Bastadin 10 Stabilizes the Open Conformation of the Ryanodine-sensitive Ca$^{2+}$ Channel in an FKBP12-dependent Manner*

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Lili Chen‡, Tadeusz F. Molinski§, and Isaac N. Pessah¶

From the ‡Department of Molecular Biosciences, School of Veterinary Medicine, Graduate Program in Neuroscience, and the ¶Department of Chemistry, University of California, Davis, California 95616

The marine sponge Ianthella basta synthesizes at least 25 tetrameric bromotyrosine structures that possess a stringent structural requirement for modifying the gating behavior of ryanodine-sensitive Ca$^{2+}$ channels (ryanodine receptors) (RyR). Bastadin 5 (B5) was shown to stabilize open and closed channel states with little influence on the sensitivity of the channel to activation by Ca$^{2+}$ (Mack, M. M., Molinski, T. F., Buck, E. D., and Pessah, I. N. (1994) J. Biol. Chem. 269, 23236–23249). In the present paper, we utilize single channel analysis and measurements of Ca$^{2+}$ flux across the sarcoplasmic reticulum to identify bastadin 10 (B10) as the structural congener responsible for dramatically stabilizing the open conformation of the RyR channel, possibly by reducing the free energy associated with closed to open channel transitions ($\Delta G^\circ \rightarrow o$). The stability of the channel open state induced by B10 sensitized the channel to activation by Ca$^{2+}$ to such an extent that it essentially obviated regulation by physiological concentrations of Ca$^{2+}$ and relieved inhibition by physiological Mg$^{2+}$. These actions of B10 were produced only on the cytoplasmic face of the channel, were selectively eliminated by pretreatment of channels with FK506 or rapamycin, and were reconstituted by human recombinant FKBP12. The actions of B10 were found to be reversible. A structure-activity model is proposed by which substitutions on the Eastern and Western hemispheres of the bastarane macrocycle may confer specificity toward the RyR1-FKBP12 complex to stabilize either the closed or open channel conformation. These results indicate that RyR1-FKBP12 complexes possesses a novel binding domain for phenoxyacetechols and raise the possibility of molecular recognition of an endogenous ligand.

Immunophilins are a family of proteins that function to regulate Ca$^{2+}$-dependent and Ca$^{2+}$-independent cellular signaling cascades within immune, myogenic, and neuronal cells (1–3). Although immunophilins represent a structurally heterogeneous family of proteins of molecular mass ranging from 12 to 56 kDa, they share several common functional and pharmacological features. All possess rotamase (peptidylprolyl cis/trans-isomerase) activity which is inhibited by nanomolar concentrations of immunosuppressant drugs including FK506, its analogs, and rapamycin. The 12-kDa FK506-binding protein (FKBP12) is perhaps the best studied immunophilin and is expressed in yeast (4, 5) and most widely expressed in mammalian cells (1–3). Our current understanding of how FKBP12 regulates cellular signal processing largely derives from studies that examine how immunosuppressants influence gain or loss of functions through their ability to promote or disrupt physical associations between FKBP12 and key mediators of cell signaling. FK506 and rapamycin share mutually exclusive high affinity binding sites on FKBP12 but promote associations with distinct cytosolic signaling proteins (6, 7). For example, FK506 binds to FKBP12 and promotes an association with calcineurin thereby inhibiting phosphatase 2B activity that, in turn, is essential for regulating the phosphoryl state of a wide variety of Ca$^{2+}$-dependent mediators of cell signaling including Ca$^{2+}$/calmodulin-dependent kinase, nitric oxide synthase, and nuclear factor activating T-cells (1–3). By contrast, the rapamycin-FKBP12 complex promotes formation of a ternary complex with mTOR (also known as FRAP), a non-receptor protein kinase that shares C-terminal domain homology with lipid phosphatidylinositol 3-kinases, thereby inhibiting its kinase activity (8). Formation of a FKBP12-rapamycin-mTOR complex and inhibition of mTOR kinase activity prevents hyperphosphorylation of PHAS I, thereby blocking an essential step linking activation of growth factor receptors and initiation of protein translation.

FKBP12 also indirectly regulates Ca$^{2+}$ transport by forming tight associations with the skeletal isoform of ryanodine receptor (RyR1) Ca$^{2+}$ channel complex, and Ca$^{2+}$ channels activated by inositol 1,4,5-trisphosphate receptors localized within sarcoplasmic (SR) and endoplasmic reticulum membranes (2, 3). The functional significance of FKBP12 is perhaps best understood in striated muscle where its association with the skeletal (RyR1) or cardiac (RyR2) isoforms of RyR appears to regulate the fidelity of intracellular Ca$^{2+}$ transport, although the molecular details and physiological significance of this regulation remain unclear. Deletion of FKBP12 expression using gene targeting in mice produces severe deficits of skeletal and cardiac muscle which underscores the developmental and functional importance of FKBP12 to striated muscle (9). In native skeletal muscle, each RyR1 homotetramer interacts with up to four molecules of FKBP12 (10) at domains that reside within a cytoplasmic extension of the transmembrane assembly (11). Most of our understanding of how FKBP12 regulates RyR1
function originates from studies utilizing immunosuppressants FK506 and rapamycin which, in micromolar concentration, promote dissociation of the immunophilin from the RyR1 homotrimer. Dissociation of the RyR-FKBP12 heterocomplex with immunosuppressant, or expression of RyR1 in Sf9 cells that lack constitutive expression of FKBP12, alters channel conductance from a characteristically large ~500 pS for Ca\(^{2+}\) and 100 pS for Ca\(^{2+}\) to gating transitions more frequently exhibiting 1/2, 1/3, and 1/4 subconductances (12, 13). Furthermore it has been recently suggested that FKBP12 may be responsible for cooperative gating between neighboring channels (14). The RyR1 complex deficient of FKBP12 appears to be more sensitive to activation by caffeine and Ca\(^{2+}\) (10, 15) and enhances the sensitivity of fibers to depolarization and caffeine (16).

Although immunosuppressants such as FK506 and rapamycin have been proved essential in focusing our understanding of how FKBP12 regulates RyR1, and in a broader sense cellular functions, our insight into the endogenous function of immunophilins has been limited to a gain or loss of function produced by these fungal compounds. Essentially their actions of promoting or breaking physical associations with key mediators of cell signaling have been correlated with altered function. Therefore compounds that mediate their effects in an FKBP12-dependent manner without dissociating the physical interaction with the target signaling protein, e.g. RyR1, may provide unique insight into the following: 1) the possible endogenous functions of FKBP12 in regulating SR/endoplasmic reticulum Ca\(^{2+}\) transport, and 2) the chemical structure of natural ligands and their binding sites within the RyR1-FKBP12 complex. We previously reported that bromotyrosine derivatives known as bastadins isolated from the marine sponge Ianthella basta (Pallas) dramatically alter SR Ca\(^{2+}\) transport and channel gating behavior of RyR1 in a manner that appears to require the integrity of the FKBP12-RyR complex, and these actions exhibited a stringent structure-activity relationship (17, 18). In the presence of physiological concentrations of K\(^{+}\) and Na\(^{+}\), bastadin 5 (B5) was shown to allosterically enhance maximal occupancy of \(^{1}H\)ryanodine to high affinity sites and diminished the tendency for low affinity binding sites in junctional SR. In bilayer lipid membrane (BLM) studies, B5 dramatically slowed single channel gating kinetics without significantly altering unitary conductance or open probability. The unique actions of B5 were eliminated by either FK506 or rapamycin, suggesting the pharmacological actions of bastadins were mediated by a novel modulatory site that requires the structural integrity of the FKBP12-RyR channel complex. More recently, B5 and ryanodine in combination were shown to enhance steady-state Ca\(^{2+}\) loading capacity of junctional SR vesicles by 2–3-fold, revealing a possible role of RyR1-FKBP12 complexes in regulating the filling capacity of the SR Ca\(^{2+}\) store by influencing a “leak” conformation of RyR1 (19).

In the present paper we utilize single channels reconstituted in BLM and measurements of macroscopic Ca\(^{2+}\) transport to report the following: 1) B10 essentially obviates channel regulation by physiological concentrations of Ca\(^{2+}\); 2) B10 relieves inhibition by physiological Mg\(^{2+}\); 3) these actions of B10 are selectively eliminated by FK506 or rapamycin and reconstituted by recombinant FKBP12; and 4) the actions of B10 are rapidly reversible. The unique actions of bastadin 10 (B10) were originally predicted to exist from results obtained with extracts containing a mixture of bastadins (17, 19). A structure-activity model is proposed by which substitutions on the Eastman and Western hemispheres of the bastarane macrocycle confer specificity toward the RyR1-FKBP12 complex to stabilize the closed or open channel conformation. These results indicate that RyR1-FKBP12 complexes possess a novel binding domain for phenoxyacetate and raise the possibility of molecular recognition of an endogenous ligand.

**EXPERIMENTAL PROCEDURES**

**Materials—**\(^{1}H\)Ryanodine was obtained from NEN Life Science Products with specific activity of 60–80 Ci/mmol and purity >90%. Unlabeled ryanodine was purchased from Calbiochem. Purified natural phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Human recombinant FKBP12 was a generous gift from Dr. Ernie Vilafraanca, Agouron Pharmaceuticals. FK506 was obtained from Signal Transduction Inc. (San Diego, CA). All other chemicals were commercially obtained at the highest purity available.

**Isolation of Bastadins 5 and 10**—Bastadins were extracted from lyophilized samples of I. basta (Pallas) as described previously (17, 18, 31). The active constituents were identified by bioassay-guided screening of fractions for activity toward Ca\(^{2+}\) release from skeletal SR vesicles and enhancement of \(^{1}H\)ryanodine binding. Bastadins 5 and 10 were identified from \(^1\)H NMR, \(^13\)C NMR, and matrix-assisted laser desorption, ionization Fourier transfer mass spectrometry data and compared with literature data (20).

**Preparation of Skeletal Muscle SR Membranes**—Membrane vesicles enriched in RyR1-FKBP12 complex, calsequestrin, and triadin were prepared from adult rabbit fast-twitch skeletal muscle based on the method of Saito and co-workers (21). Briefly, freshly ground muscle was homogenized in ice-cold buffer containing 5 mM imidazole HCl, pH 7.4, 300 mM sucrose, 10 mM leupeptin, and 100 mM phenylmethylsulfonyl fluoride. Differential centrifugation was performed to obtain a heavy SR fraction, and junctional SR was collected from the 38–45% (v/v) interface of a discontinuous sucrose gradient. The junctional SR was then resuspended to 3–5 mg/ml (22), frozen in liquid N\(_2\), and stored at −80 °C until needed.

**Macroscopic Ca\(^{2+}\) Transport Measurement—**Ca\(^{2+}\) transport across SR vesicles was measured with the membrane-impermeant Ca\(^{2+}\)-sensitive dye, antipyrilazo III, using a diode array spectrophotometer (model 8452, Hewlett-Packard, Palo Alto, CA). Skeletal SR vesicles (50 μg/ml) were added to 1.15 ml of ATP-regenerating buffer consisting of 90 mM KCl, 20 mM potassium MOPS, 7.5 mM sodium pyrophosphate (23), 250 μM antipyrilazo III, 12 μg of creatine phosphokinase, 5 μM phosphocreatine, and 1 mM MgATP, pH 7.0 (final volume of 1.2 ml). Transport assays were performed at 37 °C in temperature-controlled cuvettes with constant stirring. SR vesicles were loaded with five sequential additions of 24 nmol of CaCl\(_2\) which constituted approximately 80% of their loading capacity. Net Ca\(^{2+}\) fluxes across SR vesicles were measured by monitoring extravesicular Ca\(^{2+}\) changes in free Ca\(^{2+}\) by subtracting the absorbance of antipyrilazo III at 790 nm from absorbance at 710 nm at 2–4-s intervals. At the end of each experiment, the total intravesicular Ca\(^{2+}\) was determined by addition of 3 μM of the Ca\(^{2+}\) ionophore A23187, and the absorbance signals were calibrated by addition of 12 or 24 nmol of CaCl\(_2\) from a National Bureau of Standard stock solution. The actions of bastadin 5 and 10 were studied by adding the compound after the loading phase was complete in the presence or absence of FK506 or rapamycin.

**\(^{1}H\)Ryanodine Binding Assay—**Specific binding of \(^{1}H\)ryanodine to high affinity sites on skeletal SR vesicles was determined by incubating 15 μg of protein with 1 nM \(^{1}H\)ryanodine. The binding assays were performed with two distinct assay protocols. Protocol A used a buffer composed of 500 mM CsCl, 20 mM HEPES, pH 7.0, and incubation was performed at 25 °C for 3 h. Protocol B used a buffer composed of 140 mM KCl, 15 mM NaCl, 20 mM HEPES, 10% sucrose, pH 7.1, and incubation was at 37 °C for 3 h. With either protocol, free Ca\(^{2+}\) concentration in either assay condition was adjusted by addition of CaCl\(_2\) and EGTA-based calculations from the “Bound and Determined” software (24). Additions of test compounds were made to the radiolabeled assay buffer, singly or in combination, prior to addition of SR as described in the figure legends. Separation of bound and free \(^{1}H\)ryanodine was performed by filtration though Whatman G/F glass fiber filter using a Brandel (Gaithersburg, MD) cell harvester. Filters were washed three times with 0.5 ml of ice-cold buffer containing 20 mM Tris–HCl, 250 mM KCl, 15 mM NaCl, 50 μM Ca\(^{2+}\), pH 7.1. Filters were then soaked overnight in 5 ml of scintillation mixture (Ready Safe, Beckman), and bound radioactivity was determined by scintillation spectrometry. Non-specific binding was determined in the presence of 100-fold excess unlabeled ryanodine. Each experiment was performed in duplicate or triplicate and repeated at least two times.

**Single Channel Recording—**Cs\(^{+}\) current through single RyR1 channels incorporated into planar BLM was measured in an asymmetric CsCl (5:1 cis/trans) solution. The BLM was formed from a mixture of
phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine (5:3:2, w/w) at 50 mg/ml in decane, across a 150–300 μm aperture in a 1.0-ml polystyrene cup. SR vesicles were added to the cis side of chamber at a final concentration of 0.1–10 μg/ml. The cis solution contained 500 mM CaCl₂, 200 μM CaCl₂, 20 mM HEPES, pH 7.0 or pH 7.4, and the trans solution contained 100 mM CaCl₂, 7 μM free Ca²⁺, 20 mM HEPES, pH 7.4. After a single fusion event, 300 μM EGTA was added to the cis chamber and subsequently perfused by an identical buffer with no added Ca²⁺ and EGTA. Single channel current was measured under voltage clamp using a Dagan 3900 amplifier (Dagan Instruments, Minneapolis, MN). Holding potentials were with respect to the trans (ground) chamber, and positive currents were defined as current flowing from cis to trans. Current signals were captured at 10 kHz and filtered at 1 or 2 kHz using a four-pole Bessel filter. Data were digitized with a Digidata 1200 interface (Axon Instruments, Burlingame, CA) and stored on computer for subsequent analysis. The experiments were performed at room temperature and were replicated at least three times. Ca²⁺ in the cis chamber was adjusted as described above for binding experiments (24). Unless otherwise stated, test chemicals were sequentially added to the cis solution after an initial period of recording control channel behavior.

Single channel activity was analyzed with pCLAMP 6.0 (Axon Instruments). Open events were defined as intervals at which the currents exceeded 50% of maximum open level. Open probability (Pₒ) was calculated from 60 to 90 s of continuous record using the Pstat program (Axon Instruments). Data with long closure >600 ms were excluded from the analysis because the mean close time of channel is far less than 100 ms in both bastadin-modified or control channels. Current levels were analyzed by mean variance analysis, and peaks in the all-points amplitude histogram were fitted with Gaussian functions. To account for both the fast and slow component of channel gating kinetics, dwell time distribution histograms were constructed with 0.2- and 10-ms bin widths. Dwell open and close time were calculated from least square fits of biexponential function using the Pstat software. To prevent skewing of the statistical fit, the first two bins were excluded from the analysis when using a 10-ms bin width.

RESULTS AND DISCUSSION

Bastadin 10 Stabilizes the Full Open State of RyR1 in a Reversible Manner—Previously we reported that one or more components of a mixture of bromotyrosine derivatives isolated in methanolic extracts of the marine sponge I. basto induced rapid Ca²⁺ release from actively loaded skeletal SR vesicles. Ca²⁺ release from actively loaded SR vesicles elicited by addition of a mixture of bastadins was shown to be mediated through a ryanodine-sensitive pathway and could be induced even when extravesicular Ca²⁺ was as low as 150 nM (17, 19). However, this apparent Ca²⁺-independent activity was not attributable to purified bastadin 5 (B5), whose novel activity did not include a significant shift in the sensitivity of RyR1 channels to activation by Ca²⁺. Instead, the activity of B5 decreased the inhibitory potency of millimolar Ca²⁺ and Mg²⁺ by 5- and 8-fold, respectively. Further work aimed at elucidating the structure-activity of several purified components of the bastadin mixture (18) revealed that bastadin 10 (Fig. 1) could fully account for the dramatic shift in the Ca²⁺ dependence of RyR1 channels and is the subject of this report. B10 differs structurally from B5 as follows: 1) the substitutions about each bromotyrosine rings in the “Eastern” and “Western” hemisphere of the bastarane macrocycle are equivalent, 2) the C-6 is hydroxylated, and 3) the B10 molecule is chiral (Fig. 1). The interaction of B10 with RyR1 was studied in detail by measuring macroscopic Ca²⁺ transport, single channel gating behavior, and [3H]ryanodine binding.

The mechanism by which B10 alters SR Ca²⁺ transport was elucidated in the presence of low extravesicular free Ca²⁺ to reduce the activity of RyR1 channels and minimize Ca²⁺-activated Ca²⁺ efflux from SR. After the Ca²⁺ loading phase was complete and extravesicular Ca²⁺ returned to baseline (~150 nM free Ca²⁺), addition of 10 μM B10 rapidly induced Ca²⁺ release from loaded vesicles (Fig. 2A, trace a). Fig. 2A (trace b) shows that addition of ryanodine (500 μM) into a cuvette containing Ca²⁺-loaded SR vesicles first activated RyR1, resulting in a net Ca²⁺ efflux, and then blocked RyR1 permitting reaccumulation of Ca²⁺. These results are consistent with a sequential mechanism by which ryanodine alters channel function (25). Addition of B10 subsequent to channel blockade with ryanodine failed to induce Ca²⁺ release. In separate experiments, once B10-initiated Ca²⁺ release nearing completion, addition of either 500 μM ryanodine (Fig. 2B, trace c) or 10 μM ruthenium red (Fig. 2B, trace b) to block Ca²⁺ release channels caused rapid reaccumulation of extravesicular Ca²⁺ into SR.

![Fig. 1. Chemical structures of bastadin 10 (B10) and bastadin 5 (B5).](image)

![Fig. 2. Bastadin 10 induces Ca²⁺ release from SR by stabilizing the full open state of RyR1 Ca²⁺ channels. A](image)
FKBP12-dependent Mechanism for Bastadin 10

These results suggest that B10 induces SR Ca\(^{2+}\) release by selectively activating RyR1 in the absence of stimulatory concentrations of extravascular Ca\(^{2+}\). To better understand how B10 stimulated SR Ca\(^{2+}\) release, individual Ca\(^{2+}\) channels were reconstituted in BLM, and their gating behavior was directly analyzed in the presence or absence of B10 as described under "Experimental Procedures." RyR1 channels were identified by their large conductance for Cs\(^{+}\), their sensitivity to cytoplasmic (cis) Ca\(^{2+}\), and responses to known modulators. Fig. 2C (1st trace) shows a typical rapidly gating Ca\(^{2+}\) channel in the presence of 7 μM Ca\(^{2+}\) cis. Under these conditions the mean \(P_{\text{o}}\) was 0.19 ± 0.04 (mean ± S.E., \(n = 16\) channels). Addition of B10 (7.5 μM; Fig. 2C, 2nd trace) to the cytoplasmic face of the channel (cis chamber) increased channel \(P_{\text{o}}\) to near unity within seconds, apparently by stabilizing the full open state. Subsequent additions of ryanodine (10 μM) and ruthenium red (10 μM) to the B10-modified channel produced a characteristic half-conductance state and fully closed state, respectively (Fig. 2C, 3rd and 4th traces, respectively). These results indicated that B10 mediated its actions on channel gating through a site distinct from the ryanodine/ruthenium red effector sites and that the B10-modified channel remained responsive to agents thought to interact near the pore (26–28).

The degree to which B10 stimulated Ca\(^{2+}\) release from actively loaded SR and enhanced channel \(P_{\text{o}}\) was concentration-dependent in the range of 1–15 μM, yielding comparable EC\(_{50}\) values of 5.4 and 2.8 μM, respectively, and exhibiting positive cooperativity with \(n_{H} = 2.0\) (Fig. 3, A–C).

To understand the mechanism underlying the B10-modified channel, analysis of gating kinetics was performed in two ways. First, open and closed transition events were categorized by setting the bin width to 0.2 ms. With these constraints, native channels exhibited open and closed dwell times that were best described by double exponential fits thereby giving two time constants for each parameter (\(\tau_{i1}\), \(\tau_{i2}\), and \(\tau_{o1}\), \(\tau_{o2}\); respectively; where \(\tau_{i}\) indicates mean open dwell time; \(\tau_{o}\) indicates mean closed dwell time). As summarized in Table I, B10 increased \(\tau_{i1}\) and \(\tau_{i2}\) by 3.4- and 8.7-fold and decreased \(\tau_{o1}\) and \(\tau_{o2}\) by 2.3- and 2.2-fold, respectively. However, analysis using bin widths of 0.2 ms (fitting the range of 0–25 ms) clearly ignored a significant number of very long open events observed with B10-modified channels having a distribution of open events >50 ms duration. Omission of these events from the analysis resulted in underestimates of \(\tau_{i}\) and \(\tau_{o}\) for the B10-modified channels. When the data were reanalyzed with bin widths set at 10 ms (fitting the range of 0–1000 ms) to better account for these frequent long open events, only B10-modified channels exhibited substantial long open events (\(\tau_{i1} = 13.9 ± 2.6\) ms, \(\tau_{i2} = 76.6 ± 8.5\) ms). Most transitions seen with control channels without bastadin 10 exhibited maximum open dwell time less than 50 ms. By contrast, B10 does not promote long close events of ryanodine receptor channel but instead significantly shortens closed dwell times (Table I). Therefore, B10 modified RyR1 channels by prolonging open dwell times with a more subtle although significant shortening of closed dwell times. The B10-modified channels exhibited two additional noteworthy properties as follows: 1) the stabilized full open state persisted for as long as 20–30 min (the typical length of a BLM experiment) without

**Table I**

|                | Mean open time 1 | Mean open time 2 | Mean close time 1 | Mean close time 2 |
|----------------|------------------|------------------|-------------------|------------------|
|                | \(\text{ms}\)     | \(\text{ms}\)     | \(\text{ms}\)     | \(\text{ms}\)     |
| Control \((n = 12)\) | 0.34 ± 0.03      | 1.34 ± 0.12      | 1.7 ± 0.14        | 4.72 ± 1.0       |
| bastadin 10 \((n = 12)\) | 1.15 ± 0.19\(^a\) | 13.9 ± 2.6       | 76.6 ± 8.5        | 2.15 ± 0.22\(^b\) |

\(^a p < 0.001.\)
\(^b p < 0.05.\)

FIG. 3. Bastadin 10 induces SR Ca\(^{2+}\) release and channel activation in a concentration-dependent manner. The SR Ca\(^{2+}\) transport (A) and single channel (B) measurements were performed as described in Fig. 2. A, the B10 concentration added to each cuvette was (from slowest to fastest release rate) as follows. The experiment was repeated three times with two different SR preparations. B, the B10 concentration was increased stepwise in the cis chamber from 1 to 7.5 μM after a period of measuring the native channel with 7 μM free cis-Ca\(^{2+}\).

Data shown are representative traces from a single channel which was recorded for at least 2 min at each condition. This experiment was repeated four times with three different preparations. C, shows summary data for SR transport plotted as rate constants (bin 5 ms), and single channel open probability (○) is represented as mean ± S.E. The numbers in parentheses indicate the number of channels measured at each B10 concentration.

The data were represented as mean ± S.E. of 12 channels before and after adding bastadin 10 (4.5 or 7.5 μM) with 7 μM free cis Ca\(^{2+}\). It is demonstrated that bastadin 10 significantly increased the mean open time 1 and 2 (one-way ANOVA, \(p < 0.001\)) and significantly decreased the mean close time 1 (\(p < 0.001\)) and 2 (\(p < 0.05\)) of ryanodine receptor channel gating kinetics when analyzed with 0.2-ms bin width. To account for the long openings induced by bastadin 10, dwell time histogram is also constructed with 10 ms bin width.
any evidence of rundown, and 2) subconductance transitions were not apparent despite the long open times. The actions of B10 on channel gating kinetics differ from those previously reported with B5 that prolonged both full open and full closed dwell times about equally (45- and 60-fold, respectively; see Ref. 17) without significantly increasing open probability. These original results with B5 have been verified on the same SR preparations from which B10 data were obtained for the present study, suggesting that differences between B5 and B10 toward modifying channel kinetics may stem from differences in their structures, especially substitutions about the dityrosine ring moieties as discussed below.

Although B10 dramatically increased channel $P_o$, it did not alter the unitary conductance of channels for Cs$^+$ (Fig. 4A; gCs$^+ = 466 \pm 5.8$ pS with and without B10). To address if the actions of B10 were reversible, B10 was removed from the cis chamber by perfusion with several volumes of buffer lacking the drug (Fig. 4B). The B10-modified channel ($P_o = 0.99$) was restored essentially to control behavior after removal of the bastadin by perfusion ($P_o = 0.39$ versus 0.42 before and after removal of B10, respectively). A consistent observation in reversibility experiments was that once perfusion was completed, 3–5 min were required to fully restore $P_o$ to control levels. Re-introduction of 4.5 μM B10 in the cis chamber restored the $P_o$ to near unity proving that the channel-modifying actions of B10 were fully reversible (Fig. 4B, last trace).

**Fig. 4.** B10 acts reversibly without altering unitary conductance. A, current-voltage relationship of a single channel before (●) and after (○) addition of 7.5 μM B10. The control channel and B10-modified channel has the same conductance (466 ± 5.8 pS with and without B10). The R value for the linear regression is 0.99. B, the dramatic effects of B10 on channel gating kinetics is reversed by removing B10 from the cis chamber with a 10× volume of B10-free solution. Typically it took 3–5 min for the channel to regain $P_o$ values not significantly different from control. A second addition of B10 (4.5 μM) increased $P_o$ to 0.99 within 30 s. Data are representative traces from the same channel. Each condition was recorded for at least 2 min. This experiment was repeated five times with similar results.

**Fig. 5.** Bastadin 10 relieves dependence of RyR1 channel on physiological Ca$^{2+}$ concentrations. Ca$^{2+}$ concentrations were adjusted on the cytoplasmic face (cis chamber) of the channel using EGTA/CaCl$_2$ as described under “Experimental Procedures.” A, representative data traces from a single channel with Ca$^{2+}$ adjusted to 7 μM ($P_o = 0.34$) and 300 nM ($P_o = 0.01$) in the cis chamber illustrating the dependence of channel activation on micromolar Ca$^{2+}$. B10 (1.5 μM) added to the cis chamber restored channel activity ($P_o = 0.37$). B, summary curves showing Ca$^{2+}$ dependence for control (●) and B10-modified (7.5 μM; ○) channels. Note that B10-modified channels are still fully activated with 100 nM Ca$^{2+}$ and maintain appreciable activity with Ca$^{2+}$ < 10 nM. The summary data represent data from n = 4 channels. C, Ca$^{2+}$ dependence of the binding of [3H]ryanodine to high affinity binding sites found on skeletal SR is significantly shifted to the left in the presence of B10 (5.0 μM) compared with control (●). In consonance with single channel behavior, Ca$^{2+}$ < 10 nM fails to completely eliminate receptor binding in B10-modified channels. Binding experiments were performed in the same buffer as BLM experiments, containing 500 mM CsCl, 20 mM HEPES, pH 7.0. Data shown are the mean ± S.E. of three determinations performed in triplicate on two different preparations.

Bastadin 10 Relieves Ca$^{2+}$ and Mg$^{2+}$ Dependence of RyR1—
The observation that B10 stabilized the open state of the chan-
nel and induced Ca$^{2+}$ release from SR at low extravesicular Ca$^{2+}$ concentration raised the possibility that B10 might alter the Ca$^{2+}$ dependence of channel activation through allosteric means. This hypothesis was tested by measuring the relationship between channel activity and the concentration of Ca$^{2+}$ in the cis (cytoplasmic) chamber. Fig. 5A (1st trace) shows the activity of a representative channel in the presence of 7 μM Ca$^{2+}$ cis ($P_o = 0.34$). The $P_o$ declines to 0.01 when the Ca$^{2+}$ cis is reduced to 300 nM by chelation with EGTA (2nd trace). A significant finding was that despite the low Ca$^{2+}$, introduction of as little as 1.5 μM B10 essentially restores $P_o$ to 0.37 (Fig. 5A, 3rd trace). Titration of free Ca$^{2+}$ in the cis chamber between 3 nM and 100 μM revealed the dramatic extent to which B10 modified the dependence of channel gating on this important physiological modulator (Fig. 5B). With physiological levels of cytoplasmatic Ca$^{2+}$ found in resting muscle (100 nM), the presence of saturating B10 (7.5 μM) maintained a channel $P_o$ close to 1.0. $P_o$ exhibited a steep dependence on Ca$^{2+}$ between 20 and 100 nM (EC$_{50}$ ~ 25 nM), although the B10-modified channel maintained moderate activity ($P_o$ of ~0.2) even with 3 nM Ca$^{2+}$ cis. This behavior is in marked contrast to the typical unmodified channel whose EC$_{50}$ was 10 μM and which exhibited little activity ($P_o$ ~ 0) at Ca$^{2+}$ < 1 μM (Fig. 5B). Since the level of $[^{3}H]$ryanodine occupancy to high affinity binding sites has been shown to be generally correlated with the open state of the channels, we used radioligand binding analysis as an independent method for assessing changes in sensitivity to Ca$^{2+}$. The same conditions were used to measure the binding of $[^{3}H]$ryanodine to RyR1 as those used in BLM studies to avoid possible confounding influences of buffer composition and temperature. Compared with typical results obtained in low (250 mM) KCl, the Ca$^{2+}$ dependence of binding fell off sharply just below 1 μM in the presence of 500 mM CaCl (Fig. 5C). However, consistent with results obtained from BLM studies, B10 produced a dramatic upward and leftward shift in the Ca$^{2+}$ dependence of $[^{3}H]$ryanodine binding, and substantial $[^{3}H]$ryanodine occupancy was maintained even in the presence of 1 nM Ca$^{2+}$. B10 was also found to mitigate the inhibitory effects of Mg$^{2+}$. Fig. 6A shows that 1 mM Mg$^{2+}$ reduced the $P_o$ of a representative channel from 0.29 to 0.04. Addition of B10 (4.5 μM cis) rapidly restored channel activity ($P_o = 0.75$). Mitigation of channel inhibition by Mg$^{2+}$ was a consistent property of B10-modified channels (Fig. 6B).

The dramatic sensitization of the B10-modified channel to Ca$^{2+}$ is unique to this bastadin, since purified B5 only enhanced Ca$^{2+}$-induced Ca$^{2+}$ release (i.e. required nominally activating cytoplasmic Ca$^{2+}$) and failed to significantly shift the Ca$^{2+}$ activation curve for $[^{3}H]$ryanodine to the left (17, 19). These results can fully account for why methanolic extracts of I. basta were originally observed to efficaciously release accumulated SR Ca$^{2+}$ in a manner apparently independent of cytoplasmic Ca$^{2+}$ (17, 19).

The Actions of B10 Require the Integrity of the RyR1-FKBP12 Complex—Perhaps the most significant observation regarding the actions of bastadins toward the RyR1 complex is the role of FKBP12 in mediating their rather unique effects (17, 19). We therefore utilized micromolar concentration of the immunosuppressant FK506 to promote dissociation of the RyR1-FKBP12 heterocomplex (2, 29), and we assessed changes in the ability B10 to modify single channel kinetics and SR Ca$^{2+}$ transport. FK506 (5–50 μM) added to SR membranes had little direct effect on steady-state Ca$^{2+}$ transport across actively loaded SR vesicles (Fig. 7A). However, addition of B10 to Ca$^{2+}$-loaded vesicles 2 min after FK506 was introduced resulted in concentration-dependent elimination of B10-induced Ca$^{2+}$ release (Fig. 7A) with an IC$_{50}$ of 15 μM (Fig. 7B). B10-induced Ca$^{2+}$ release could also be eliminated with rapamycin (not shown). The importance of the RyR1-FKBP12 heterocomplex in mediating the actions of B10 could also be demonstrated at the level of single channels reconstituted in BLM. Fig. 7C shows that addition of FK506 to B10-modified channels essentially eliminated the overt effects of B10 and resulted in channels that exhibited significant subconductance fluctuations (compare 2nd trace to 3rd trace). Upon perfusion of the cis chamber to remove free FK506-FKBP12 complex and B10, the channel maintained its subconductance fluctuations, and subsequent re-introduction of B10 failed to restore gating behavior to that characteristic of a B10-modified channel (Fig. 7C, 4th and 5th traces). Previously we showed that under identical assay conditions neither FK506 nor rapamycin inhibited the ability of SR actively loaded with Ca$^{2+}$ to respond to Ca$^{2+}$-induced Ca$^{2+}$ release, caffeine, or ryanodine, demonstrating the continued integrity of the FKBP12-deficient channels to direct modulators of the RyR1 protein (30). In this regard, although dissociation of the RyR1-FKBP12 complex with FK506 eliminated responses to B10, the channel remained fully responsive to 10 mM caffeine (Fig. 8A, compare 3rd and 4th traces). Utilizing receptor binding analysis with $[^{3}H]$ryanodine as an indicator of
channel activity, FK506 (50 μM) was observed to significantly inhibit B10-enhanced occupancy but not caffeine or AMP-PCP-activated binding (Fig. 7B).

To assess further if channel associations with FKBP12 were essential for imparting sensitivity to B10, junctional SR vesicles were pretreated with 50–100 μM FK506 at 37 °C to more fully dissociate endogenous FKBP12 from RyR1. FKBP12-deficient channels were reconstituted in BLM and found to exhibit a characteristic high incidence of subconductance gating transitions (Fig. 9, A and B, top traces). Addition of human recombinant FKBP12 to the cis chamber prior to addition of B10 significantly decreased the tendency for subconductance gating behavior, and the channel maintained its rapid gating kinetics (Fig. 9, A and B, middle traces). Subsequent addition of B10 to the reconstituted channel dramatically stabilized the open state (bottom traces). Occasionally, FKBP12-deficient channels exhibited a tendency to rundown in activity over several minutes as shown in Fig. 10A (compare 1st and 2nd traces), and addition of B10 failed to recover channel activity (3rd trace). Importantly, addition of recombinant FKBP12 (4 μM) in the presence of B10 restored the characteristic gating kinetics of the B10-modified channel within 1–6 min after addition of immunophilin (4th trace). Addition of B10 to FKBP12-deficient channels prior to rundown was also ineffective in rescuing channel function (Fig. 10B, 1st to 3rd traces). In fact, on several occasions addition of B10 to FKBP12-deficient channels appeared to accelerate rundown. However, even if the channel was exhibiting complete rundown in the presence of B10 (failure to gate), reconstitution of the RyR1-FKBP12 complex with exogenous immunophilin restored the B10-modified channel. The B10-modified channel complex reconstituted with recombinant FKBP12 exhibited gating characteristics that were indistinguishable from those of the B10-modified native complex including 1) very frequent long-lived full open transitions, 2) significantly enhanced channel open probability, 3) the absence of subconductance behavior, and 4) a lack of noticeable rundown.

Taken together these results directly demonstrate that a functional RyR1-FKBP12 complex is necessary to express the actions of B10. Disruption of the RyR1-FKBP12 complex selectively eliminated a bastadin effector domain that may reside, at least in part, within critical regions of contact between the immunophilin and RyR1. Consistent with this hypothesis, BLM experiments performed with B10 added to the trans (luminal) side of the channel failed to alter gating behavior. The current results suggest that the principal action of B10 may be to alter relative free energies associated with closed and open states of RyR1 (Fig. 11).
with very brief transitions to the open state (open dwell times typically <1ms) suggesting a higher free energy for the open state relative to the closed and a large $\Delta G^\circ_c \rightarrow o$. By contrast, the B10-modified channel exhibits significantly longer open dwell times which is consistent with a more favorable free energy for the open state and an associated decrease in $\Delta G^\circ_c \rightarrow o$ (Fig. 11). A B10-induced decrease in free energy associated with the open conformation of the channel could also account for the dramatic increase apparent sensitivity of the channel to activation by Ca$^{2+}$.

**Structure-Activity Analysis of Bastadin 10 and Bastadin 5—*I. basta* synthesizes at least 25 tetrameric bromotyrosine structures (18),; however, bastadins display a stringent structural requirement not only for potency and efficacy toward the RyR1-FKBP12 complex but also for the exact type of modulation conferred. Bastadin 5 (B5) seems to stabilize both open and closed channel states in BLM experiments but has little effect on the apparent sensitivity of the channel to activation by Ca$^{2+}$ in [$^3$H]ryanodine-binding experiments (17). Bastadin 10 is a very weak activator of the RyR1-FKBP12 complex and can inhibit B5-enhanced binding of [$^3$H]ryanodine compete (17). In contrast, B10 stabilizes primarily the open conformation of the channel and sensitizes the channel to activation by Ca$^{2+}$ to such an extent that it essentially eliminates regulation in the physiologic range of this ion. Both B5 and B10 alleviate inhibition by physiologic concentration of Mg$^{2+}$. Structural differences between B5 and B10 undoubtedly underlie both their ability to interact with a novel binding domain requiring the integrity of the RyR1-FKBP12 complex and the different manner in which each alters channel function. The solution conformation of each molecule can be approximated by the x-ray structure of B5 tetra-O-methyl ether which has been previously reported (20). Each solution conformation is, of necessity, non-planar as a consequence of non-bonded steric interactions between aryl ring substituents (especially bromine) and torsional constraints within the macrocyclic ring (Fig. 1). The idea that non-coplanarity in the bastadin structure is essential for activity toward the RyR1-FKBP12 complex is supported by the recent evidence that non-coplanar, but not coplanar, polychlorinated biphenyls (PCBs) with two (e.g., 2,2'-dichlorobiphenyl) or three (e.g., 2,2',3,5',6-pentachlorobiphenyl) ortho-chlorine substitutions possess nanomolar potency toward mobilizing Ca$^{2+}$ from microsomes isolated from skeletal SR (30, 32, 33). The actions of non-coplanar PCBs toward Ca$^{2+}$ channel function were found to be indistinguishable from those of B10 with respect to their ability to 1) profoundly alter sensitivity to both

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**Fig. 9. Human recombinant FKBP12 restores B10 sensitivity.** A, representative data trace from a single channel pretreated with FK506 (50 μM) prior to reconstitution in BLM. Pretreated SR (3–5 μl) was added to the cis chamber thereby diluting any residual FK506 >100-fold. The cis chamber was then perfused with 10 volumes of solution before data were recorded. FK506-treated channel (1st panel) showed frequent occurrence of subconductance states, indicative of the removal of endogenous FKBP12. Recombinant FKBP12 (4 μM, 2nd panel) increased the occurrence of full conductance events and decreased the substrates. The open probability of full conductance before and after addition of 4 μM FKBP12 is 0.002 and 0.032, respectively (a 15-fold increase). Subsequent addition of B10 (4.5 μM) immediately increased $P_o$ ($P_o$ = 0.58) for the full conductance fluctuation. The experiment was repeated for three times with similar results. B, summary plots showing amplitude histograms constructed from mean variance analysis of corresponding recording in A. Note that this FK506-treated channel exhibited 1⁄2 states most frequently.
activation and inhibition by Ca\(^{2+}\) and Mg\(^{2+}\), and 2) the dependence of this activity on the integrity of the RyR1-FKBP12 complex (30). An important similarity between bastadins and non-planar PCBs is that neither structure promotes dissociation of the RyR-FKBP12 complex, suggesting the existence of a novel, yet unidentified modulatory site. Thus the present results show that the actions of B10 require the integrity of the RyR1-FKBP12 heterocomplex (\(n = 4\)).

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