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Biochemical Pharmacology 69 (2005) 1177–1186
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

2-Aminoethoxydiphenyl borate perturbs hormone-sensitive calcium stores and blocks store-operated calcium influx pathways independent of cytoskeletal disruption in human A549 lung cancer cells

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Received 25 September 2004; accepted 24 January 2005

Abstract

Recent studies have identified novel actions for 2-aminoethoxydiphenyl borate (2-APB) in triggering calcium release and enhancing calcium influx induced by the depletion of intracellular calcium stores. In this study, we have examined the effects of 2-APB on the human lung adenocarcinoma A549 cell line, which we have previously shown displays a unique calcium influx response, when ER calcium stores are depleted by thapsigargin (TG) treatment. Here, we show that low concentrations of 2-APB failed to induce the rapid augmentation of TG-activated calcium influx previously reported for other cell types. We observed that store-operated calcium (SOC) channels in the A549 cell line exhibited short-term sensitivity to low doses of 2-APB, perhaps reflecting a delayed augmentation of SOC channel activity or the recruitment of 2-APB-insensitive SOC channels. In both intact and permeabilized cells, 2-APB effectively discharged a subset of A549 calcium pools corresponding to the hormone-sensitive intracellular calcium stores. The 2-APB-induced calcium release produced a long-lasting perturbation of the adenosine triphosphate (ATP)-releasable calcium pools, effectively uncoupling ATP-activated calcium release even, when stores are replenished with calcium. In contrast to previous reports, we found that disruption of either the actin or microtubule-based cytoskeleton failed to block the 2-APB-induced effects on calcium signaling in A549 cells. Our study describes novel cytoskeletal-independent effects of 2-APB on Ca^{2+}-signaling pathways, revealing differentially sensitive Ca^{2+}-influx pathways and long-term perturbation of hormone-sensitive Ca^{2+} stores.

Keywords: 2-Aminoethoxydiphenyl borate; Store-operated calcium influx; Calcium stores; A549 lung cells; Inositol 1,4,5-trisphosphate receptor; Cytoskeleton

1. Introduction

Calcium plays a pivotal role in controlling numerous cellular functions ranging from short-term effects such as muscle contraction and neurotransmitter secretion to long-term changes in apoptosis, cellular proliferation and differentiation [1,2]. Ca^{2+} mobilization is initiated by cell membrane receptors coupled to phospholipase C (PLC) isoforms resulting in the production of IP_3, which releases Ca^{2+} from intracellular stores in the ER. In the majority of cells examined, depletion of the ER Ca^{2+} stores is coupled to the activation of Ca^{2+}-influx channels, a process referred to as capacitative Ca^{2+} entry (CCE) or store-operated Ca^{2+} entry [3–5]. The compound 2-APB was first characterized as a cell-permeable non-competitive inhibitor of IP_3Rs, binding to a site separate from the IP_3-binding site [6]. More recently, 2-APB has been shown to be a potent store-operated calcium (SOC) blocker in many cell lines and has been widely used to probe the actions of both IP_3Rs and SOC channels in Ca^{2+}-signaling pathways [2,7]. 2-APB has aided in our understanding of the mechanisms cells use to couple ER stores depletion to Ca^{2+} influx, although many features of this process remain unknown. Despite reports of multiple targets for 2-APB's actions [7,8], the

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; SOC, store-operated Ca^{2+} channel; 2-APB, 2-aminoethoxydiphenyl borate; ER, endoplasmic reticulum; PM, plasma membrane; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; TG, thapsigargin; $[Ca^{2+}]_i$, intracellular Ca^{2+}; HBSS, Hank's balanced salt solution

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doi:10.1016/j.bcp.2005.01.011

compound represents an important tool in understanding intracellular Ca^{2+}-signaling pathways.

Recent studies have reported intriguing actions of 2-APB on Ca^{2+}-signaling pathways that depend on ER Ca^{2+} store depletion and on ER/PM interactions requiring the function of an intact cytoskeleton [9,10]. These findings suggest that 2-APB or related compounds may help provide a better understanding of the mechanism that couples depleted Ca^{2+} stores to Ca^{2+} influx through PM SOC channels.

We have recently described a unique Ca^{2+}-signaling phenotype in the human lung adenocarcinoma cell line A549, which appears to become altered, when these cells develop chemoresistance to the cancer drug paclitaxel [11]. In particular, A549 cells exhibit a unique Ca^{2+}-influx response, when ER stores are depleted using the SERCA blocker TG, characterized by a sequential fast transient and slower sustained Ca^{2+}-influx component. In the present study, we sought to characterize the actions of 2-APB in A549 cells to better understand the coupling mechanisms mediating Ca^{2+} influx in these cells. Here, we report novel dose and stimulus-specific effects of 2-APB on Ca^{2+}-influx responses in A549 cells that suggest differential SOC sensitivity to the inhibitory action of this compound. In addition, we find that 2-APB is a potent inducer of Ca^{2+} mobilization from intracellular stores, specifically discharging hormone-sensitive compartments. Moreover, in contrast to previous reports [9,10] we find that 2-APB's actions to modulate both Ca^{2+} influx and release are not affected by perturbation of the actin or microtubule-based cytoskeleton.

2. Materials and methods

2.1. Cell culture and materials

The lung adenocarcinoma cell line A549 (ATCC CCL-185) was cultured as a monolayer in DMEM-F12 medium, supplemented with 1% L-glutamine, 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate at 37 °C with 95% air and 5% CO_2. For all experiments, cells in log phase were used. All reagents were from commercial sources, including cell culture reagents, paclitaxel, ATP, creatine kinase, creatine phosphate (Sigma Chemicals), Fura-2 AM, Fluo-3, BODIPY FL phallacidin (Molecular Probes Inc.), Hank's Balanced Salt Solution (HBSS) (Cambrex), digitonin, 2-APB (Calbiochem), TG (LC laboratories) and cytochalasin D (Tocris). Stock solutions of TG (1.5 mM), 2-APB (75 mM), paclitaxel (1 mM), cytochalasin D (10 mM) and Fluo-3 (3 mM) were prepared in 100% DMSO, while Fura-2 AM (1.5 mM) was prepared in DMSO containing 20% pluronic acid and stored at −20 °C.

2.2. Intracellular calcium measurements

Cells grown on poly-L-lysine glass coverslips were rinsed twice with HBSS (20 mM HEPES, 10 mM glucose, 150 mM NaCl, 1.2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4). Cells were loaded with 1.5 μM Fura-2, AM in 1 ml of HBSS from a stock solution of 1.5 mM Fura-2, AM in DMSO containing 20% pluronic acid, at room temperature for 30 min and subsequently rinsed twice with HBSS. The cells were incubated in HBSS for an additional 30 min to allow complete de-esterification of the dye. In some cases, cells were pre-treated with paclitaxel (5 μM) or cytochalasin D (10 μM) for 4 h and 30 min, respectively. Average fluorescence changes in Fura-2-loaded cell populations were measured by using continuous rapid alternating monochromator excitation (340 and 380 nm) and monochromoter emission (510 nm) in a fluorescence spectrophotometer equipped with a 75 Watt xenon arc lamp (Quantamaster Model C-61/2000, PTI). Fluorescence ratios were acquired every 0.1 s using data-capture software specific for the instrument (Felix Fluorescence Analysis Software, PTI). Changes in intracellular Ca^{2+} concentration are reported either as peak amplitude fluorescence ratio units or as initial rate changes in fluorescence units per minute.

2.3. Transient transfection

Cells were cultured overnight in two-well Lab-Tek® tissue culture chamber slides (Nunc International Corp.) with 1×10^5 cells per well in 2 ml of culture medium. Two micrograms of the enhanced green fluorescent protein (EGFP)-alpha-tubulin expression construct (ClonTech) in 75 μl of DMEM was mixed with 7.5 μl of SuperFect™ (Qiagen), incubated for 10 min at room temperature and then diluted with 400 μl of complete culture media. Cell monolayers were washed and the DNA–SuperFect mixture was added to the cells. After incubation at 37 °C for 3 h to allow uptake of DNA–SuperFect complexes, 2 ml of fresh complete culture media was added to the chamber. Expression of EGFP was monitored by fluorescence microscopy. In general, subsequent experiments were done 24 h after transfection.

2.4. Confocal microscopy

Cells were seeded on poly-L-lysine coated glass coverslips (10 mm × 22 mm) and incubated in the presence or absence of cytochalasin D (10 μM) for a period of 30 min. Cells were then fixed with formaldehyde (3.7% in PBS) for 15 min at room temperature and permeabilized with acetone at −20 °C for 10 min. After three washes with PBS, cells were stained with BODIPY FL phallacidin (1 μg/ml in PBS, Molecular Probes) for 15 min at room temperature. Excess dye was rinsed with PBS and the coverslips were mounted onto glass slides with Cytoseal-60 (Richard-Allen Scientific). Cells were observed using a confocal microscope equipped with an argon ion laser scanning head (TCS NT, Leica Microsystems) fitted with a 100× oil immersion lens (N.A. = 1.40). Fluorescent images were

collected with an approximately 1.3 nm pinhole aperture. Images acquired are averages of 16 scans of the same plane in the *z*-axis.

2.5. Fluorescence microscopy

Subconfluent monolayers of cells transfected with C-terminal EGFP tagged alpha-tubulin (Clontech) were treated with Paclitaxel (5 μM) or vehicle for 5 h. Fluorescence images were captured with ImagePro® software (Media Cybernetics) using a CCD camera (Optronics) attached to a Nikon TE 200 microscope.

2.6. Measurement of Ca^{2+}-release responses in permeabilized A549 cells

Cell permeabilization and fluorescence measurements were conducted as previously described [12]. Briefly, cells were suspended (15×10^6 cells/ml) in 2 ml of an intracellular-like medium containing 140 mM KCl, 2.4 mM $MgCl_2$, 10 mM HEPES, 5 mM NaCl, 5 mM sodium acetate, 50 I.U. creatine kinase, 25 mM creatine phosphate, 2 mM ATP and 3 μM Fluo-3, pH 7.4. Treatment with digitonin (50 μM) was used to disrupt the plasma membrane. The addition of 50 μM digitonin resulted in approximately 98% permeabilization of cells, as determined by trypan blue dye incorporation. The Fluo-3 fluorescence signals (excitation, 503 nm; emission, 530 nm) were measured using a PTI fluorescence spectrophotometer and recorded as a function of time at 37 °C. Changes in Ca^{2+} concentration in permeabilized cells are reported either as peak amplitude Fluo-3 fluorescence units or as initial rate changes in Fluo-3 fluorescence units per second. All the solutions and the intracellular-like buffer required for the assay were prepared using deionized ultra filtered water (Fischer Scientific).

2.7. Statistical analysis

The data are presented as the mean experimental values with statistical variation indicated by the standard error of the means ± S.E.M. with the number of experimental repetitions indicated in parentheses. Statistical significance was determined by using the paired Student's *t*-test for comparison of means.

3. Results

3.1. 2-APB exerts differential blockade on TG- and hormone-induced Ca^{2+} influx

We have previously observed a unique Ca^{2+}-response phenotype in TG-stimulated A549 human lung cancer cells [11], suggesting activation of novel Ca^{2+}-influx pathways. In this study, we sought to further clarify the nature of Ca^{2+}-signaling responses in A549 cells by using the SOC pathway regulator 2-APB. Fig. 1A shows the A549 cell response to the SERCA inhibitor TG, which induces a unique 'triphasic' Ca^{2+} signal in these cells. The first phase

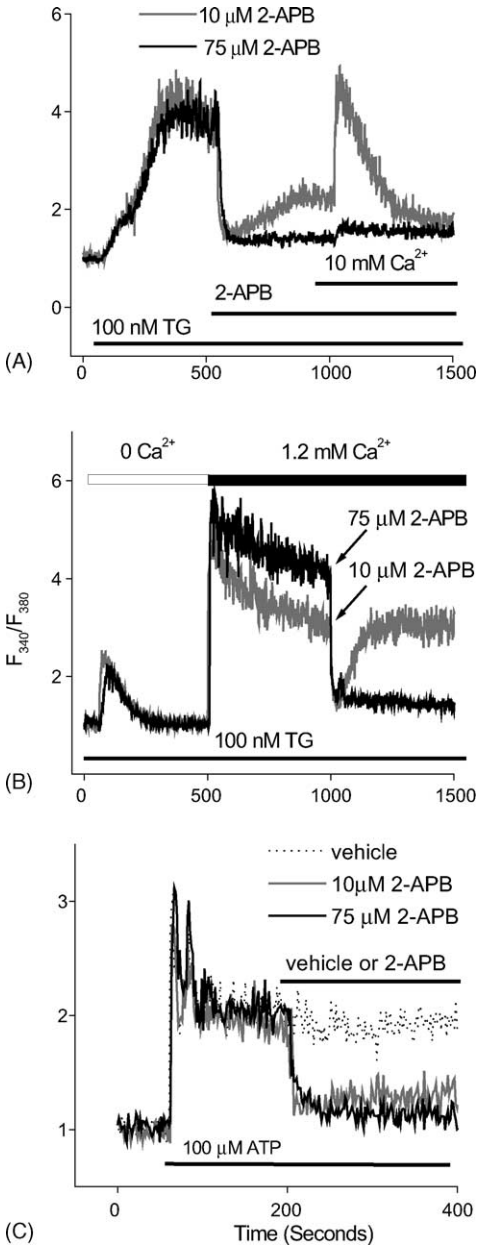

Fig. 1. 2-APB blocks calcium influx in a dose- and stimulus-specific manner in A549 cells: (A) TG (100 nM) in the presence of extracellular Ca^{2+} (1.2 mM) induced a triphasic Ca^{2+} signal with Ca^{2+}-release coupling to a rapid Ca^{2+} influx phase that reached a sustained plateau of Ca^{2+} entry. The sustained phase of Ca^{2+} entry was rapidly inhibited by both 10 μM and 75 μM 2-APB. Inhibition of Ca^{2+} influx by 10 μM 2-APB was transient in the TG-stimulated cells and further addition of Ca^{2+} (10 mM) produced an increase in the F340/380 fluorescence signal. (B) In the absence of extracellular Ca^{2+}, TG (100 nM) induced a transient Ca^{2+}-release response. Ca^{2+} influx was observed after restoration of 1.2 mM $Ca^{2+}$and was inhibited by 2-APB (10 μM and 75 μM) but as in (A) Ca^{2+} influx re-appeared, when 10 μM 2-APB was applied. (C) ATP (100 μM) in the presence of extracellular Ca^{2+} (1.2 mM) induced a sustained Ca^{2+}-influx response that was inhibited by both 10 and 75 μM 2-APB.

of the TG-induced response resulted in an increase in the F340/380 ratio corresponding to 0.64 ± 0.06 fluorescence ratio units/min ($n = 9$), which plateaus briefly (shoulder of initial peak) before developing into a rapid second phase (0.85 ± 0.08 fluorescence ratio units/min) increase in the F340/380 fluorescence signal that leveled off to the third phase sustained elevated fluorescence peak (Fig. 1A). Fig. 1B and our previous studies revealed that the TG-induced initial rise in $[Ca^{2+}]_i$ is mainly due to release from intracellular Ca^{2+} stores, whereas the secondary more slowly developing responses consisting of a rapid increase in $[Ca^{2+}]_i$ followed by the sustained elevated plateau phase are due to Ca^{2+} influx [11]. 2-APB treatment at both high (75 μM) and low (10 μM) concentrations rapidly abolished the TG-induced sustained phase of Ca^{2+} influx (Fig. 1A). Surprisingly, low-dose 2-APB (10 μM) treatment exerted only a transient inhibition of the SOC Ca^{2+}-influx response induced by TG treatment. Ca^{2+} influx gradually recovered (0.16 ± 0.06 fluorescence ratio units/min; $n = 7$) to a sustained plateau phase after maximal inhibition induced by 2-APB. Indeed, SOC channels appeared to recover full sensitivity in the presence of 10 μM 2-APB as the addition of high Ca^{2+} (10 mM) to the external medium elicited a rapid, albeit transient, increase in $[Ca^{2+}]_i$ (Fig. 1A). The recovery of Ca^{2+} influx following low-dose (10 μM) 2-APB-induced blockade was even more pronounced (0.87 ± 0.12 fluorescence ratio units/min; $n = 6$) in A549 cells treated with 100 nM TG in Ca^{2+}-free medium (Fig. 1B). The maximal TG-releasable pool based on peak amplitude responses in these experiments was 1.37 ± 0.32 fluorescence units ($n = 12$). Fig. 1C shows that A549 cells treated with 100 μM ATP undergo a biphasic Ca^{2+} response characterized by a rapid initial discharge from intracellular stores followed by a sustained plateau phase of Ca^{2+} influx. The addition of 2-APB at both high (75 μM) and low (10 μM) concentrations during the ATP-induced sustained Ca^{2+}-entry phase rapidly abolished the influx component (Fig. 1A). In contrast to TG stimulation, Ca^{2+}-influx responses failed to recover when cells were treated with either low or high doses of 2-APB, suggesting a stimulus-specific sensitivity of Ca^{2+}-influx pathways to the compound (Fig. 1C).

3.2. 2-APB induces release from intracellular Ca^{2+} stores in A549 cells in a dose-dependent manner

Given previous reports of the dual actions of 2-APB on both SOC channels and IP_3Rs [9,10], we investigated whether the compound could mediate release from intracellular Ca^{2+} stores in A549 cells. Fig. 2A shows that 2-APB when applied at 75 μM induced a $[Ca^{2+}]_i$ transient (peak fluorescence increase of 0.87 ± 0.21 fluorescence units) in the absence of extracellular Ca^{2+}, indicating the ability of 2-APB to mediate Ca^{2+} discharge from intracellular stores. Restoration of extracellular Ca^{2+} to 1.2 mM following the 2-APB-induced Ca^{2+} transient failed to

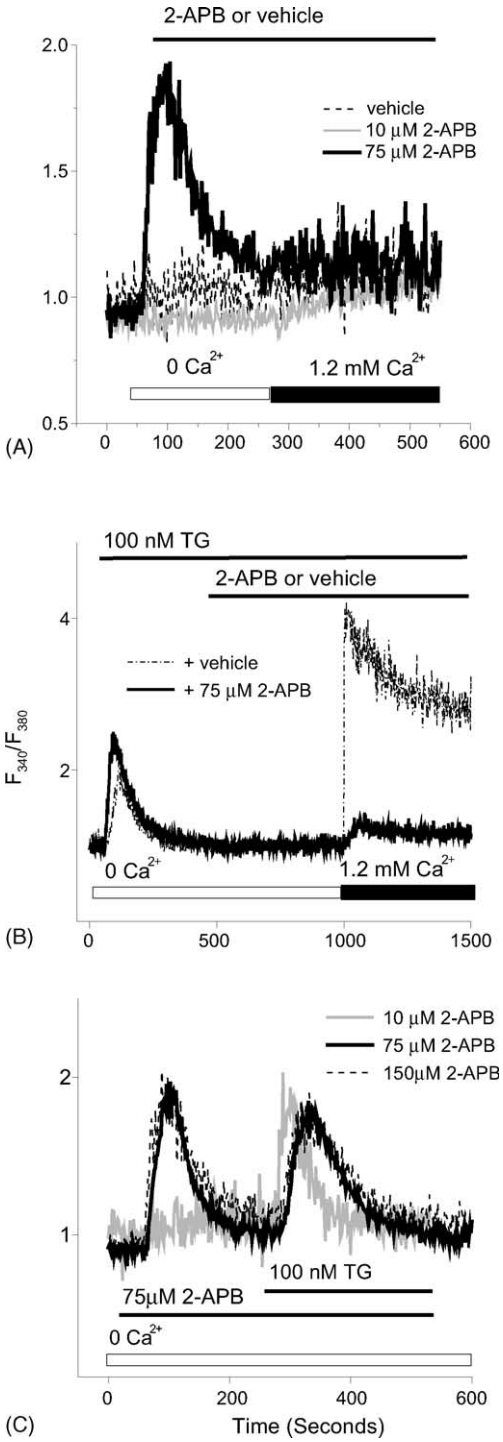

Fig. 2. 2-APB releases Ca^{2+} from a subcompartment of the TG-releasable Ca^{2+} pools in A549 cells: (A) 2-APB (75 μM) increased $[Ca^{2+}]_i$ in a Ca^{2+}-free medium, while 10 μM 2-APB and vehicle did not. Depletion of internal stores by 2-APB (75 μM) failed to induce Ca^{2+} influx upon restoration of extracellular Ca^{2+} (1.2 mM). (B) TG (100 nM)-induced Ca^{2+} release was followed by the addition of 2-APB (75 μM) in a Ca^{2+}-free medium. Restoration of extracellular Ca^{2+} (1.2 mM) resulted in Ca^{2+} influx that was inhibited by 2-APB (75 μM). (C) The addition of 2-APB (75 and 150 μM) increases $[Ca^{2+}]_c$ in a Ca^{2+}-free medium. Subsequent addition of TG (100 nM) was able to induce additional Ca^{2+} release. Application of 2-APB (10 μM) failed to release Ca^{2+}, while TG-induced Ca^{2+} release remained unaltered.

stimulate Ca^{2+} influx, consistent with 2-APB's ability to block SOC-mediated Ca^{2+} influx (Fig. 2A). In contrast, the application of 2-APB at 10 μM did not induce a $[Ca^{2+}]_i$ transient (Fig. 2A), although this concentration was effective at blocking SOC-mediated Ca^{2+} influx (Fig. 1).

To further characterize the 2-APB-mediated Ca^{2+}-release response we examined the relationship between the 2-APB- and TG-releasable Ca^{2+} pools. Fig. 2B shows that pre-treatment with 100 nM TG in Ca^{2+}-free conditions completely abolishes A549 cell responses to 75 μM 2-APB. The application of 2-APB in this experiment abrogates the TG-induced Ca^{2+}-influx response observable by restoration of extracellular Ca^{2+} (1.2 mM) (Fig. 2B). Conversely, pre-treatment with 2-APB (75 μM) does not abolish A549 cell responses to the subsequent application of 100 nM TG (Fig. 2C). We examined varying 2-APB concentrations (10, 75 and 150 μM) using Ca^{2+}-free conditions, all of which failed to significantly alter the peak amplitude of TG-inducible responses at 0.73 ± 0.14 fluorescence units ($p < 0.001$; $n = 6$) (Fig. 2C).

3.3. 2-APB induces heparin-insensitive Ca^{2+} release in permeabilized A549 cells

Our results suggest that 2-APB's target to stimulate Ca^{2+} release is not the SERCA pumps but rather a hormone-like physiological target that permits discharge of a subcompartment of the global TG-sensitive Ca^{2+} store. Thus, we examined the nature of 2-APB-mediated Ca^{2+} release by investigating possible intracellular targets in A549 cells. For these experiments, we employed a permeabilized cell assay using suspensions of A549 cells. Fig. 3A shows a representative experiment in which A549 cells were suspended in an intracellular-like medium and the plasma membranes were permeabilized by the addition of 50 μM digitonin. Digitonin induced a rapid decline in the Fluo-3 fluorescence signals as Ca^{2+} is sequestered into intracellular storage compartments [12]. The application of IP_3 (500 nM) to digitonin-permeabilized A549 cells resulted in a rapid Ca^{2+} transient (initial Ca^{2+} release rate of 0.53 ± 0.18 fluorescence units/s; $n = 8$) from IP_3-sensitive stores, presumably in the ER (Fig. 3A). The IP_3-induced response decayed to pre-stimulus levels by 200 ± 22 s ($n = 8$). The addition of the SERCA blocker TG (100 nM) resulted in a very different type of response in which Ca^{2+} release proceeded more slowly and was longer lasting (0.0076 ± 0.0017 fluorescence units/s; sustained response for >300 s; $n = 12$), consistent with the actions of a pump inhibitor. Subsequent addition of the Ca^{2+} ionophore ionomycin mobilized Ca^{2+} stored in the remaining TG-insensitive pools presumably including mitochondrial Ca^{2+} stores (Fig. 3A). Fig. 3B shows that the direct application of 2-APB (75 μM) to permeabilized A549 cells also induced a rapid Ca^{2+} transient (initial rate of Ca^{2+} release 0.36 ± 0.13 fluorescence units/s) and a peak signal response of 0.46 ± 0.16 fluorescence units ($n = 7$). The addition of IP_3 (500 nM) following the decay of the 2-APB-mediated response stimulated an additional Ca^{2+} transient (0.34 ± 0.09 fluorescence units/s) closely resembling 2-APB-induced Ca^{2+} signals in peak signal amplitude as well as signal decay properties (0.49 ± 0.11

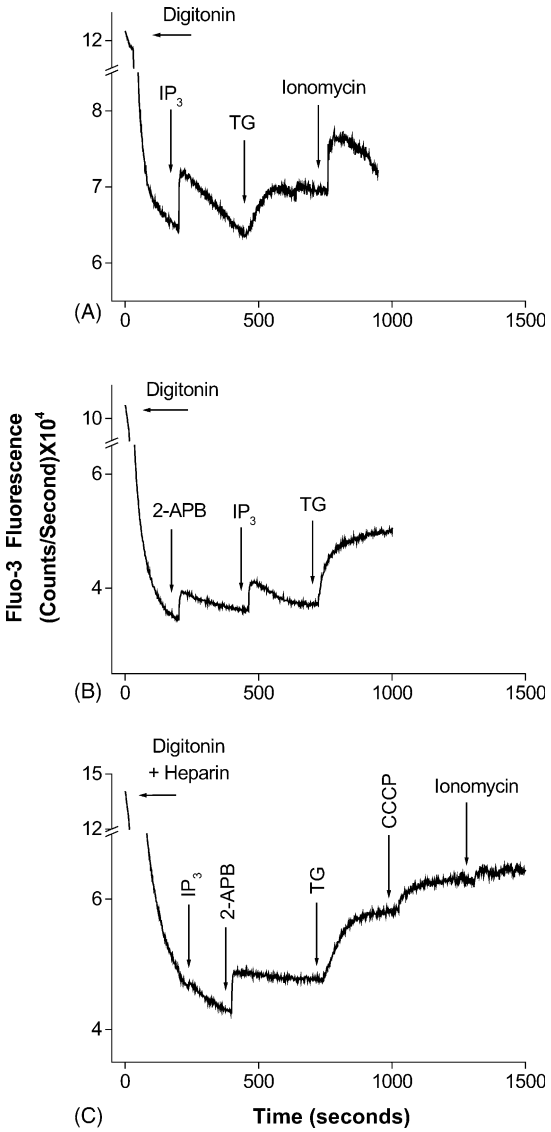

Fig. 3. 2-APB induces heparin-insensitive Ca^{2+} release in permeabilized A549 cells. Cells were suspended in an intracellular-like buffer (as described in Section 2) and permeabilized using digitonin (50 μM): (A) following permeabilization, stable fluorescence signals (using Fluo-3 as the Ca^{2+} indicator) were obtained. The addition of IP_3 (500 nM) produced a transient increase in $[Ca^{2+}]_i$. TG (100 nM) produced a sustained increase in $[Ca^{2+}]_i$. Ionomycin (1 μM) discharged Ca^{2+} from additional Ca^{2+} stores. (B) subsequent to permeabilization, 2-APB (75 μM) induced a rapid increase in $[Ca^{2+}]_i$. The addition of IP_3 (500 nM) also produced a transient increase in $[Ca^{2+}]_i$. TG (100 nM) raised $[Ca^{2+}]_i$ levels in a more gradual manner. (C) Cells were suspended in an intracellular-like buffer and permeabilized using digitonin (50 μM) with the IP_3R blocker, heparin sulfate (60 μg/ml). Heparin abolished IP_3 (500 nM)-induced responses but failed to inhibit 2-APB-mediated Ca^{2+} release. TG (100 nM) caused a further increase in $[Ca^{2+}]_i$ levels. The addition of CCCP (1 μM) discharged the mitochondrial Ca^{2+}-releasable stores. The addition of ionomycin (1 μM) induced a very small $[Ca^{2+}]_i$ transient suggesting previous depletion of the internal Ca^{2+} stores. Each trace is representative of three individual experiments.

fluorescence units and decay of approximately 80% of the response by 200 s; $n = 7$) (Fig. 3B). Fig. 3B also shows that the TG (100 nM)-releasable pools are intact after the sequential discharge responses induced by 2-APB and IP_3 treatment.

To determine if 2-APB's effects were due to direct interactions on the IP_3Rs in A549 cells, we tested the sensitivity of 2-APB-mediated responses to heparin, a well-known IP_3R competitive inhibitor. Fig. 3C shows that in the permeabilized cell assay 2-APB's actions to release Ca^{2+} were insensitive to heparin treatment (60 μg/ml). Heparin pre-treatment abolished IP_3 responses, yet 2-APB induced a robust Ca^{2+} discharge (initial Ca^{2+} release rate of 0.44 ± 0.12 fluorescence units/s; $n = 5$) that decayed more slowly than in permeabilized cells not treated with heparin given only approximately 20% of the response decayed by 300 s (Fig. 3B cf. C; $p < 0.001$). Fig. 3C also shows that the sequential addition of TG (100 nM), the protonophore CCCP (1 μM) and ionomycin (1 μM) effectively discharged the ER, mitochondrial and other Ca^{2+} pools in A549 cells, indicating that heparin treatment did not affect Ca^{2+} uptake into, or release from these storage compartments.

3.4. 2-APB abolishes ATP-mediated Ca^{2+} release resulting in long-lasting perturbation of ATP-sensitive Ca^{2+} pools

The preceding experiments suggest that 2-APB may be acting selectively on the IP_3 pathway, specifically targeting IP_3-sensitive Ca^{2+} stores perhaps by direct interaction with the IP_3R or other proteins regulating IP_3R function. To investigate the effects of 2-APB on IP_3/IP_3R pathways in A549 cells, we examined the effects of the compound on ATP-induced Ca^{2+}-signaling responses. We have shown (Fig. 1C) that ATP-induced Ca^{2+} influx is blocked by 2-APB at all concentrations tested. In contrast, TG-induced Ca^{2+} influx was less sensitive to low-dose 2-APB (10 μM), with recovery of influx responses gradually developing following an initial phase of inhibition. We tested the effects of 2-APB pre-treatment on ATP-induced Ca^{2+} release and influx responses. As shown in Fig. 4A, low concentrations of 2-APB (10 μM) did not induce Ca^{2+} release in A549 cells (Fig. 2A) nor did they block the actions of ATP (100 μM) to release Ca^{2+} from intracellular stores. As noted earlier (Fig. 1C), however, 2-APB applied at 10 μM did block Ca^{2+}-influx responses stimulated by ATP treatment, as compared to vehicle-treated control A549 cells (Fig. 4A). Unexpectedly, the application of higher doses of 2-APB (75 μM) to A549 cells completely abolished responses to the subsequent addition of ATP (Fig. 4A). Similar results were found, when this experiment was repeated using bradykinin (BK), another hormone pathway coupled to phosphoinositide hydrolysis and Ca^{2+} mobilization (data not shown). As noted previously (Fig. 2A), 2-APB (at 75 μM) by itself induced Ca^{2+} release

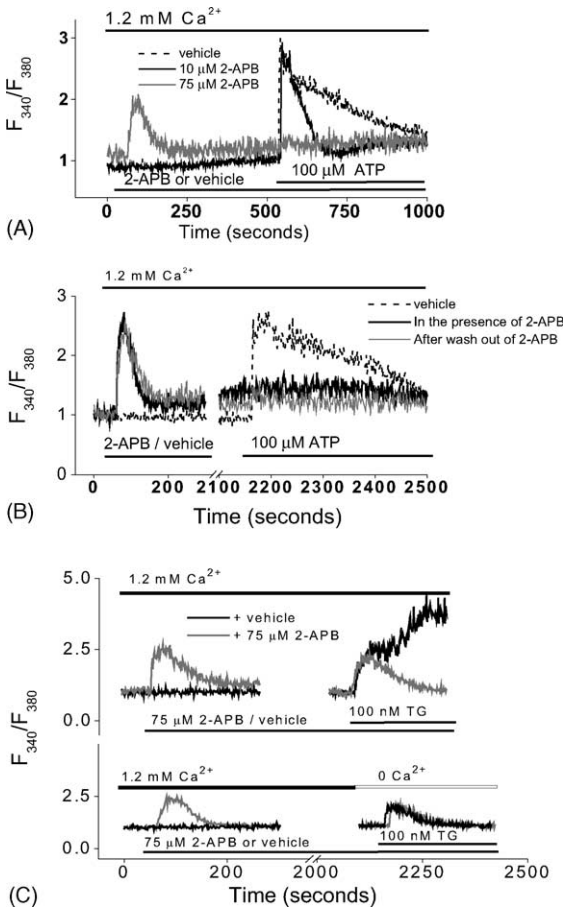

Fig. 4. 2-APB treatment results in long-lasting perturbation of ATP-sensitive Ca^{2+} pools: (A) in the absence of 2-APB, ATP (100 μM)-induced Ca^{2+} release and produced a sustained Ca^{2+} influx. The addition of 2-APB (10 μM) failed to increase $[Ca^{2+}]_i$, but was able to inhibit Ca^{2+} influx after the addition of ATP. 2-APB (75 μM) transiently increased $[Ca^{2+}]_I$, while the addition of ATP failed to increase $[Ca^{2+}]_i$. (B) In the presence of extracellular Ca^{2+}, cells grown on coverslips were pre-treated with vehicle or 2-APB (75 μM, 5 min). Coverslips were then washed with HBSS and incubated in fresh HBSS in the presence or absence of 2-APB for 30 min. In cells pre-treated with vehicle, ATP (100 μM) produced a rapid but prolonged increase in $[Ca^{2+}]_i$. Following pre-treatment with 2-APB (75 μM), ATP failed to produce an increase in $[Ca^{2+}]_i$ both in the continued presence of or after wash out of 2-APB. (C) In Ca^{2+} containing medium, cells pre-treated with 2-APB (75 μM) produced a transient increase in $[Ca^{2+}]_i$. Following the incubation in HBSS for 30 min, the addition of TG (100 nM) produced a transient $[Ca^{2+}]_i$ increase in the presence of 2-APB (C, upper panel). In the absence of 2-APB, TG produced a sustained Ca^{2+} influx (C, upper panel). The addition of TG (100 nM) in a Ca^{2+}-free medium, following incubation in HBSS for 30 min, elicited transient increases in $[Ca^{2+}]_i$ both in the presence (C, lower panel) or absence of 2-APB (C, lower panel).

(Fig. 4A). However, it is not likely that 75 μM 2-APB blocks ATP responses by depleting Ca^{2+} stores, given TG responses are still preserved following application of 2-APB at this dose and at concentrations up to 150 μM (Fig. 2C). 2-APB may specifically deplete Ca^{2+} from a discrete Ca^{2+} pool that includes the hormone (eg., ATP, BK)-sensitive compartments, leaving other TG-sensitive Ca^{2+} pools intact. To investigate this possibility, we performed experiments in which ATP responses were

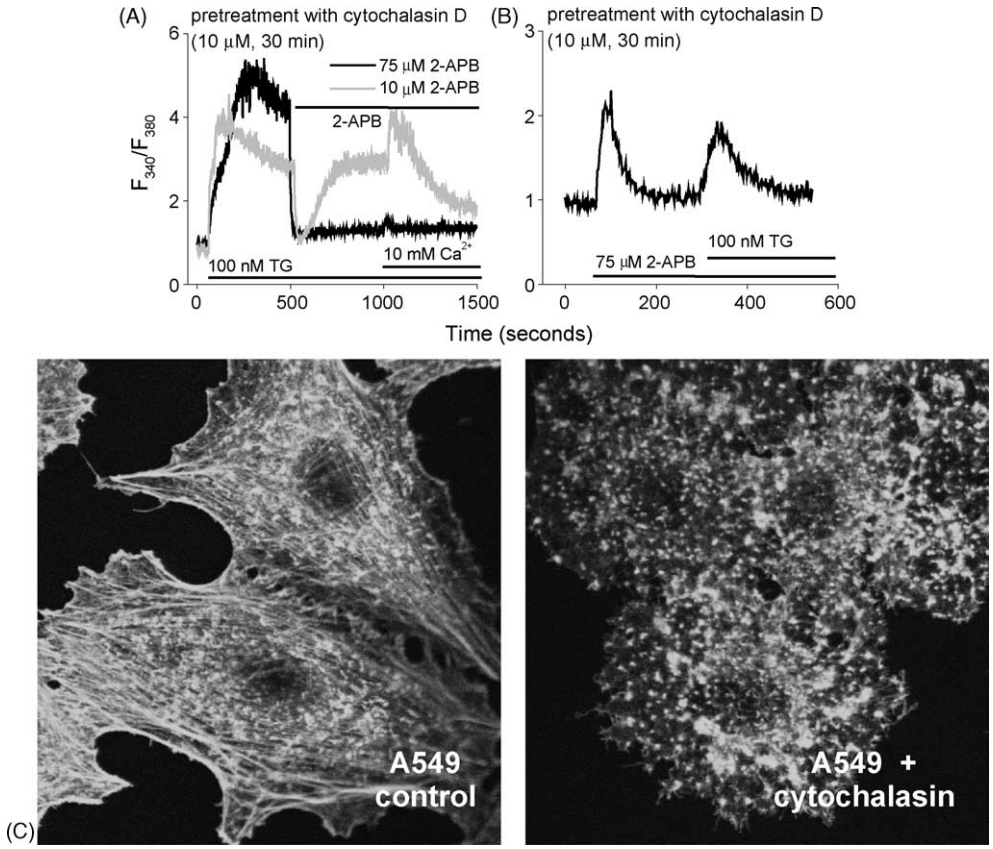

Fig. 5. Actions of 2-APB to block Ca^{2+} influx and promote Ca^{2+} release are not affected in actin damaged A549 cells. Cells grown on poly-L-lysine coated coverslips were pre-treated with cytochalasin D (10 μM) in HBSS for 30 min: (A) store-operated Ca^{2+} entry, induced by TG (100 nM) was inhibited by the addition of 2-APB (75 μM). Addition of Ca^{2+} (10 mM) failed to increase $[Ca^{2+}]_i$. Ca^{2+} influx was also inhibited by 10 μM 2-APB, but responses gradually re-appeared. High extracellular Ca^{2+} (10 mM) further stimulated Ca^{2+} influx, suggesting recovery of channel activation at low-dose (10 μM) 2-APB. (B) addition of 2-APB (75 μM), in a Ca^{2+}-free medium was able to transiently increase $[Ca^{2+}]_i$ levels. Addition of TG (100 nM) also elicited a transient increase in $[Ca^{2+}]_i$. (C) Confocal microscopy of the actin cytoskeleton. Cells were treated with cytochalasin D (10 μM) for 30 min and stained with BODIPY FL phallacidin. Confocal microscopy revealed extensive damage to the actin cytoskeleton in response to cytochalasin D treatment. Magnification 100×.

measured following 30 min of recovery from the application of 75 μM 2-APB, to allow replenishment of the ATP-sensitive Ca^{2+} stores. Surprisingly, we observed that ATP responses remained absent 30 min after application of 2-APB (Fig. 4B) and could not be restored after A549 cells were washed free of 2-APB (Fig. 4B). This long-lasting effect induced by 2-APB treatment was again specific for the ATP-sensitive Ca^{2+} stores, since TG responses remained intact after a 30 min recovery from the application of 75 (M 2-APB (Fig. 4C). Fig. 4C shows that TG responses were detected after 30 min of 2-APB treatment either in the presence or absence of Ca^{2+} in the extracellular medium.

3.5. 2-APB's actions to block Ca^{2+} influx or to stimulate Ca^{2+} release in A549 cells do not depend on cytoskeletal integrity

As noted, 2-APB does not block IP_3-induced Ca^{2+} release in A549-permeabilized cells (Fig. 3B), yet it does effectively abolish ATP-induced Ca^{2+} release in intact cells at 75 μM (Fig. 4A), an effect maintained for at least 30 min (Fig. 4B). We hypothesized that the differential effects of 2-APB in permeabilized cells as compared to intact cells may result from de-coupled ER/PM interactions due to physical disruption of the digitonin-permeabilized plasma membrane. To test the role of the cytoskeleton in regulating 2-APB's actions in A549 cells, we used cytochalasin D and paclitaxel to perturb actin and microtubule function, respectively. Fig. 5A shows that 30 min pre-treatment of A549 cells with 10 μM cytochalasin D did not prevent 2-APB-mediated inhibition of TG-induced Ca^{2+} influx. Actin perturbation did alter the TG-response profile in reducing the delay phase between TG-induced Ca^{2+} release and activation of Ca^{2+} influx (Fig. 5A cf. Fig. 1A). Also, TG-induced Ca^{2+}influx recovered more quickly (0.62 ± 0.15 fluorescence ratio units/min, $n = 5$), when 10 μM 2-APB was applied compared to cells not exposed to cytochalasin D (Fig. 5A cf. Fig. 1A, $p < 0.001$). Further, cytochalasin D-mediated actin damage also did not prevent 2-APB's actions to release Ca^{2+} from intracellular stores (Fig. 5B). As before, Fig. 5B shows that 75 μM 2-APB fails to discharge all of the A549 Ca^{2+} pools, since a component of TG-releasable Ca^{2+} remains following 2-APB treat-

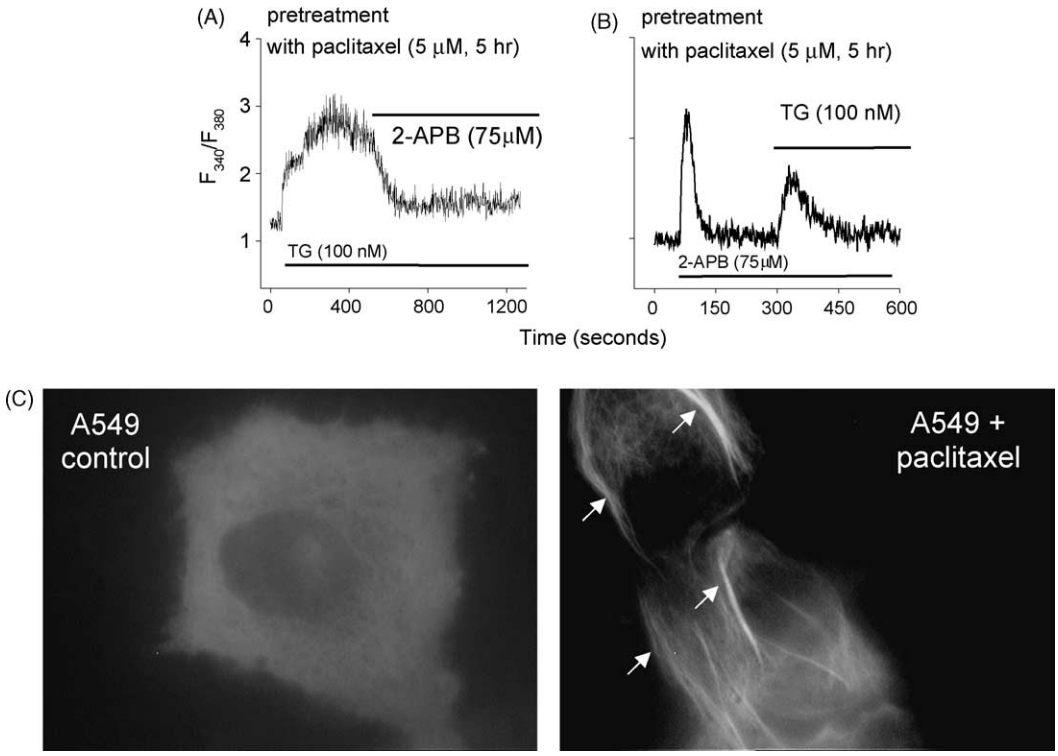

Fig. 6. Actions of 2-APB to block Ca^{2+} influx and promote Ca^{2+} release are not affected in microtubule damaged A549 cells. Cells grown on poly-L-lysine coated coverslips were treated with paclitaxel (5 μM) for 5 h: (A) store-operated Ca^{2+} entry, induced by TG (100 nM) was inhibited by the addition of 2-APB (75 μM). (B) Addition of 2-APB (75 μM), in a Ca^{2+}-free medium was able to transiently increase $[Ca^{2+}]_i$ levels. The addition of TG (100 nM) also elicited a transient increase in $[Ca^{2+}]_i$. (C) Fluorescent images of pEGFP-alpha-tubulin-expressing cells. A uniform network of microtubules was observed throughout the cytoplasm in untreated cells. Treatment with paclitaxel (5 μM, 5 h) resulted in increased stabilization and bundling (arrows) indicative of microtubular damage.

ment. To verify that cytochalasin D treatment was perturbing actin function, we used confocal microscopy and phallacidin fluorescence staining to monitor F-actin structure in A549 cells. As shown in Fig. 5C, cells treated with cytochalasin D for 30 min showed pronounced disruption of F-actin networks clearly discernible in control A549 cells, verifying that the concentration and duration of cytochalasin D treatment in these experiments was perturbing filamentous actin formation.

Similarly, we investigated the effects of microtubule perturbation on 2-APB responses by exposing A549 cells to paclitaxel (5 μM). Fig. 6A shows that a 5 h pre-treatment with paclitaxel does not prevent 2-APB from inhibiting TG-induced Ca^{2+} influx, although paclitaxel does alter the TG-induced Ca^{2+} response as we have previously observed [11]. Specifically, the peak amplitude TG response is reduced approximately 50% following paclitaxel exposure (peak fluorescence ratio of 1.59 ± 0.23 versus 2.94 ± 0.27, Fig. 6A cf. Fig. 1A). Fig. 6A also shows that the delay between Ca^{2+} release and activation of Ca^{2+} influx is increased and the initial rate of Ca^{2+} influx is reduced to 0.25 ± 0.09 fluorescence ratio units/min ($n = 5$) compared to cells not exposed to paclitaxel (0.85 ± 0.08 fluorescence ratio units/min, $p < 0.001$). In addition, we did not observe the gradual recovery of Ca^{2+} influx in 10 μM 2-APB-treated cells exposed to paclitaxel (data not shown), as was noted in cytochalasin D and untreated A549 cells (Fig. 1A and B and Fig. 5A). As observed for cytochalasin D, paclitaxel treatment does not alter the ability of 2-APB (75 μM) to induce Ca^{2+} release in A549 cells (Fig. 6B). To ensure that paclitaxel treatment was perturbing microtubule function, we employed conventional fluorescence microscopy using cells transfected with an expression vector encoding green fluorescent protein labeled α tubulin (pEGFP-α tubulin). Fig. 6C shows that control A549 cells expressing EGFP-α tubulin reveal a uniform distribution of microtubule fibers throughout the cytoplasm. In contrast, paclitaxel-treated cells show an appreciable non-uniform bundling of microtubules consistent with the known action of paclitaxel to stabilize tubulin polymers and thereby to interfere with the dynamic assembly and disassembly of microtubules.

4. Discussion

We have previously reported a novel pattern of Ca^{2+} influx induced by TG treatment in the A549 cell line [11]. Ca^{2+} influx induced by TG appears to proceed in two distinct phases in A549 cells: a rapid transient phase followed by a comparatively slower long-lasting phase [11]. Thus, we proposed that the A549 cell would display a unique response profile to 2-APB, given the preliminary suggestions that the compound sensitively targets ER/PM coupling events that precede Ca^{2+} influx.

We found that 2-APB is a potent blocker of Ca^{2+} influx in A549 cells stimulated by either activators of phosphatidylinositol hydrolysis or by TG-induced global ER Ca^{2+} depletion. Even at low concentrations of 2-APB, we observed rapid inhibition of TG or ATP-induced Ca^{2+} influx. This result contrasts significantly with the reported pronounced augmentation of Ca^{2+} influx induced by low 2-APB concentrations (peak effect at 10 μM) in B lymphocytes and other cell types [9]. However, we do observe that TG-stimulated Ca^{2+}-influx responses exhibit a transient sensitivity to lower 2-APB concentrations (10 μM), with the gradual restoration of Ca^{2+} influx even in the continued presence of the compound (Fig. 1A and B). Lower doses of 2-APB may, therefore, promote the recruitment of more SOC channels to the plasma membrane, as previously proposed [9] or activate certain types of SOC channels as it has been recently reported that TRP channels of the vanilloid family are activated by 2-APB [13,14]. These events may occur more slowly in the A549 cell line than in B lymphocytes and other cell types, explaining the gradual re-appearance of the influx response following its initial rapid eradication with low-dose 2-APB application. Indeed, it may be a more protracted coupling mechanism in A549 cells that accounts for the slower secondary phase of Ca^{2+} influx unique to these cells, when intracellular stores are depleted by TG treatment. Alternatively, it is possible that A549 cells do not express the TRP channel isoforms or TRP heteromeric combinations that confer this property of 2-APB-induced augmentation of Ca^{2+} influx. Nevertheless, this unique mode of Ca^{2+} influx in the A549 cells suggest that this may be a useful cell line for investigating store-depletion-induced coupling mechanisms underlying Ca^{2+}-influx activation.

An intriguing observation we report in this study is the stimulus-specificity of 2-APB's actions on Ca^{2+} influx. In contrast to TG-induced Ca^{2+} influx, ATP-stimulated responses failed to recover, when low doses of 2-APB were used. A recent study reported a similar finding in neutrophils, showing that Ca^{2+} influx induced by receptor activation was more sensitive to 2-APB than Ca^{2+} influx induced by TG treatment [15]. IP_3Rs of the ATP pathway specifically may interact with certain TRP channel isoforms that result in a functional channel complex highly sensitive to 2-APB, such that even at lower doses these channels remain inhibited. It is interesting to note that pretreatment with 2-APB can abolish ATP-induced Ca^{2+}-release responses, further suggesting the presence of a highly sensitive 2-APB target that may regulate both Ca^{2+} release and influx pathways. Even after washing out 2-APB and allowing time for ATP-sensitive pools to refill, we still observed that ATP fails to induce Ca^{2+} discharge, perhaps indicating long-lasting perturbation of the IP_3R/SOC channel complexes due to a heightened sensitivity to 2-APB modulation. These observations suggest that IP_3R/SOC channel interactions may operate in bidirectional modes regulating both Ca^{2+} release and Ca^{2+} influx, analogous to dihydropyridine receptor/ryanodine receptor complexes in skeletal muscle [16,17].

Previous studies have found that 2-APB's Ca^{2+}-releasing actions require ER/PM interactions that depend on cytoskeletal functions [9,10]. These observations further suggest a close physical juxtaposition between ER and PM proteins, possibly IP_3R/SOC channels and other proteins that represent the target(s) for 2-APB regulation. However, we report that neither membrane-permeabilization nor cytoskeletal disruption prevents 2-APB-induced Ca^{2+} release in A549 cells. We found that 2-APB-induced Ca^{2+} release in a manner similar to IP_3 in permeabilized A549 cells. Moreover, 2-APB fails to inhibit IP_3 responses in permeabilized A549 cells, in contrast to earlier reports describing 2-APB as an inhibitor of IP_3R function [6,18]. Our results suggest that 2-APB induces Ca^{2+} release by acting on a site of the IP_3R separate from the IP_3-binding site, as heparin fails to block 2-APB responses yet does appear to alter 2-APB-induced Ca^{2+} release in permeabilized cells. 2-APB may modulate the actions of a protein regulator of IP_3R function, perhaps SOC channels or other proteins that interact with non-IP_3-binding sites.

One possible interpretation of the apparent interaction of 2-APB with both IP_3Rs and SOC channels is that the target of the compound is a regulator of both IP_3Rs and SOC channels. Perhaps, the component targeted by 2-APB functions to regulate the coupling process proposed to occur [1,2] between IP_3Rs and SOC channels during agonist-induced Ca^{2+} influx. This mechanism may be dependent on dynamic cytoskeletal changes permitting close physical positioning of the ER and PM, where IP_3R/SOC channel interaction could occur, representing the coupling event between the ER Ca^{2+} stores and the PM SOC channels. We sought to examine whether cytoskeleton function was needed for the 2-APB-induced effects as previously described [9]. We found that perturbation of either the actin or microtubule-based cytoskeleton does not significantly affect 2-APB's actions to either block Ca^{2+} influx or to release intracellular Ca^{2+} stores. These findings suggest that in A549 cells 2-APB may be interacting separately with IP_3Rs and SOC channels to produce the observed effects. Alternatively, it is possible that 2-APB targets a signaling module comprised of multiple proteins capable of regulating both Ca^{2+} release and influx, as recently proposed for 2-APB's potential influence on phospholipase C activity and IP_3 production [10,19]. This form of 2-APB regulation could control the production of IP_3 and may be less sensitive to cytoskeletal impairment.

In summary, our results using 2-APB in A549 cells contrast significantly with previous reports [9,10]. We found that low-dose (10 μM) 2-APB blocks Ca^{2+} influx rather than augmenting it as reported in other studies. However, we observed that 2-APB's effects were different, when cells were treated with different stimuli, revealing that global Ca^{2+}-store depletion due to TG treatment

gradually re-established functional coupling to Ca^{2+} influx following the initial 2-APB-mediated blockade of influx. In contrast, ATP-induced Ca^{2+} influx did not recover after low-dose 2-APB treatment. We suggest that this difference likely reflects the use of different pathways in A549 cells coupling to Ca^{2+} influx depending on whether TG or ATP are the agents acting to discharge Ca^{2+} stores. Our results suggest these differential pathways also have a differential sensitivity to 2-APB, with the ATP pathway being more sensitive than the TG pathway such that long-term inhibition of ATP-mediated Ca^{2+} release follows 2-APB treatment. The overlapping effects we observe in our study may be explained if 2-APB is targeting a multifunctional signaling module that regulates both Ca^{2+} release and Ca^{2+} influx processes yet is insensitive to cytoskeletal damage. Thus, in A549 cells, 2-APB may interact with the signaling module to promote Ca^{2+} release but also produces a complex that blocks Ca^{2+} influx. Our study underscores the diverse pathways cells employ to couple Ca^{2+} release to the activation of Ca^{2+} influx and is consistent with findings in other cell types that indicate 2-APB may be a useful probe to better understand this coupling process.

Acknowledgments

We express our gratitude to Dr. Paul A. Richmond, Department of Biology, University of the Pacific for plasmids encoding EGFP-alpha-tubulin and EYFP-mito. We also thank Dr. Janey Youngblom, California State University, Stanislaus for allowing us to use the confocal microscopy facility. This work was supported by a New Investigator Program Award from the American Association of Colleges of Pharmacy to D.W.T.

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