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BASTADIN 12 AND RYANODINE REVEAL SIMILARITIES BETWEEN THAPSIGARGIN- AND TETRABROMOBISPHENOL A-INDUCED INTRACELLULAR Ca^{2+} RELEASE IN CULTURED CEREBELLAR GRANULE CELLS

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Tetrabromobisphenol A (TBBPA) is a commonly used brominated flame retardant with recognized neuro- and cytotoxic properties that are presumably mediated by intracellular Ca^{2+} release. Other studies have demonstrated that ryanodine is able to inhibit Ca^{2+} efflux from skeletal sarcoplasmic reticulum (SR) membranes in response to the known Ca^{2+} releaser thapsigargin, provided that the macrocyclic brominated tyrosine derivative bastadin 5 is also present. Similar effects supporting the role of ryanodine receptors in thapsigargin-evoked Ca^{2+} release have been observed in primary cultures of rat cerebellar granule cells (CGCs). Here, we used CGCs and the fluorescent intracellular Ca^{2+} probe fluo-3 to test the following hypotheses: (1) TBBPA shares Ca^{2+} releasing properties with thapsigargin, and (2) synthetic bastadin 12 can replace bastadin 5 as a pharmacological tool to identify these similarities. The results demonstrated that either 200 nM thapsigargin or 30 μM bastadin 12 alone induced an increase in the intracellular Ca^{2+} level in CGCs, whereas 2.5 and 10 μM bastadin 12 had no effect on the basal Ca^{2+} concentration. The thapsigargin-induced Ca^{2+} release was partially reduced by co-administration of either 2.5 μM bastadin 12 or 200 μM ryanodine, and the release of Ca^{2+} was nearly completely attenuated by these compounds when they were given together. TBBPA (5, 10 and 25 μM) administration caused a concentration-dependent increase in CGC Ca^{2+} levels. Administration of 2.5 μM bastadin 12 with 200 μM ryanodine blocked the increase in intracellular Ca^{2+} evoked by 10 μM TBBPA, although these compounds were ineffective when applied separately. These results indicate that bastadin 12 may replace bastadin 5 when testing the ability of ryanodine to inhibit Ca^{2+} release from the intracellular stores of cultured neurons, and our findings support the hypothesis that TBBPA and thapsigargin induce intracellular Ca^{2+} release through a common mechanism.

Key words: *bastadins, brominated flame retardants, calcium releasers, neuronal cultures, ryanodine receptors, cerebellar granule cells, calcium pump*

INTRODUCTION

Tetrabromobisphenol A (TBBPA) is a hydrophobic phenolic heat-resistant compound classified as a brominated flame retardant (BFR) that has been commonly used in the electronics, textiles and building industries to reduce the ignition of various products. Although early concerns regarding the environmental and toxicological significance of TBBPA (1, 2) appear to have been highly exaggerated (3, 4), recent data from *in vivo* and *in vitro* studies have demonstrated endocrine disruption and multiple toxic effects of TBBPA, including cytotoxicity in cultured neurons and neurotoxicity affecting brain functions *in vivo* (5-8). Calcium imbalance resulting either from enhanced Ca^{2+} influx to neurons *via* glutamate receptors (7) and/or Ca^{2+} release from intracellular stores (9) has been proposed to contribute to the cytotoxicity of TBBPA. Our previous results demonstrated that 7.5 μM TBBPA increases intracellular Ca^{2+} levels in primary cultures of rat cerebellar granule cells (CGCs) without activating ^{45}Ca influx into neurons (10). TBBPA, as well as other BFRs, was demonstrated to inhibit SERCA Ca^{2+} pumps in the skeletal muscle and cerebellar microsomes and induce intracellular Ca^{2+} release in cultured TM4 Sertoli, SH-SY5Y neuroblastoma or PC12 pheochromocytoma cell lines (9, 11-15). The aforementioned general characteristics of TBBPA resemble the well-known properties of the established calcium releaser thapsigargin, which inhibits the SERCA Ca^{2+} pump, releases Ca^{2+} from intracellular stores and exhibits a significant potential for cytotoxicity (16, 17). Further studies using new pharmacological tools are needed to test the hypothesis that TBBPA and thapsigargin induce Ca^{2+} release through a common mechanism.

Bastadins are cyclic tetramers of brominated tyrosine derivatives isolated from the marine sponge *Ianthella basta*. Some of these compounds modulate ryanodine receptor (RyR) channel gating and show an affinity for the complex formed by RyR and the immunophilin protein FKBP12 (18, 19). FKBP12 and FKBP12.6 are bound to the skeletal and cardiac ryanodine receptors (RyR1 and RyR2, respectively) and regulate the function of these intracellular Ca^{2+} release channels (20, 21). Using skeletal

muscle sarcoplasmic reticulum (SR) membranes, 5 and 7.5 μM bastadin 10 were shown to increase the high-affinity binding of [^{3}H]ryanodine, stabilize the open conformation of RyRs and sensitize the channel to Ca^{2+}, promoting Ca^{2+} release from SR (19). A similar concentration range of bastadin 5 (*Fig. 1C*) was shown to promote a high-affinity ryanodine-binding conformation in RyRs and stabilize both the open and closed RyR channel state while only slightly affecting the sensitivity of the channel to Ca^{2+} activation (18). In the same study, bastadin 5 enhanced Ca^{2+}-induced calcium release (CICR) from SR. Further studies using skeletal SR membranes and thapsigargin to inhibit the SERCA calcium pump and induce calcium release revealed Ca^{2+} efflux from SR that was insensitive to the RyR inhibitors ryanodine or ruthenium red, as well as to 10 μM bastadin 5 administered alone, whereas bastadin 5 co-administered with ryanodine or ruthenium red completely attenuated the Ca^{2+} release induced by thapsigargin (22). A similar effect was reproduced in the BC_3H1 cell line; this effect was interpreted to indicate that thapsigargin unmasked ryanodine-insensitive leak channels converted by bastadins into ryanodine-sensitive Ca^{2+} channels (22). In the latter experiment, a bastadin mixture was administered at a concentration of 80 μM, which is able to independently induce Ca^{2+} release. More recent studies of skeletal myotubes consequently used 20 μM bastadin 5 as a pharmacological tool to convert the leak conformation of RyR1, which is insensitive to ryanodine, into a ryanodine-sensitive conformation, resulting in a significant reduction of the resting Ca^{2+} level in dystrophic myotubes (23, 24). We previously used primary cultures of rat CGCs to demonstrate that synthetic analogues of bastadins, including 20 and 30 μM bastadin 5 and 15 μM bastadin 10, increase the intracellular Ca^{2+} level; we found that this effect was inhibited by FK-506 in a concentration-dependent manner (25, 26). Moreover, 5 μM bastadin 5 (which does not affect the basal Ca^{2+} level in neurons) co-administered with 200 μM ryanodine completely prevents the calcium release evoked by 200 nM thapsigargin in CGCs (25).

Thus far, only bastadin 5 or an unspecified bastadin mixture co-administered with ryanodine has been shown to inhibit thapsigargin-induced Ca^{2+} release (22-25). However, our unpublished data indicated that other biologically active bastadins could produce similar results. Verification of this hypothesis could support the use of various bastadins as pharmacological tools to identify calcium leak channels. In the present study, we investigated the ability of synthetic bastadin 12, which at low concentrations does not induce Ca^{2+} release *per se*, to inhibit thapsigargin-evoked Ca^{2+} release when co-administered with ryanodine in primary rat CGC cultures. We then applied these results toward investigating the TBBPA-evoked increase in intracellular Ca^{2+} levels.

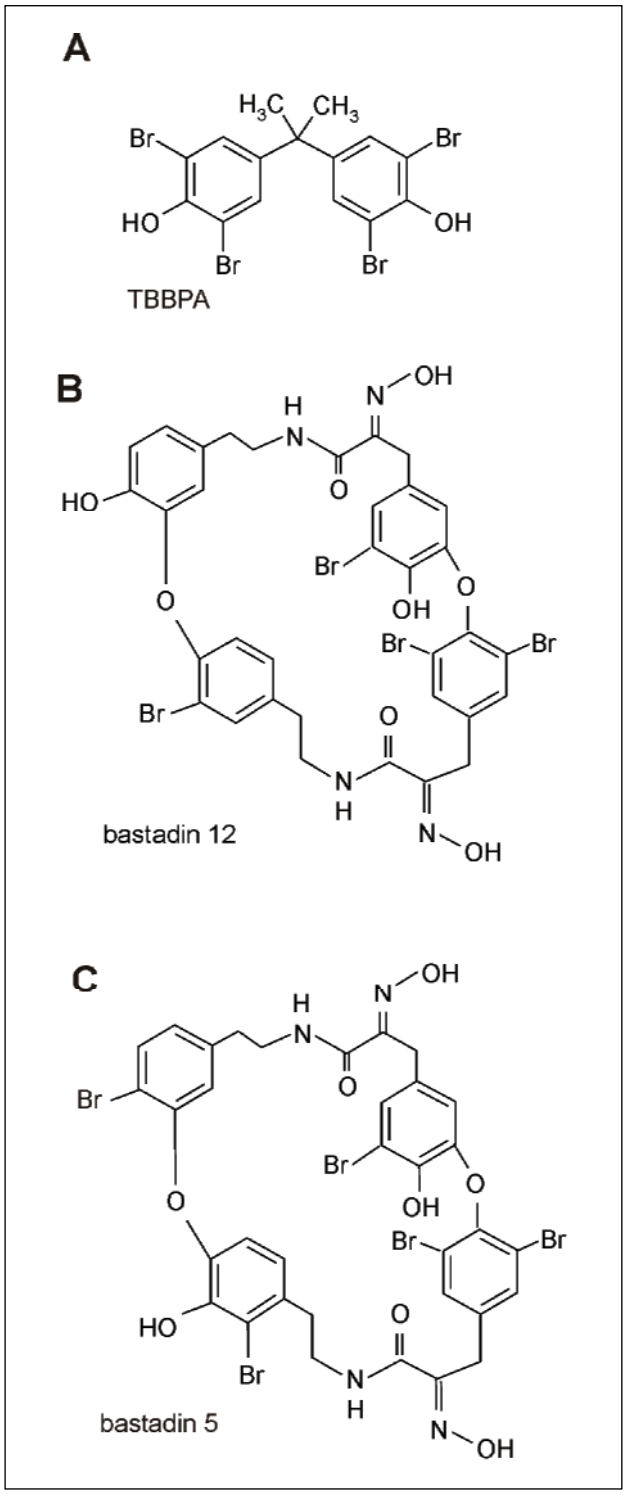

Fig. 1. The chemical structures of TBBPA (A), bastadin 12 (B) and bastadin 5 (C).

MATERIALS AND METHODS

Materials

Tetrabromobisphenol A (TBBPA) (*Fig. 1A*) was purchased from LGC Standards (Lomianki, Poland). Ryanodine, dimethyl sulphoxide (DMSO), fetal bovine serum and other materials for cell culture were purchased from Sigma-Aldrich (Poznan, Poland). Bastadin 12 (*Fig. 1B*) was synthesized at the Institute of Physical Chemistry, Laboratory of Natural Products Synthesis and Bioorganic Chemistry NCSR 'DEMOKRITOS' (Athens, Greece) according to the procedure described by Couladouros *et al.* (27); the compound was supplied by Dr. Emmanuel N. Pitsinos. Bastadin 12 was dissolved in DMSO. The stock solution was further diluted with this solvent to obtain a final concentration of 0.5% DMSO in the incubation media. Thapsigargin was purchased from Calbiochem (La Jolla, CA, USA). Fluo-3 AM was produced by Molecular Probes, Inc. (Paisley, UK). Analytical grade chemicals were used in all other cases.

Primary cultures of rat cerebellar granule cells

Primary cultures of cerebellar granule cells were prepared from 7-day-old Wistar rats. The procedure was approved by the Forth Local Ethical Committee in Warsaw and was performed in accordance with EC Directive 86/609/EEC from 24 November

1986, making all efforts to reduce the number of animals used and to minimize their suffering.

Cerebellar granule cells (CGC) were isolated and cultured according to the method of Schousboe *et al.* (28) as previously described (29, 30). The cerebella were cut into slices and incubated at 37°C in ionic buffer containing 0.025% trypsin and 0.05% DNase 1. After 15 min, trypsin inhibitor (0.04%) was added; the cerebellar slices were then collected *via* centrifugation, triturated and re-centrifuged. After the cells were suspended in basal medium eagle (BME) supplemented with 10% fetal calf serum, 25 mM KCl, 4 mM glutamine, streptomycin (50 µg/ml) and penicillin (50 U/ml), they were seeded onto 12-well plates coated with poly-L-lysine (NUNC) at a density of 2×10^6 cells per well. Two days after plating, 7.5 µM cytosine arabinofuranoside was applied to prevent non-neuronal cells from replicating. The CGCs were cultured for 7 days.

Intracellular Ca^{2+} levels

Changes in intracellular Ca^{2+} concentration were measured fluorometrically as previously described (25). The fluorescent calcium-sensitive probe fluo-3 AM (4 µM) was applied to CGCs cultured in the original growth medium for 30 min at 37°C. Then, after washing, the growth medium was substituted with Locke's 5 buffer containing 154 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 4 mM $NaHCO_3$, 5 mM glucose and 5 mM HEPES (pH 7.4).

Fluorescence from the cell-entrapped fluo-3 was measured using a LSM 510 confocal microscope with LSM 510 data acquisition software, version 3.2 (Carl Zeiss AG, Jena, Germany). The fluorescence of fluo-3 (excited using a 488-nm argon laser) was measured at 530 nm every 30 s. Representative data for each well included the mean values of 15 randomly selected objects (cells or cell agglomerates). The autofluorescence background remained stable throughout the experiment and did not exceed 10% of the basal fluorescence emitted by CGCs loaded with fluo-3 AM, whereas experimentally manipulated increases in fluorescence reached 400–600% of the basal level. Therefore, we did not apply any correction for the background during calculations because it was essentially insignificant. The results are presented in the figures as percent changes in fluorescence intensity relative to the basal level (F/F_o) vs. the duration of the incubation. Moreover, to more precisely evaluate the effects of the drugs on the rapid phase of Ca^{2+} release, the cumulative rise in fluo-3 fluorescence over the basal level during the initial 90 s after thapsigargin and TBBPA application was calculated and presented in *Table 1* and in the Results section.

Statistics

All results presented in this paper were collected from three separate experiments, each of which tested a separate preparation of CGCs. In each experiment, five wells were used

Table 1. Modulation of increases in intracellular Ca^{2+} concentration in cerebellar granule cells induced by thapsigargin and TBBPA.

Modulating agents	Cumulative rise in fluo-3 F/Fo above the baseline, induced by the Ca^{2+} releasing substances (%)	
	Thapsigargin (200 nM)	TBBPA (10 µM)
None	1076 ± 70	406 ± 36
Ryanodine 200 µM	141 ± 24*	425 ± 26
Bastadin 12 2.5 µM	256 ± 35*	376 ± 20
Bastadin + ryanodine	95 ± 14*	-5 ± 5*

The results presented in *Figs 3* and *5* were recalculated to obtain the cumulative increases in fluo-3 fluorescence above the basal level during the initial 90 s after the application of thapsigargin or TBBPA. Other information as described in legends to corresponding figures. Results are means ± S.E.M. (n=15).

* - results significantly different from the corresponding groups untreated with modulating agents (one-way ANOVA test, $P<0.05$).

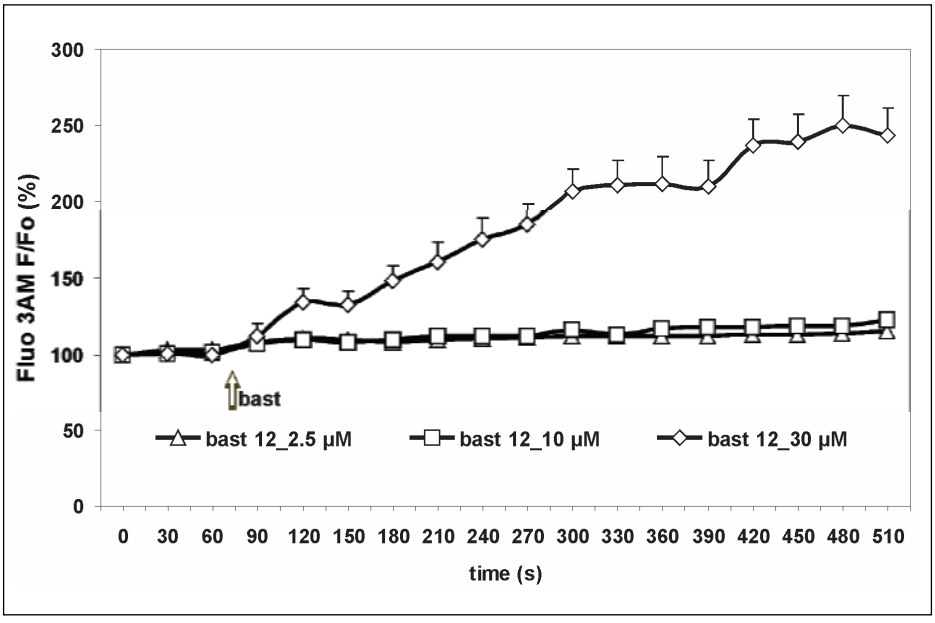

Fig. 2. Concentration-dependent effects of bastadin 12 on intracellular Ca^{2+} levels in primary cultures of rat cerebellar granule cells. After measuring the baseline fluorescence of fluo-3, bastadin 12 was applied at concentrations of 2.5, 10 and 30 µM at the time indicated by an arrow. The results are expressed as the percent changes in fluorescence intensity relative to the baseline (means ± S.E.M., n=15). Analysis of the significant differences between individual points is presented in the main text.

per treatment. Because the corresponding data for each point did not differ significantly among the three independent experiments (one-way ANOVA, $P<0.05$), the obtained data were combined and analyzed as a single set. The results are presented as the means ± S.E.M. of the number of repetitions (n=15). Statistical analysis was performed using the Statistica software program (ver.10, StatSoft). One-way ANOVA followed by Dunn's method correction and the Wilcoxon rank test were used to compare differences between experimental data points and basal levels. $P<0.05$ was considered significant for all tests.

RESULTS

Figs. 2-5 show changes in fluo-3 fluorescence in the primary CGC cultures, indicating alterations in the intracellular concentration of free Ca^{2+} ions. The initial experiments were designed to identify the concentration below which bastadin 12 does not interfere with intracellular Ca^{2+} levels in CGCs (*Fig. 2*). Application of the vehicle for bastadin 12 (0.5% DMSO) does not significantly change the basal fluorescence level (for graphical clarity, this result is omitted from *Fig. 2*; see *Fig. 4*). Bastadin 12 applied in concentrations of 2.5 and 10 µM had no significant effect on the level of Ca^{2+} compared with the basal level or that of vehicle control, whereas administration of 30 µM bastadin 12 significantly increased the intracellular Ca^{2+} concentration in CGCs relative both to the basal state (Wilcoxon rank test, $P<0.05$) and to those induced by lower concentrations of this substance plus or the vehicle (one-way ANOVA, $P<0.05$).

The results presented in *Fig. 3* show the effect of 200 nM thapsigargin, an established calcium-releasing agent, on the intracellular Ca^{2+} levels of CGCs and the effects of 2.5 µM bastadin 12 and 200 µM ryanodine on the thapsigargin-altered levels. In agreement with the data shown in *Fig. 2*, bastadin 12 at the given concentration failed to significantly affect basal fluorescence (one-way ANOVA, $P<0.05$). In this and other experiments (*Fig. 4*), we

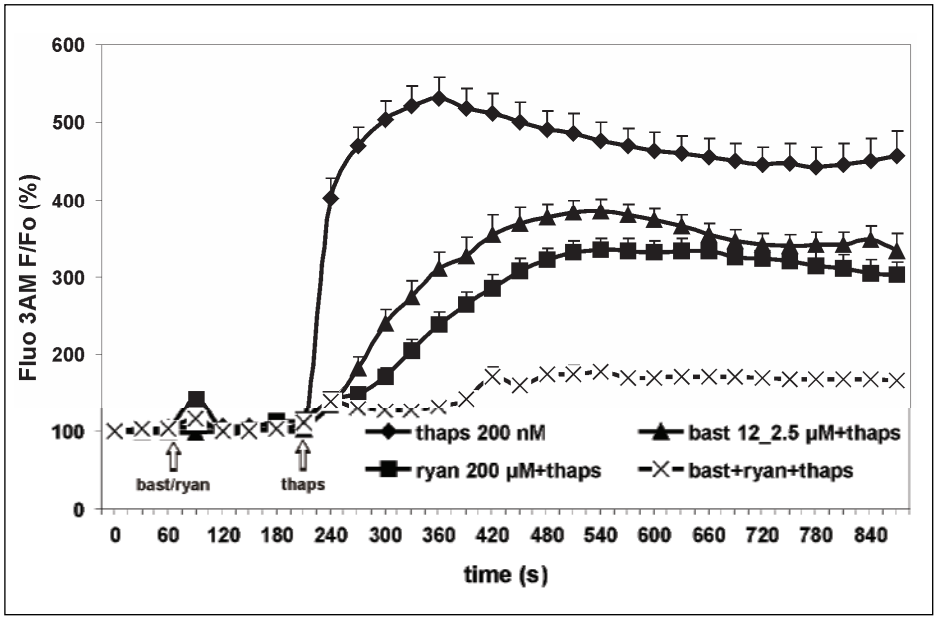

Fig. 3. Effects of 2.5 µM bastadin 12 and 200 µM ryanodine applied separately or in combination on the thapsigargin-induced increase in intracellular Ca^{2+} levels in primary cultures of rat cerebellar granule cells. Bastadin 12 and/or ryanodine were applied after a 60-s measurement of the baseline, and 200 nM thapsigargin was added 150 s later. See *Fig. 2* for additional information.

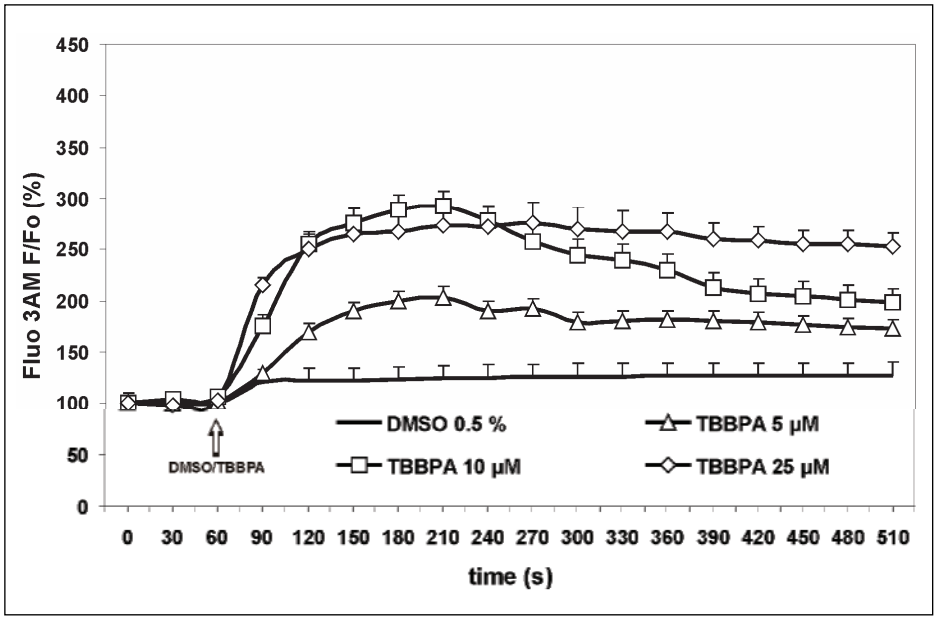

Fig. 4. TBBPA-induced increase in intracellular Ca^{2+} concentration in primary cultures of rat cerebellar granule cells. After a 60-s measurement of the baseline fluorescence of the fluo-3 AM-loaded neurons, 5, 10 or 25 µM TBBPA or vehicle (0.5% DMSO) was administered. Changes in the intracellular Ca^{2+} concentration were evaluated by measuring the fluo-3 fluorescence and are expressed as a percent of the basal level. See *Fig. 2* for additional information.

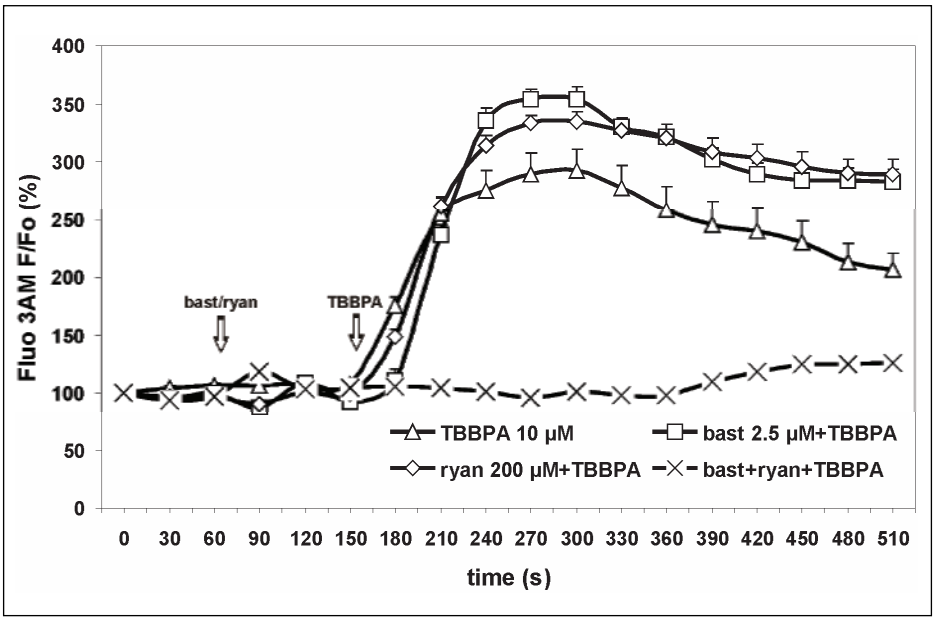

Fig. 5. Effects of bastadin 12 and ryanodine on the increase in intracellular Ca^{2+} concentration in cerebellar granule cells induced by 10 µM TBBPA. Bastadin 12 (2.5 µM) and/or 200 µM ryanodine were applied 60 s after incubation began, and 10 µM TBBPA was given 90 s later. See *Fig. 2* for additional information.

observed a brief reversible spike in fluorescence immediately after the administration of ryanodine; however, this previously reported effect (31) was not detected in other experiments that we performed (*Fig. 5*), most likely because of the low temporal resolution of the readings. The data in *Fig. 3* and *Table 1* confirm that thapsigargin administration induces an immediate and potent rise in CGC intracellular Ca^{2+} above the basal level (Wilcoxon test, $P<0.05$). This effect is partially inhibited by the application of either 200 µM ryanodine or 2.5 µM bastadin 12, whereas bastadin 12 co-administered with ryanodine nearly completely suppresses the Ca^{2+} release evoked by thapsigargin. These inhibitory effects were significant (one-way ANOVA, $P<0.05$).

The results shown in of *Fig. 4* reveal that TBBPA applied at 5, 10 and 25 µM induces a rapid and concentration-dependent rise in intracellular Ca^{2+} levels. The cumulative rises in fluo-3 fluorescence above the basal level during the initial 90 sec after the application of 5, 10 and 25 µM TBBPA were 259 ± 26%, 406 ± 36%, and 431 ± 38%, respectively. These increases were significantly different from the baseline (Wilcoxon test, $P<0.05$) and the response to vehicle (one-way ANOVA, $P<0.05$).

As with thapsigargin (see above), we evaluated the effects of ryanodine and bastadin 12 on calcium transients evoked by 10 µM TBBPA (*Fig. 5* and *Table 1*). The results indicated that application of either 200 µM ryanodine or 2.5 µM bastadin 12 alone failed to significantly interfere with the increase in fluo-3 fluorescence induced by 10 µM TBBPA, although a tendency toward potentiation was observed (one-way ANOVA, $P>0.05$). However, the results shown in *Fig. 5* and *Table 1* demonstrate that co-administration of bastadin 12 with ryanodine prior to 10 µM TBBPA completely inhibits the TBBPA-induced release of intracellular Ca^{2+} in CGCs (one-way ANOVA, $P<0.05$).

DISCUSSION

In this study, we tested the effects of bastadin 12 on steady-state Ca^{2+} levels in CGCs and examined its combined influence with ryanodine on thapsigargin-induced intracellular Ca^{2+} release. Our results demonstrate that bastadin 12 applied at a concentration of 30 µM increases the intracellular concentration of Ca^{2+}, whereas 2.5 µM bastadin 12 (which does not change intracellular Ca^{2+} levels *per se*) co-administered with 200 µM ryanodine potently inhibits the Ca^{2+} release induced by thapsigargin. Synergism with ryanodine to inhibit thapsigargin-induced Ca^{2+} release has been reported previously for bastadin 5 (22-25), although the present report represents the first time these properties have been described for bastadin 12. Consequently, we hypothesized that bastadin 12 may serve as a substitute for bastadin 5 in experiments to characterize the release of Ca^{2+} induced by TBBPA. We showed that the increase in intracellular Ca^{2+} levels in response to 10 µM TBBPA may be completely inhibited by co-administration of bastadin 12 and ryanodine but not by either of these substances alone. Our data are consistent with the hypothesis that TBBPA and thapsigargin release calcium through a common mechanism.

In the present experiments, primary cultures of CGCs served as *in vitro* model neurons. These cells were previously studied to assess TBBPA cytotoxicity and describe the effects of bastadins on Ca^{2+} homeostasis in neurons (7, 10, 25, 26). A number of previous studies demonstrated calcium releases in CGCs from stores in the endoplasmic reticulum (ER) that were evoked by either pharmacological stimulation or toxins that interfere with the activity of ryanodine receptors and inhibit the SERCA Ca^{2+} pump (25, 26, 32-34). In the present experiments, thapsigargin was applied as a reference Ca^{2+}-releasing agent. This selective inhibitor of sarco(endo)plasmic reticulum P-type Ca^{2+} ATPase (SERCA) causes Ca^{2+} to be released from intracellular stores and has been widely employed as a pharmacological tool to deplete the endoplasmic reticulum pool of Ca^{2+} (35, 36).

Several biologically active natural and synthetic bastadins, including bastadin 5 and 10, interact with FKBP12-RyR complex at concentrations that exceed 10–20 µM, stabilizing the open conformation of RyRs to release intracellular Ca^{2+} (19, 25, 26). Our present results are consistent with these data, demonstrating that 30 µM bastadin 12 also induces Ca^{2+} release. As reported above, bastadin 5 (and other antagonists of ryanodine receptors (RyRs)), ryanodine or ruthenium red when given alone are known to be unable to inhibit the Ca^{2+} release induced by thapsigargin in skeletal SR, myotubes, BC_3H1 cells or primary CGC cultures, whereas RyR inhibitors in combination with bastadin 5 completely block this effect (22-25). These data have been interpreted as evidence of an unmasking effect by thapsigargin on the ryanodine-insensitive Ca^{2+}-leak conformation of RyRs; they have also been interpreted to indicate the ability of bastadin 5, which interacts with the FKBP12-RyR complex and stabilizes open and closed channel

states, to convert these ryanodine-insensitive leak channels into ryanodine-sensitive channels (22). The results of the present experiments demonstrate that either 2.5 μM bastadin 12 or 200 μM ryanodine significantly inhibits thapsigargin-evoked Ca^{2+} release in CGCs when applied separately and that these substances nearly completely abolish this effect when applied in combination. Unlike previous studies that used bastadin 5 at relatively high calcium-releasing concentrations to investigate leak Ca^{2+} channels in skeletal SR membranes or myotubes (22-24), we used 2.5 μM bastadin 12 in our experiments, which has no effect on the basal Ca^{2+} level *per se* in CGCs.

Our present results suggest that bastadin 12 can replace bastadin 5 when studying the role of the leak conformation of RyRs in the mechanisms of intracellular Ca^{2+} release. However, the selectivity of even specific tools (such as tetrodotoxin) has been challenged (37), and therefore, the selectivity of bastadin 12 for RyR requires consideration. Primary cultures of rat CGCs are known to express functional G_q-coupled muscarinic M_3 receptors, among various other receptors, which release IP3 following application of the agonist oxotremorine-M (38, 39). Bastadins are known to interact with FKBP12 proteins (18), and these immunophilins regulate not only RyRs but also IP3Rs (20, 21). Moreover, interactions between RyR- and IP3-mediated mechanisms of Ca^{2+} release in rat CGCs are well known (40). Thus, bastadins may interfere with IP3 receptor-mediated Ca^{2+} release. However, there are discrepancies in the literature concerning the regulation of the IP3R by FKBPs (20), and interactions between bastadins and the IP3R-FKBP12 complex may not inhibit the activity of IP3-dependent calcium channels. In accordance with this hypothesis, Mack *et al.* (18) failed to find an effect of 10 μM bastadin 5 on heparin-sensitive, IP3-induced Ca^{2+} release from cerebellar membrane vesicles. Although the interaction of bastadin 12 with IP3R requires further examination, we can assume based on the findings of Mack *et al.* (18) that the combination of bastadin 12 and ryanodine inhibits the intracellular Ca^{2+} release mediated by RyR leak channels. The present study legitimizes the use of these compounds as tools to further study the role of intracellular Ca^{2+} release in the cytotoxicity of TBBPA.

TBBPA at low concentrations is known to increase the intracellular Ca^{2+} concentration. Reistad *et al.* (7) suggested that glutamate receptors and Ca^{2+} influx into CGCs might play roles in the mechanism of TBBPA-evoked calcium transients. Other studies in various neural cell lines have demonstrated that TBBPA releases Ca^{2+} from ryanodine-sensitive stores and inhibits the activity of the SERCA Ca^{2+} pump in microsomal preparations (9, 11-15). These responses are similar to the effects of thapsigargin (35). Here, we confirmed that TBBPA increases the intracellular Ca^{2+} level in CGCs in a concentration-dependent manner. Moreover, as presented in *Fig. 5*, increases in the intracellular Ca^{2+} concentration induced by 10 μM TBBPA are completely inhibited by 2.5 μM bastadin 12 co-administered with 200 μM ryanodine. This result clearly indicates that release from intracellular stores is the primary mechanism of the calcium transients induced by 10 μM TBBPA; however, our previous results indicated that 25 μM TBBPA induces an increase in Ca^{2+} uptake in neurons (10).

We propose that the Ca^{2+} transients in CGCs evoked by 10 μM TBBPA reflect Ca^{2+} efflux from the ER *via* leak RyR channels. This explanation is based on the interpretation of the mechanisms of thapsigargin-induced intracellular Ca^{2+} release and its inhibition by co-administration of bastadin 5 and ryanodine (which has been proposed by Pessah and colleagues (18, 22)) together with our findings that bastadin 12 can replace bastadin 5 and that this compound completely inhibits the effect of TBBPA when co-administered with ryanodine. However, identifying the efflux channels does not explain the primary mechanism that triggers TBBPA-induced Ca^{2+} imbalance in CGCs. Several possibilities should be considered. TBBPA is known to induce oxidative stress and to activate various protein kinases in CGCs and other cell types (7, 41). Redox modulation (42, 43) and phosphorylation of the RyR2 protein (44) have been demonstrated to regulate the RyR open channel probability. Another attractive possibility is that TBBPA could directly interact with the RyR-FKBP complex in a manner similar to that of non-coplanar 2,2',3,5',6-pentachlorobiphenyl (PCB 95) (34, 45). Further studies are required to more precisely elucidate the primary mechanism responsible for the TBBPA-evoked intracellular Ca^{2+} imbalance in CGCs.

Our present results demonstrate that low concentrations of bastadin 12 (which fail to release Ca^{2+} from intracellular stores when administered alone) may replace the previously used bastadin 5 when co-administered with ryanodine during the study of Ca^{2+} release mechanisms from ryanodine-sensitive intracellular stores in response to various agents. Our present data confirm that TBBPA induces a concentration-dependent increase in the intracellular Ca^{2+} level in CGCs. This effect was completely blocked by co-administration of 2.5 μM bastadin 12 and ryanodine but not by either of these substances applied alone. We assert that, similarly to the effects of thapsigargin, TBBPA releases Ca^{2+} *via* ryanodine-insensitive Ca^{2+} leak channels and that bastadin 12 converts these channels into classical ryanodine-sensitive receptors. Bastadin 12 with ryanodine may be useful in studies of the role of intracellular Ca^{2+} release in the mechanism of TBBPA-induced cyto- and neurotoxicity.

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Conflicts of interest: None declared.

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