Common Immunophilin Mechanism for Noncoplanar PCBs and Naturally Occurring Bromotyrosines from *Ianthella basta*

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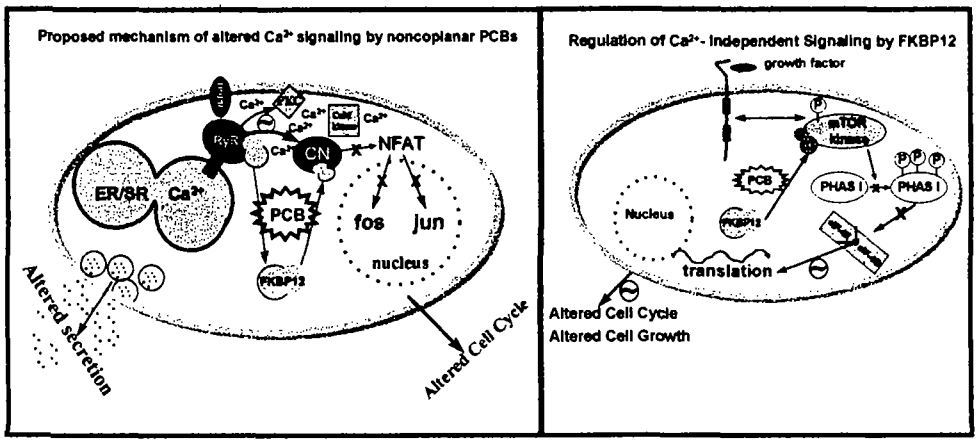
INTRODUCTION

Immunophilins are a family of proteins which function to regulate Ca^{2+}-dependent and Ca^{2+}-independent cellular signaling cascades within immune and neuronal cells. Immunophilins which bind the immunosuppressant drug FK506 have been named "FK506-binding proteins" (FKBP). FKBP12, the major T-cell immunophilin, has been shown to be tightly associated with ryanodine-sensitive and IP_3-sensitive Ca^{2+} channels (*i.e.*, ryanodine receptors (RyR) and IP_3 receptors, respectively) which are localized to endoplasmic reticulum (ER) of all mammalian cells. FKBP12 regulates the fidelity of cellular Ca^{2+} signaling through its association with RyR. In the presence of immunosuppressant FK506, FKBP12 dissociates from RyR resulting in a dramatic change in channel gating characteristics. Concomitantly the FK506/FKBP12 complex associates with calcineurin (CN) thereby blocking phosphatase activity essential to several cellular functions including secretion, cytokine production and cell growth. In addition to regulating Ca^{2+}-dependent signaling pathways, FKBP12 has also been shown to regulate Ca^{2+}-independent cascade essential for initiation of protein translation. The key regulatory step in this cascade is the non-receptor protein kinase mTOR (mammalian target of rapamycin) which posses C-terminal domain homology with PI-3-kinase. Cell activation by growth factor activates mTOR by receptor-mediated phosphorylation. mTOR in turn catalyzes hyperphosphorylation of PHAS I which in its activated form dissociates from elongation initiation factor (eIF) 4E, an essential regulatory component of 5'-untranslated regions (UTRs) of mRNA which possesses helicase activity. Free eIF-4E associates with a multifunctional scaffolding protein eIF-4G which stimulates protein synthesis. Immunophilin FKBP12 therefore is essential in regulating the coupling of growth factor receptor to eIF-4E-dependent protein synthesis. Xenobiotics which alter the cellular regulatory functions of FKBP12 and its related immunophilins would be expected to significantly alter Ca^{2+}-dependent and independent signaling in nerve cells, either directly or indirectly by alter immune cell secretion of cytokines.



We recently provided evidence that noncoplanar PCBs with two (*e.g.*, PCB 4) or three (*e.g.*, PCB 95) *ortho*-chlorine substitutions possess nanomolar potency toward mobilizing Ca^{2+} from microsomes isolated from rat hippocampus, cerebral cortex, and cerebellum (1-3). A highly selective, structurally specific interaction between noncoplanar PCBs and RyR was found to fully account for altered microsomal Ca^{2+} transport. These actions of non-coplanar PCBs are mediated through interaction with FKBP12 since PCB-induced Ca^{2+} mobilization could be selectively eliminated by the immunosuppressant agents FK506 and rapamycin which affect dissociation of the FKBP12/RyR heterocomplex (3). An important observation is that unlike immunosuppressants FK506 and rapamycin, noncoplanar PCBs do not promote dissociation of the FKBP12/RyR complex, suggesting the existence of a novel modulatory site. We report here that the molecular mechanism by which noncoplanar PCBs alter Ca^{2+} regulation at the level of single channels and disrupt Ca^{2+} signaling in intact cells in a manner indistinguishable from those measured with naturally occurring bromotyrosine derivatives from the marine sponge *Ianthella basta* (4-6).

METHODS

Isolation of Bastadins: Bastadins were extracted directly from *Ianthella basta* collected from Western Australia (6). Bastadin 5 and 10 were isolated and purified as previously described (6) and their structures elucidated by NMR and mass spectral analyses (6).

Electorphysiological Planar Lipid Bilayer Experiments: Microsomal membranes were isolated from either male rabbit skeletal muscle or whole male rat brains. Tissues were homogenized in 250 mM sucrose, 10 mM HEPES, 1 mM EDTA, 1 mM DTT benzamidine (1 mM), leupeptin (1 μg/ml), pepstatin A (0.7 μg/ml), and PMSF (0.1 mM), (pH 7.4) using a PowerGen 700D. Muscle and brain microsomes were isolated as previously described (2) at 6-10 mg/ml in 10 % sucrose, 20 mM HEPES, pH 7.2), frozen in liquid nitrogen, and stored at -80°C.

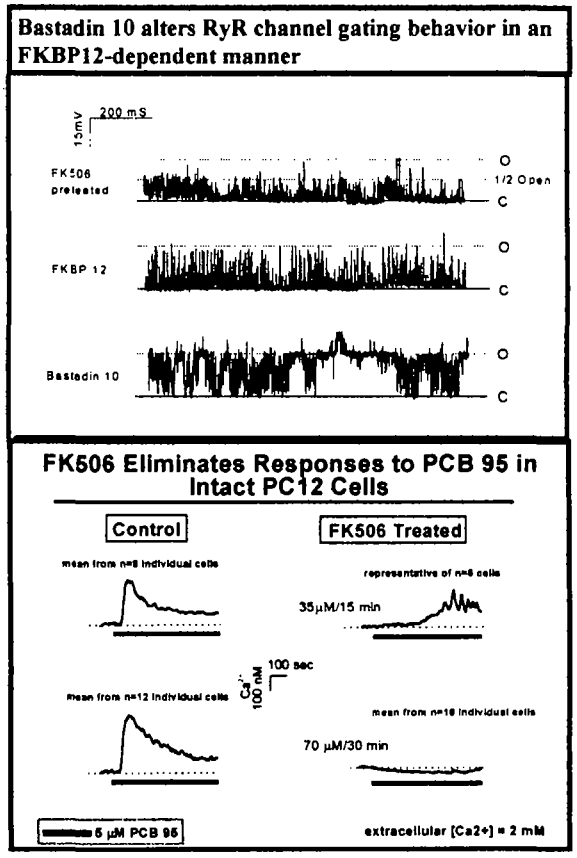
Planar lipid bilayer (BLM) reconstitution experiments were carried out with vesicles prepared as described above fused into a BLM made from a 5:3:2 mixture of phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylcholine (PC) suspended in decane. The BLM was formed across a 250 μm hole in a polystyrene cup separating two chambers of 0.7 ml each. Membrane vesicles were added to the *cis* chamber in the presence of Ca^{2+} CsCl, Hepes, pH 7.4. The trans chamber contained CsCl, HEPES, pH 7.4. After vesicle fusion EGTA was added to the *cis* chamber of the BLM to prevent any additional fusion events. In some experiments the *cis* and *trans* chambers were perfused with a solution composed of Hepes and Cs^{+} as either the chloride or methane sulfonate salt (symmetrical conditions). In other experiments the *cis* chamber was perfused with CsCl (asymmetrical 5:1 *cis:trans* conditions). A Dagan patch clamp amplifier was used to measure currents through a single channel. The single channel data was filtered at 2-5 kHz using a Digidata 1200 and stored on computer. Depending on the experimental conditions, PCBs or bastadins were added to the *cis* chamber and stirred. Subsequent channel gating behavior was recorded using Axotape software for 1 to 5 min. At the end of each experiment, ryanodine or ruthenium red was added to block channel function and confirm the source of the gating currents. Single-channel data from BLM experiments were analyzed using the pCLAMP software. Open and closed time constants were determined by least squares fit to double exponentials and steady state channel open probability determined.

Mechanism of noncoplanar PCBs and bastadins in PC12 Cells: PC12 cells express both FKBP12 and RyR and were utilized to examine further the interaction of noncoplanar PCBs or bastadins with RyR and immunophillins. Intact PC12 cells were cultured in RPMI 1640 with L-

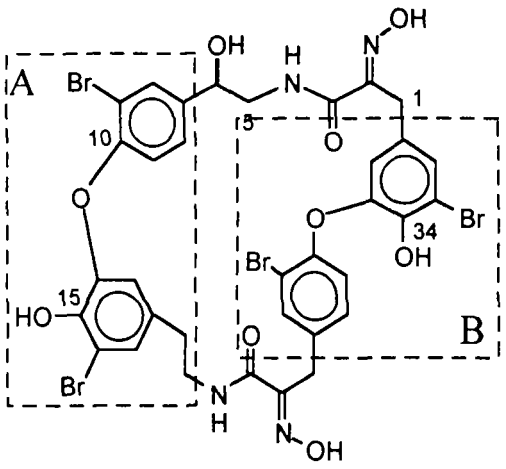
glutamine. 10% HIHS, 5%FBS, 1%P/S; 37°C in 5% CO_2 (<20 passages). Cells were loaded with fura-2 (using 5 mM of the AM ester in assay buffer for 20 min at 37°C. Intracellular Ca^{2+} was measured from 5-17 individual cells per field using ratio fluorescence imaging. Responses from individual cells were averaged from within a field and across replicate trials.

RESULTS AND DISCUSSION

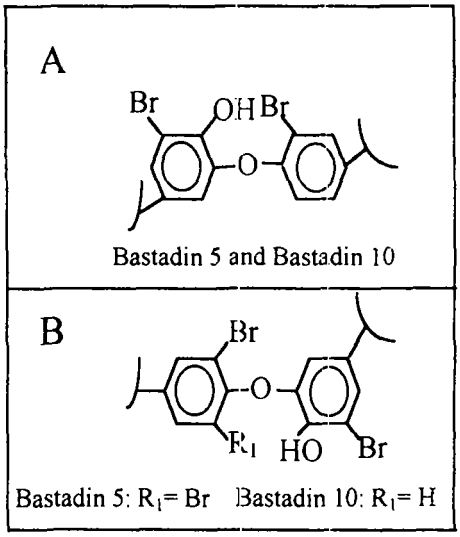
Bastadin10, a macrocyclic bromotyrosine derivative from *Ianthella basta*, was found to be a novel modulator of the Ca^{2+} release channel (RyR) from skeletal muscle and brain. We have shown that bastadin 10 released accumulated Ca^{2+} from microsomal vesicles in a ryanodine and ruthenium red dependent manner. Bastadin 10 dramatically relieved the dependence of channel activation on Ca^{2+} in the physiological concentration range in both radioligand receptor binding and single channel experiments. Most important, the dramatic actions of bastadin 10, like those of PCB 95 on microsomal Ca^{2+} efflux and channel open time were completely and selectively eliminated by the immunosuppressant FK506, implying the actions of bastadin 10 are mediated by FKBP12. These properties are indistinguishable from those observed with 2,2',3,5',6-pentachlorobiphenyl (PCB 95) (1-3). Measurements of single channel currents made with Cs^{+} (5:1, *cis:trans*), reveal that the actions of bastadin 10 are reversible. Further FK506 (50 μM) added *cis* fully abrogates all actions of bastadin 10 at the level single channels. SR pretreated (15min/37°C and 3hr/ 4°C) with FK506 effectively removes FKBP12 from RyR receptor. Although FKBP12-deficient Ca^{2+} channels exhibit frequent subconductances having ¾, ½., and ¼ transitions and lack responses to bastadin 10, they retain sensitivity to caffeine and adenine nucleotide. Addition of human recombinant FKBP12 (2-4 μM) to the *cis* chamber restores rapid gating transition having full conductance and the sensitivity of the channel to bastadin 10. Addition FKBP12 (4 μM) to a native channel does not alter channel behavior. These results demonstrate that bastadins, like noncoplanar PCBs, through their modulatory action on the FKBP12/RyR complex, can reversibly modulate gating kinetics and channel sensitivity to activation by Ca^{2+}, possibly by reducing the free energy barrier associated with attainment of the full open state of the channel. PC12 cells express both FKBP12 and RyR and were utilized to examine further the interaction of non-coplanar PCBs and bastadins with RyR and immunophillins in intact



cells. Ratio fluorescence imaging revealed that PCB95 altered Ca^{2+}-signaling in PC12 cells. These actions were eliminated by pretreatment with FK506 or rapamycin or RyR blockers. These results show that *ortho*-substituted PCBs alter microsomal Ca^{2+} transport by a receptor-mediated mechanism involving the major T-cell immunophilin FKBP12.



Bastadin-10



Proposed toxophores of bastadins 5 and 10

These results suggest that PCB related changes in neuroplasticityand spatial learning and memory we have shown to occur (7,8) could, at least in part, be mediated by alterations in FKBP12/RyR function. Common features inherent within the structure of noncoplanar PCBs and naturally occurring bastadins may underlie a common mechanism by which they alter Ca^{2+} dependent cell signaling by interacting with FKBP12. Bastadins are bromotyrosine derivatives which possess *ortho*-hydroxy and *ortho*-bromo substitutents at the two diphenyl ethers. We propose that these groups represent the analogous toxophores present with *ortho*-chloro moieties found in the most active noncoplanar PCBs. Therefore the novel action of naturally occurring bastadins and xenobiotic noncoplanar PCBs toward immunophilin regulated signaling pathways are proposed have a common chemical basis. RyR are closely associated with memory acquisition in water maze-trained rats. These results suggest that PCB related changes in neuroplasticity (7), and spatial learning and memory (8) could be mediated by alterations in FKBP12/RyR function. The significance of Ca^{2+} dependent and independent mechanisms involving immunophilin FKBP12 will be discussed. Supported by NIEHS ES05707-06 and 1RO1ES05002-07.

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