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Synthetic Bastadins Modify the Activity of Ryanodine Receptors in Cultured Cerebellar Granule Cells

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Key Words
Caffeine · Calcium mobilization · Neurons · Primary culture · Thapsigargin

Abstract
Although the interactions of several natural bastadins with the RyR1 isoform of the ryanodine receptor in sarcoplasmic reticulum has been described, their structure-dependent interference with the RyR2 isoform, mainly expressed in cardiac muscle and brain neurons, has not been studied. In this work, we examined calcium transients induced by natural bastadin 10 and several synthetic bastadins in cultured cerebellar granule cells known to contain RyR2. The fluorescent calcium indicator fluo-3 and confocal microscopy were used to evaluate changes in the intracellular Ca^{2+} concentration (Ca_i), and the involvement of ryanodine receptors was assessed using pharmacological tools. Our results demonstrate that apart from the inactive BAST218F6 (a bisdebromo analogue of bastadin 10), synthetic bastadin 5, and synthetic analogues BAST217B, BAST240 and BAST268 (at concentrations >20 μM) increased Ca_i in a concentration-dependent, ryanodine- and FK-506-sensitive way, with a potency significantly exceeding that of 20 mM caffeine. Moreover, the same active bastadins at a concentration of 5 μM in the presence of ryanodine prevented a thapsigargin-induced increase in Ca_i. These results indicate that bastadins, acting in a structure-dependent manner, modify the activity of RyR2 in primary neuronal culture and provide new information about structure-related pharmacological properties of bastadins.

Introduction

Calcium is a second messenger mediating several basic neuronal functions and a potential trigger of neuronal injury [1–3]. These roles have been associated with the entry of extracellular calcium into neurons and/or the mobilization of intracellular calcium stores. Calcium from the neuronal endoplasmic reticulum (ER) may be released via the channels of ryanodine or IP3 receptors [4]. The influx of extracellular calcium into neurons via

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NMDA receptors triggers mobilization of calcium from ryanodine-sensitive ER stores, in a process known as calcium-induced calcium release (CICR) [5]. Imbalance in ER calcium stores may play a crucial role in the mechanisms of neurodegeneration [6–9].

Three distinct isoforms of ryanodine receptor (RyR), encoded by different genes, have been identified: RyR1, a skeletal muscle type; RyR2, a mainly cardiac type that is also expressed in brain neurons, and RyR3, a so-called brain type, although it has been detected in many tissues [10, 11]. The immunophilin FKBP 12, a member of a family of proteins binding FK-506 (tacrolimus), regulates the activity of ryanodine receptors in different tissues including neurons, with possible consequences for neuronal survival and/or injury [12, 13]. New pharmacological tools are required to gain a better understanding of these important interactions. It has been shown that several bastadins interfere with the activity of ryanodine receptors in the sarcoplasmic reticulum (SR) by binding to FKBP 12 [14, 15].

Bastadins are an ever-growing family of natural compounds isolated from marine sponges of the order Verongida [16]. Apart from open-chain bastadins 1 and 2 and biphenylyl bastadin 3, they are macrocyclic bis-diaryl ethers that are further classified either as bastaranes or isobastaranes. They feature unique α-oximino amides, both of E-configuration, and an unprecedented mixture of mono- and di-*o*-brominated diarylethers, biosynthetically derived via oxidative phenolic coupling of tyramine-tyrosine units in an apparently combinatorial fashion [17, 18]. Some natural bastadins, which have affinity for FKBP 12, modify the activity of RyR1 in SR [15, 19]. In particular, bastadin 10 promotes the release of calcium [15], whereas bastadin 5 in the presence of ryanodine inhibits calcium leakage induced by thapsigargin [19]. Other bastadins exhibit antibacterial, anti-inflammatory, antiangiogenic or cytotoxic activities [20–23].

Studies of the effect of bastadins on the activity of ryanodine receptors have mainly concentrated on the RyR1 (skeletal muscle) subtype in isolated SR vesicles [14, 15, 19, 24, 25], or in the BC_3H_1 cell line differentiating into a myogenic phenotype [19]. In addition, their effects on the activity of the RyR3 (brain) isoform in the salivary gland have also been characterized [26]. Until now, the interference by bastadins with the RyR2 (cardiac) isoform expressed in abundance in the brain [27] has not been studied. Since several differences in the properties of the individual RyRs subtypes have been detected [28, 29], it is important to close this gap in our knowledge.

Although stringent structural requirements for the biological activities of bastadins have been demonstrated, few systematic studies on their structure-related properties have been performed due to the limited availability of the natural substances, which are isolated from marine sponges [14]. Recently, an efficient and versatile synthetic methodology for the preparation of all members of the bastadin family has been developed [30–33].

Primary cultures of rat cerebellar granule cells (CGC) are a useful model of glutamatergic neurons for studying calcium transients and calcium-dependent mechanisms of neurodegeneration and neuroprotection [34–37]. Ryanodine receptors of the RyR2 subtype and a functional ryanodine- and caffeine-sensitive intracellular calcium pool has been demonstrated in these cells [38, 39].

The aim of the current study was to investigate the effects of synthetic bastadins on the activity of RyRs in neurons, and to determine the relationship between the structure of bastadins and their biological activity. Utilizing CGC, we tested the effects of natural bastadin 10, synthetic bastadin 5 and several synthetic analogues. Activation of the ryanodine-sensitive intracellular calcium pool in CGC was assessed by changes in the intracellular calcium level (Ca_i) measured using the fluo-3 fluorescent calcium indicator and verified using specific pharmacological tools. Attention was focused on the effects of bastadins observed previously in experiments with RyR1 from SR [15, 19], namely the release of calcium from the intracellular caffeine and ryanodine-sensitive pool, and the inhibition in the presence of ryanodine of thapsigargin-induced calcium release.

Materials and Methods

Materials

Natural bastadin 10 extracted and purified from *Ianthella basta* [40], FK-506 and thapsigargin were purchased from Calbiochem, La Jolla, Calif., USA. Synthetic bastadins 5, BAST240, BAST268, BAST217B and BAST218F6 (fig. 1) were prepared in the Institute of Physical Chemistry, Laboratory of Natural Products Synthesis and Bioorganic Chemistry NCSR 'DEMOKRITOS', Athens, Greece, as previously described [30]. The bastadins were dissolved and diluted in dimethyl sulphoxide (DMSO). The final concentration of this solvent in the incubation medium was usually $\leq$0.15%; however, in some experiments it reached 1.5%. Vehicle control experiments using equivalent concentrations of DMSO were therefore performed. Fluo-3 AM was obtained from Molecular Probes, Leiden, The Netherlands. Caffeine was supplied by Fluka Chemie AG, Buchs, Switzerland. Other chemicals and materials for cell culture were purchased from Sigma Chemical Company, St. Louis, Mo., USA. All reagents were of analytical grade.

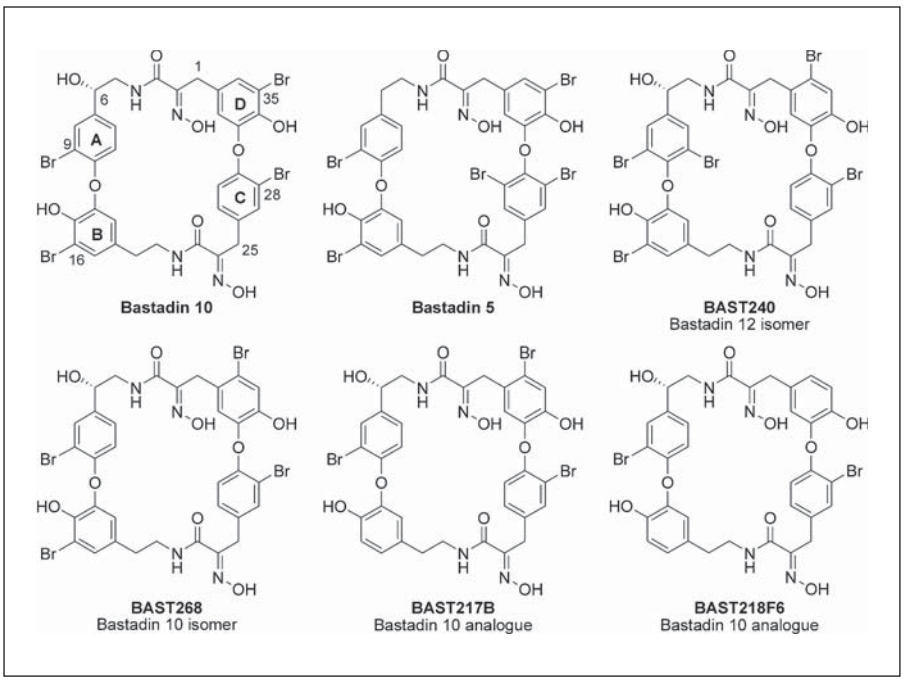

Fig. 1. Structure of bastadins used in this study.

Cell Culture

Primary cultures of CGC were prepared from 7-day-old Wistar rats according to the method of Schousboe et al. [41], with slight modifications as described previously [34, 35]. The use of rat pups was in accordance with Polish governmental regulations concerning experiments on animals (Dz.U.97.111.724 and Dz. U.05.289.2143) and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The procedure was approved by the First Local Ethical Committee in Warsaw and all efforts were made to reduce the number of animals used and to minimize suffering. The rat pups were decapitated, and after separation of the vessels, the collected cerebella were cut into 400-μm cubes. The tissue was incubated for 15 min at 37°C in ionic buffer containing 0.025% trypsin and 0.05% DNase 1. The incubation was terminated by the addition of trypsin inhibitor (0.04%) and centrifugation. The cells were separated by trituration of the pellet and further centrifugation. A cell suspension in basal Eagle's medium supplemented with 10% fetal calf serum (Sigma), 25 mM KCl, 4 mM glutamine, streptomycin (50 μg/ml) and penicillin (50 U/ml) was used to seed 12-well plates coated with poly-L-lysine (NUNC), at a density of 2×10^6 cells per well. To prevent the replication of non-neuronal cells, 7.5 μM cytosine arabinofuranoside was added to the cultures 36 h after plating. The CGC were used in experiments after 7 days in vitro (DIV).

Loading of Cells with Fluo-3 AM and Fluorescence Measurements

CGC were loaded with 16 μM fluo-3 AM at 37°C for 30 min in the original growth medium. Loading was terminated by washing the cells four times with medium containing 154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 8.6 mM HEPES (pH 7.4) and 5.6 mM glucose. Cell-entrapped fluo-3 was excited with an argon laser at 488 nm and fluorescence was measured at 530 nm using a Zeiss LSM 510 confocal microscope with LSM 510 data acquisition software, version 3.2. Scans were collected every 30 s during each 15-min measurement period. Initially, three scans (at 0, 30 and 60 s) were taken to establish the control (basal) fluorescence level, presented in graphs as 100%. Compounds triggering calcium release (caffeine, thapsigargin or bastadins) were added after the third scan, while inhibitors (ryanodine or FK-506) were administered before the first scan. The results were presented as percent changes in the intensity of fluorescence compared with the basal level (F/F_0). The presented data were derived from one experiment representative for three independent trials using separate cultures that gave qualitatively identical results. They are presented as means from 10 randomly selected objects (cells or cell agglomerates).

Data Analysis

The results are presented as mean ± SD (n = 10). Statistical analyses were performed using the program Statistica 7.0. Three nonparametric tests were used: Mann-Whitney, Friedman and Wilcoxon signed-rank test. For all the tests, $p < 0.05$ was considered significant. The Mann-Whitney test was used to analyze the differences between the same time points in two parallel experiments. The Friedman test was used to analyze differences in one experiment and the Wilcoxon test was used to compare differences between experimental data points and the basal level.

Results

Caffeine-Evoked Increases in Intracellular Calcium Levels in CGC: Role of Ryanodine Receptors

In order to demonstrate the effects of activation of RyRs in CGC on intracellular calcium levels measured

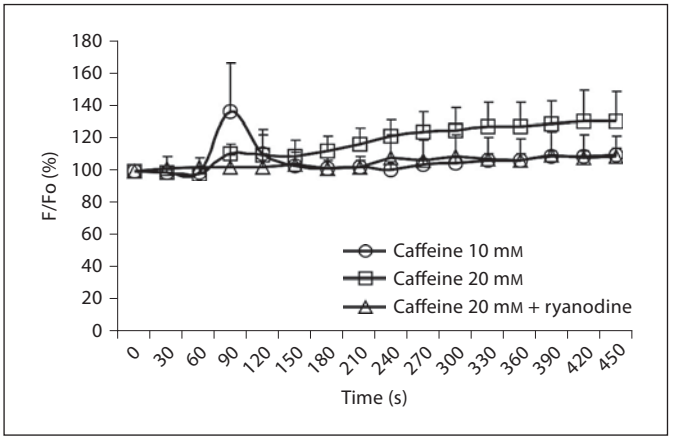

Fig. 2. Caffeine-induced increase in the intracellular calcium concentration in primary cultures of rat cerebellar granule cells. Cells were stimulated with 10 or 20 mM caffeine applied after 60 s of incubation; 200 μM ryanodine was added at time 0, when present. Results are means ± SD (n = 10). All results (20 mM caffeine) are statistically different from the basal level (Wilcoxon test, $p < 0.05$). All results (20 mM caffeine + ryanodine), except the points at 120 and 150 s, are significantly different from the effect of caffeine applied alone (Mann-Whitney U test, $p < 0.05$).

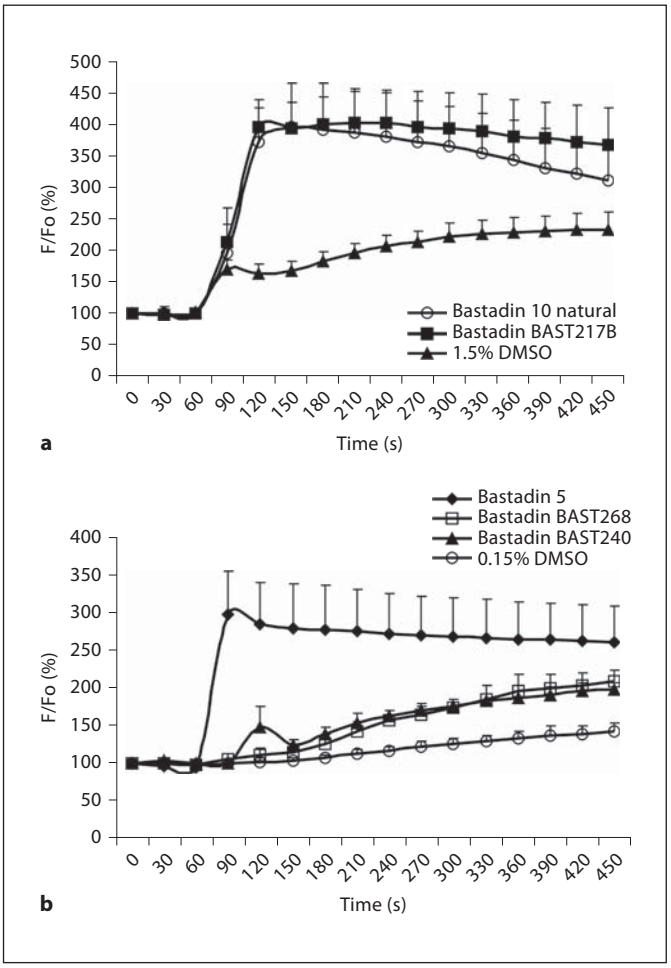

Fig. 3. Effect of bastadins on the intracellular calcium level in primary cultures of rat cerebellar granule cells. Natural bastadin 10 (15 μM), synthetic bastadin BAST217B and vehicle 1.5% DMSO (**a**), as well as synthetic bastadins 5, BAST268, BAST240 and vehicle 0.15% DMSO (**b**), were applied after 60 s of incubation. Results are means ± SD (n = 10). The presented data are from one of three independent experiments using different cultures that gave qualitatively identical results. All results presented in **a** and **b** are statistically different from the basal level, with the exception of points at 90 and 120 s (DMSO) and a point at 90 s (BAST240) in **b** (Wilcoxon test $p < 0.05$). All results are significantly different from the corresponding vehicle controls (DMSO) with the exception of points at 120, 420 and 450 s (natural bastadin 10; in **a**), and at 90 s (BAST240; in **b**) (Mann-Whitney U test, $p < 0.05$).

with the fluo-3 calcium-sensitive fluorescent probe, caffeine was used as a reference substance. As shown in figure 2, addition of 10 mM caffeine to the incubation medium induced a modest one-phase transient (lasting only 1 min) increase in fluo-3 fluorescence, indicative of a rise in the intracellular calcium level. Caffeine applied at 20 mM induced a slowly developing prolonged increase in intracellular calcium. This effect was completely prevented by the application of 200 μM ryanodine, demonstrating the role of ryanodine receptors in the caffeine-induced mobilization of intracellular calcium.

Effects of Bastadins on Intracellular Calcium Levels in CGC: Role of Ryanodine Receptors and FKBP

Some natural bastadins, particularly bastadins 10 and 5, stimulate the release of calcium via RyR1 from the membranes of SR isolated from skeletal muscles [15, 19]. In this study, the calcium mobilizing potential of bastadins in CGC was examined for the natural bastadin 10 and its synthetic isomer BAST217B (fig. 3a), as well as the bisdebromo-bastadin 10 analogue, BAST218F6 (results not shown). We also tested synthetic bastadin 5, an isomer of bastadin 10 (BAST268), and BAST240, a bastadin 12 isomer (fig. 3b). Synthetic compounds BAST268 and BAST240 are isomers of bastadin 10 and bastadin 12, respectively (ring D bears a bromine substituent at C-36 instead of C-35). Compound BAST217B is an analogue of bastadin 10 which lacks a bromine substituent on ring B while ring D bears a bromine substituent at C-36 instead of C-35. Analogue BAST218F6 is devoid of a bromo substituent on both rings B and D. Figure 3a demonstrates that 15 μM natural bastadin 10 induced a rapid and pro-

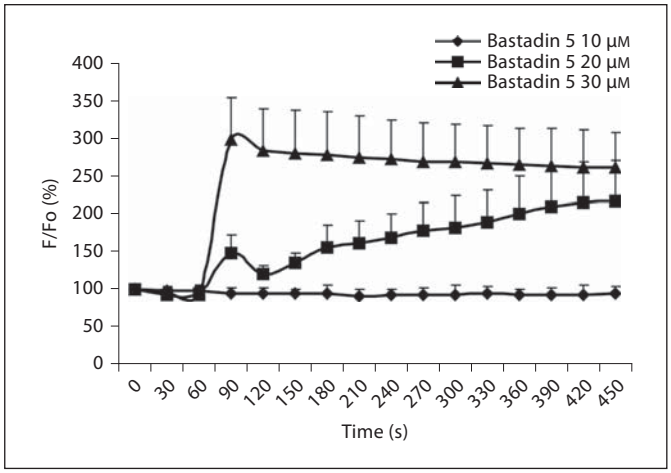

Fig. 4. Dose-dependent increase in the intracellular calcium concentration in primary cultures of rat cerebellar granule cells evoked by synthetic bastadin 5 applied at concentrations of 10, 20 and 30 μM. For the effect of vehicle 0.15% DMSO corresponding to 30 μM bastadin 5, see figure 3b. Results are means ± SD (n = 10). Bastadin 5 applied at a 10-μM concentration produced no statistically significant effect (Friedman test, $p < 0.05$), while all the results representing effects of 20 and 30 μM bastadin 5 were significantly different within groups (Friedman test, $p < 0.05$) and from their basal levels (Wilcoxon test, $p < 0.05$). At the corresponding experimental time points, all the results for the bastadin 5 at 10, 20 and 30 μM concentrations, except the basal levels and points at 420 and 450 s, are significantly different (Mann-Whitney U test, $p < 0.05$).

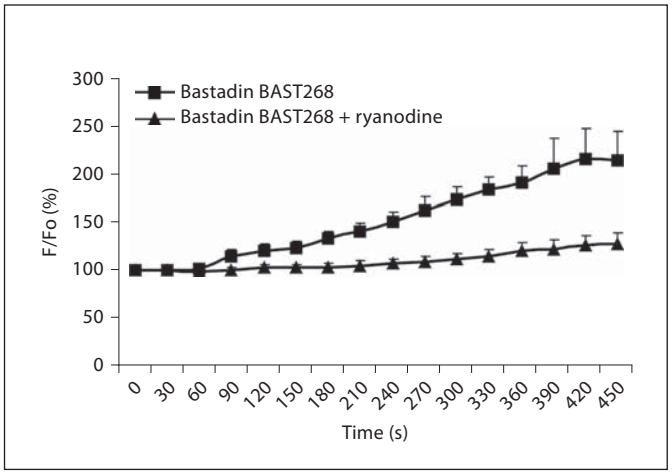

Fig. 5. Ryanodine inhibits the synthetic bastadin BAST268-evoked increase in the intracellular calcium concentration in primary cultures of rat cerebellar granule cells. Cells were stimulated with 30 μM BAST268 applied after 60 s of incubation; 200 μM ryanodine was added at time 0, when present. For the effect of vehicle 0.15% DMSO, see figure 3b. Results are means ± SD (n = 10). All the presented results are statistically different from the basal level with the exception of a point at 90 s (BAST268 + ryanodine) (Wilcoxon test, $p < 0.05$). All results (BAST268 + ryanodine), except a point at 90 s, are significantly different from the effect of bastadin 10 applied alone (Mann-Whitney U test, $p < 0.05$).

longed increase in the intracellular calcium level in CGC, and a similar effect was also produced by the synthetic bastadin BAST217B applied at 30 μM. However, the solvent vehicle used for these compounds, 1.5% DMSO, induced a significant increase in the intracellular calcium level by itself, and this effect could partially contribute to the rapid and potent responses induced by these bastadins. Synthetic bastadins 5, BAST268 and BAST240 applied at 30 μM also increased the intracellular calcium level in CGC (fig. 3b). The effect of bastadin 5 was well pronounced, whereas the responses to synthetic bastadins BAST268 and BAST240 developed more slowly and the intracellular calcium reached a lower level. Vehicle control experiments revealed that, in this case, only an insignificant portion of the bastadin-evoked increase in intracellular calcium level might be ascribed to the effect of the solvent, 0.15% DMSO (fig. 3b). The only compound not stimulating a release of calcium was BAST218F6, which is devoid of a bromo substituent on ring D, suggesting that this moiety is important for function (results not shown).

Dose-response relationships were studied for the calcium-releasing synthetic bastadins. The results presented in figure 4 using bastadin 5 as an example indicate that administration of these compounds at concentrations higher than 20 μM provoked calcium release in CGC. As shown in figure 5, 200 μM ryanodine prevented increases in the intracellular calcium concentration evoked by BAST268. The same effect was also observed for bastadin 5, BAST217B and BAST240 (results not shown). This phenomenon resembles the ryanodine-mediated inhibition of caffeine-induced calcium release (fig. 2), and confirms the role of RyRs in the mobilization of intracellular calcium induced by the administration of bastadins.

Previous studies [14] demonstrated that the FK-506 binding protein immunophilin FKBP12 is the target for bastadins in the RyR1 complex in the SR. Other subtypes of RyRs expressed in the cardiac muscle, brain and other tissues also utilize FK-506 binding proteins. In the heart, the FK-506 binding protein calstabin-2 or FKBP12.6 is known to stabilize the cardiac RyR2 receptor complex and to prevent calcium leakage through this channel [42].

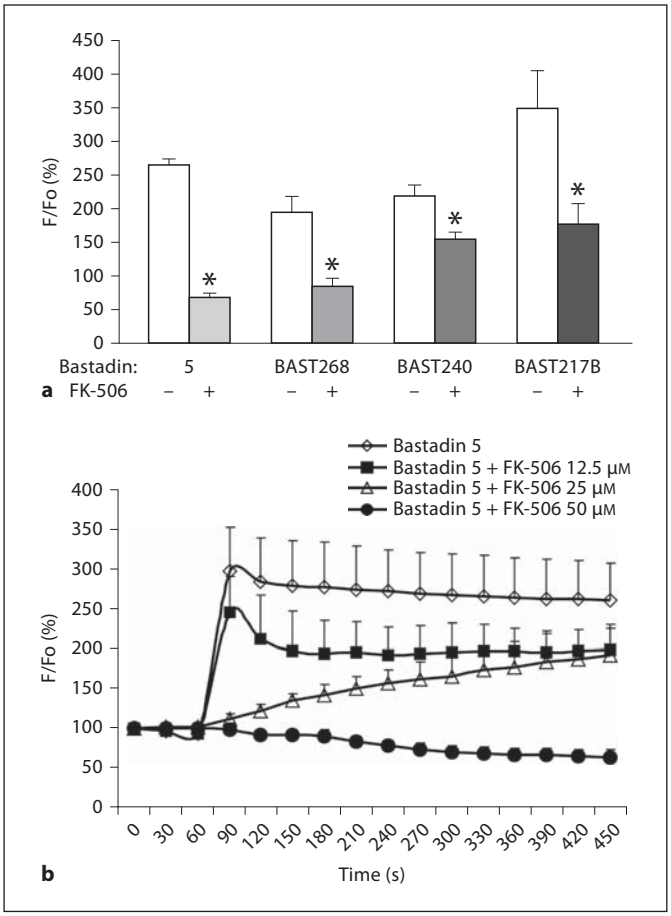

Fig. 6. Effect of FK-506 on bastadin-evoked increases in the intracellular calcium concentration in primary cultures of rat cerebellar granule cells. Increases in the intracellular calcium concentration induced by 30 μM synthetic bastadins 5, BAST268, BAST240 and BAST217B, presented as peak responses, were significantly inhibited by 50 μM FK-506 (**a**). FK-506 applied at 12.5, 25 and 50 μM inhibited the increase in intracellular calcium concentration evoked by 30 μM synthetic bastadin 5 in a dose-dependent manner (**b**). Cells were stimulated with 30 μM synthetic bastadins applied after 60 s of incubation; FK-506 was added at time 0, when present. Results are means ± SD (n = 10). All results are statistically different from the basal level with the exception of a point at 90 s (bastadin 5 + FK-506 50 μM) (Wilcoxon test, $p < 0.05$). All results are significantly different from the effect of bastadins applied alone (Mann-Whitney U test, $p < 0.05$). * Results in **a** significantly different from the effects of bastadins applied alone (Mann-Whitney U test, $p < 0.05$).

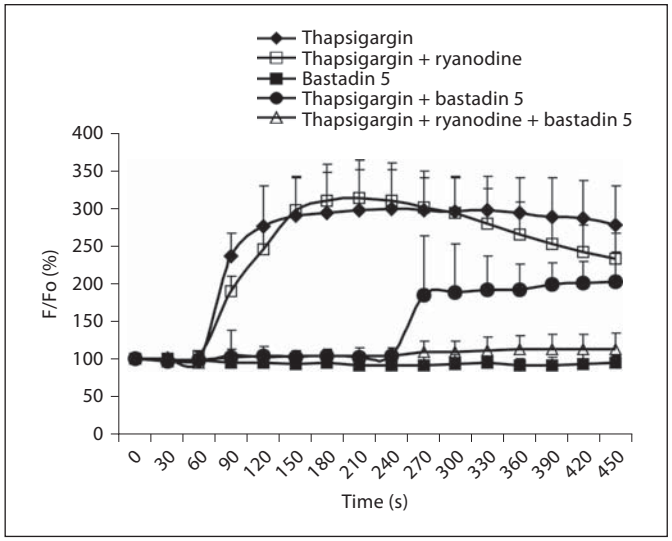

Fig. 7. Effects of bastadin 5 and ryanodine on the thapsigargin-induced increase in the intracellular calcium concentration in primary cultures of rat cerebellar granule cells. Bastadin 5 (5 μM) and/or 200 μM ryanodine were applied at time 0; 200 nM thapsigargin was added after 60 s or 170 s of incubation. Results are means ± SD (n = 10). Bastadin 5 at the given concentration produced no statistically significant effect (Friedman test, $p < 0.05$). All the other results are statistically different from their basal levels with the exception of points at 120 and 210 s (thapsigargin + bastadin 5) and a point at 240 s (thapsigargin + ryanodine + bastadin 5) (Wilcoxon test, $p < 0.05$). At the corresponding experimental time points, all results for the bastadin 5, thapsigargin + bastadin 5, and thapsigargin + ryanodine + bastadin 5 groups are significantly different (Mann-Whitney U test, $p < 0.05$).

To verify the role of the FKBP in the bastadin-evoked activation of RyR2 calcium channels in brain neurons, we tested the effect of the immunosuppressive agent FK-506 on the increase in intracellular calcium levels in CGC caused by bastadin 5, BAST268, BAST240 and BAST217B. FK-506 given alone at micromolar concentrations induced a negligible rise in intracellular calcium levels (results not shown). As shown in figure 6a, 50 μM FK-506 completely inhibited the increases in intracellular calcium evoked by bastadin 5 and BAST268, and significantly, but incompletely suppressed the effects of BAST217B and BAST240. The dose-dependent inhibition of calcium release evoked by bastadin 5 (30 μM), produced by FK-506 at concentrations of 12.5, 25 and 50 μM, is shown in figure 6b.

Interference of Bastadins with Thapsigargin-Induced Intracellular Calcium Release in CGC

Thapsigargin is a selective inhibitor of sarco(endo)-plasmic reticulum P-type Ca^{2+} ATPase (SERCA) and acts to deplete calcium stores in the ER [43]. Data presented by Pessah et al. [19] demonstrated that bastadin 5 given at low micromolar concentrations in the presence of RyR

antagonists ryanodine or ruthenium red inhibits thapsigargin-induced calcium release from the sarcoplasmic reticulum. Here we checked whether this phenomenon may be reproduced in CGC, and if bastadins other than bastadin 5 can also inhibit thapsigargin-induced calcium release in neurons. The results presented in figure 7 demonstrate that application of 200 nM thapsigargin induced an instant increase in the intracellular calcium level in CGC. This phenomenon was not affected by 200 μM ryanodine. Application of bastadin 5 at a lower concentration (5 μM) did not increase the intracellular calcium level; however, under these conditions the Ca^{2+}-releasing effect of thapsigargin was suppressed by about 50%, and it was completely inhibited when 200 μM ryanodine was also present (fig. 7). Further experiments demonstrated that this effect may be obtained by application of bastadin 5 in the 2.5–10 μM concentration range, and that synthetic bastadins BAST268, BAST217B and BAST240 given at a concentration of 5 μM in the presence of 200 μM ryanodine induced exactly the same effect (results not shown). The only synthetic bastadin derivative that had no effect on thapsigargin-induced calcium release was the bisdebromo analogue of bastadin 10, BAST218F6 (results not shown). This result points to the importance of a bromo substituent on ring D for this function.

Discussion

The results of this study confirm that primary cultures of CGC are a useful model for studying mobilization of intracellular calcium, and they demonstrate their utility in research on the regulation of RyRs by bastadins. Using this model, we demonstrated the interference by bastadins with the activity of RyR2 (a 'cardiac' isoform), which is mainly present in brain neurons. Our findings have extended the list of known bastadins that activate ryanodine receptors and release calcium from ER stores in a ryanodine- and FK-506-sensitive manner, and which, in the presence of ryanodine, inhibit the release of calcium evoked by thapsigargin. We detected structure-related properties of bastadins, as bastadin BAST218F6, a bisdebromo analogue of bastadin 10, appeared to be inactive suggesting that bromo-substitution on ring D is important for function.

The calcium-sensitive fluorescent probe used in the present work, fluo-3, has been exploited for more than a decade in studies of intracellular calcium transients in cultured neurons, including cerebellar granule cells [35, 44, 45]. Although ratiometric calcium measurements with an alternative calcium-sensitive fluorescent probe, fura-2, are less sensitive to interfering factors [39], this dye reportedly obstructs activation of ryanodine receptors in CGC [45]. Our experimental model, primary cultures of rat cerebellar granule neurons, has previously been used in numerous basic neurobiological studies as well as in pharmacological and neurotoxicological tests [46, 47]. Moreover, CGC in culture may be considered an adequate model for studies selectively aimed at RyR2, which is the main ryanodine receptor isoform present in brain neurons [48]. Although Martin et al. [49], utilizing RT-PCR, detected mRNAs encoding all three isoforms in rat CGC, Genazzani et al. [38] were only able to demonstrate the presence of the RyR2 protein in these cells. Several studies have successfully used primary cultures of CGC to examine the phenomenon of CICR [39, 45, 50–52].

We observed a relatively weak effect of caffeine on the intracellular calcium level in CGC. It is known that caffeine at 10 mM or 1 μM ryanodine activates CICR from ER stores via ryanodine receptors/channels, whereas 100 μM ryanodine inhibits these receptors and the calcium release provoked by caffeine [53–56]. However, previous studies have demonstrated that CGC in culture change their responsiveness to caffeine depending on the duration of growth in vitro; the maximal sensitivity of ryanodine receptors to caffeine was detected at DIV 3 and it decreases significantly by DIV 7 [39, 45]. In our experiments, we used 1-week-old cultures, because at this age they express fully mature glutamate receptors including NMDA receptors, which are important players in intracellular calcium homeostasis [41, 57]. Furthermore, in agreement with the findings of Gafni et al. [39], our control experiments with caffeine clearly demonstrated that caffeine-induced release of calcium from CGC is extremely sensitive to high concentrations of ryanodine, confirming specific involvement of ryanodine receptors in calcium transients.

We also tested whether RyR2 receptors/channels in neurons respond to bastadins in a similar way to RyR1. Previous studies by Pessah and coworkers, mainly using isolated SR membranes, characterized the interference by several natural bastadins, isolated from the marine sponge *I. basta*, with the RyR1 subtype of ryanodine receptor [14, 15, 19, 24]. These studies demonstrated potentiation by some of these compounds of CICR and pointed to the FK-506-binding protein FKBP as the target for bastadins causing interference with the FKBP-RyR1 receptor complex [14]. Further experiments identified bastadin 10 as particularly potent in triggering the release of intracellular calcium via stabilization of the open

conformation of the RyR1 channel, in a process dependent on an intact FKBP-RyR1 complex. FK-506 inhibited calcium transients induced by bastadin 10 by disrupting this complex and releasing FKBP [15]. In agreement with the findings of Pessah and coworkers, our results demonstrated that natural bastadin 10, synthetic bastadin 5, and some previously unstudied analogues of bastadins 10 and 12, applied at concentrations exceeding 20 μM, caused the release of calcium in cultured cerebellar granule neurons. This effect was more pronounced than the mobilization of calcium induced by caffeine, the classical promoter of calcium release, applied at concentrations 3 orders of magnitude higher than the bastadins. In agreement with data from experiments with SR membranes [14, 15], our results also demonstrated that both ryanodine, applied at high micromolar concentration, and immunosuppressing agent FK-506, inhibit the release of intracellular calcium evoked by bastadins in CGC. This indicates that in neurons, as in skeletal muscles, several bastadins cause the release of calcium via ryanodine receptors/channels in a process mediated by FKBP.

In this study, we have also reproduced in CGC another important property of bastadin 5, initially discovered by Pessah et al. [19], namely inhibition of thapsigargin-induced calcium release in the presence of ryanodine. This effect has been interpreted as interference by bastadin 5 with the ryanodine-insensitive leak channels unmasked by thapsigargin [19]. Thapsigargin is a well-known ER P-type Ca^{2+} ATPase inhibitor that causes calcium release from intracellular stores [43, 58, 59]. Our results extend the findings of Pessah's group, since not only synthetic bastadin 5, but also other bastadins that activate mobilization of intracellular calcium in CGC, prevented the thapsigargin-induced leakage of calcium from the ER in the presence of ryanodine. It is noteworthy that interference by synthetic bastadins with RyR2 in CGC is clearly a structure-dependent phenomenon. BAST218F6, a bisdebromo derivative of bastadin 10, failed to provoke calcium release and did not interfere with thapsigargin-induced calcium transients in CGC, indicating that bromo-substitution on ring D is important for function. These findings complement those from experiments conducted with natural bastadins by Mack et al. [14], who identified differences in the potency of different isomers of bastadin 5 in their interference with RyR1. Furthermore, conservation of the dibromocatechol ether moiety was found important for the interaction of bastadin 5 analogues with the RyR1/FKBP12 complex by a recent study of Masuno et al. [60].

A role for the destabilization of RyRs resulting in dysfunction of intracellular calcium stores has been suggested among the mechanisms of neurodegeneration during various pathological conditions [61–63]. Thus, pharmacological destabilization of FKBP-RyR complexes leading to calcium leakage through neuronal RyR channels may serve as a model in studies on the mechanisms of neuronal death evoked by ER disruption. Previous studies have shown that hexachlorocyclohexane and non-planar 2,2′,3,5′,6-pentachlorobiphenyl induce neurotoxicity and destabilize intracellular calcium via a mechanism involving RyRs [39, 50, 51]. Mariussen et al. [64] demonstrated a role of NMDA receptors in neurotoxic effects of polychlorinated biphenyls. Our results suggest that several bastadins may also be used as tools to disrupt the FKBP-RyR complex and thus disturb ER calcium homeostasis in neurons, which may lead to neuronal injury. Our manuscript describing the neurotoxic properties of bastadins including involvement of NMDA receptors is currently in preparation.

Using primary cultures of rat cerebellar granule neurons expressing the RyR2 subtype of ryanodine receptor, we have demonstrated that natural bastadin 10 and several synthetic bastadins produce an effect identical to that observed with the RyR1 subtype in the sarcoplasmic reticulum of skeletal muscle. Synthetic bastadin 5 and bastadin analogues BAST268, BAST217B and BAST240 release calcium from stores in the ER of neurons in a ryanodine- and FK-506-sensitive way. Moreover, in the presence of ryanodine, the same compounds completely prevent thapsigargin-induced calcium release. These effects are structure-dependent, since a bisdebromo analogue of bastadin 10, BAST218F6, is completely devoid of these properties. Thus, a common mechanism appears to be responsible for the interference by bastadins with FKBP-RyR complexes in both sarcoplasmic reticulum expressing RyR1 and neurons expressing RyR2. We suggest that bastadins may be useful as a tool in future studies on the role of FKBP-RyR disruption in the process of neurodegeneration.

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