JPET Fast Forward. Published on July 13, 2006 as DOI: 10.1124/jpet.106.108209

BASILIOLIDES, A CLASS OF TETRACYCLIC C-19 DILACTONES FROM *THAPSIA GARGANICA*, RELEASE $Ca2^{+}$ FROM THE ENDOPLASMIC RETICULUM AND REGULATE THE ACTIVITY OF THE TRANSCRIPTION FACTORS NFAT, NF-κB AND AP-1 IN T LYMPHOCYTES

Carmen Navarrete, Rocío Sancho, Francisco J. Caballero, Federica Pollastro, Bernd L. Fiebich, Olov Sterner, Giovanni Appendino, and Eduardo Muñoz

Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Córdoba. Facultad de Medicina. Avda. de Menendez Pidal s/n, 14004 Córdoba, Spain (C.N., R.S., F.J.C., E.M.); Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università del Piemonte Orientale, Via Bovio 6, 28100 Novara, Italy (F.P., G.A.); Vivacell Biotechnology GmbH, Ferdinand Porsche-Str.5 D-79211 Denzlingen, Germany (B.L.F.); and Department of Organic Chemistry, Lund University, P.O.Box 124, 221 00 Lund, Sweden (O.S.).

JPET Fast Forward. Published on July 13, 2006 as DOI: 10.1124/jpet.106.108209
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***RUNNING TITLE*:** Basiliolides regulate ER calcium homeostasis

***CORRESPONDING AUTHOR*:** Dr. Eduardo Muñoz, Dpto. de Biología Celular, Fisiología e Inmunología. Facultad de Medicina. Avda. de Menéndez Pidal s/n, 14004 Córdoba, Spain. Tel.: 34 957 218267; Fax: 34 957 218229; E-mail: fi1muble@uco.es

***NUMBER OF TEXT PAGES:* 24**

***NUMBER OF TABLES:* 0**

***NUMBER OF FIGURES:* 7**

***NUMBER OF REFERENCES:* 32**

NUMBER OF WORDS:

Abstract: **236**

Introduction: **676**

Discussion: **966**

ABBREVIATIONS: **AP-1**, activator protein-1; **CRAC**, calcium release activated Ca^{2+} channels; **ER**, endoplasmic reticulum; **ERK**, extracellular regulated kinase; **IκB**, κB inhibitor; **InsP_3R**, inositol (1,4,5) triphosphate receptor; **JNK**, c-Jun N-terminal kinase; **NFAT**, nuclear factor of activated cells; **NF-κB,** nuclear factor *kappa* B; **SERCA**, sarcoplasmatic-endoplasmatic reticulum Ca^{2+}-ATPases; **TCR**, T cell receptor; **TG**, Thapsigargin.

RECOMMENDED SECTION ASSIGMENT: Inflammation and Immunopharmacology

ABSTRACT

Calcium concentration within the endoplasmic reticulum (ER) plays an essential role in cell physiology. We have investigated the effects of basiliolides, a novel class of C-19 dilactones isolated from *Thapsia garganica*, on Ca^{2+} mobilization in T cells. Basiliolide A1 induced a rapid mobilization of intracellular Ca^{2+} in the leukemia T-cell line, Jurkat. First, a rapid calcium peak was observed and inhibited by BAPTA-AM. This initial calcium mobilization was followed by a sustained elevation, mediated by the entry of extracellular calcium through store-operated calcium release-activated Ca^{2+} (CRAC) channels and sensitive to inhibition by EGTA and by the CRAC channel inhibitor BTP-2. Basiliolide A1 mobilized $Ca2^{+}$ from ER stores but in contrast to thapsigargin did not induce apoptosis. Basiliolide A1 induced NFAT1 dephosphorylation and activation that was inhibited by BTP-2 and CsA. In addition, we found that Basiliolide A1 alone did not mediate IκBα degradation nor RelA phosphorylation (ser536) but it synergized with PMA to induce a complete degradation of the NF-κB inhibitory protein and to activate the c-Jun N-terminal Kinase (JNK). Moroever, Basiliolide A1 regulated both IL-2 and TNF-α gene expression at the transcriptional levels. In Basiliolide B, oxidation of one of the two geminal methyls to a carboxymethyl group retained most of the activity of Basiliolide A1. In contrast, Basiliolide C, where the 15-carbon is oxidized to an acetoxymethine, was much less active. These findings qualify these compounds as new probes to investigate intracellular calcium homeostasis.

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INTRODUCTION

The sesquiterpene lactone thapsigargin (TG) is an irreversible inhibitor of sarcoplasmatic-endoplasmatic reticulum Ca^{2+}-ATPases (SERCA), and has become an indispensable tool to study calcium homeostasis (Treiman et al., 1998). TG causes a net transfer of Ca^{2+} from the ER to cytosol, elevating $[Ca^{2+}]_i$ (Rooney and Meldolesi, 1996); (Thastrup et al., 1990) and inducing apoptosis via ER stress. We have recently shown that, apart from TG, *T. garganica* also contain further compounds capable to mobilize Ca^{2+} from the ER. These were identified in a series of unique C-19 dilactones named Basiliolides (Appendino et al., 2005)

In biological systems, calcium ions (Ca^{2+}) function as ubiquitous messengers that play essential role in signal transduction and control a wide array of cellular functions. In T cells the signal transduction pathways triggered by the activation of the T cell receptor (TCR)/CD3 complex lead to the immediate activation of transcription factors that regulate a variety of activation-associated genes. Many of them are cytokines and surface receptors that play an important role in coordinating the immune response (Crabtree and Clipstone, 1994). The signal transduction pathways involved in T cell activation are initiated by the activation of PLCγ by specific tyrosine kinases at the lipid rafts resulting in the hydrolysis of the phosphatidylinositol 4,5 bisphosphate and the generation of inositol (1,4,5) triphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ binds to the $InsP_3$ receptor ($InsP_3R$) in the membrane of the endoplasmic reticulum (ER), which is the main intracellular Ca^{2+} store, and initiates release of the stored Ca^{2+}. Depletion of the ER of Ca^{2+} activates store-operated calcium release-activated Ca^{2+} (CRAC) channels in the plasma membrane that is an essential step during T-lymphocyte activation (Lewis, 2001). The Ca^{2+} influx operated through those channels is critical to induce an effective immune response (Feske et al., 2001).

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Nuclear Factor of activated T cells (NFAT) is a family of transcription factors present in cells and tissues both inside and outside of the immune system and is composed of at least four structurally related members: NFAT1, NFAT2, NFAT3, and NFAT4 that are expressed in the cytoplasm of the resting cells as well as the constitutively nuclear NFAT5 member (Hogan et al., 2003). As a consequence of an increase of $[Ca^{2+}]_i$ levels, calcineurin is activated. This Ca^{2+}-calmodulin-dependent protein phosphatase subsequently dephosphorylates the nuclear factor of activated T cells (NFAT), triggering its nuclear shuttling. Once in the nucleus, NFAT binds to the DNA either alone or in conjunction with other transcriptional partners (Macian, 2005). The activator protein 1 (AP-1) is considered the major interacting partner of NFAT and is also controlled by TCR-dependent Ca^{2+} signals through Ca^{2+}-dependent kinases (Rao et al., 1997). NFAT was first described as an inducible regulatory complex critical for transcriptional induction of IL-2 gene in activated T cells (Shaw et al., 1988) but was subsequently shown to regulate the transcription of many other cytokines and T-cell activation-induced proteins (Macian, 2005).

The transcription factor nuclear factor *kappa* B (NF-κB) is one of the key regulators of genes involved in the immune/inflammatory response as well as in survival from apoptosis. NF-κB is an inducible transcription factor that interacts with a family of inhibitory IκB proteins, of which IκBα is the best characterized (Karin and Ben-Neriah, 2000). In most cell types, these proteins sequester NF-κB in the cytoplasm by masking its nuclear localization sequence. Antigen stimulation in T cells triggers a signaling pathway that results in the phosphorylation, ubiquitination, and subsequent degradation of IκB proteins, with the eventual translocation of NF-κB from the cytoplasm to the nucleus (Karin and Ben-Neriah, 2000). Although the signaling pathways leading to NF-κB activation downstream to TCR engagement are not fully understood, it has been shown that NF-κB activity is also influenced by a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Feng et al., 2002).

Since basiliolides are structurally unrelated to TG, it was interesting to further investigate their biological profile, and compare it with that of TG. We now report that basiliolides mobilize Ca^{2+} from the ER with a mechanism apparently different from that of TG, which qualifies these compounds as new probes to investigate intracellular calcium homeostasis.

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METHODS

Cell lines and reagents. Jurkat cells (ATCC, Rockville, MD) were maintained in exponential growth in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1 mM Hepes and the antibiotics penicillin 100 U/ml and streptomycin 100 μg/ml (Invitrogen). Cells were maintained in a humid chamber at 37ºC under 5% CO_2. The anti-IκBα mAb was a gift from R. T. Hay (St. Andrews, Scotland), and the rabbit polyclonal anti-NFAT1 was a gift from J.M. Redondo (CBM, Madrid, Spain). The anti phospho-ERK 1+2 (sc-7383) was from Santa Cruz Biotechnology (CA, USA), the mAbs anti-phospho-JNK (9255S) and anti-phospho-p65 (3031S) were from New England Biolabs (Hitchin, UK). Thapsigargin and the tetracyclic C-19 dilactones Basiliolide A1, B and C were isolated from *Thapsia garganica* as previously described (Appendino et al., 2005). BTP2 (N-{4-[3,5-bis(Trifluoromethyl)-1H-pyrazol-1-yl]phenyl}-4-methyl-1,2,3-thiadiazole-5-carboxamide) was from Calbiochem. All other reagents were from Sigma Chemical (St. Louis, MO).

Measurement of TNF-α synthesis. Jurkat cells (10^6/ml) were treated as indicated for 6 h in complete medium. After culture, supernatants were harvested and centrifuged for 10 min at 10,000 x g, and the levels of TNF-α in the supernatant were measured by ELISA (Immunotools, Germany) according to the manufacturer's instructions. Experiments were carried out in triplicate. Analysis was performed using analysis of variance followed by the Student-Newman-Keuls method with values of $p < 0.05$ considered to be significant.

Cell Cycle Analysis and Cytotoxicity Assays. The percentage of cells in each phase of the cell cycle was determined by flow cytometry. Briefly cells were collected after treatments, washed twice with PBS, and fixed with ethanol (70%) for 24 h at 4 °C. The cells were then washed twice with phosphate buffered saline (PBS) solution and subjected to RNA digestion (RNAse-A, 50 U/ml) and PI (20 μg/ml) staining in PBS for 1 h at RT, and analyzed

by cytofluorimetry. Under these conditions, low molecular weight DNA leaks from the ethanol-fixed cells and the subsequent staining allows the determination of the percentage of apoptotic cells (sub-G_0/G_1 fraction). For cytotoxicity analysis, Jurkat cells were seeded in 96-well plates in complete medium and treated with increasing doses of either thapsigargin or Basiliolide A1 for the indicated period of time. Samples were then diluted with 300 µl of PBS and incubated for 1 min at room temperature in the presence of propidium iodide (10 µg/ml). After incubation, cells were immediately analyzed by flow cytometry.

Ca^{2+} mobilization assay in Jurkat cells. Jurkat cells were incubated for 1 h at 37 ºC in Tyrode's salt solution (137 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12.0 mM $NaHCO_3$, 5.6 mM D-glucose) containing 5 µM Indo1-AM (Invitrogen) for 30 min at 37°C in the dark. Cells were then harvested, washed three times with buffer to remove extracellular Indo1 dye, readjusted to 10^6 cells/ml in the appropriate buffer and analyzed in a spectrofluorimeter operated in the ratio mode (Hitachi F-2500 model, Hitachi Ltd.) under continuous stirring and at a constant temperature of 37 ºC using a water-jacketed device. After five min accommodation to equilibrate temperatures, samples were excited at 338 nm and emission was collected at 405 and 485 nm, corresponding to the fluorescence emitted by Ca^{2+}-bound and free Indo-1, respectively. $[Ca^{2+}]_i$ was calculated using the ratio values between bound- and free-Indo-1 fluorescence, and assuming an Indo-1 K_d for Ca^{2+} of 0.23 µM. Maximum and minimum ratio values for calculations were determined by the addition at the end of the measurements of 10 µM ionomycin or 4 mM EGTA, respectively. $[Ca^{2+}]_i$ changes are presented as changes in the ratio of bound-to-free calcium (340 nm/380 nm). To determine the rate of Ca^{2+} entry, Indo-1-loaded cells were suspended in nominally Ca^{2+}-free buffer (50 mM Hepes, pH 7.5, 200 mM NaCl) and stimulated with Basiliolide A as indicated. Then, $CaCl_2$ (1 mM) was introduced in the medium and the ensuing increase in $[Ca^{2+}]_i$ was monitored.

Transient transfections and luciferase activity. Jurkat cells (10^6) were transfected with the 100 ng of the reporter plasmids by using Lipofectamin™ Reagent (Invitrogen) following the manufacture´s instructions. Twenty four h after transfection the cells were stimulated as indicated for 6 hours and then lysed in 25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 7% glycerol. Luciferase activity was measured using an Autolumat LB 9501 (EG&G Berthold, USA) following the instructions of the luciferase assay kit (Promega, Madison, WI) and protein concentration was measured by the Bradford method. The background obtained with the lysis buffer was subtracted in each experimental value and the specific transactivation expressed as total RLU induction. All the experiments were repeated at least three times. Analysis was performed using analysis of variance followed by the Student-Newman-Keuls method with values of $p < 0.05$ considered to be significant. **Plasmids.** The AP-1-Luc, NFAT-Luc, KBF-luc, IL-2-Luc and TNF-Luc plasmids have been previously described (Sancho et al., 2004).

Western Blots. Jurkat cells (10^6 cells/ml) were stimulated as indicated and then washed with PBS and resuspended in lysis buffer (20 mM Hepes pH 8.0, 0.35 M NaCl, 0.1 mM EGTA, 0.5 mM EDTA, 1 mM $MgCl_2$, 20% Glycerol, 1mM DTT, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, 0.5 μg/ml apronitin, and 1 mM PMSF) containing 0.5% NP-40. Cells were incubated for 15 min at 4 ºC and cellular proteins were obtained by centrifugation. Protein concentration was determined by Bradford assay (Bio-Rad, Richmond, CA) and 30 μg of proteins were boiled in Laemmli buffer and electrophoresed in 10% SDS polyacrylamide gels or in 6% SDS polyacrylamide gels (for NFAT detection). Separated proteins were transferred to nitrocellulose membranes (0.5 A at 100 V; 4 ºC) for 1 h. Blots were blocked in TBS solution containing 0.1 % Tween 20 and 5% non-fat dry milk overnight at 4 ºC, and immunodetection of specific proteins was carried out with primary antibodies using an ECL system (GE Healthcare).

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RESULTS

Effects of Basiliolide A1 on $[Ca^{2+}]_i$ mobilization. We have previously shown that basiliolides mobilise $[Ca^{2+}]_i$ in sea urchin eggs (Appendino et al., 2005). To study the effects of these novel compounds in mammalian cells the $[Ca^{2+}]_i$ in Indo-1-loaded Jurkat cells was analysed. Addition of increasing concentrations of Basiliolide A1 to the cells resulted in a rapid increase in $[Ca^{2+}]$i comparable to the one induced by TG (Figure 1A, 1B). The Ca^{2+} increase was maintained throughout the recording in response to both compounds although TG was slightly more potent than Basiliolide A1 (Figure 1A, 1B). When ER intracellular stores were depleted by thapsigargin, Basiliolide A1 was no longer able to mobilise $[Ca^{2+}]_i$ (Figure 1 C), but TG could still mobilise $[Ca^{2+}]_i$ in Basiliolide A1-treated cells (Figure 1D). These observations suggest that thapsigargin and Basiliolide A1 target the Ca^{2+} ER-stores through different mechanisms.

The rapid and sustained effect of Basiliolide A on $[Ca^{2+}]_i$ mobilization suggests the participation of CRAC in such mobilization. In order to dissect the biochemical mechanism for this Basiliolide A-induced calcium mobilization, we examined the effects of EGTA, an extracellular calcium chelator, and BAPTA-AM, a compound that can enter cells in a ester form, but that is then hydrolyzed by intracellular esterases and retained in the cytoplasm, acting as a specific calcium chelator. A different pattern of basililoide A-induced calcium mobilization was observed in the presence of either EGTA or BAPTA-AM (Fig. 2A). When cells were treated with BAPTA-AM, the early elevation of intracellular calcium disappeared but a slow calcium accumulation persisted. On the contrary, cells treated with EGTA, which cannot enter the cell, showed the rapid and early phase of calcium mobilization in response to Basiliolide A, but it disappeared within seconds until it reached the basal levels. These data indicate that Basiliolide A induces calcium mobilization by at least two coupled mechanisms,

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the first one would be mediated by the release of calcium from intracellular stores (inhibited by BAPTA-AM), and the second one mediated by the entry of extracellular calcium, inhibited by EGTA and probably induced by the opening of cell surface calcium channels. The effects of BAPTA as calcium chelator may be sufficient to quench a limited amount of the calcium release to the cytosol (internal stores), but it is not able to quench completely the high $[Ca^{2+}]_i$ induced by Basiliolide A in Jurkat cells. Next, we reasoned that Basiliolide A1-induced Ca^{2+} depletion of the ER should activate CRAC channels in the plasma membrane and to address this point we preincubated the cells for 24 h with the potent CRAC channel inhibitor, BTP2 (Zitt et al., 2004). We found that Basiliolide A1 induced the early phase of calcium mobilization but the late phase was completely prevented by BTP2 (Fig. 2A). To further confirm the activity of Basiliolide A1 on calcium mobilization from ER stores and CRAC channels we performed experiments in nominally free calcium and we observed that Basiliolide A induces an immediate $[Ca^{2+}]_i$ mobilization that was further enhanced by the addition of $CaCl_2$ to the cuvette (Fig. 2B). As expected the $[Ca^{2+}]_i$ increase mediated by the addition of $CaCl_2$ was almost completely inhibited by pre-incubation of the cells with BTP2 (Fig. 2C) but not by the L-type calcium channels blockers verapamil and nifedipine (data not shown).

Basiliolide A1 does not induce apoptosis in Jurkat cells. It have been shown that depletion of intracellular $[Ca^{2+}]_i$ store itself may be a major factor in thapsigargin-induced ER stress and apoptosis in a wide variety of mammalian cells (Jiang et al., 1994; Kaneko and Tsukamoto, 1994). Since Basiliolide A also depletes ER intracellular $[Ca^{2+}]_i$ stores in Jurkat cells we investigated its potential pro-apoptotic activity. The cells were incubated with Basiliolide A (5 μM), thapsigargin (1 μM) or the calcium ionophore ionomycin (1 μM) for 18 h and the hypodiploidy (i.e., loss of fragmented DNA) was detected, using propidium iodide staining, as a marker for apoptosis. In figure 3A is shown that both thapsigargin and

ionomycin were able to induce a clear increase in the percentage of hyplodiploid cells (57,85 % and 23.5 % respectively). By the contrary, Basiliolide A1 lack apoptotic activity indicating that this compound do not induce the ER stress required to activate the apoptotic programme (Lang et al., 2005). The rise of $[Ca^{2+}]_i$ is also involved in the necrotic pathway of cell death that can not be measured by cell cycle analysis and therefore we sough to study the effects of Basiliolide A1 and TG on cellular toxicity that was evaluated by propidium iodide staining and flow cytometry. Jurkat cells were treated with increasing doses of both compounds and cell viability was tested after 6, 12, and 18 h. As depicted in figure 3B Basiliolide A1 lacked cytotoxic effects at the concentrations and times analyzed. In contrast TG clearly induced cell death with all the concentration tested.

Effects of Basiliolide A1 on NFAT activation. Transcriptional activation of NFAT requires its translocation to the nucleus where it binds to specific consensus sites in the promoter region of the target genes. Since NFAT activation is tightly regulated by $[Ca^{2+}]_i$ mobilization we studied the role of Basiliolide A on NFAT transactivation activity. Jurkat cells were transfected with a luciferase reporter construct under the control of minimal promoter containing three NFAT binding sites and 24 h later the cells were stimulated for 6 h with PMA alone or in the presence of increasing concentrations of Basiliolide A or thapsigargin and the results shown in figure 4A demonstrated that basiliode A effectively induces the luciferase expression driven by the artificial NFAT promoter in PMA-stimulated cells and in a concentration-dependent manner. As expected neither Basiliolide A nor PMA were able to induce NFAT-dependent luciferase activity. Next, we examined NFAT1 expression and phosphorylation in Basiliolide A-stimulated Jurkat cells by immunoblots of total extracts with a polyclonal anti-NFAT1 antiserum. Similarly to ionomycin the stimulation of cells with Basiliolide A alone or in combination with PMA for 30 min caused a marked NFAT dephosphorylation that was prevented by pre-incubation with CsA (Fig. 4B).

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According to our previous results with the $[Ca^{2+}]_i$ measurements (Fig. 2B and 2C), BTP2 inhibited the Basiliolide A-induced NFAT dephosphorylation and transcriptional activity (Fig. 4C and insert). In contrast dantrolene, an inhibitor of ER ryanodine receptor, did not modify the NFAT transactivation activity (Fig. 4C).

Effects of Basiliolide A1 in the signaling pathways leading to NF-κB and AP-1 activation. Whilst $[Ca^{2+}]_i$ is clearly involved in NFAT dephosphorylation and nuclear translocation, its effect on NF-κB and AP-1 activation is more indirect (Rao et al., 1997). However, ER-stress induced by thapsigargin has been shown to activate the NF-κB and MAPK signaling pathway (Leonardi et al., 2002; Engedal et al., 2002; Maloney et al., 1999; Rosado and Sage, 2001). Thus, we investigated some of major biochemical pathways activated by Basiliolide-A leading to NF-κB and AP-1 activation in Jurkat cells. We found that Basiliolide A1 alone did not induce IκBα degradation nor RelA (NF-κB subunit) phosphorylation (ser 536) but it synergize with PMA to induce a complete degradation of this NF-κB inhibitory protein (Fig. 5A). We also detected that Basiliolide A1 and ionomycin increased PMA-mediated JNK1 and JNK2 phosphorylation while PMA-induced ERK 1 and ERK2 phosphorylation was not affected (Fig. 5A). Accordingly, Basiliolide A1 synergized with PMA to induce NF-κB- and AP-1-dependent transcriptional activation (Fig. 5B and 5C).

IL-2 and TNF-α gene promoter are regulated by the coordinated action of NFAT, NF-κB and AP-1 transcription factors that are activated by antigen receptor engagement plus an accessory signal usually supplied by the antigen-presenting cell (Crabtree and Clipstone, 1994). Agents that bypass these receptors, such as PMA and ionomycin, can mimic T-cell activation in the Jurkat cells. Thus, the co-stimulatory effect of Basiliolide A1 was studied by transfecting Jurkat cells with the reporter plasmids IL-2-Luc and TNF-α-Luc. After transfection, cells were treated with PMA alone or in combination with Basiliolide A1 or ionomycin as a control for six h and tested for luciferase activity. In figure 6 it is shown that

Basiliolide A1 is a potent co-activator with PMA to induce the luciferase expression driven by both calcium-dependent cellular gene promoters (6A and 6B). Interestingly, Basiliolide A1 was more effective than ionomycin to induce TNF-α release from the cells (Fig. 6C) suggesting that Basiliolide A1 may control the release of cytokines at both transcriptional and post-transcriptional levels.

The position 15 of basiliolide structure is critical for NFAT activation in Jurkat cells. To get insights on the structure-activity relationships of basiliolides, the activity of Basiliolide A1 was compared to that of its two more oxidized analogues, basiliolides B and C (Fig. 7). While basiliolide B, differing for the oxidation of one of the two geminal methyls to a carboxymethyl group retained most of the activity of Basiliolide A1, basiliolide C, where the 15-carbon is oxidized to an acetoxymethine, was much less active. In contrast to basiliolide A, high concentrations of thapsigargin were less effective than lower concentrations to induce NFAT-dependent luciferase activity.

DISCUSSION

Control of cytosolic Ca^{2+} levels involves the coordination of diverse intracellular and extracellular Ca^{2+} currents, and plays a critical role in a wide range of cellular functions including apoptosis. We show here that Basiliolide A1 increases cytosolic $[Ca^{2+}]_i$ by inducing Ca^{2+} transfer from ER to cytosol and eventual activation of CRAC channels. The sustained $[Ca^{2+}]_i$ elevation in Basiliolide A1-treated Jurkat cells is sufficient to activate gene expression but not to induce apoptosis. Conversely, both the calcium ionophore ionomycin and the irreversible SERCA inhibitor thapsigargin were able to induce apoptosis in these cells, suggesting that basilioide A1 mobilizes intracellular Ca^{2+} trough a different mechanism.

Under physiological conditions, Ca^{2+} release from ER to cytosol can be induced in T cells by the direct binding of the second messengers $InsP_3$ and cyclic adenosine diphosphoribose via the $InsP_3R$ and the ryanodin receptor (RyR), respectively (Quintana et al., 2005). Basiliolide A1 might interfere with upstream mechanisms that lead to the generation of some of these second messengers. However, Basiliolide A1 could induce calcium mobilization both in the parental Jurkat clone and in a PLCγ1-defficient line (Irvin et al., 2000) (data not shown) while the RyR antagonist dantrolene, was unable to prevent both $[Ca^{2+}]_i$ elevation and NFAT activation induced by basiliolide. These observations rule out a direct activation of these ER receptors by Basiliolide A1, suggesting an indirect interaction with them. Notwithstanding at the present we can not discard the possibility that basiliolide A interacts directly either with $InsP_3R$ or RyR. It has been shown recently that Homer, a scaffold protein, physically associates with the ryanodine receptors type 1 (RyR1) regulating gating responses to Ca^{2+} and caffeine (Feng et al., 2002) as well as NFAT-dependent signaling (Stiber et al., 2005). It is therefore not unconceivable that, just like caffeine, also Basiliolide A1 modulates RyR in a Homer-dependent manner (Feng et al., 2002).

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SERCA is a critical enzyme that pumps Ca^{2+} from the cytosol into the ER lumen to maintain a low $[Ca^{2+}]_{i.}$ The sustained inhibition of this activity causes depletion of intracellular Ca^{2+} stores and activation of capacitative Ca^{2+} entry, generating supramolecular $[Ca^{2+}]_i$ in the cytosol. In Jurkat and related cell types, this activates apoptotic pathways (Jayaraman and Marks, 1997). The very tight binding of thapsigargin to all currently known SERCAs causes an irreversible inhibition, that persists after removal of the excess inhibitor (Waldron et al., 1994) an observation that can explain, or contribute to, the potent apoptotic activity of this compound. Conversely, Basiliolide A1 was unable to induce apoptosis in Jurkat cells, despite its alleged SERCA inhibitory activity (Appendino et al., 2005). It is therefore tempting to suggest that Basiliolide A1 acts as a reversible SERCA inhibitor, not unlike 2,,5-di-(*tert*-butyl)-1,4-benzohydroquinone (tBuBHQ), a SERCA-blocker that protects HeLa cells from ceramide-induced apoptosis (Pinton et al., 2001). The reversible activity of Basiliolide A1 is supported by the observation that Jurkat cells treated with this compound for one hour, and then washed and cultured again for 12 h in calcium-containing medium, were still sensible to the $[Ca^{2+}]$ mobilizing properties of Basiliolide A1 (data not shown). To conciliate these observation, we can assume that the reduction of the ER- $[Ca^{2+}]_i$ by Basiliolide A1 leads to a constant release of Ca^{2+} to the cytosol and reuptake by the ER, whose long kinetics makes it possible for calcium to equilibrate between different intracellular organelles. This calcium leakage can be translated into calcium-dependent gene transcription and other cellular functions, but not in the induction of cell death. Thapsigargin and ionomycin generate instead very high $[Ca^{2+}]_i$ and quickly induce apoptosis. We are currently investigating wether Basiliolide A1 can protect cells from ceramide-induced apoptosis in T cells and in other cell types. Another interesting difference between Basiliolide A1 and thapsigargin is that NFAT activation by thaspigargin has a complex kinetics, with high concentrations being less efficient lower concentrations. This could be explained in part

by an increase in apoptosis, since a higher percentage of cell death was found in Jurkat cells treated with 5 μM TG compared to cells treated with lower concentrations of this SERCA inhibitor. Interestingly, it has been proposed that high concentrations of thapsigargin can also have inhibitory effect on Ca^{2+} or Mn^{2+} entry from the medium (Mason et al., 1991). Taken together, these observations qualify basiliolides as a novel class of molecular probes to study calcium homeostasis, characterized by lack of apoptotic and CRAC inhibitory activity.

NFAT, NF-κB and AP-1 are probably the three most important transcription factor families in T cells, all of them being activated downstream from TCR engagement in a Ca^{2+}-dependent manner. Whereas intracellular calcium ($[Ca^{2+}]_i$) is clearly involved in activation of NFAT, the role of $[Ca^{2+}]_i$ may be regarded as being more indirect for NF-κB and AP-1 (Feske et al., 2001; Li and Verma, 2002). Accordingly, we found that Basiliolide A1 is potent co-stimulator of the NF-κB and the AP-1 pathways in T cells but this activity was not restricted to T cells since we observed that basiliolides also regulates NFAT and NF-κB activity in neuronal cells (unpublished results). This is of special relevance, since both transcription factors can protect neurons from cell death both “in vivo” and “in vitro” (Benedito et al., 2005; Fridmacher et al., 2003). Non-cytotoxic compounds that mobilize calcium by targeting the ER are currently of great interest for the treatment of neurodegenerative diseases

The differences between the activity of Basiliolides A1, B and C demonstrate the existence of definite structure-activity relationships within these compounds, and should spur activity aimed at the isolation of further members of this class of compounds and/or to their semisynthetic modification. As a final observation, it is interesting to remark that *Thapsia garganica* is one of the oldest medicinal plants, and it is therefore surprising that this treasure trove of bioactive compounds was overlooked for so long in terms of phytochemical and pharmacological investigations.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Juan M. Redondo (CBM-UAM, Madrid, Spain) for the anti-NFAT1 antisera.

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FOOTNOTES

This work was supported by Ministerio de Educación y Ciencia Grant SAF2004-00926 to E.M.

JPET Fast Forward. Published on July 13, 2006 as DOI: 10.1124/jpet.106.108209
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LEGENDS FOR FIGURES

Figure 1. Effect of Basiliolide A1 on calcium mobilization. A,B) Jurkat cells were loaded with Indo1-AM, treated with increasing concentrations of either Basiliolide A1 (BSD-A1) or Thapsigargin, or solvent alone (DMSO) and the calcium mobilization measured by ratiometric fluorescence as indicated in materials and methods. C, D) The concentrations of BSD-A1 and thapsigargin used were 5 μM and 1 μM respectively. Vertical arrow indicates the time of compound addition. After 5 min recording, ionomycin (1 μg/ml) was added to standardize results. $[Ca^{2+}]_i$ changes are presented as changes in the ratio of bound to free calcium (340 nm/380 nm). The calcium traces are representative of at least five independent experiments.

Figure 2. Effect of Basililolide A1 on ER-induced Ca^{2+} release and CRAC channels. A) Jurkat cells were loaded with Indo1-AM as in figure 1 and then incubated with either BAPTA-AM (5 μM), EGTA (2 mM) for 30 min or BTP-2 for 24 h prior to Basiliolide A1 treatment (5 μM) (vertical arrow). B) Indo-A loaded Jurkat cells were incubated in nominally Ca^{2+}-free buffer and treated or not with BSD-A1 (5 μM) at the indicated time followed by addition of Ca Cl_2 (1 mM). C) Jurkat T cells were pre-incubated during 24 h with the CRAC channel inhibitor, BTP-2 (11,8 nM) and then the $[Ca^{2+}]$ measurement was performed as in B. $[Ca^{2+}]$i changes are presented as changes in the ratio of bound to free calcium (340 nm/380 nm). The calcium traces are representative of at least three independent experiments.

Figure 3. A) Basiliolide A1 does not induce apoptosis in Jurkat cells. Jurkat T cells were incubated with BSD-A1 (5 μM), Thapsigargin (1 μM) or the calcium ionophore ionomycin (1 μM) for 18 h. After treatments cells were harvested, and the percentage of cell in each cell

cycle phase determined by propidium iodide staining followed by flow cytometry analysis. The percentages of cells in sub Go/Gs phase of the cell cycle are indicated. B) **Effects of Basiliolide A1 aand TG on cell viability.** Jurkat cells were treated with increasing concentrations of either Basiliolide A1 or TG for 6, 12, and 18 h. After treatments cells were harvested, and the percentage of cell death was determined by propidium iodide staining followed by flow cytometry analysis. Results are represented as the percentage of cell viability and are the mean ± S.D. of three different experiments.

Figure 4. Effect of Basiliolide A1 on NFAT activation. A) Jurkat cells transiently transfected with NFAT-luc reporter plasmid were treated with the indicated doses of Basiliolide A1 and PMA (50 ng/ml) for six h, and luciferase activity measured in the cell lysates. Results are the mean ± SE of three determinations expressed as fold induction (experimental RLU-background RLU/basal RLU-background RLU) * $p<0.05$ and ** $p< 0.01$ compared to PMA treatment. B) Jurkat cells were stimulated with BSD-A1 (1 μM), PMA (50 ng/ml) and ionomycin (1 μM) as indicated and the phosphorylation status of NFAT detected in the cellular extracts. C) NFAT-luc transfected Jurkat cells were pretreated or not with either BTP2 or dantrolene followed by stimulation or not with ionomycin (1 μM), PMA (50 ng/ml) plus BSD-A1 (1 μM) for 6 h as shown the figure. Results are the mean ± S.E. of three determinations expressed as fold induction. Insert. Jurkat cells preincubated (1) or not (2) with BTP-2 for 24 h, then treated with BSD-A1 (1 μM) for 30 min and NFAT1 detected by western blot.

Figure 5. Effect of Basiliolide A1 on the MAPK and the NF-κB activation pathways. A) Jurkat cells were treated with BSD-A1 (1 μM), ionomycin (1 μM) or PMA (50 ng/ml) as indicated and the steady state levels of IκBα and the phosphorylation status of p65 (Ser^{536}),

JNK and ERK analyzed by Western blot using specific mAbs. BSD-A1 regulates NF-κB and AP-1 transcriptional activities. Jurkat T cells were transiently transfected with the luciferase reporter plasmids KBF-Luc (B), AP-1-Luc (C), as described in *Materials and Methods*. Cells were stimulated with BSD-A1 (1μM), PMA (50 ng/ml) or ionomycin (1μM) for 6 h and the luciferase activity measured. The results are the mean ± SE of three determinations and expressed as relative light units (RLU).

Figure 6. Effect of Basiliolide A1 on IL-2 and TNFα gene promoter activity and TNFα release. Jurkat T cells transfected with either the IL-2 promoter luciferase reporter plasmid (A) or the TNF-α promoter luciferase reporter plasmid (B) and 24 h later stimulated for 6 h with BSD-A1 (1 μM), ionomycin (1 μM) or PMA (50ng/ml) as indicated, and luciferase activity measured in the cell lysates. Results are the mean ± SE of three determinations expressed as fold induction (experimental RLU-background RLU/basal RLU-background RLU). C) Jurkat cells were stimulated for 6 h with BSD-A1 (1 μM), ionomycin (1 μM) or PMA (50 ng/ml) and the TNF-α release measured in the supernatants by ELISA. The results are the mean ± SE of three determinations. * $p<0.01$ compared to PMA treatment and ** $p< 0.01$ compared to PMA plus Ionomycin treatment.

Figure 7. Critical role of the position 15 in the biological activity of Basiliolide A1. Chemical structures of BSD-A1 compared to that of its two oxidized analogues, BSD-B and BSD-C (top). Jurkat cells transiently transfected with the NFAT-luc reporter plasmid were treated with PMA (50 ng/ml) alone or in combination with either ionomycin (1 μM), BSD-A1, BSD-B, BSD-C or Thapsigargin (TG) for 6h and the luciferase activity measured and expressed as fold induction. Results are the mean ± S.E. of three determinations.

Figure 1

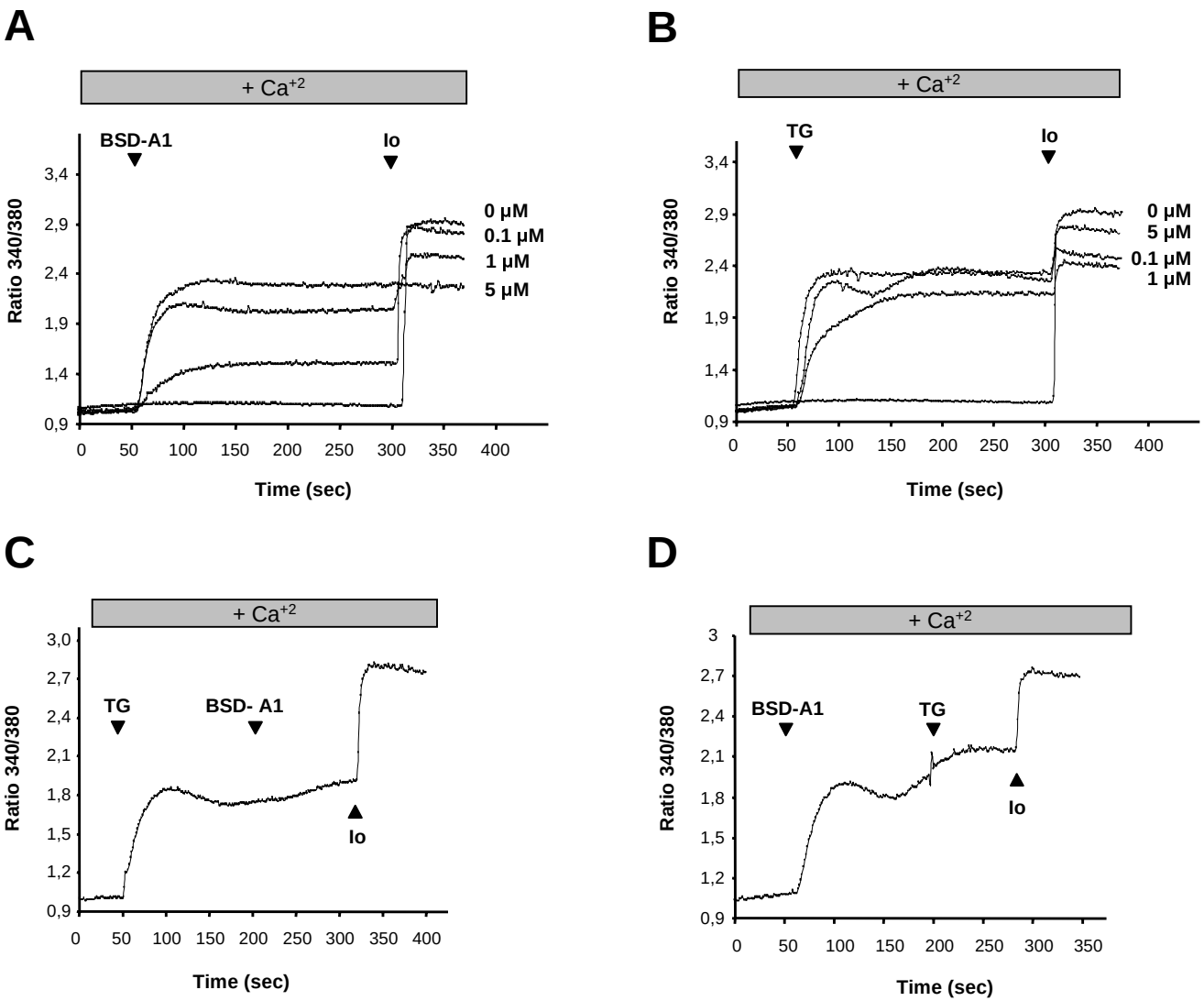


Figure 2

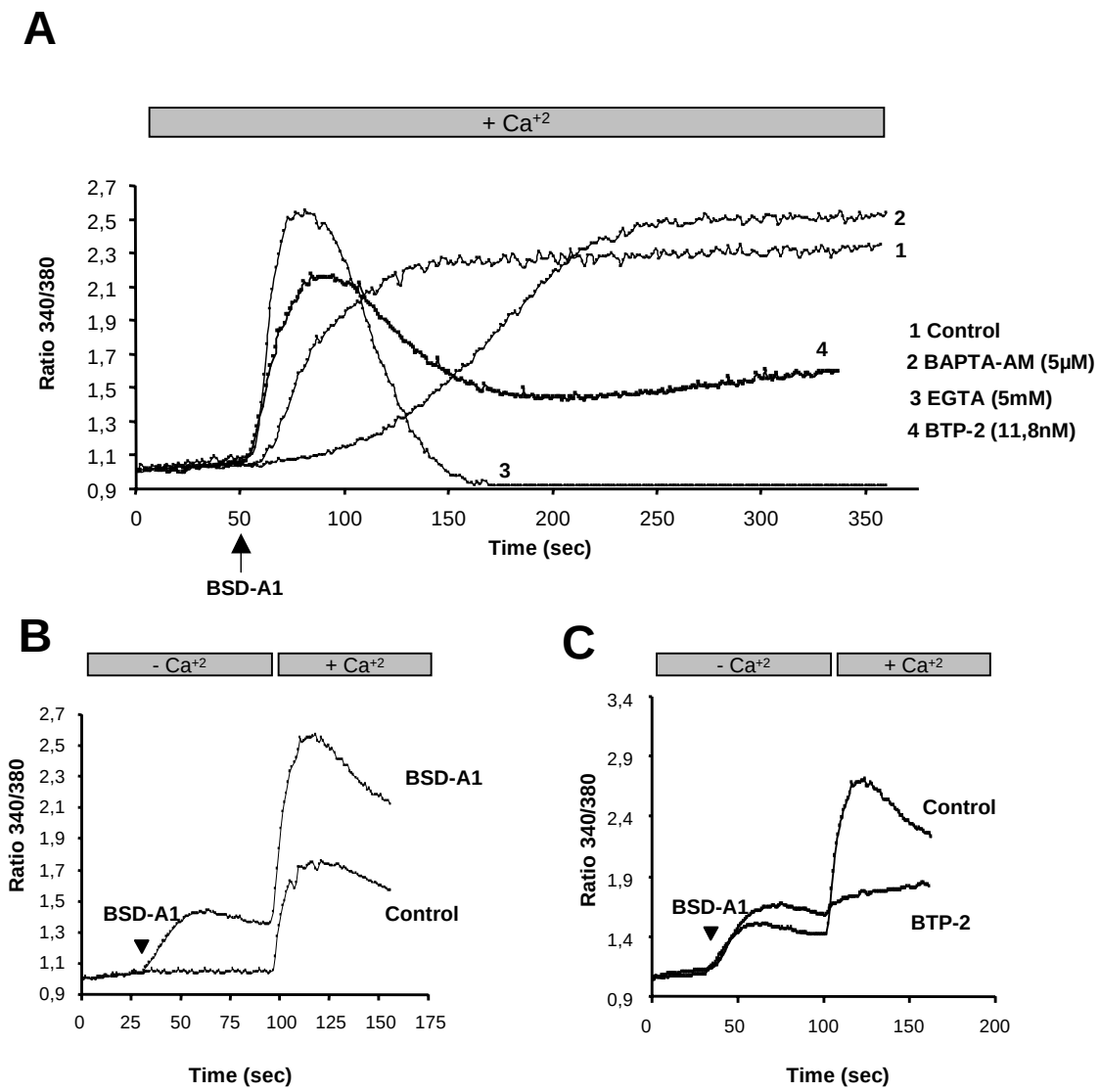


Figure 3

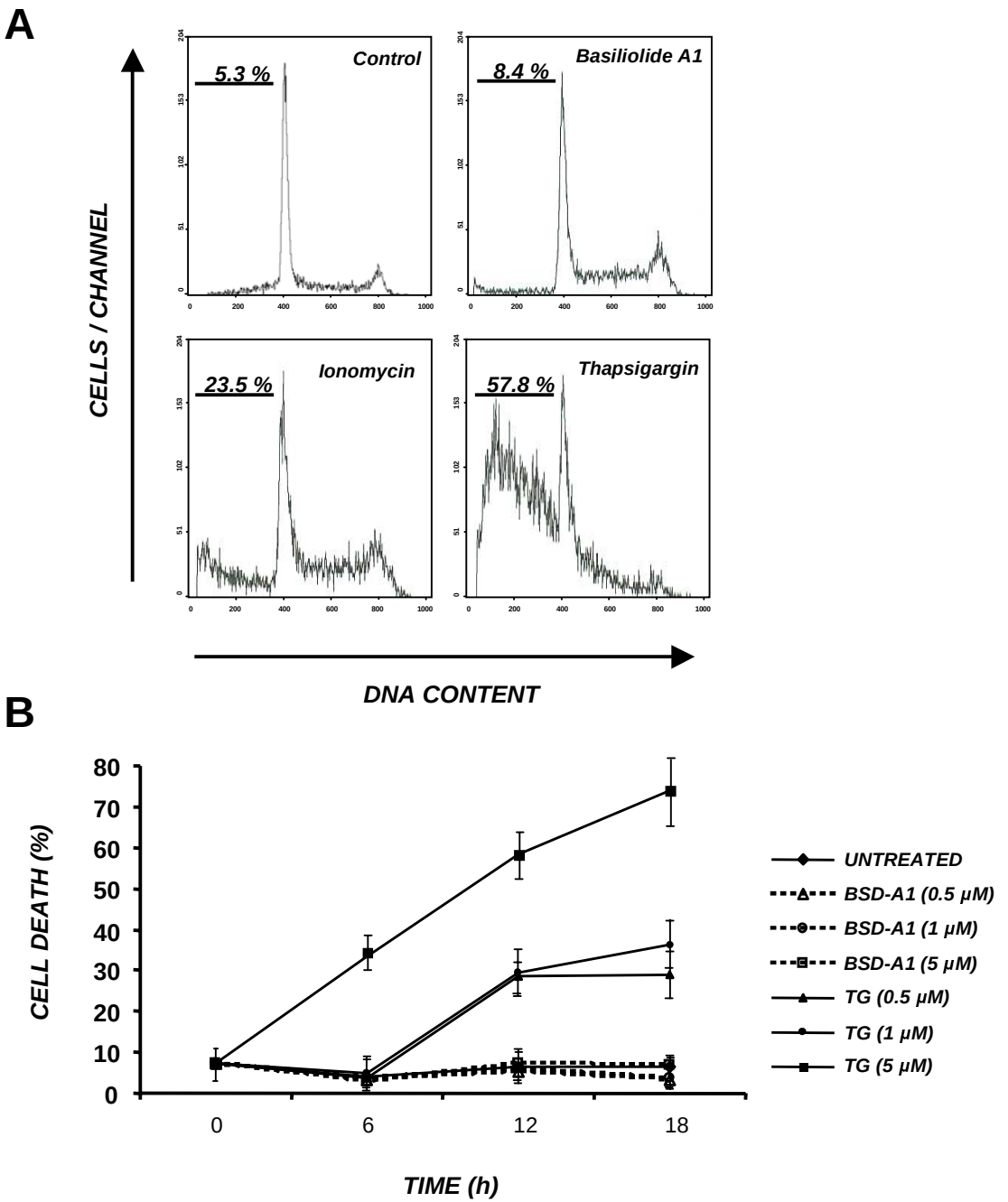


Figure 4

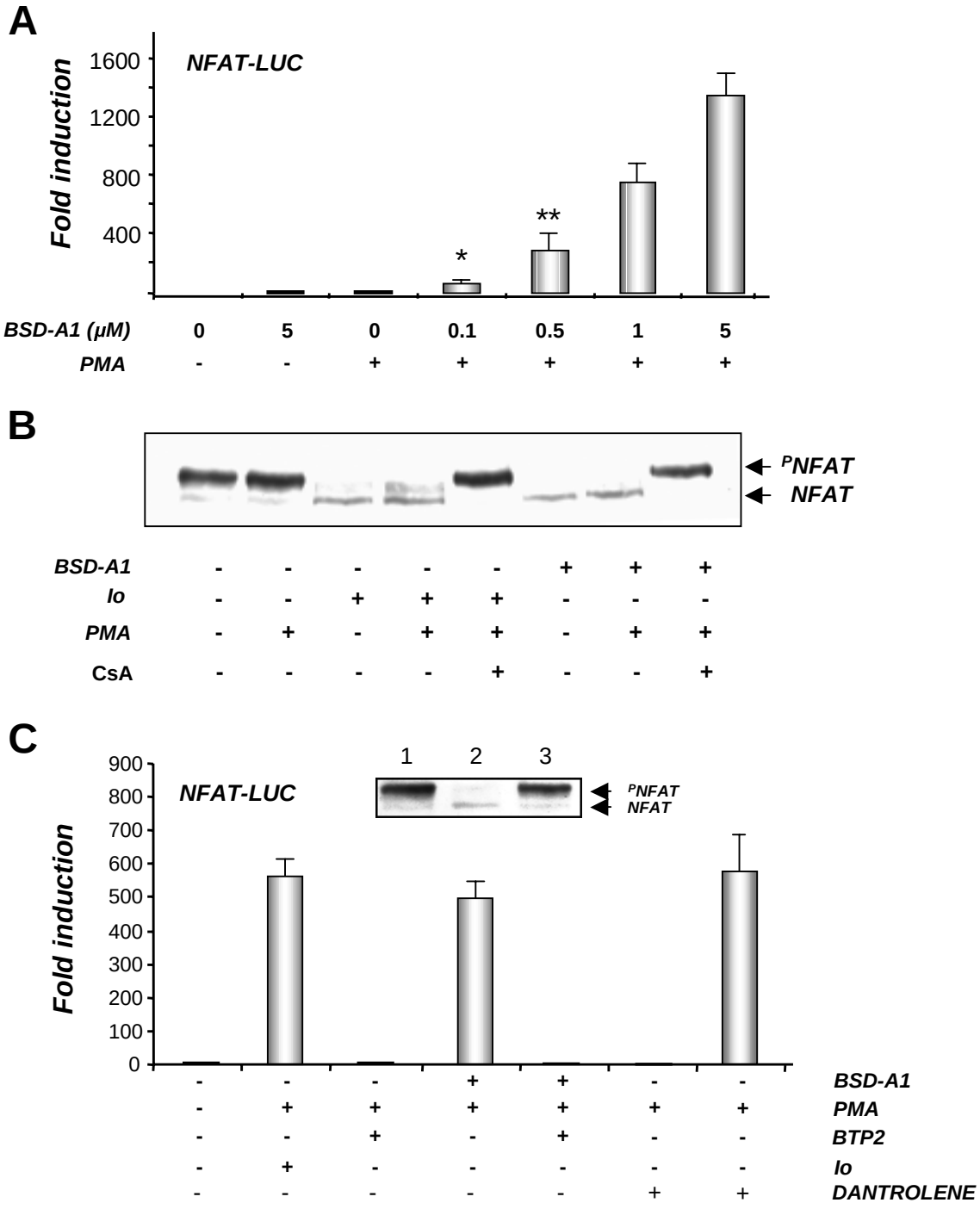
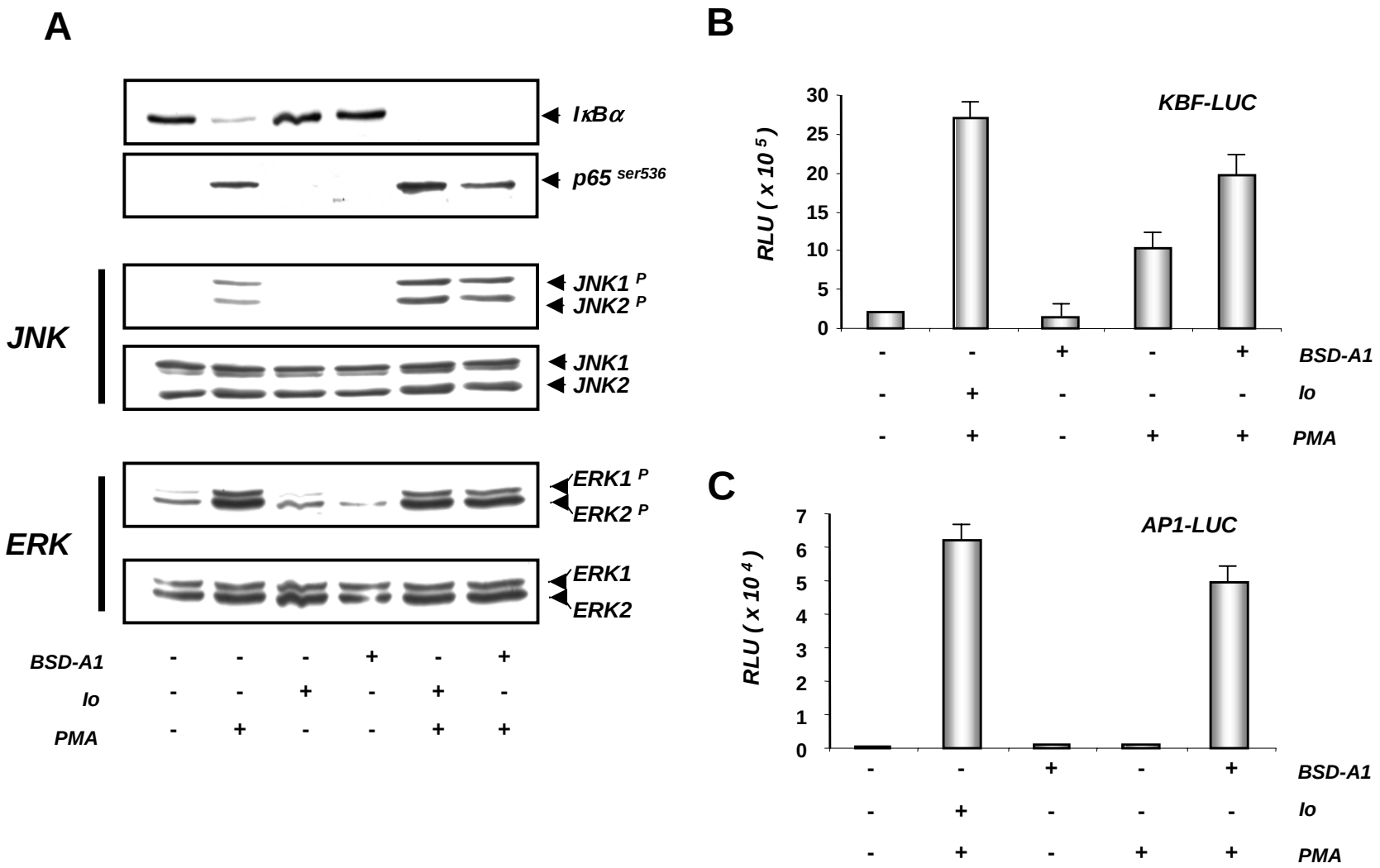


Figure 5



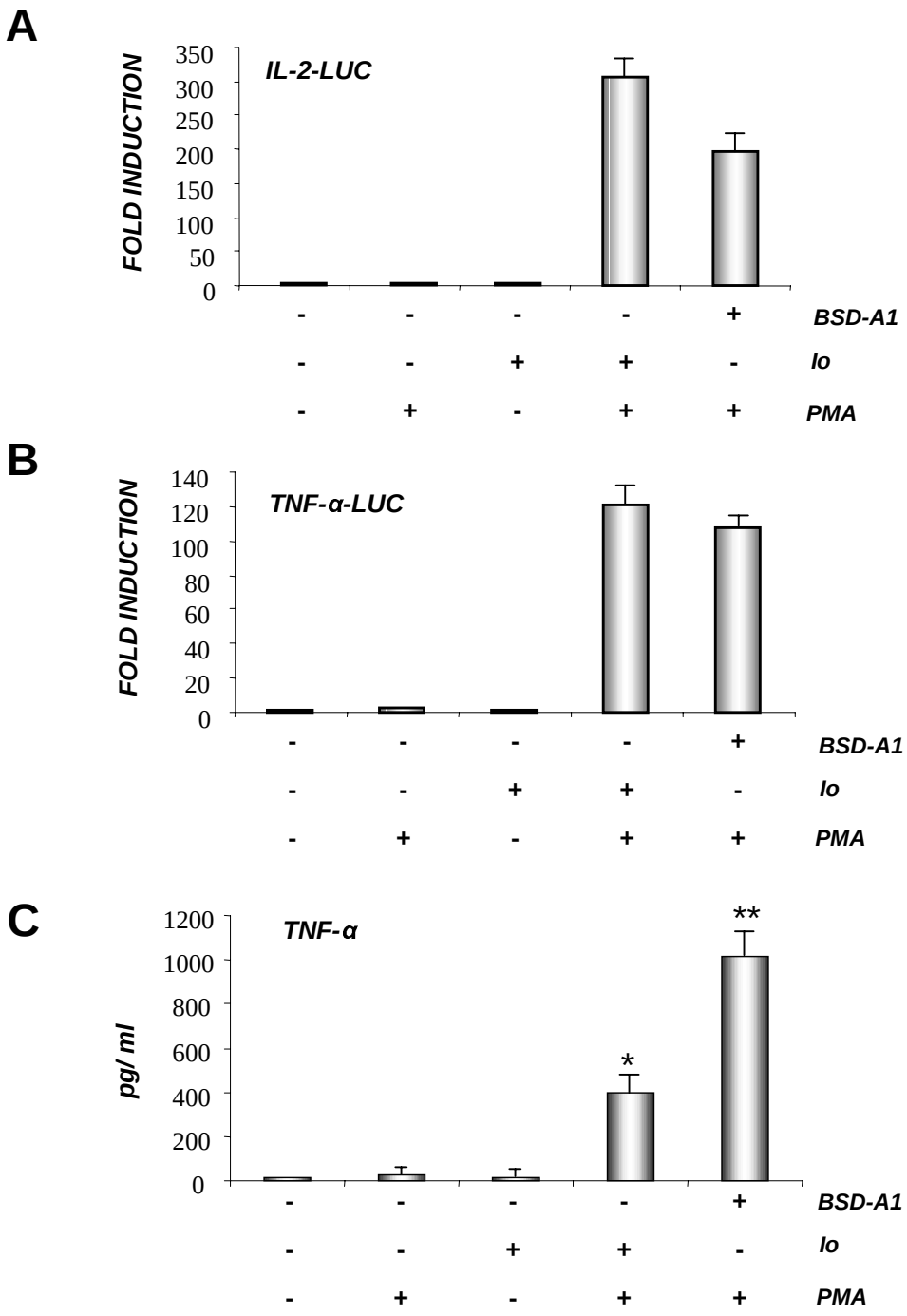


Figure 6

Figure 7