. We chose to investigate the effects of the compound on T lymphocyte Ca^{2+} stores, using the well-described Jurkat T lymphocyte as a reliable cell system for Ca^{2+} signaling pathways. Our study identified the lowest concentrations of the SERCA inhibitors thapsigargin (TG) and 2,5-di-(*tert* butyl)-1,4-benzohydroquinone (tBHQ) capable of releasing Ca^{2+}, permitting the differentiation of the TG-sensitive SERCA 2b Ca^{2+} store from the tBHQ-sensitive SERCA 3 Ca^{2+} store. We proceeded to test the effects of CDN1163 on Ca^{2+} stores, examining specific actions on the SERCA 2b and SERCA 3 Ca^{2+} pools using our low dose SERCA blocker regimen. In contrast to previous work, we find CDN1163 exerts complex time-sensitive and SERCA isoform-specific actions on Ca^{2+} stores. Surprisingly, short-term exposure (0-30 minutes) to CDN1163 perturbs T cell Ca^{2+} stores by suppressing Ca^{2+} uptake with diminished Ca^{2+} release from the SERCA 2b-controlled store. Concomitantly, we find evidence for a SERCA-activating effect of CDN1163 on the SERCA 3 regulated store, given the observation of increased Ca^{2+} release inducible by low dose tBHQ. Intriguingly, longer-term (>12 hours) CDN1163 exposure reversed this pattern, with increased Ca^{2+} release from SERCA 2b-regulated pools, yet decreased Ca^{2+} release responses from tBHQ-sensitive SERCA 3 pool. Our results reveal differential effects of CDN1163 on Ca^{2+} stores regulated by distinct SERCA isoforms, prompting the need for careful interpretation of the compound's effects, particularly in cells expressing multiple SERCA isoforms.

Keywords: Ca^{2+} Stores; SERCA Regulation; ER Ca^{2+} Pools; T Cell Activation

1. Introduction

The T lymphocyte is the chief orchestrator of the adaptive immune system, coordinating and regulating the multi-level interactions and deployment of both cell-mediated immunity and antibody responses [1]. T cells initiate the critical early stages of an immune response via the signaling reactions induced by T cell receptor (TCR) binding to an antigenic stimulus. A major component of the antigen-activated TCR signaling pathway is an early rapid increase in cytosolic Ca^{2+} levels deriving from intracellular Ca^{2+} storage sites as well as activation of tightly coupled Ca^{2+} influx pathways [2–4]. T cell activation, thus relies heavily on the functional integrity of intracellular Ca^{2+} stores, generally thought to reside in the endoplasmic reticulum (ER). Regulation and maintenance of ER Ca^{2+} levels is therefore essential for activation of the TCR pathway, and the central family of ion-transporting enzymes mediating these functions are the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) [4–7]. The SERCA Ca^{2+}-ATPases or Ca^{2+} pumps have attracted much interest as potential

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targets for drug modulation in disease states given their prominent role in contributing to Ca^{2+} release/uptake events integrated within the TCR-induced signaling framework [8–16].

In this study we have sought to further characterize the pharmacology of SERCA-regulated Ca^{2+} stores in T lymphocytes. There is a clear imperative to gain a better understanding of the roles played by Ca^{2+} stores as essential regulators of the complex spatiotemporal Ca^{2+} signal underlying critical early signaling events driving T cell activation [17–19]. A powerful approach to probing Ca^{2+} store functions in T cell signaling networks is to modulate SERCA pump function using an array of small molecule pharmacological agents that can potentially provide a means for fine control of the various SERCA pump states as the primary regulators determining ER Ca^{2+} store levels [9,11,12,16,19].

Much has been learned about Ca^{2+} stores regulation using the classic thapsigargin, cyclopiazonic acid and 2,5-di-(*tert* butyl)-1,4-benzohydroquinone trio of SERCA blockers, clearly validating the profitable application of SERCA pharmacology in efforts to dissect Ca^{2+} signaling mechanisms [20–23]. Thus to further augment our tools for SERCA pump modulation, there is compelling interest to complement the SERCA inhibitors with compounds that can increase SERCA activity. We can potentially achieve a greater insight into the complex roles and functions of ER Ca^{2+} stores by utilizing pharmacological agents that can both downregulate as well as upregulate SERCA functional activity. At present, CDN1163 appears to be the best pharmacological agent with the capacity to increase SERCA activity, having been shown to exert a significant boost in SERCA enzymatic action in muscle and nonmuscle SERCA isoforms [8,12,15,19,24–26]. We were thus motivated to examine the effects of CDN1163 on Ca^{2+} stores in T cells, the critical central coordinator of the adaptive immune system with a uniquely pronounced dependency on ER Ca^{2+} stores and Ca^{2+} influx pathways underlying T cell activation.

2. Materials and Methods

2.1. Materials

Fura 2/AM (fura 2 acetoxymethylester), Fluo-3 pentapotassium salt, pluronic acid, RPMI-160, fetal bovine serum (FBS), streptomycin, and penicillin were obtained from Thermo Fisher. Ryanodine, oligomycin, thapsigargin, cytochalasin D were obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas). D-myo-Inositol 1,4,5 trisphosphate K salt (IP3), nicotinic acid adenine dinucleotide phosphate sodium salt (NAADP), phytohemagglutinin (PHA), 2,5-di-(*tert* butyl)-1,4-benzohydroquinone (tBHQ), thrombin, creatine phosphokinase (CPK), phosphocreatine disodium salt hydrate, adenosine 5′-triphosphate disodium salt hydrate (ATP), DTT, and saponin were obtained from Sigma. Sterile Cell Strainers (100 μm), 50 ml syringe tubes and 60 mm cell culture dishes were from Fisher Scientific. CDN1163 was from Bio-Techne (Minneapolis, USA).

2.2. Cell Culture

Jurkat cells (Clone E6-1, ATCC TIB-152) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) and grown at 37 °C in a humidified atmosphere (95% air,5% CO_2). Cells were maintained and expanded in either 25 cm^2 (T25) or 75 cm^2 (T75) tissue culture flasks (Fisher Scientific). Cell density was not allowed to exceed 3 X 10^6 cells/mL and cultures were maintained at a cell concentration between 1 x 10^5 and 1 X 10^6 viable cells/mL. Fresh medium was added every 2 to 3 days depending on cell density.

2.3. Splenocyte Isolation

The use of animals for these experiments was conducted in accord with protocols approved by the institutional animal care and use committees at the University of the Pacific. Spleen and lymph nodes were aseptically isolated from adult Sprague-Dawley (SD) rats (8 weeks old, N=4). Briefly, rats were anesthetized and spleens were aseptically removed and placed into 60-mm cell culture dishes containing ice-cold HBSS (Hanks Balanced Salt Solution) and minced into small pieces with a scissor. Tissue fragments were dissected and passed through a 100-μm cell strainer using a 10-ml syringe

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plunger and ice-cold HBSS into a 50-ml conical tube and then centrifuged (200g at 4°C) for 10 min. The supernatant was discarded, and the pellet was resuspended in 5 ml of a red blood cell (RBC) lysis buffer containing 155 mM NH_4Cl (9 parts) in 130 mM Tris base pH 7.65 (1 part) and incubated at 37°C for 5 min. RBC lysis was halted by the addition of 10 ml ice-cold complete cell culture medium and cells were then centrifuged (200g at 4°C) for 10 min. The supernatant was discarded, and the pellet was resuspended in 10 ml complete cell culture medium and maintained in a humidified atmosphere (37°C, 95% air and 5% CO_2).

2.4. Cell Calcium Assays

Cells (approximately 1×10^6 cells/ml) were washed in Ca^{2+}-containing (1.8 mM) HBSS (Hanks Balanced Salt Solution) and loaded with 1.5 μM fura-2/AM in 20% (w/v) Pluronic F-127 and incubated for one hour at 37°C. After loading, the cells were washed twice with HBSS and incubated at 37°C for an additional 30 min to allow for de-esterification of the dye. Cells loaded with fura 2/AM were kept in the dark at room temperature throughout the experiments. Changes in cytosolic Ca^{2+} were measured in cell population experiments using a fluorescence spectrophotometer equipped with a thermostatically controlled sample compartment, permitting continuous stirring of samples in the cuvette. All measurements were carried out at room temperature (25°C). To achieve Ca^{2+}-free conditions, EGTA (2 mM) was added to chelate extracellular Ca^{2+} just before the addition of Ca^{2+} mobilizing agonists (1-2 min). Ca^{2+} changes in Jurkat cells and rat splenocytes loaded with fura 2/AM were measured via rapid alternation of the excitation monochromator between 340 and 380 nm, with fluorescence emission measured at 510 nm using a ratiometric spectrofluorimiter (PTI). Cytosolic Ca^{2+} responses are presented as the changes in the fluorescence ratio values measured at 340/380 nm for Fura-2, or as non-ratiometric Fluo-3 fluorescence changes for the excitation/emission (503/530 nm) wavelength pair. The data are reported as either peak amplitude changes in fluorescence values or as initial rates of fluorescence changes and presented as the means ± S.E.M., with the number of experimental repetitions indicated in parentheses.

2.5. Permeabilized Cell Assays

For preparation of permeabilized cells, 4×10^7 cells were washed twice and resuspended in 2 ml of an intracellular-like medium (110 mM-KCl, 10 mM-NaCl, 2 mM-$MgCl_2$, 20 mM-Hepes, 5 mM-KH_2PO_4, pH 7.5) in the presence of 1 mM DTT. Saponin (20 μg/ml) was added, and the cell suspension was incubated for 5 min at 37°C to complete permeabilization. An ATP-regenerating system consisting of creatine kinase (40 units/ml) and phosphocreatine (20 mM) was added. Oligomycin (10 μg/ml) was also included to inhibit the mitochondrial ATPase. Following cell permeabilization, Fluo-3 (0.5 μM), was added to the cuvette. Subsequent addition of ATP to a final concentration of 1 mM resulted in a decrease in the fluorescence, indicating Ca^{2+} uptake by the intracellular stores. After baseline stabilization, drugs were added according to the experimental plan. Ca^{2+} release from intracellular stores was measured from cells suspended in cuvettes using a fluorescence spectrophotometer equipped with a thermostatically controlled sample compartment maintained at 37°C with continuous stirring. Fluorescence changes with Fluo-3 in permeabilized cell suspensions were measured with excitation wavelength settings of 503 nm and 530 nm for the emission wavelength.

2.6. Statistical Analysis

analysis of statistical significance was performed using the Student's T test. P values ≤ 0.05 were considered to represent significant differences in the results

3. Results

3.1. Low Concentrations of TG and tBHQ Induce Ca^{2+} Release in T Cells and Establish a Pharmacological Regimen for Specific Blockade of SERCA 2b and SERCA 3

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Jurkat lymphocytes and rat splenocytes were loaded with Fura 2 to measure intracellular Ca^{2+} changes in response to SERCA drugs and other agents that mobilize cytoplasmic Ca^{2+}. Experiments were conducted in the absence of extracellular Ca^{2+}, except where indicated, to directly assess Ca^{2+} release events and Ca^{2+} store status without the need to consider Ca^{2+} elevation due to Ca^{2+} influx. To acquire additional information on Ca^{2+} release and Ca^{2+} store content as well as to utilize Ca^{2+} mobilizing agents impermeable to plasma membrane (PM) we also conducted experiments using Jurkat cells in permeabilized membrane assays.

In order to investigate the effects of the SERCA activator CDN1163 on T cell Ca^{2+} stores we sought to characterize SERCA regulated Ca^{2+} store function using Jurkat lymphocytes as the model system for T cell Ca^{2+} signaling [27–31]

Previous work has revealed that T lymphocytes express both SERCA 2b and SERCA 3 Ca^{2+} pump isoforms [32–35], yet it is not known what the specific function of these two SERCA pumps is with respect to regulating Ca^{2+} signaling events in T cell function.

Figure 1 shows the establishment of the lowest concentrations of the SERCA inhibitors thapsigargin (TG) and 2,5-di-(*tert* butyl)-1,4-benzohydroquinone (tBHQ) capable of inducing detectable Ca^{2+} release responses in Jurkat lymphocytes with our methods. Figure 1A shows responses to the sequential application of TG (100 pM), tBHQ (1 μM) and ionomycin (1 μM) in Jurkat cells loaded with Fura 2 and suspended in a Ca^{2+}-free medium. Following TG-induced Ca^{2+} release (ΔF340/380= 0.16 ± 0.04 ratio units, n=12), the further addition of low concentration tBHQ induces an additional increment of Ca^{2+} discharge on top of the elevated TG response (ΔF340/380= 0.10 ± 0.007, n=10) from intracellular stores, suggesting that the two SERCA blockers are acting on distinct Ca^{2+} storage sites. Lastly, the addition of ionomycin (1 μM) induces the release of remaining sequestered Ca^{2+} (ΔF340/380= 0.28 ± 0.07, n=12), suggesting that the releasable Ca^{2+} induced by low dose TG and tBHQ is approximately half of the total Ca^{2+} stored in the Jurkat lymphocyte. Previous studies using a variety of hematopoietic-derived cells including platelets and Jurkat lymphocytes have revealed that low concentrations of TG specifically block SERCA 2b pumps whereas low doses of tBHQ specifically target the SERCA 3 isoform [32,36–38].

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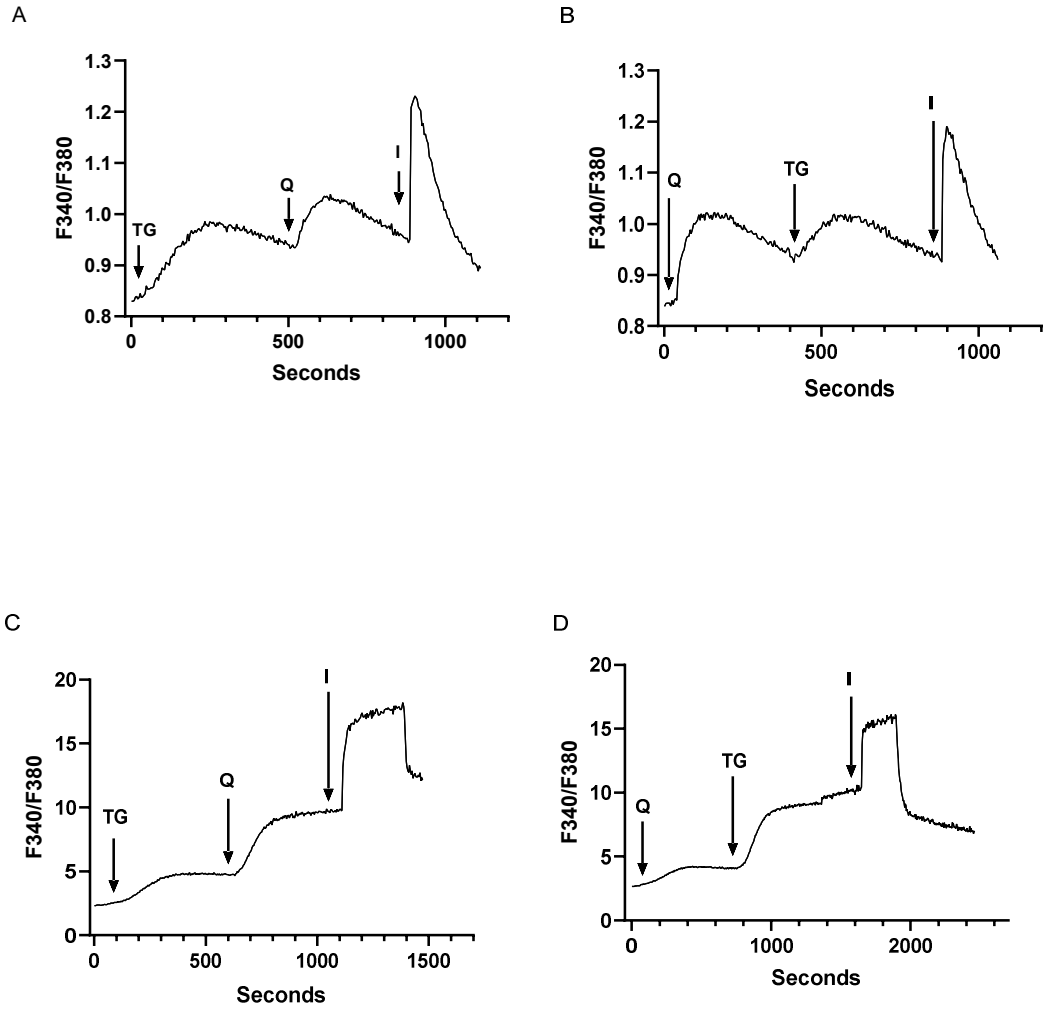

Figure 1. Low concentrations of the SERCA blockers thapsigargin (TG) and 2,5, di-(*tert*-butyl) 1,4-benzohydroquinone (tBHQ) can specifically induce Ca^{2+} release from distinct Ca^{2+} stores in Jurkat and Rat T lymphocytes. For A and B, Jurkat T lymphocytes were loaded with Fura-2 and suspended in Ca^{2+}-free media (balanced salt solution plus 2 mM EGTA). *A*, Jurkat cell Ca^{2+} release responses induced by the sequential application (*arrows*) of TG (100 pM), tBHQ (*Q*, 1 μM) and ionomycin (*I*, 1 μM) as determined by the ratio of fluorescence changes at 340 and 380 nm (F340/380). *B*, the same experiment as in *A* but with the reverse application to Jurkat cells of tBHQ (*Q*, 1 μM), TG (100 pM) and ionomycin (*I*, μM). *C*, Rat spleen T cells loaded with Fura 2 and stimulated with TG (100 pM), tBHQ (*Q*, μM) and ionomycin (*I*, μM) in a balanced salt solution containing Ca^{2+}. *D*, Rat spleen T cells stimulated with tBHQ (*Q*, μM), TG (100 pM) and ionomycin (*I*, μM) in Ca^{2+}-containing media. Fluorescence traces shown are representative of ten separate experiments.

Figure 1B shows a similar experimental outcome where we reversed the order of application of the SERCA blockers, adding tBHQ first followed by TG addition. This result is consistent with the interpretation that the drugs used at these concentrations are revealing that the SERCA 2b and SERCA 3 regulated Ca^{2+} stores represent separate units of Ca^{2+} release in the Jurkat lymphocyte, given Ca^{2+} release responses are similar in magnitude (tBHQ: 0.18 ± 0.06, TG: 0.08 ± 0.003 ratio units, n=6) and independent of the order of application of the SERCA blockers.

Figure 1C and 1D show Ca^{2+} responses to TG and tBHQ application in rat primary lymphocytes. As above, we used TG (100 pM) and tBHQ (1 μM) at low concentrations determined to work in Jurkat cell Ca^{2+} release experiments. We find that a similar effect of TG and tBHQ treatment is observed in primary lymphocytes, regardless of the order of drug application and with similar relative

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proportions of releasable Ca^{2+} pools. We could not however perform these experiments in Ca^{2+}-free media as the Ca^{2+} release responses were too small to measure; thus the experiments shown in Figures 1C and 1D reflect Ca^{2+} responses in cells suspended in Ca^{2+}-containing media, which resulted in larger fluorescence responses compared to Jurkat cell experiments. The Ca^{2+} release responses induced by TG and tBHQ, albeit larger due to contributions from Ca^{2+} influx (Fig 1C, TG induced response: ΔF340/380= 2.6 ± 0.62, n=5; tBHQ induced response: ΔF340/380= 5.2 ± 1.1, n=5), suggest that primary lymphocytes contain the same Ca^{2+} pool profile as we observe in Jurkat lymphocytes with low concentrations of TG and tBHQ inducing release from SERCA 2b and SERCA 3 Ca^{2+} pools respectively, and thus validate the usage of the Jurkat lymphocyte as a good model system for investigating SERCA regulation of T cell Ca^{2+} signaling networks.

3.2. Low Dose SERCA Blockers and Agonist-Induced Ca^{2+} Release Patterns Suggest a Complex T Cell Ca^{2+} Pool Profile with up to Five Distinct Ca^{2+} Store Compartments

In order to investigate the effects of the SERCA activator CDN1163 on T cell Ca^{2+} stores, we sought to further characterize the properties and relationships of the SERCA 2b and SERCA3-regulated Ca^{2+} pools in Jurkat lymphocytes as revealed by treatment with low doses of TG and tBHQ.

Previous work has identified at least four distinct Ca^{2+} stores in Jurkat lymphocytes comprising inositol 1,4,5-trisphosphate (IP3), TG, ryanodine receptor (RyR) and ionomycin releasable Ca^{2+} pools [27]. The recognition that low tBHQ concentrations release Ca^{2+} from a SERCA 3-regulated Ca^{2+} pool suggests one additional Ca^{2+} store and thus increases to at least five the number of distinct Ca^{2+} pools in the T lymphocyte Ca^{2+} signaling paradigm. Indeed, we show in Figure 2A the sequential release of Ca^{2+} induced by the application of low dose tBHQ (1 μM, 0.13 ± 0.03 ratio units) and TG (100 pM, 0.04 ± 0.008 ratio units) followed by a further Ca^{2+} release response induced by the addition of the T cell receptor crosslinker phytohemagglutinin A (PHA, 10 μg/ml, 0.07 ± 0.004 ratio units), which is known to mobilize Ca^{2+} from the IP3 sensitive stores. Along with the ionomycin-releasable Ca^{2+} pool (1 μM, 0.17 ± 0.04 ratio units), Figure 2A suggests the presence of four intracellular Ca^{2+} pools in the Jurkat lymphocyte. Figure 2B shows that we can further induce Ca^{2+} release in this sequential application scheme by the inclusion of 30 μM ryanodine (0.04 ± 0.002 ratio units), revealing RyR, tBHQ, TG, PHA (IP3) and ionomycin releasable stores, thereby accounting for five distinct Ca^{2+} pools in the Jurkat lymphocyte. The final ionomycin induced release in Figure 2B is approximately 35% reduced compared to Figure 2A (0.17 vs 0.11 ratio units), consistent with the more extensive discharge of intracellular Ca^{2+} pools via application of both SERCA blockers and RyR/IP3R activators.

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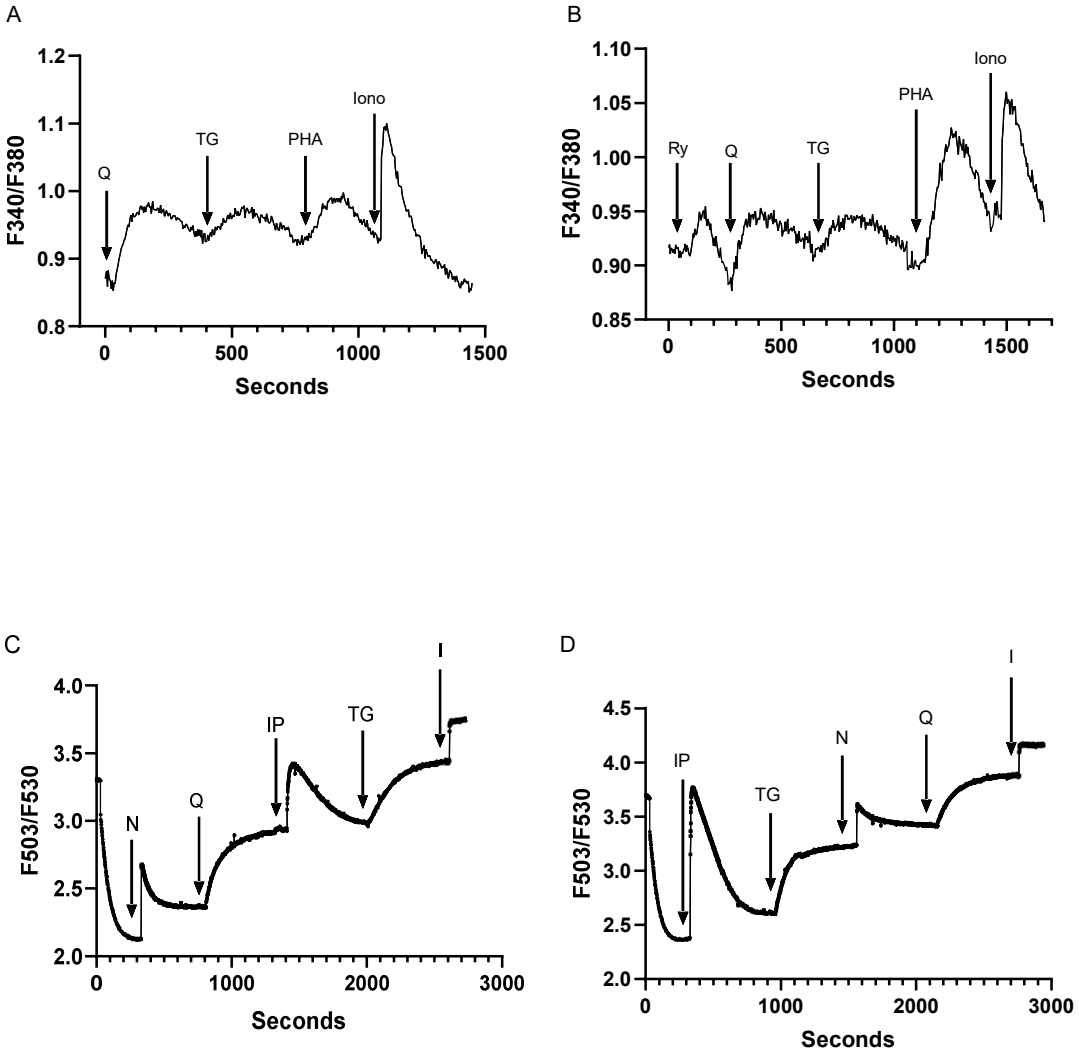

Figure 2. Low dose SERCA blockers and Ca^{2+} release agonists reveal at least five distinct Ca^{2+} pools in intact and membrane permeabilized Jurkat T lymphocytes. For *A* and *B*, Jurkat T lymphocytes were loaded with Fura-2 and suspended in Ca^{2+}-free media (balanced salt solution plus 2 mM EGTA). *A*, Jurkat cell Ca^{2+} release responses induced by the sequential application (*arrows*) of tBHQ (*Q*, 1 μM), TG (100 pM), PHA (10 μg/ml) and ionomycin (*I*, 1 μM) as determined by the ratio of fluorescence changes at 340 and 380 nm (F340/380). *B*, the same experiment as in *A* but with the sequential application to Jurkat cells of ryanodine (*Ry*, 30 μM), tBHQ (*Q*, 1 μM), TG (100 pM), PHA (10 μg/ml) and ionomycin (*I*, μM). *C* and *D* show experiments using saponin permeabilized Jurkat cells, depicting Ca^{2+} release responses as detected by Fuo-3 fluorescence changes. *C*, permeabilized cell responses to the sequential addition of NAADP (*N*, 400 μM), tBHQ (*Q*, 1 μM), IP3 (*IP*, 0.5 μM), TG (1.5 nM) and ionomycin (*I*, 1 μM). *D*. same experiment as shown in *C* but with the sequential addition of IP3 (*IP*, 0.5 μM), TG (1.5 nM), NAADP (*N*, 400 μM), tBHQ (*Q*, 1 μM)and ionomycin (*I*, 1 μM). Fluorescence traces shown are representative of six to ten individual experiments.

To examine the intracellular Ca^{2+} store profile in T lympocytes more closely we conducted experiments using permeabilized Jurkat T cells allowing investigation of direct Ca^{2+} release responses induced by agonists impermeant to the plasma membrane. For these experiments we used NAADP to release Ca^{2+} from RyR-sensitive stores and the direct application of IP3 to release Ca^{2+} from TCR (PHA)-coupled IP3R-sensitive stores in addition to the SERCA blockers and ionomycin. Figure 2C shows the detection of Ca^{2+} release from internal pools by the sequential application of NAADP (peak ΔF: 0.55 ± 0.03), tBHQ (ΔF: 0.59 ± 0.04), IP3 (ΔF: 0.49 ± 0.02), TG (ΔF: 0.46 ± 0.05) and ionomycin (ΔF:

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0.31 ± 0.02). Figure 2D shows that regardless of the order of application these Ca^{2+} release activators can induce release from five distinct intracellular storage sites. Thus, Figures 2C and 2D confirm the results observed from intact cell experiments indicating the presence of five releasable Ca^{2+} pools in T lymphocytes.

3.3. Assessment of Inter-Relationships of Agonist Releasable Ca^{2+} Pools in T Lymphocytes

We explored the nature of the relationships between the SERCA 2b and SERCA 3 regulated Ca^{2+} stores to gain further insight into the functional roles of these distinct Ca^{2+} pools in T lymphocytes. Previous studies have reported that different Ca^{2+} mobilizing agents demonstrate the capacity to specifically induce Ca^{2+} release from SERCA 2b or SERCA 3 storage compartments, which may reveal recruitment of distinct Ca^{2+} release pathways corresponding to either SERCA 2b or SERCA 3 gated pools to subserve specific signaling functions in T cells, as has been shown previously for ADP secretion in platelets [39–42].

We chose to examine further a select group of Ca^{2+} mobilizing agonists that operate through the TCR, RyR and GPCR Ca^{2+} release pathways. As above, we utilized both intact and permeabilized Ca^{2+} assays to determine the effects of SERCA 2b and SERCA 3 modulation on PHA, ryanodine and thrombin responses in intact T lymphocytes and, correspondingly, IP3 and the RyR activator NAADP in permeablized cells.

In agreement with previous experiments using Jurkat lymphocytes [27], we find that the IP3 sensitive Ca^{2+} pool is a subcompartment of the larger TG releasable Ca^{2+} store given we still observe TG (1.5 nM)-mediated Ca^{2+} release following IP3 (0.5 μM)-induced responses in permeabilized cells (Fig 2C & 2D). However, by adding TG first and by gradually increasing its concentration up to just 15 nM we can abolish the IP3 induced Ca^{2+} release responses (Figures 3A-C), verifying that with more aggressive SERCA 2b inhibition we deplete the IP3-sensitive Ca^{2+} pool. Indeed, by increasing TG levels to 15 nM we find that we can abolish tBHQ responses as well, indicating loss of the ability to pharmacologically discriminate between SERCA 2b and SERCA 3- regulated stores at this TG concentration (Figure 3C). These observations provide assurance we can perturb TCR-coupled IP3 sensitive Ca^{2+} stores via specific pharmacologic modulation of SERCA 2b function with low dose TG.

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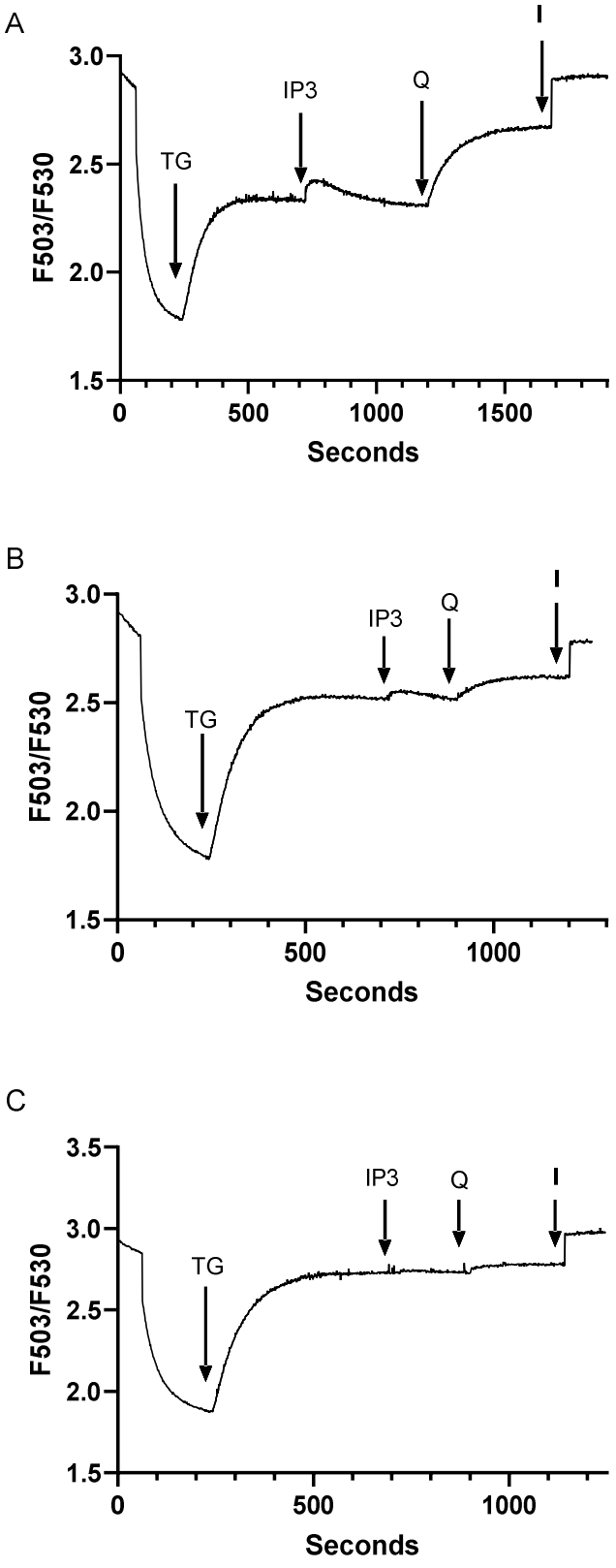

Figure 3. Increasing TG-induced SERCA inhibition depletes the IP3 and tBHQ- releasable Ca^{2+} pools establishing TG concentration range permitting SERCA pool specific modulation in Jurkat T lymphocytes. *A-C* show experiments using saponin permeabilized Jurkat cells, depicting Ca^{2+} release responses as detected by Fuo-3 fluorescence changes. *A*, permeabilized cell responses to the sequential

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addition of TG (2 nM), IP3 (0.5 μM), tBHQ (Q, 1 μM) and ionomycin (I, 1 μM). *B*, Ca^{2+} release responses induced in permeabilized Jurkat lymphocytes by the sequential application of TG (10 nM), IP3 (0.5 μM), tBHQ (Q, 1 μM), and ionomycin (I, 1 μM). *C*, Ca^{2+} release responses induced in permeabilized Jurkat lymphocytes by the sequential application of TG (15 nM), IP3 (0.5 μM), tBHQ (Q, 1 μM), and ionomycin (I, 1 μM). Fluorescence traces shown are representative of four to eight individual experiments.

RyR signaling in T cells has been marked by a relatively high degree of irresolution given mostly low expression levels of the receptor in lymphocytes [43–45]. Nonetheless, recent work claims a prominent role for RyR in shaping the earliest Ca^{2+} signals essential for T cell activation [43,44,46]. Jurkat T lymphocytes as a clonal homogeneous population have been useful in clarifying the roles of RyR in T cell signaling given their expression of RyRs, albeit at low levels [28,47,48]. Studies using Jurkat lymphocytes have revealed that the Ca^{2+} mobilizing agonist NAADP acts on RyRs to induce Ca^{2+} release from the ER and not from a unique separate acidic Ca^{2+} store compartment as has been observed in platelets[49,50]. Moreover, platelet studies have indicated that the SERCA 3 Ca^{2+} pump and not SERCA 2b isoform controls the NAADP Ca^{2+} releasable store [39,51]. Thus, we performed experiments to determine whether T cell RyR regulated Ca^{2+} stores, like platelet NAADP releasable pools, are affiliated specifically with SERCA 3 pumps using our low dose TG and tBHQ SERCA blocker regimen. Figure 4A shows that ryanodine (30 μM) induces a small Ca^{2+} transient (0.06 ± 0.005 ratio units, n=5) in Jurkat cells suspended in a Ca^{2+} free medium which rapidly decays in the presence of functional SERCA 2b/3 Ca^{2+} pump activity. The ryanodine inducible responses have no effect on subsequent Ca^{2+} release responses induced by tBHQ (0.12 ± 0.008 ratio units) and TG (0.08 ± 0.006 ratio units) suggesting that RyR activation in the Jurkat lymphocyte is inducing release from a smaller subcompartment of the larger tBHQ and/or TG releasable pools. However, when we reverse the order of application and increase the concentration of tBHQ we find that we can abolish the ryanodine inducible response (Figure 4B, $p<0.05$, n=5) with little effect on the subsequent TG induced Ca^{2+} release response (Figure 4B). This finding suggests that, similar to platelets, NAADP releasable Ca^{2+} stores are replenished by SERCA 3 Ca^{2+} pumps in T cells.

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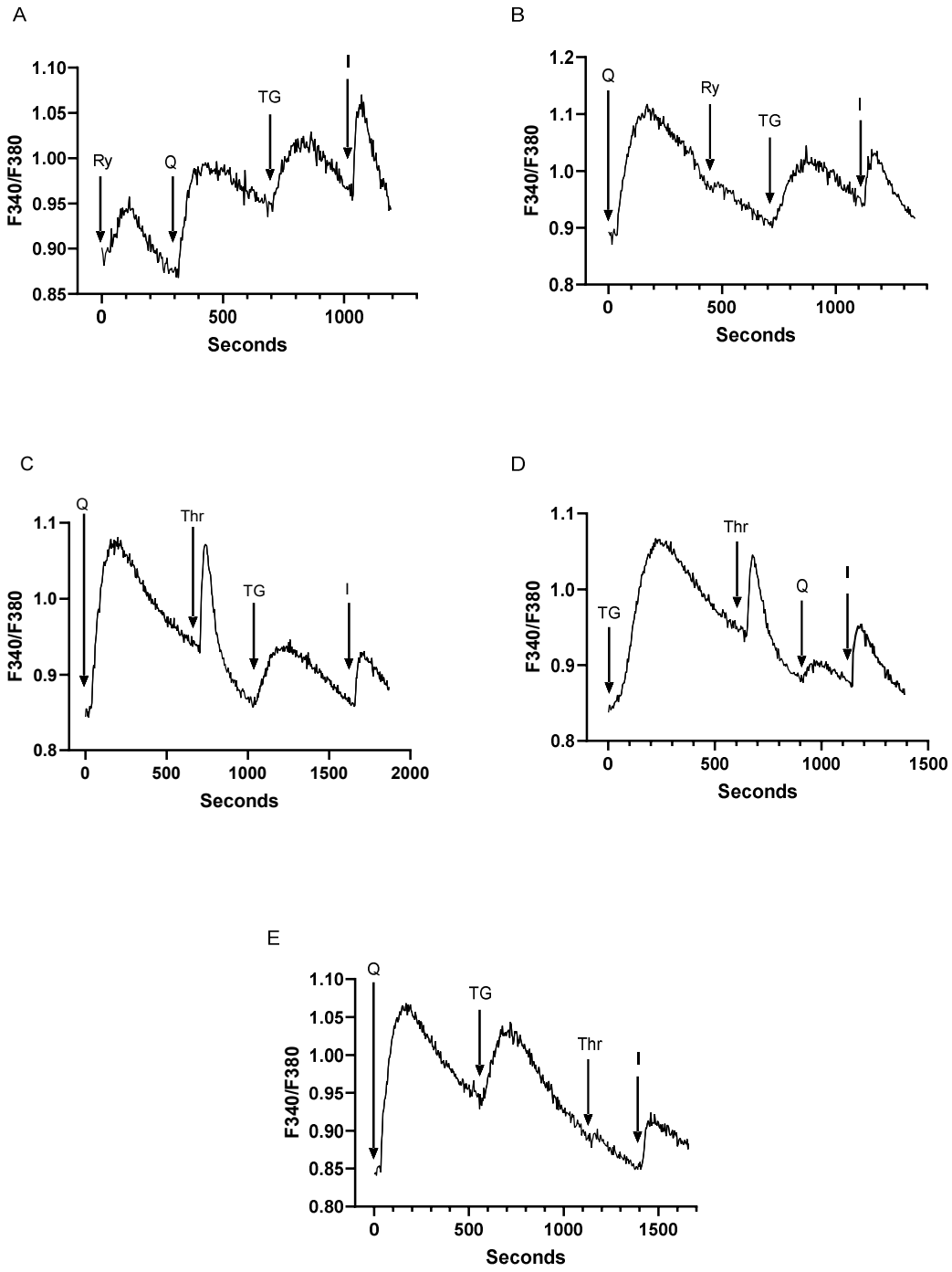

Figure 4. Relationships of the Ryanodine and Thrombin releasable Ca^{2+} pools to the low dose TG SERCA 2b and low dose tBHQ SERCA 3 regulated Ca^{2+} stores. For *A*-E, Jurkat T lymphocytes were loaded with Fura-2 and suspended in Ca^{2+}-free media (balanced salt solution plus 2 mM EGTA). *A*, Jurkat cell Ca^{2+} release responses induced by the sequential application (*arrows*) of ryanodine (Ry, 30 μM) tBHQ (*Q*, 2 μM), TG (200 pM), and ionomycin (*I*, 1 μM) as determined by the ratio of fluorescence changes at 340 and 380 nm (F340/380). *B*, the same experiment as in *A* but with the sequential application to Jurkat cells of ryanodine tBHQ (*Q*, 2 μM), ryanodine (*Ry*, 30 μM), TG (200 pM), and ionomycin (*I*, μM). *C*, Ca^{2+} release responses to the sequential addition of tBHQ (Q, 2 μM), thrombin (Thr, 0.1 U/ml), TG (200 pM) and ionomycin (1 μM). *D*, Ca^{2+} release responses to the sequential addition of TG (200 pM), thrombin (Thr, 0.1 U/ml), tBHQ (Q, 2 μM), , and ionomycin (1 μM). *E*, Ca^{2+}

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release responses to the sequential addition of tBHQ (Q, 2 μM), TG (200 pM), thrombin (Thr, 0.1 U/ml), and ionomycin (1 μM). Fluorescence traces shown are representative of four to seven individual experiments.

We investigated whether we could alter thrombin responses in T cells using low dose TG and tBHQ to establish specific linkage of thrombin releasable Ca^{2+} stores with SERCA 2b or SERCA 3 Ca^{2+} pools. Thrombin acts on G protein-coupled receptors (GPCRs) and has been observed in platelet studies to be coupled to two distinct signaling functions [40,41]: thrombin induced Ca^{2+} release from SERCA 3-regulated pools comprised an early platelet signal to generate ADP secretion which was amplified by a secondary thrombin-induced Ca^{2+} signal deriving from SERCA 2b-regulated stores [52]. It was proposed that the thrombin activated GPCR pathway produced two second messengers in the platelet system: IP3 moblized Ca^{2+} from the SERCA 2b Ca^{2+} stores whereas NAADP released Ca^{2+} as the initial early signal from SERCA 3 Ca^{2+} stores [52]. We find that an apparent similar mechanism may be working in T cells (Figures 4C-E). Figure 4C shows that exposure of Jurkat lymphocytes to tBHQ (2 μM) fails to abolish thrombin responses (0.13 ± 0.03 ratio units, n=5), while also leaving intact subsequent TG responses (0.09 ± 0.006 ratio units) representing the SERCA 2b Ca^{2+} pool. Similarly, the addition of TG (200 pM) first also does not eradicate the thrombin induced Ca^{2+} release response (0.11 ± 0.008 ratio units, n=5), while subsequent tBHQ responses are also still inducible albeit significantly reduced ($p<0.05$, n=5), suggesting that most of the thrombin releasable Ca^{2+} is contained in the SERCA 2b regulated stores (Figure 4D). Conversely, if T cells are treated first with tBHQ (2 μM) followed by TG (200 pM) thrombin responses are abolished (Figure 4E). This result is consistent with the platelet observations suggesting that thrombin in T lymphocytes appears to release Ca^{2+} from both SERCA 2b and SERCA 3 Ca^{2+} stores.

3.4. Despite Differences in SERCA Blocker Sensitivities and Agonist-Mobilizable Ca^{2+} Responses, the SERCA 2b and SERCA 3-Regulated Ca^{2+} Stores Exhibit Similar Ca^{2+} Influx Coupling Actions with Similar Sensitivity to Actin Cytoskeletal Disruption

T cell activation and gene expression pathways require that SERCA regulated Ca^{2+} stores communicate with PM Orai channels to mediate Ca^{2+} influx [3,7]. Thus, we sought to investigate whether the TG and tBHQ releasable Ca^{2+} stores described above exhibited similar features in regulating Ca^{2+} influx pathways. The platelet Ca^{2+} signaling system provides a useful framework for understanding the T cell signaling network which also appears to contain multiple distinct SERCA 2b and SERCA 3 regulated intracellular Ca^{2+} stores. Platelet studies have revealed differences in the SERCA 2b and SERCA 3 regulated Ca^{2+} stores, with the SERCA 3 Ca^{2+} stores demonstrating weaker coupling to Ca^{2+} influx pathways but less sensitivity to cytoskeletal disruption as compared to SERCA 2b Ca^{2+} stores [53]. Our experiments using T cells reveal differences to the platelet system with respect to Ca^{2+} store regulation of Ca^{2+} influx pathways. Figure 5A shows that tBHQ-mediated SERCA 3 blockade and pool depletion activates Ca^{2+} influx responses (2.3 ± 0.52 ratio units, n=12) similar to those induced by TG treatment (2.4 ± 0.71 ratio units, n=15), suggesting that, unlike platelets, the SERCA 3 and SERCA 2b- regulated stores exhibit similar coupling sensitivities to depletion-induced Ca^{2+} influx responses in T lymphocytes. However, in contrast to the platelet system, we find that treating Jurkat cells with the actin cytoskeletal disruptor cytochalasin D (cytD,10 μM) increases the ability of both SERCA 2b and SERCA 3 Ca^{2+} stores to couple to Ca^{2+} influx responses, as we observed larger Ca^{2+} influx responses in both TG (6.9 ± 2.5 control *vs* 9.0 ± 2.1 cytD ratio units, n=4) and tBHQ (2.1 ± 0.88 control *vs* 5.5 ± 1.2 cytD ratio, n=4) treated cells (Figures 5B and 5C). Thus, in T cells the two Ca^{2+} stores both appear to be negatively regulated by actin polymerization, suggesting that actin dynamics may be interfering with SERCA 2b and SERCA 3 vesicle trafficking to the plasma membrane Ca^{2+} channels. In platelets the insensitivity of the SERCA 3 Ca^{2+} store to cytD induced actin disruption led to speculation that this Ca^{2+} pool may reside in close junctional apposition to the plasma membrane [53], which our experiments suggest may not be the case in the T cell system. Intriguingly, we find that pre-treating Jurkat lymphocytes with cytD significantly reduces the TG (0.25 ± 0.03 control *vs* 0.14 ± 0.05 cytD ratio units, $p<0.05$, n=4) and tBHQ (0.16 ± 0.008 *vs* 0.10 ± 0.006

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cytD ratio units, $p<0.05$, n=4) inducible Ca^{2+} release responses (Figures 5D and 5E) suggesting that stable actin filament networks may also be essential for supporting Ca^{2+} release structures, a feature that was not observed in platelet experiments. Indeed, this effect of cytoskeletal perturbation to attenuate Ca^{2+} release may reflect a more depleted Ca^{2+} pool state and thus explain a more robust Ca^{2+} influx coupling response following cytD exposure (Figures 5B and 5C).

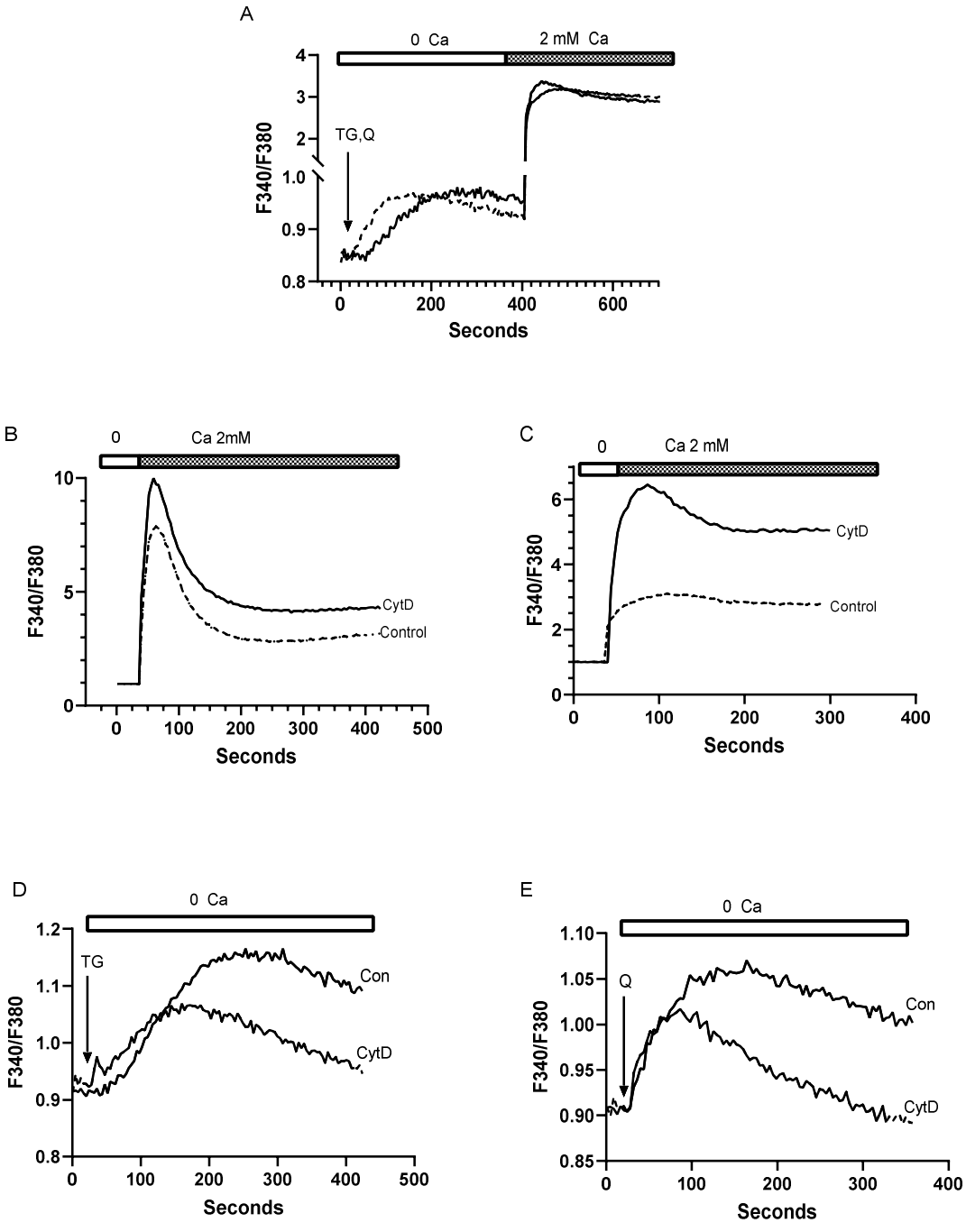

Figure 5. T lymphocyte SERCA 2b and SERCA 3-regulated Ca^{2+} stores reveal similar sensitivities to cytoskeletal disruption with enhanced Ca^{2+} influx responses and reduced Ca^{2+} release activity. For *A-E*, Jurkat T lymphocytes were loaded with Fura-2 and suspended in Ca^{2+}-free media (balanced salt solution plus 2 mM EGTA). *A*, Cells were suspended in Ca^{2+}-free conditions (open bar) and stimulated (arrow) with TG (100 pM) or tBHQ (Q, 1 μM). After approximately 400 seconds of Ca^{2+} release activity, Ca^{2+} levels were increased to 2 mM to elicit Ca^{2+} influx responses (hatched bar). *B* and *C*, Ca^{2+} influx

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responses were induced as in *A* (hatched bars) in cell populations pre-incubated for 60 minutes in the presence (solid lines) or absence (dashed lines) of cytochalasin D (10 μM). *D* and *E*, Jurkat lymphocytes suspended in Ca^{2+}-free media were stimulated with TG (*D*, 200 pM) and tBHQ (*E*, 2 μM) in the presence (CytD) or absence (Con) of cytochalasin D (60 min, 10 μM). Fluorescence traces shown are representative of four to six individual experiments.

3.5. The SERCA Activator CDN1163 Exerts Complex Short and Long-Term Effects on T Cell Ca^{2+} Stores Revealing a Differential Regulatory Action on SERCA 2b versus SERCA 3 Ca^{2+} Pools

We next focused on exploring the effects of the recently identified SERCA activator molecule CDN1163 on T cell Ca^{2+} stores. As mentioned, there is much interest in identifying and characterizing a small molecule complement to the group of SERCA blockers that can achieve SERCA activation. Our foregoing experiments have further characterized some of the additional complexity in T cell Ca^{2+} stores in revealing distinct SERCA 2b and SERCA 3 Ca^{2+} pools that appear to be recruited to produce Ca^{2+} responses tailored to distinct signaling triggers, including TCR activation, thrombin and RyR-mediated signals. Thus, given previous work elucidating the salutary effects of CDN1163 attributable to increased activity of SERCA function[8,12,16,26], we anticipated that the compound would produce an augmented Ca^{2+} store condition with perhaps greater Ca^{2+} release responses in the Jurkat T cell system. However when we measured intact Jurkat cell Ca^{2+} responses treated with varying concentrations of CDN1163 we did not uniformly observe this effect.

Indeed, Figure 6A shows that a 20 minute pre-incubation of T cells with CDN1163 (10 μM) significantly reduced TCR-mediated Ca^{2+} release as measured by PHA treatment in Ca^{2+}-free media (Peak ratio units: 0.29 ± 0.06 untreated *vs* 0.15 ± 0.04 CDN treated, $p< 0.05$, n=7), which represents the IP3 releasable component of the larger TG sensitive Ca^{2+} stores. We observed the same effect of short-term (<30 minutes) CDN1163 exposure when cells were treated with low dose TG (100 pM), revealing reduced Ca^{2+} release responses from this pharmacologically defined SERCA 2b Ca^{2+} pool (Figure 6B). Surprisingly, this effect was not observed in the tBHQ-sensitive SERCA 3 Ca^{2+} store. Figure 6C shows that Jurkat lymphocytes exposed to CDN1163 (10 μM) for 20 minutes exhibit significantly increased Ca^{2+} release responses when treated with low dose tBHQ (peak ratio units: 0.17 ± 0.008 untreated *vs* 0.35 ± 0.06 CDN treated, $p<0.05$, n=8). And thus our low dose TG/tBHQ treatment regimen reveals reciprocal effects of short-term CDN1163 exposure on T cell SERCA 2b versus SERCA 3-regulated Ca^{2+} stores. These findings suggest the intriguing possibility that CDN1163 can exert opposing differential regulatory influences on the SERCA pumps, perturbations induced by short-term exposure to the compound that produce diminished Ca^{2+} loading in the SERCA 2b pool with concurrent augmentation of the SERCA 3 Ca^{2+} pool.

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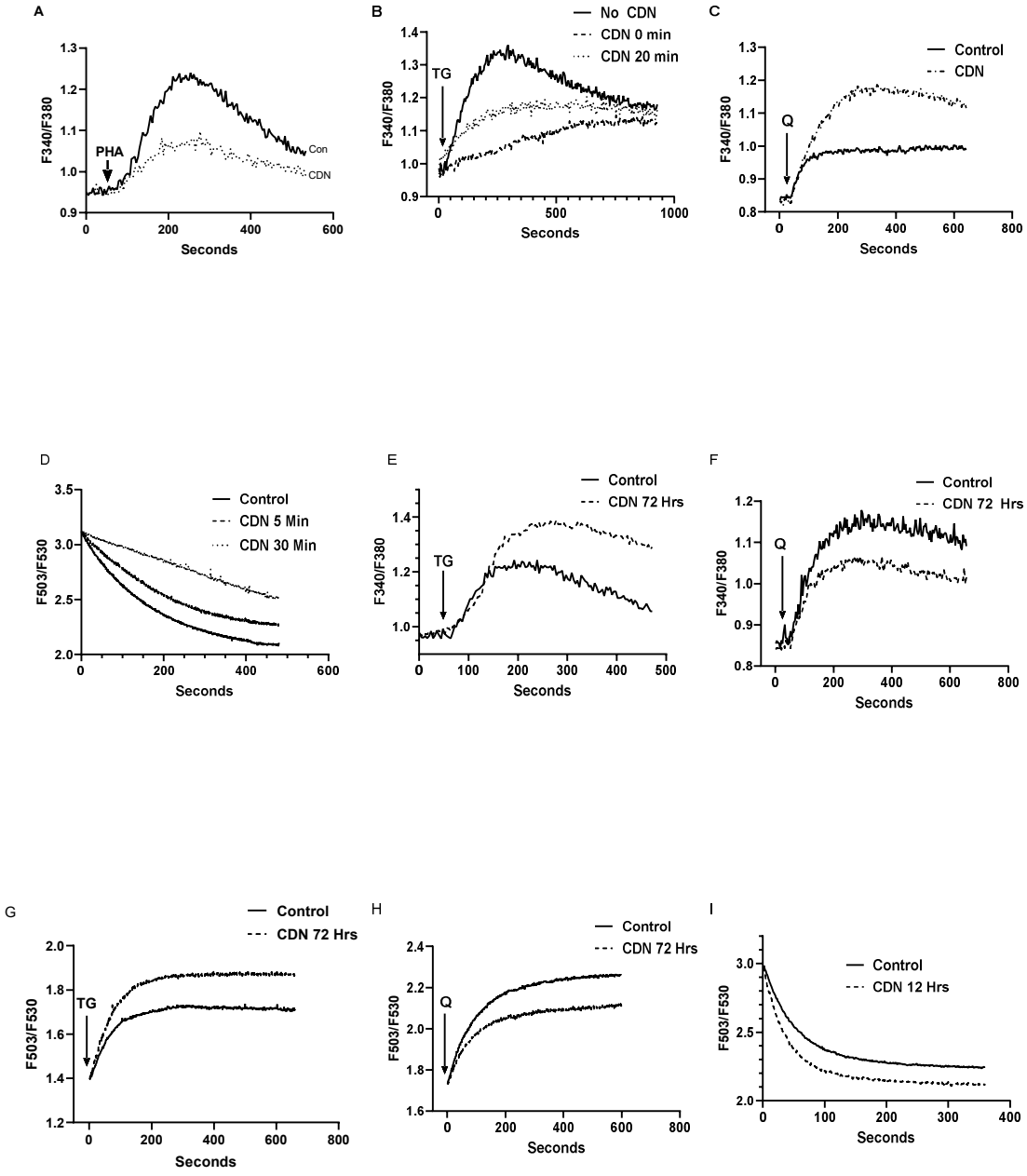

Figure 6. The SERCA activator CDN1163 exerts differential time-dependent effects on T cell SERCA 2b and SERCA 3 Ca^{2+} stores. For *A-C* Jurkat T lymphocytes were loaded with Fura-2 and suspended in Ca^{2+}-free media (balanced salt solution plus 2 mM EGTA). *A*, Ca^{2+} release responses induced by treatment with PHA (10 μg/ml) in the presence (dashed trace) or absence (solid trace) of CDN1163 (10 μM, 20 minutes). *B*, TG (100 pM) induced Ca^{2+} release in the presence (dashed traces, 0 and 20 min) or absence (solid trace) of CDN1163 (10 μM). *C*, tBHQ (1 μM) induced Ca^{2+} release in the presence (dashed trace, 20 min) or absence (solid trace) of CDN1163 (10 μM). *D*, Fluo-3 fluorescence Ca^{2+} uptake assay. Ca^{2+} uptake in ER stores was initiated by the addition of ATP (see Materials & Methods section) in saponin permeabilized Jurkat lymphocytes incubated in the presence (dashed curves, 5 and 30 minutes) or absence (solid curve) of CDN1163 (25 μM). Rate of Ca^{2+} uptake was estimated based on the linear intial rate of Fluo-3 fluorescene decay. *E* and *F*, Ca^{2+} release responses induced by TG and tBHQ in Jurkat lymphocytes incubated for longer durations with CDN1163. *E*, Ca^{2+} release induced by TG (100 pM) in the presence (dashed trace, 72 hours) or absence (solid trace) of CDN1163 (10 μM). *F*, Ca^{2+} release induced by tBHQ (1 μM) in the presence (dashed trace, 72 hours) or absence (solid trace) of CDN1163 (10 μM). *G* and *H* show experiments using saponin permeabilized

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Jurkat cells, depicting Ca^{2+} release responses as detected by Fuo-3 fluorescence changes. *G*, Ca^{2+} release induced in permeabilized cells treated with TG (1 nM) in the presence (dashed trace, 72 hours) or absence (solid trace) of CDN1163 (10 μM). *H*, Ca^{2+} release induced in permeabilized cells treated with tBHQ (1 μM) in the presence (dashed trace, 72 hours) or absence (solid trace) of CDN1163 (10 μM). *I*, Ca^{2+} uptake in ER stores was initiated by the addition of ATP (see Materials & Methods section) in saponin permeabilized Jurkat lymphocytes incubated in the presence (dashed curve, 12 hours) or absence (solid curve) of CDN1163 (25 μM). Rate of Ca^{2+} uptake was estimated based on the linear intial rate of Fluo-3 fluorescene decay.

Our experiments with CDN1163 in T cells thus yielded somewhat paradoxical effects, given we appear to observe diminished levels of stored Ca^{2+} in the major SERCA 2b-regulated pool after treatment with a putative SERCA pump activator. To further explore CDN1163's effects on T cell Ca^{2+} stores, we used saponin-permeabilization assays to measure Ca^{2+} uptake responses directly. We incubated Jurkat lymphocytes with CDN1163 (25 μM) over a short time interval (≤ 30 min) and then proceeded to induce PM permeabilization using low concentrations of saponin. Calcium uptake into ER stores was then initiated by the addition of ATP in a cuvette-based assay, tracking Ca^{2+} uptake into stores by the decline in Fluo-3 fluorescence. Indeed, Figure 6D shows that CDN1163-treated permeabilized Jurkat cells revealed pronounced inhibition of Ca^{2+} uptake as determined by linear initial rates of fluorescence decay (ΔF/sec: 5.2 x 10^{-3} untreated *vs* 1.5 x 10^{-3} 30 min CDN treated, n=8), suggesting a possible mechanism to explain the observed attenuated Ca^{2+} release responses from the SERCA 2b-regulated Ca^{2+} stores. This result however does not explain the apparent augmentation of Ca^{2+} stored in the tBHQ-sensitive SERCA 3-regulated Ca^{2+} pool. It is worth noting that previous studies using Jurkat cells and platelets have revealed that the SERCA 2b-regulated Ca^{2+} store is likely to be larger than the SERCA 3 Ca^{2+} store [27,51,53]. Thus, it is possible that CDN1163 short-term exposure is specifically perturbing the SERCA 2b-mediated Ca^{2+} uptake which would comprise the dominant effect in our permeabilized cell assays masking a smaller contribution from increased SERCA 3-mediated Ca^{2+} uptake. We also observed that application of CDN1163 directly to Fura 2 loaded Jurkat lymphocytes induced a gradual small increase in cytoplasmic Ca^{2+} levels (data not shown) further suggesting that the compound over this time interval blocks aggregate SERCA activity and thereby induces Ca^{2+} leakage from the ER Ca^{2+} stores leading to a relatively depleted state in the SERCA 2b Ca^{2+} store. As above, this result may be explained by the larger effect on the SERCA 2b Ca^{2+} pool experiencing a CDN1163-induced downregulatory action even while the compound exerts a modest increase in Ca^{2+} loading into the SERCA 3 pool. Notably, this ability of CDN1163 to induce gradual increases in cytosolic Ca^{2+} levels with short-term exposure was also recently reported in experiments measuring Ca^{2+} changes in A549 lung epithelial cells [54,55].

Given CDN1163's well-described action as an allosteric SERCA pump activator [8], we tested whether the compound may require longer periods of exposure to T lymphocytes to increase SERCA activity globally and augment Ca^{2+} store levels in SERCA 2b and SERCA 3 regulated stores. Indeed, we observed that longer exposure to CDN1163 (72 hours, Figure 6E) increased Ca^{2+} store levels in the SERCA 2b pool, as revealed by the application of low dose TG (peak ratio units: 0.33 ± 0.06 untreated *vs* 0.49 ± 0.07 72 hr CDN treated, $p<0.05$, n=7). Yet, surprisingly, Ca^{2+} store levels in the SERCA 3-regulated stores moved in the opposite direction exhibiting reduced levels with long-term CDN1163 treatment (Figure 6F), as the low dose tBHQ-induced Ca^{2+} release responses revealed (peak ratio units: 0.54 ± 0.05 untreated *vs* 0.39 ± 0.04 72 hr CDN treated, n=9). Thus, as with the short-term incubation experiments, we also find in the longer-term exposures a remarkable asymmetry in CDN1163's effects on the SERCA 2b and SERCA 3-regulated Ca^{2+} stores, with an augmented SERCA 2b and a concomitant reduced SERCA 3 pool with longer CDN1163 treatment. We conducted additional experiments using the permeabilized Jurkat T cell assay to determine if this reversal effect due to longer-term CDN1163 exposure could be observed in direct Ca^{2+} release assays and on Ca^{2+} uptake responses. Indeed, we observed the same pattern in these experiments with permeabilized T lymphocytes as was found using intact cells, detecting increased Ca^{2+} release directly with TG application (Figure 6G) and, conversely, diminished Ca^{2+} release inducible by tBHQ application (Figure 6H) in cells incubated with CDN1163 (10 μM) for 72 hours. Intriguingly, it appears that, like

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TG and tBHQ, CDN1163 exerts differential effects on SERCA 2b and SERCA 3 Ca^{2+} pump isoforms. And thus, our findings suggest that the stimulatory effect of CDN1163 on T cell Ca^{2+} stores is multiplex, requiring prolonged incubation to reverse an initial inhibitory action and augment the SERCA 2b Ca^{2+} store, yet over the extended incubation period the compound exerts a gradual downregulation of Ca^{2+} replenishment of the SERCA 3 regulated store. Using the Ca^{2+} uptake assay in permeabilized cells over a range of different CDN1163 exposure periods, we found that by approximately 12 hours Ca^{2+} uptake activity had been restored (Figure 6I), as well as perhaps slightly elevated (ΔF/sec: 7.2 x 10^{-3} untreated *vs* 8.8 x 10^{-3} CDN 12 hr treated, n=8). This time frame for the restoration of ATP-activated Ca^{2+} uptake responses in the presence of CDN1163 aligns well with the general time frame required for the shift in Ca^{2+} release responses to occur in the intact cell experiments.

4. Discussion

To better assess novel actions of potential SERCA activators within the Ca^{2+} signaling landscape of T cell functions, we were motivated to extend seminal previous work characterizing the major intracellular Ca^{2+} stores. We have employed the strategy of using low concentrations of TG and tBHQ in our experiments, an approach successfully applied to probe the functions SERCA 2b and SERCA 3- regulated Ca^{2+} pools in human platelets. We applied this strategy using the Jurkat T lymphocyte model which has been used less extensively than platelets to characterize properties of the intracellular Ca^{2+} stores. We identified the lowest concentrations of SERCA blockers that elicited measurable Ca^{2+} release responses in cells incubated in Ca^{2+}-free media, thus providing greater assurances of using these agents to specifically modulate the SERCA 2b and SERCA 3 pump isoforms. We validated the use of the Jurkat T cell line by verifying that these same effects could be produced in primary lymphocytes isolated from rat splenocytes, confirming that Jurkat cells share the same pharmacological phenotype as primary T lymphocytes when treated with low dose TG and tBHQ. Indeed, use of the Jurkat T lymphocyte provides a significant experimental advantage given their clonal homogeneous responses and the ability to cultivate large numbers of cells, features which greatly assist analysis of measuring relatively weak signals due to modest SERCA perturbations. Moreover, Jurkat lymphocytes continue to be used as powerful T cell model systems given the strong validation and close overlap with primary T cells in the signaling representation of the TCR pathway as the primary upstream activator of ER Ca^{2+} release along with the tightly coupled Ca^{2+} influx pathway [28,30,31,46].

We have examined the Ca^{2+} pool profile in Jurkat lymphocytes with the added discriminatory refinement of employing low concentrations of TG and tBHQ, referencing previous work in human platelets in which this approach has been productively used to gain insight into SERCA 2b and SERCA 3- regulated Ca^{2+} stores [36,39–41,51,53]. Using low dose TG and tBHQ in Ca^{2+}-free cell suspensions we have determined Ca^{2+} release specifically from SERCA 2b versus SERCA 3-regulated Ca^{2+} stores in T lymphocytes. Indeed, our experiments extend earlier investigations to describe at least five distinct Ca^{2+}-releasable storage sites in T cells: a TG-sensitive SERCA 2b pool, a subcompartment of the TG-sensitive pool releasable by IP3, a tBHQ-sensitive SERCA 3 pool, a pool dischargeable by agonists of RyRs, a pool releasable by GPCR agonists and finally the remaining Ca^{2+} storage pool releasable by ionomycin application. These are clearly approximate estimations given agonists, such as thrombin, can release Ca^{2+} from multiple SERCA-controlled stores; and, moreover, Ca^{2+} stores in T cells are likely to contain built-in interconnectivity with Ca^{2+} release from one compartment being captured by a neighboring SERCA-regulated compartment, as has been shown for the RyR expressing Ca^{2+} pools in T lymphocytes [27,28,43,56]. These observations suggest the intriguing scenario of a complex and dynamic interrelationship among the various intracellular Ca^{2+} stores whereby rapid exchange and flow of Ca^{2+} ions through discrete regional space of the larger ER organelle, whether phsyically separated or not, may establish *de novo* spatially localized gradients adapted to accommodate specific T cell signaling functions. These experiments have further characterized the complex array of Ca^{2+} storage compartments and functions in T lymphocytes and

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have provided a useful foundation of SERCA-specific actions (inducible via low dose TG and tBHQ) to examine the effects of the novel SERCA-activating compound CDN1163.

In contrast to most CDN1163 studies, we did not observe a clear and unambiguous stimulatory effect of the compound on T cell SERCA activity. Indeed, our experiments revealed a surprising short and long-term acting dichotomy in which an initial period of apparent SERCA inhibition and Ca^{2+} stores depletion gradually shifts to SERCA activation and Ca^{2+} stores repletion. This discrepancy with previous reports may be due to a more complex SERCA pump expression profile in T cells, given that these cells rely on a minimum of at least two distinct SERCA pump isoforms to manage intracellular Ca^{2+} signaling dynamics. Jurkat T lymphocytes, an often used surrogate for T cell function, are well known for their expression of multiple SERCA isoforms with the predominant pump species being the SERCA 2b and SERCA 3 isoforms [32,57]. And although the specific protein functions are unknown, Jurkat T cells appear to tap into an extensive diversity in SERCA gene expression with earlier studies revealing the expression of all six SERCA 3 pump isoforms (SERCA 3a-f) along with the SERCA 2b isoform [35,58], which suggests a high degree of precisioned and specialized control built in for regulation of T cell Ca^{2+} store functions.

Indeed, some of the apparent incongruous effects we observe with CDN1163 may be due to this complex SERCA environment in T cells, with distinct pump isoforms working in diverse groups of interacting protein partners within heterogeneous ER/PM locales. We find, for example, that when Jurkat lymphocytes are exposed to the putative SERCA activator CDN1163 for short durations (≤ 30 minutes) Ca^{2+} release induced by the IP3 pathway or by low dose TG treatment are significantly reduced. We observed this effect in Ca^{2+} responses measured in both intact and permeabilized cells, a result which paradoxically suggests that CDN1163 may be acting to perturb SERCA function, initiate ER Ca^{2+} leak pathways and promote loss of ER Ca^{2+} levels. This interpretation is consistent with our experiments using permeabilized cells in which we observed that short duration CDN1163 exposure suppressed Ca^{2+} uptake. These actions of CDN1163 to impair ER Ca^{2+} uptake produced a gradual increase in Ca^{2+} release observable in our permeabilized cell assays which was the likely cause of reduced IP3 and TG- inducible Ca^{2+} release.

Our approach in this study to use low concentration TG and tBHQ to specifically target SERCA 2b and SERCA 3 has provided insight into the novel actions of CDN1163. We report here intriguing differences using these two SERCA blockers in the sensitivity of SERCA 2b and SERCA 3 to the effects of the SERCA activator CDN1163. In our experiments CDN1163 appears to perturb SERCA 2b regulated Ca^{2+} stores to a greater extent than the low dose tBHQ sensitive SERCA 3 Ca^{2+} store. It is worth noting that CDN1163 has been shown in previous studies to bind to and modulate SERCA 2 isoforms in various cells and tissues, but no clear evidence has emerged for the compound's effects on the SERCA 3 isoform [8,12,26]. Indeed, we find that CDN1163 attenuates the low dose tBHQ releasable Ca^{2+} store albeit with a less pronounced effect as compared to the TG sensitive pool. Furthermore, we have reported that long-term (>24 hours) CDN1163 exposure fails to produce a stimulatory effect with improved Ca^{2+} release inducible by tBHQ unlike what is observed in the long-term incubation experiments with the TG-releasable Ca^{2+} pool. These findings suggest that there are likely not uniform stimulatory effects induced by CDN1163 across all SERCA pump isoforms; and indeed, these experiments suggest that clear unambiguous stimulation of SERCA function may be difficult to achieve in T cells and other cells that express multiple SERCA pump isoforms.

Our work does appear to align with previous investigations characterizing CDN1163 as a SERCA activator, albeit acting on an enigmatically slower timeframe in our T lymphocyte model. As mentioned, this effect may be attributable to differential actions of the compound on the different SERCA pump isoforms expressed in the T lymphocyte, with the global cellular SERCA activity being the sum of complex stimulatory and inhibitory effects on SERCA 2b and SERCA 3. Intriguingly, however, CDN1163's time-dependent augmentation of SERCA 2b Ca^{2+} stores may be hinting at the compound's ability to react to and promote differential SERCA states and/or SERCA-pumping environments. Indeed, CDN1163 was initially identified in a chemical library screen for its ability to interfere with SERCA binding interactions with phospholamban [8], the cardiac protein regulator of the SERCA 2a pump isoform. Perhaps CDN1163 is targeting a similar site of regulatory control in the

SERCA 2b pump explaining an initial early period of perturbation on Ca^{2+} transport activity with accompanying Ca^{2+} leak expression; yet with prolonged incubation, regulatory control possibly arising from time-dependent SERCA-associated protein partners re-configures ER systems to enhance Ca^{2+} uptake.

It has been reported that hematopoietic and other cell types can recruit opposing SERCA actions with downregulation of SERCA 3 activity linked to time-dependent upregulation of SERCA 2b expression/function, an observation clearly identified during T cell activation [57,59,60]. Indeed, other studies have identified STIM1 as a candidate potential SERCA regulator, such that when ER Ca^{2+} stores experience relative depletion STIM1, as an ER Ca^{2+} sensor, may participate in SERCA activating functions to replenish ER Ca^{2+} levels [61]. This may also explain the time delay we observe in our T cell model given the initial CDN1163-mediated ER Ca^{2+} leak as a relatively weak, gradual depletion-activating signal may utlimately couple to STIM1 or other protein regulators to promote greater SERCA activity with increased ER Ca^{2+} transport. Thus, continued interrogation and characterization of CDN1163 may offer an additional SERCA pharmacological tool to probe novel SERCA regulatory networks that appear to play multi-layered Ca^{2+} signaling roles in T lymphocyte signaling.

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