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Development of a Fluorescence Assay for the Characterization of Brevenal Binding to Rat Brain Synaptosomes

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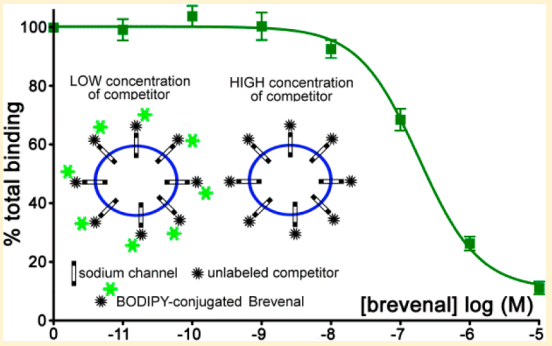
Supporting Information

ABSTRACT: The marine dinoflagellate *Karenia brevis* produces a family of neurotoxins known as brevetoxins. Brevetoxins elicit their effects by binding to and activating voltage-sensitive sodium channels (VSSCs) in cell membranes. *K. brevis* also produces brevenal, a brevetoxin antagonist, which is able to inhibit and/or negate many of the detrimental effects of brevetoxins. Brevenal binding to VSSCs has yet to be fully characterized, in part due to the difficulty and expense of current techniques. In this study, we have developed a novel fluorescence binding assay for the brevenal binding site. Several fluorescent compounds were conjugated to brevenal to assess their effects on brevenal binding. The assay was validated against the radioligand assay for the brevenal binding site and yielded comparable equilibrium inhibition constants. The fluorescence-based assay was shown to be quicker and far less expensive and did not generate radioactive waste or need facilities for handling radioactive materials. In-depth studies using the brevenal conjugates showed that, while brevenal conjugates do bind to a binding site in the VSSC protein complex, they are not displaced by known VSSC site specific ligands. As such, brevenal elicits its action through a novel mechanism and/or currently unknown receptor site on VSSCs.



Karenia brevis, the dinoflagellate responsible for the majority of Florida red tides, produces a number of ladder frame polyether compounds. The most abundant of these are the brevetoxins (PbTxs),[1] but other fused-ring polyether compounds isolated from *K. brevis* cultures include hemibrevetoxin B,[2] brevisin,[3] brevisamide,[4] tamulamides A and B,[5] and brevenal.[6] In the United States, *K. brevis* blooms are frequent, nearly annual occurrences off the coast of Florida, but also occur in North Carolina and other regions of the Gulf of Mexico. The PbTxs produced during these blooms are potent neurotoxins, which have caused massive mortalities in fish, seabirds, sea turtles, and marine mammals.[7−10] Consumption of contaminated shellfish may result in the development of neurotoxic shellfish poisoning. Patients afflicted with neurotoxic shellfish poisoning can experience gastroenteritis, sensory abnormalities, and cranial nerve dysfunction, and in severe cases may require treatment in intensive care to prevent respiratory failure.[11,12]

Brevenal was the first natural nontoxic ligand that displaces PbTxs from binding to voltage-sensitive sodium channels (VSSCs).[13] *K. brevis* produces brevenal both in culture and in the environment during blooms.[6,13] This was the first documented case of a toxin-producing organism also producing its own antagonist. Studies with fish have found that brevenal is nontoxic at micromolar concentrations, which is interesting considering the LD_{50} values of PbTxs are in the nanomolar range.[6,13] Brevenal has also been found to negate many of the deleterious physiological effects of PbTxs, including inhibiting PbTx-induced bronchoconstriction,[14] antagonizing PbTx-induced elevations in intracellular calcium levels,[15] and reducing cell death caused by highly toxic concentrations of PbTxs.[16] Furthermore, brevenal concentrations during Florida red tides are inversely correlated with human respiratory effects, in that concentrations are high when health effects are low and low when health effects are high.[13]

Brevenal has been found to competitively displace tritiated PbTx from synaptosomes in a radioligand binding assay.[13] However, PbTxs are unable to displace tritiated brevenal in the reverse competitive binding assays.[17] In addition, brevenal does not elicit the same effects as PbTxs in vivo, in that it has much lower toxicity to fish,[6] it is able to attenuate the harmful effects of PbTxs in fish and sheep,[6,14] it increases mucociliary transport in sheep,[14] and it does not appear to have any effect on VSSCs in patch-clamp experiments.[13] These findings suggest that brevenal acts as an antagonist to PbTx by binding to a site that is distinctly different from the PbTx binding location. It is believed that binding of brevenal to its receptor can effect allosteric modulation of the PbTx binding site, resulting in an attenuation of the PbTx response of the organism or cell. Because of brevenal's antagonistic activity against PbTx and ability to increase mucociliary transport via a putative new binding site on VSSCs, it is of interest therapeutically for PbTx

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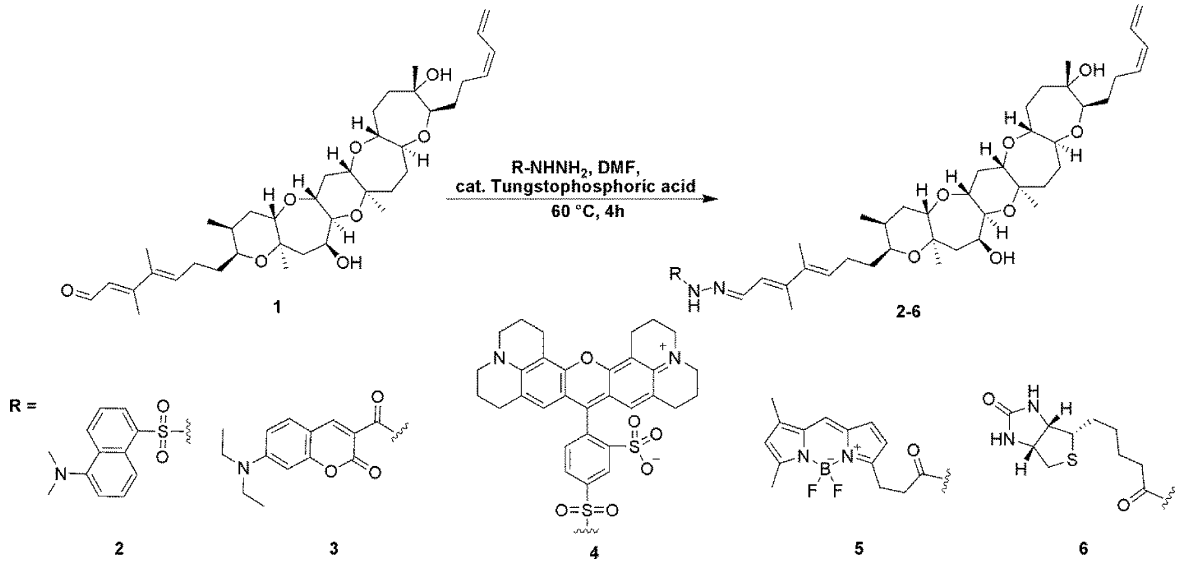
Scheme 1. Chemical Structures of the Brevenal Derivatives: (1) Brevenal, (2) Dansyl-brevenal, (3) Coumarin-brevenal, (4) Texas Red-brevenal, (5) BODIPY FL-brevenal, (6) Biotin-brevenal



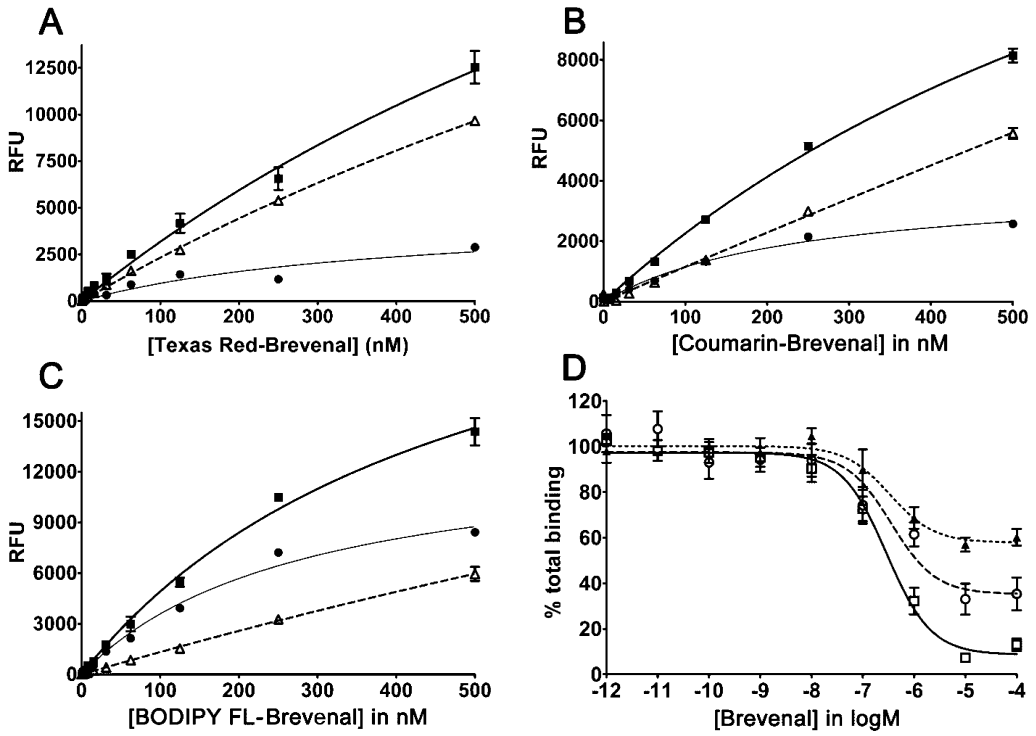


Figure 1. Saturation binding and inhibition of Texas Red-brevenal (A), coumarin-brevenal (B), and BODIPY FL-brevenal (C) binding to rat brain synaptosomes. Total binding without competition (■) and nonspecific binding with competition from 10 μM brevenal (△) was measured using relative fluorescence units (RFUs). Specific binding (●) was calculated from the difference between mean total binding and mean nonspecific binding at each indicated concentration of the fluorescent brevenal ligands. Representative experiment shown (n = 2 replicates per fluorescent brevenal concentration). (D) Competition for binding was assayed using Texas Red-brevenal (▲, dotted line, 10 nM, n = 6), coumarin-brevenal (○, dashed line, 100 nM, n = 6), and BODIPY FL-brevenal (□, solid line, 10 nM, n = 12) against varying concentrations of unlabeled brevenal (10 pM to 10 μM) to determine inhibition of binding.

poisoning and as a treatment for mucociliary disorders. Therefore, the development of brevenal probes is important to study brevenal's interaction at its biding site, isolation, and determination of the brevenal's binding site, and in the search for novel brevenal ligands.

A significant barrier to investigations into the brevenal binding site is the reliance on radioactivity for the current receptor binding assay. The generation of radioactive ligands is cumbersome and expensive, and there are increasing restrictions and regulations associated with the use and disposal of radioactive materials. In addition, great care must be taken with radioactive materials to avoid or limit contamination. As such, there is considerable need for a nonradioactive platform for use in examining the affinity of compounds for the brevenal

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binding site. In recent years, the use of fluorescence techniques has revolutionized the study of receptor−ligand interactions and drug discovery research. Fluorescence-based assays generally have lower nonspecific binding and background effects than their radioligand counterparts. Because of this, fluorescent assays may be able to detect interactions between natural product ligands and their receptors that had previously been overlooked because the conventional radioligand assays are plagued with high background.[18] Recently, we reported development of a fluorescence-based receptor binding assay for PbTxs.[19] This assay yields comparable results to the traditional radioligand assay and is much faster, less expensive, and safer and does not require the specialized equipment necessary for experiments involving radioactive labeled ligands.[19]

As an extension of this previous work, it was our goal to develop an effective receptor binding assay using a fluorescent ligand for the study of the brevenal binding site. This fluorescence-based assay allows specific detection of compounds that bind to the brevenal binding site and allows for rapid screening of novel brevenal analogues that may be used as a treatment for the effects of PbTxs and/or respiratory diseases such as cystic fibrosis, for which brevenal is currently under development.

RESULTS AND DISCUSSION

Synthesis, Binding Affinities, and Optimal Wavelengths of the Fluorescent Brevenal Conjugates. Fluorophores representing a variety of scaffolds and excitation/emission spectra were selected for this research. Using a modified Fischer condensation reaction, brevenal (**1**) was reacted with fluorophore hydrazides to give the brevenal fluorophore conjugates **2**−**5** and biotinylated brevenal (**6**) (Scheme 1).

The brevenal conjugates were then evaluated for their affinity for the brevenal binding site using the radioligand binding assay. The dansyl derivative (**2**) did not bind effectively to the brevenal binding site and was eliminated from further research. The coumarin (**3**), Texas Red (**4**), and BODIPY FL (**5**) fluorescent conjugates and the biotin analogue (**6**) showed good affinity for the binding site (K_i = 236, 156, 340, and 318 nM, respectively), as compared to brevenal (K_i = 149 nM).

To determine the optimal excitation and emission wavelengths for the brevenal conjugates, the fluorescent brevenal ligands were diluted to a concentration of 10 μM in assay buffer, and the absorbance and emission spectra were measured. Optimal excitation (ex) and emission (em) wavelengths were chosen as follows: ex:438 nm;em:474 nm for coumarin-brevenal (**3**), ex:495 nm;em:525 nm for BODIPY FL-brevenal (**5**), and ex:585 nm;em:620 nm for Texas Red-brevenal (**4**).

Equilibrium Dissociation Constants of Fluorescent Brevenal Conjugates. In order to characterize binding and determine the equilibrium dissociation constant (K_d) of compounds **3**−**5**, saturation binding experiments were performed using serial dilutions of the fluorescent ligands in the presence and absence of brevenal (10 μM). All three fluorescent brevenal ligands bound to rat brain synaptosomes (Figure 1). Total binding of the conjugates was concentration-dependent and nonlinear, while nonspecific binding of each was concentration-dependent and linear (Figure 1A−C). Subtraction of nonspecific binding from total binding yielded the specific saturation binding component. Nonspecific binding was determined to be highest for the Texas Red-brevenal conjugate (**4**) and lowest for BODIPY FL-brevenal (**5**). To ensure saturation had been achieved, the concentrations were increased for each fluorescent ligand to at least 1 μM, but there was no resulting increase in specific binding curves (data not shown).

To identify which fluorescent ligand would be best for competition assays, given the nonspecific binding of each fluorescent ligand, we next measured the percent of total binding for brevenal displacement of Texas Red-brevenal (10 nM), coumarin-brevenal (100 nM), and BODIPY FL-brevenal (10 nM). As shown in Figure 1D, Texas Red-brevenal (**4**) exhibited the highest background and/or nonspecific binding interference, while BODIPY FL-brevenal (**5**) exhibited the lowest background and/or nonspecific binding interference. In addition, coumarin-brevenal was the only fluorescent ligand that could not be measured with a competition assay at 10 nM, likely due to lower brightness and thus less fluorescence signal at this concentration of fluorescent ligand, coupled with the lower limit of detection by the fluorescence plate reader. Because of the limitations for the Texas Red- and coumarin-brevenal derivatives and the associated lower nonspecific binding of the BODIPY FL-brevenal derivative to rat brain synaptosomes, we chose BODIPY FL-brevenal as the ligand for further characterization and comparison to the radioligand binding assay. ^{1}H NMR spectra for the compounds (CD_3OD, 500 mHz) can be viewed in the Supporting Information. Nonlinear regression analysis of the specific binding component of the saturation assays indicated that BODIPY FL-brevenal bound to rat brain synaptosomes with a K_d of 264 ± 46 nM (n = 15).

Inhibition Binding Experiments and Comparison to Radioligand Binding Assay. In order to confirm the accuracy and precision of the fluorescent ligand binding assay, a series of inhibition binding experiments were performed using biotinylated brevenal and various naturally occurring PbTx analogues (PbTx-1, PbTx-2, PbTx-3, and PbTx-9) compared to unlabeled brevenal itself. As shown in Figure 2, both biotinylated brevenal and unlabeled brevenal competed for binding to synaptosomes. None of the naturally occurring PbTx analogues were able to displace BODIPY FL-brevenal from synaptosomes. This is in agreement with

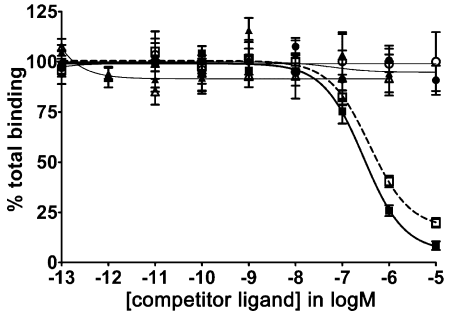

Figure 2. Inhibition of BODIPY FL-brevenal binding by various competitors. Competition for binding was assayed using BODIPY FL-brevenal (10 nM) against varying concentrations of nonfluorescent ligands: brevenal (■), biotinylated brevenal (□), PbTx-1 (▲), PbTx-2 (△), PbTx-3 (●), and PbTx-9 (○) from 1 pM to 10 μM in order to determine inhibition of BODIPY FL-brevenal binding to rat brain synaptosomes. The thick solid line denotes brevenal binding (n = 12), the dashed line denotes biotinylated brevenal binding (n = 12), and the thin lines denote the lack of binding curves generated from the various PbTx analogues (n = 6).

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previous findings using the radioligand brevenal receptor binding assay.[17]

Using the K_d of BODIPY FL-brevenal determined from saturation experiments, K_i values were calculated for brevenal and biotinylated brevenal (Table 1). In addition, a series of

Table 1. Comparison between the Radioligand [^{3}H]-Brevenal and Fluorescent Ligand BODIPY FL-Brevenal Receptor Assays

compound	K_i: fluorescent-ligand assay		K_i: radioligand assay	
brevenal	170 ± 31 nM	n = 12	149 ± 30 nM	n = 11
biotin-brevenal	324 ± 28 nM	n = 12	318 ± 55 nM	n = 7

radioligand binding experiments were also performed in order to validate the fluorescent ligand binding assay. As shown in Table 1, there was no significant difference in K_i values calculated for brevenal or biotinylated brevenal when using either the fluorescent ligand or radioligand binding assays ($p > 0.05$; Student's t-test). This indicates that the fluorescence-based assay is a viable substitute for the radioligand-based assay. Additionally, on the basis of time to complete an assay (2 days for radioligand versus less than 2 h for fluorescent ligand), this new method will allow a much higher throughput of samples.

Fluorescent-based assays may be able to detect natural product ligand−receptor interactions that may be overlooked in the traditional radioligand assays, because of the higher background associated with radioligands.[18] This is particularly the case with the brevenal receptor binding assays. The most recently described radioligand binding assay for brevenal[17] used a more tedious centrifugational method because all attempts at using a simplified, high-throughput multiwell filter plate format resulted in an assay with extraordinarily high background. The fluorescent-based multiwell filter plate assay described in this report has relatively low background and therefore may provide a platform that can discern more subtle ligand−binding site interactions. However, it is important to note that fluorescence-based assays can be problematic if the addition of a bulky fluorescent probe causes binding issues; therefore, optimization of the fluorescence-based assays is critical. Interestingly, we have previously found that the addition of the BODIPY-FL molecule to brevetoxin actually increased binding affinity when compared to the radioactive brevetoxin.[19] While the mechanisms behind this observation are currently unknown, other studies have shown that conjugating BODIPY molecules to various ligands may slightly increase or decrease binding affinity for the receptors. Even though there were small changes in the binding affinity of the BODIPY derivatives, they still remained effective in establishing binding constants.[20,21] In this study, BODIPY FL-brevenal was found to bind with a higher inhibition constant than brevenal alone on the radioligand assay (149 nM vs 340 nM, respectively). While the addition of the BODIPY FL molecule did affect the binding affinity, it was still found to bind with sufficient affinity to be used as a suitable ligand for the development of a fluorescence-based receptor binding assay. Furthermore, a fluorescent ligand with lower affinity for the brevenal binding site could conceivably be more sensitive in detecting binding by brevenal derivatives, as it is easier to displace. Indeed, most modifications to the brevenal structure have been found to decrease affinity to the binding site.[22]

Investigation of the Brevenal Binding Site. To further examine the nature of the brevenal binding site, a series of inhibition experiments were performed using other site-specific VSSC ligands, including tetrodotoxin (site 1), veratridine and aconitine (site 2), scorpion toxins from *Leiurus quinquestriatus* (site 3) and *Centruroides sculpturatus* (site 4), and deltamethrin (site 7). The site 6 VSSC ligand δ-conotoxin was unavailable for testing. As shown in Table 2, none of these ligands were

Table 2. Inhibition of BODIPY FL-Brevenal Binding to Rat Brain Synaptosomes by Various Site-Specific Sodium Channel Ligands

compound/ligand	K_i value	maximum concentration	receptor site
brevenal	170 ± 31 nM	100 μM	unknown
tetrodotoxin	no displacement	30 μM	VSSC site 1
veratridine	no displacement	10 μM	VSSC site 2
aconitine	no displacement	100 μM	VSSC site 2
α scorpion toxin from *Leiurus quinquestriatus*	no displacement	400 μg/mL	VSSC site 3
β scorpion toxin from *Centruroides sculpturatus*	no displacement	400 μg/mL	VSSC site 4
brevetoxin A (PbTx-1)	no displacement	1 μM	VSSC site 5
brevetoxin B (PbTx-2, PbTx-3, PbTx-9)	no displacement	1−10 μM	VSSC site 5
deltamethrin	no displacement	100 μM	VSSC site 7

able to displace BODIPY FL-brevenal from rat brain synaptosomes, suggesting that brevenal does not bind to VSSC sites 1−5 or 7. These data are in agreement with previous findings using the radioligand brevenal binding assay.[17]

To determine if brevenal binds to VSSCs, a receptor extraction experiment was performed to isolate the protein and/or protein complex(es) that bind to brevenal under native conditions. These experiments were conducted in nondenaturing conditions to preserve the binding site of brevenal, as it could involve several incongruent sites along the primary protein sequence when it is folded together in a secondary or tertiary structure. As shown in Figure 3A, total protein was isolated using the biotin-brevenal conjugate, resulting in an intense protein band at approximately 250 kDa in the membrane extract. This band was confirmed as positive for the alpha subunit of VSSCs using Western blot analysis (Figure 3B). The smaller band at 40 kDa in the membrane extract of the Western blot is unknown at this time, but it could be nonspecific binding by the antibody to another protein, or it could be a piece of the alpha subunit that was cleaved by native proteases in the tissue during protein extraction. These results, in conjunction with the receptor binding screening of other VSSC ligands, suggest that brevenal elicits its action through a novel mechanism, which may be through a presently unknown binding site on VSSCs or another protein in the VSSC complex (e.g., a beta subunit or other protein associated with noncovalent interactions to the alpha subunit of the VSSC). Generally, the alpha subunit of the VSSC is associated with up to three different beta subunits.[23,24] In addition, modulation of VSSCs by other receptors (e.g., acetylcholine receptors, receptor tyrosine kinases) and secondary messenger systems (e.g., calcium/calmodulin, G-protein subunits $G_{\beta\gamma}$, protein kinases, tyrosine phosphatases) indicates association of

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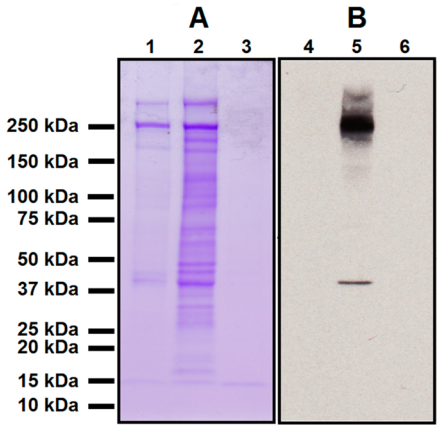

Figure 3. Coomassie stain and Western blot of the brevenal receptor protein isolated from rat brain. Protein was extracted from rat brains, and the brevenal receptor protein complex was isolated using the biotin-brevenal conjugate and streptavidin-conjugated magnetic beads. Isolated protein (cytosolic compartment, lanes 1 and 4; membrane compartment, lanes 2 and 5; nuclear compartment, lanes 3 and 6) was denatured, separated by size, and stained with Coomassie Brilliant Blue (panel A) to determine total protein bands, as well as stained with an antisodium channel antibody in Western blot analysis (panel B). Brevenal binds to a protein complex of different sized proteins (panel A), of which the approximate 250 kDa band stains positively for the alpha subunit of VSSCs (panel B).

VSSCs with other cellular and membrane proteins and/or extracellular matrix proteins.[23,24] Ongoing work in our laboratory is directed toward identifying the brevenal binding site in VSSCs.

Summary. Because of the hazards associated with radioactive material usage, there exists considerable need in drug discovery and natural product research for nonradioactive assays to examine receptor−ligand interactions. The development of novel fluorescence-based techniques has allowed for the elucidation of complex receptor interactions for a variety of important targets, such as the G-protein coupled receptors.[25−27] Fluorescence-based assays have several advantages over radioligand assays, including lower nonspecific binding and background, production of far less hazardous waste, and simpler and faster protocols. Given the advantages of the fluorescent platform, we developed a fluorescence-based receptor binding assay for the brevenal binding site on rat brain synaptosomes. This assay has already shown utility in screening for selective agonists for the brevenal binding site. In addition, work is ongoing in our laboratory to use compounds screened by the fluorescence-based brevenal receptor binding assay to aid in the further identification of the brevenal binding site and identify novel ligands for this binding site.

EXPERIMENTAL SECTION

General Experimental Procedures. Brevenal, PbTx-1, PbTx-2, PbTx-3, and PbTx-9 were purified from cultures of *Karenia brevis* (Wilson strain) as previously described.[6,13] Reagent grade sucrose, sodium phosphate dibasic, Trizma base, HEPES, choline chloride, glucose, EGTA, bovine serum albumin (BSA), glycine, protease inhibitor cocktail, veratridine, aconitine, *Leiurus quinquestriatus* scorpion venom, and *Centruroides sculpturatus* scorpion venom were purchased from Sigma-Aldrich. Tetrodotoxin was purchased from Tocris Bioscience. Deltamethrin was purchased from Supelco. Reagent grade potassium chloride, magnesium sulfate, EtOH, sodium dodecyl sulfate (SDS), glacial acetic acid, and ACS certified petroleum ether were purchased from Fisher Scientific. Alkamuls detergent was purchased from Rhone-Poulenc. HPLC grade MeOH and CH_3CN and LC-MS grade CH_3CN with 0.1% formic acid were purchased from Honeywell Burdick and Jackson. Anhydrous dimethylformamide (DMF) was purchased from Alfa Aesar. ACS certified $CHCl_3$ was purchased from Mallinckrodt Chemicals. 12-Tungstophosphoric acid hydrate was purchased from Strem Chemical. Sterile PBS and the fluorophores BODIPY FL hydrazide (D2371), diethylaminocoumarin-3-carboxylic acid hydrazide (D355), Texas Red hydrazide (T6256), and dansyl hydrazine (D100) were all purchased from Life Technologies. (+)-Biotin hydrazide was purchased from Sigma. All deuterated NMR solvents were purchased from Sigma-Aldrich.

Synthesis of the Fluorescent Brevenal and Biotinylated Brevenal Conjugates. The fluorescent dyes BODIPY FL hydrazide (D2371), 7-diethylaminocoumarin-3-carboxylic acid hydrazide (D355), and Texas Red hydrazide (T6256), dansyl hydrazine (D100), and (+)-biotin hydrazide were conjugated to brevenal using a modified Fischer reaction.[28] A one-pot reaction was performed for each fluorophore using a 1:1 molar ratio of brevenal to fluorophore in DMF. The reaction conditions were carried out using a catalytic amount of tungstophosphoric acid at 60 °C for 4 h with stirring. The DMF was evaporated to dryness under vacuum, and the residual material was resuspended in a small amount of MeOH and filtered (0.2 μm nylon syringe filter) (Fisher Scientific). After filtration the crude product was purified using reversed-phase HPLC procedures. Compounds **2**, **3**, **5**, and **6** were purified using a Phenomenex LUNA C_{18} column, with mixtures of MeOH and H_2O as the eluents. Compound **4** was purified using an Agilent Zorbax C_{18} column using a mixture of MeOH and H_2O modified with 0.1% formic acid as the eluent.

Verification of conjuguate structure was confirmed using UPLC/HRMS as previously described.[22] Structures of the fluorescent brevenal conjugates were confirmed using ^{1}H NMR experiments and were performed on a Bruker Avance 500 MHz spectrometer equipped with a 1.7 mm TXI probe at 298 K.

*Dansyl-brevenal (**2**):* pale yellow solid in 61% yield; ^{1}H NMR (CD_3OD), δ 0.89 (1H, t, J = 7 Hz), 0.92 (3H, d, J = 7 Hz), 1.04 (3H, s), 1.12 (3H, s), 1.15 (3H, s), 1.45 (4H, m), 1.65 (2H, m), 1.74 (6H, m), 1.89 (5H, m), 2.02 (5H, m), 2.16 (4H, m), 2.86 (7H, m), 3.24 (2H, m), 3.32 (2H, m), 3.49 (1H, m), 3.66 (1H, m), 3.72 (1H, m), 3.95 (1H, m), 5.09 (1H, m), 5.19 (1H, dd, J = 16 and 2 Hz), 5.44 (1H, m), 5.78 (1H, m), 6.03 (1H, m), 6.69 (1H, m), 7.26 (1H, d, J = 8 Hz), 7.58 (1H, m), 7.93 (1H, d, J = 9 Hz), 8.26 (1H, d, J = 7 Hz), 8.38 (1H, d, J = 9 Hz), 8.56 (4H, m); HRESIMS m/z 904.5140 $[M + H]^+$ (calculated for $C_{51}H_{74}N_3O_9S$, 905.5140).

*Coumarin-brevenal (**3**):* pale yellow solid in 99% yield; ^{1}H NMR (CD_3OD), δ 0.90 (4H, t, J = 7 Hz), 0.97 (3H, d, J = 7 Hz), 1.04 (3H, s), 1.13 (3H, s), 1.20 (3H, s), 1.25 (6H, t, J = 7 Hz), 1.37 (2H, m), 1.57 (3H, m), 1.64 (3H, m), 1.76 (5H, m), 1.89 (3H, s), 1.92 (2H, s), 2.05 (3H, m), 2.09 (3H, s), 2.26 (3H, m), 3.26 (2H, m), 3.34 (2H, m), 3.55 (5H, m), 3.97 (1H, m), 4.10 (3H, m), 5.09 (1H, d, J = 10 Hz), 5.19 (1H, d, J = 17 Hz), 5.43 (1H, m), 5.95 (1H, m), 6.03 (1H, t, J = 11 Hz), 6.42 (1H, d, J = 10 Hz), 6.60 (1H, d, J = 2 Hz), 6.70 (1H, m), 6.85 (1H, dd, J = 9 and 2 Hz), 7.59 (1H, d, J = 9 Hz), 8.40 (1H, m), 8.54 (1H, m), 8.72 (1H, s); HRESIMS m/z 914.5525 $[M + H]^+$ (calculated for $C_{53}H_{76}N_3O_{10}$, 914.5525).

*Texas Red-brevenal (**4**):* pink solid in 44% yield; ^{1}H NMR (CD_3OD), δ 0.56 (2H, t, J = 7 Hz), 0.90 (4H, t, J = 6 Hz), 0.95 (3H, d, J = 7 Hz), 1.04 (3H, s), 1.08 (3H, s), 1.15 (4H, m), 1.23 (2H, m), 1.49 (3H, m), 1.63 (5H, m), 1.77 (5H, m), 1.82 (5H, s), 1.89 (5H, m), 1.97 (3H, s), 2.02 (3H, m), 2.08 (4H, t), 2.22 (2H, m), 2.29 (1H, m), 2.37 (1H, m), 2.63 (4H, m), 3.05 (2H, s), 3.24 (2h, d, J = 9 Hz), 3.50 (8H, m), 3.71 (1H, m), 3.96 (1H, m), 4.06 (1H, m), 4.99 (1H, m), 5.09 (1H, d, J = 11 Hz), 5.19 (1H, d, J = 16 Hz), 5.46 (1H, m), 5.86 (1H, m), 6.04 (1H, m), 6.19 (1H, m), 6.55 (1H, s), 6.69 (1H, m), 7.43 (1H, d, J = 7 Hz), 8.03 (1H, d, J = 7 Hz), 8.12 (1H, d, J = 7 Hz), 8.66 (1H, s); HRESIMS m/z 1281.5838 $[M + H]^+$ (calculated for $C_{70}H_{90}N_4O_{13}S_2Na$, 1281.5838).

*BODIPY FL-brevenal (**5**):* green solid in 64% yield; ^{1}H NMR (CD_3OD), δ 0.96 (3H, d, J = 7 Hz), 1.14 (3H, d, J = 6 Hz), 1.22 (9H, s), 1.29 (3H, s), 1.42 (2H, m), 1.47 (2H, m, 1.54 (2H, m), 1.66 (3H,

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m), 1.76 (3H, m), 1.83 (3H, m), 1.88 (3H, s), 1.92 (2H, m), 2.01 (1H, s), 2.03 (2H, s), 2.07 (1H, m), 2.29 (5H, m), 2.52 (3H, s), 2.69 (1H, t, J = 8 Hz), 3.06 (1H, t, J = 8 Hz), 3.12 (1H, m), 3.18 (1H, dd, J = 10 and 3 Hz), 3.27 (2H, m), 3.35 (2H, s), 3.37 (1H, t), 3.46 (1H, dd, J = 12 and 4 Hz), 3.50 (1H, m), 3.62 (1H, m), 3.74 (2H, m), 3.86 (2H, m), 3.95 (1H, m), 4.08 (1H, d, J = 3 Hz), 5.87 (0.3H, t, J = 7 Hz), 5.92 (0.7H, t, J = 7 Hz), 6.23 (1H, m), 6.35 (2H, m), 7.00 (1H, d, J = 4 Hz), 7.41 (0.3H, s), 7.43 (0.7H, s), 8.04 (0.3H, d, J = 10 Hz), 8.21 (0.7H, d, J = 10 Hz); HRESIMS m/z 945.5728 $[M + H]^+$ (calculated for $C_{53}H_{76}BF_2N_4O_8$, 945.5719).

*Biotin-brevenal (**6**):* white solid in 41% yield; ^{1}H NMR (CD_3OD), δ 0.97 (3H, d, J = 7 Hz), 1.05 (3H, s), 1.13 (3H, s), 1.20 (3H, s), 1.29 (2H, s), 1.37 (3H, m), 1.51 (4H, m), 1.64 (5H, m), 1.77 (8H, m), 1.88 (5H, m), 2.05 (5H, m), 2.16 (1H, m), 2.26 (4H, m), 2.38 (1H, m), 2.69 (1H, m), 2.92 (1H, dd, J = 13 and 5 Hz), 3.25 (3H, m), 3.34 (3H, m), 3.55 (1H, m), 3.72 (1H, m), 3.98 (1H, t, J = 3 Hz), 4.08 (1H, m), 4.31 (1H, dd, J = 8 and 5 Hz), 4.49 (1H, dd, J = 8 and 5 Hz), 5.09 (1H, d, J = 11 Hz), 5.19 (1H, d, J = 17 Hz), 5.45 (1H, m), 5.90 (1H, m), 6.04 (1H, t, J = 11 Hz), 6.34 (1H, d, J = 10 Hz), 6.68 (0.4H, t, J = 9 Hz), 6.72 (0.6H, t, J = 9 Hz), 8.06 (0.4H, d, J = 10 Hz), 8.22 (0.6H, d, J = 10 Hz); HRESIMS m/z 897.5406 $[M + H]^+$ (calculated for $C_{49}H_{77}N_4O_9S$, 897.5406).

Radioligand Binding Assays. Synaptosomes were prepared as previously described.[19] [^{3}H]-brevenol was synthesized from brevenal as previously described.[17] Binding of [^{3}H]-brevenol to rat brain synaptosomes was measured independently using a rapid centrifugation technique as previously described.[17,29] All buffers, reagents, and plasticware were at ice temperature throughout each experiment except during centrifugation. All experiments were performed in standard binding medium with 1 mg/mL BSA without detergent.

Fluorescent-Ligand Binding Assays. Binding of the fluorescent brevenal conjugates was determined on rat brain synaptosomes using a rapid filtration technique as described previously.[19] All buffers, reagents, and plasticware were at ice temperature throughout each experiment. All experiments were performed in standard binding medium with 1 mg/mL BSA and 0.02% Alkamuls detergent.

Saturation binding experiments and corresponding calculation of equilibrium dissociation constants (K_d) were conducted and statistically analyzed as previously described,[19] with the exception that the fluorescent ligands in these experiments were brevenal conjugates. Briefly, a 96-well assay plate was prepared in which 50 μg (50 μL at 1 mg/mL) of synaptosomes was suspended in assay buffer plus 5 μL of EtOH (to determine total binding) or 5 μL of EtOH containing 10 μM brevenal (to determine nonspecific binding). This concentration of unlabeled brevenal was sufficient to displace the fluorescent brevenal ligands bound to the receptors, leaving the nonspecifically bound molecules on synaptosomes. Stock solutions of 500 nM BODIPY FL-brevenal, coumarin-brevenal, or Texas Red-brevenal were prepared in assay buffer and serially diluted (1:2) in the wells until the final concentration was below 1 nM.

The inhibition of binding of the fluorescent ligands was determined in the presence of various competitors ranging in concentration from 1 $\times$ 10^{-12} to 1 $\times$ 10^{-5} M. Serial dilutions (1:10) of unlabeled brevenal (control), biotinylated brevenal, natural brevetoxin derivatives (PbTx-1, PbTx-2, PbTx-3, and PbTx-9), veratridine, aconitine, and deltamethrin were prepared in EtOH. Serial dilutions (1:10) of tetrodotoxin and scorpion venom were prepared in PBS. The appropriate concentration of the competitor solution was added (5 μL) to each well with 50 μg of synaptosomes, the fluorescent ligand (10 nM final concentration BODIPY FL-brevenal and Texas Red-brevenal, 100 nM final concentration of coumarin-brevenal), and assay buffer. Competition binding experiments and the calculation of equilibrium inhibition constants (K_i) were then conducted as previously described.[19] Data were normalized to 100% total binding so as to compare the differences in binding curves and/or inhibition constants among the three fluorescent brevenals (each which yielded data in varying ranges of relative fluorescent units) and to the radioligand assay (which yielded data in counts of radioactivity per minute).

Brevenal Receptor Isolation. Rat brain protein was isolated into cytosolic, membrane, nuclear, and cytoskeletal fractions using a compartmental protein extraction kit (Millipore). Protein content of each fraction was assayed using a modified Lowry protein assay (Bio-Rad). Fractions were treated with the biotin-brevenal conjugate at a concentration of 5−10 μg/mg of protein for over 1 h on ice, followed by 1 mg of MyOne Streptavidin T1 Dynabeads (Invitrogen Life Technologies) for 30 min to equilibrate binding. Bound protein was removed with a magnet and then denatured via boiling and the addition of Laemmli sample buffer (Bio-Rad).

Coomassie Stain and Western Blot Analyses. Brevenal receptor protein samples were electrophoresed with SDS-PAGE running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS) on 7.5% Mini-PROTEAN TGX polyacrylamide gels (Bio-Rad). Gels were stained with a Coomassie Brilliant Blue R-250 40% MeOH solution (Bio-Rad) for 1−2 h and then destained with a 40% MeOH/10% glacial acetic acid solution for 2−4 h. For Western blot analysis, electrophoresed samples were transferred to Immobilon-P transfer membranes (Millipore) using transfer buffer (20 mM Tris base, 150 mM glycine, 25% methanol). Membranes were reacted with an affinity purified primary rabbit anti-PAN voltage gated sodium channel polyclonal antibody (Millipore). The anti-PAN antibody was chosen because it recognizes a conserved region among various VSSC alpha subunits so as to detect any that might be present in the sample. Blots were washed with PBS-Tween and incubated in the presence of a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Millipore). Bound enzyme was detected with the Super Signal West Pico chemiluminescence substrate system (ThermoFisher Scientific). Blots were exposed to Kodak Biomax light film (Carestream Health) for 5 min and developed.

Statistical Analysis. K_i values determined in the fluorescent and radioligand assays for brevenal and biotin-brevenal were compared using Student's t-test SAS v9.1.3 software (SAS Institute Inc.). Results are presented as the mean ± SEM and were considered statistically significant if a p-value of less than 0.05 was obtained.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR spectra for brevenal, dansyl-brevenal, coumarin-brevenal, Texas Red-brevenal, BODIPY FL-brevenal, and biotinylated-brevenal (CD_3OD, 500 mHz) can be viewed in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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