High-Throughput Screening for N-Type Calcium Channel Blockers Using a Scintillation Proximity Assay

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N-type calcium channels located on presynaptic nerve terminals regulate neurotransmitter release, including that from the spinal terminations of primary afferent nociceptors. Accordingly, N-type calcium channel blockers may have clinical utility as analgesic drugs. A selective N-type calcium channel inhibitor, ziconotide (Prialt), is a neuroactive peptide recently marketed as a novel nonopioid treatment for severe chronic pain. To develop a small-molecule N-type calcium channel blocker, the authors developed a 96-well plate high-throughput screening scintillation proximity assay (SPA) for N-type calcium channel blockers using $[\text{125I}]\omega$-conotoxin GVIA as a channel-specific ligand. Assay reagents were handled using Caliper’s Allegro automation system, and bound ligands were detected using a PerkinElmer TopCount. Using this assay, more than 150,000 compounds were screened at 10 $\mu$M and approximately 340 compounds were identified as hits, exhibiting at least 40% inhibition of $[\text{125I}]\text{GVIA}$ binding. This is the 1st demonstration of the use of $[\text{125I}]$-labeled peptides with SPA beads to provide a binding assay for the evaluation of ligand binding to calcium channels. This assay could be a useful tool for drug discovery. (Journal of Biomolecular Screening 2006:672-677)

Key words: calcium channel, SPA, $\omega$-conotoxin GVIA, HTS

INTRODUCTION

Calcium channels are believed to be essential for presynaptic neurotransmitter release. Located on presynaptic nerve terminals, N-type calcium channels regulate the influx of calcium in both the central and peripheral nervous systems. Spinal release of primary afferent peptides, such as substance P and calcitonin gene-related peptide, are regulated by N-type calcium channels, suggesting that N-type calcium channel blockers may be potential analgesic drugs. N-type calcium channel blockers have been shown to attenuate both phase 1 and phase 2 of formalin-induced hyperalgesia. Many preclinical studies have demonstrated the efficacy of calcium channel blockers on nerve-injury-induced mechanical allodynia, suggesting that N-type calcium channel inhibitors may have clinical utility in the reduction of neuropathic pain. $\omega$-conotoxin MVIIA (known as SNX-111, Ziconotide, or Prialt), a selective N-type calcium channel blocker, has been studied in about 2000 human subjects in clinical trials as a novel treatment for severe chronic pain. These studies indicate that N-type calcium channel inhibitors are potential analgesics.

There are several peptidic N-type calcium channel blockers, such as $\omega$-conotoxin GVIA, MVIIA, CVID (also known as AM336), CVIA, CVIB, TVIA, CNVIIA, PtxIIA, $\omega$-Agalla, MVIC, MVIIC, and huiwentoxin. GVIA, isolated from the venom of Conus geographus, is a 27-amino-acid peptide that is a potent and selective blocker of N-type calcium channels. MVIIA, isolated from the venom of Conus magnus, is another potent and selective N-type calcium channel blocker. MVIIC is an inhibitor for both N-type calcium channels and P/Q-type calcium channels.

GVIA and MVIIA are widely used as tools to identify N-type calcium channels. The density of N-type calcium channels in animal tissues or cell membranes has been tested using $[\text{125I}]\text{GVIA}$ or $[\text{125I}]\text{MVIIA}$ as N-type calcium channel-specific ligands. Usually, a vacuum filtration method is used to separate bound from free ligand. The bound ligand remains on the filter, and the free ligand is washed out with wash buffer.

Currently, there is no non-separation-based binding assay for these N-type calcium channels. Reducing the number of steps and avoiding wash steps are always a consideration for improving the assay especially for robotics platforms. In this study, we developed a scintillation proximity assay (SPA) for N-type calcium channels using $[\text{125I}]\text{GVIA}$ as a N-type calcium channel ligand, eliminating the need for a filtration separation.
step. Thus, this assay could be carried out in a relatively short time, facilitating its automation for high-throughput screening (HTS).

**MATERIALS AND METHODS**

**Materials**

\[^{125}\text{I}]\text{GVIA}\) (specific activity = 2200 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). Polyvinyltoluene SPA beads coated with wheat germ agglutinin (WGA-PVT) were obtained from Amersham Bioscience (Piscataway, NJ). The \(\omega\)-conotoxins GVIA, MVIIA, and MVIIC and \(\omega\)-agatoxin IVA were purchased from Sigma (St. Louis, MO). Ninety-six-well Microlite I plates were purchased from Dynex Technologies, Inc. (Chantilly, VA).

**Preparation of rat brain membranes**

Rat brain membranes were prepared from adult male Sprague-Dawley rat brain tissue according to the procedures described previously. Briefly, rat brain tissue was homogenized in ice-cold 25 mM Tris (pH 7.2), 2 mM EDTA, and 320 mM sucrose and then centrifuged at 1000 g for 10 min. The resultant supernatant was then centrifuged at 40,000 g for 20 min. The membrane pellet was resuspended in 50 mM Hepes-Tris (pH 7.4) binding buffer and stored at \(-70^\circ\text{C}\) until use. The protein concentration in the membrane preparation was determined using the Bradford Reagent (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard.

**N-type calcium channel SPA principle**

N-type calcium channel SPA involves the use of scintillation beads, which are coated with WGA, rat brain membranes, and \[^{125}\text{I}]\text{GVIA}\). First, the beads are coupled with the membranes (or cell membranes bound to WGA) and then \[^{125}\text{I}]\text{GVIA}\) binds to the membranes. The emitted radiation from \[^{125}\text{I}]\text{GVIA}\) activates the scintillant in the beads and produces light. The light is detected by the photomultiplier tube in a PerkinElmer TopCount (Wellesley, MA). The unbound \[^{125}\text{I}]\text{GVIA}\) does not produce light because its radiation energy is absorbed by the surrounding aqueous environment. Thus, the bound \[^{125}\text{I}]\text{GVIA}\) shows the light signal whereas the unbound one does not, eliminating separation steps such as filtration or centrifugation.

**Scintillation proximity assay**

A standard binding assay was performed at room temperature in a 96-well-plate with a total volume of 200 µl. Rat brain membranes were preincubated with WGA-PVT SPA beads for at least 30 min in the binding buffer containing 0.05% BSA. The precoupled SPA beads were incubated with 15 pM \[^{125}\text{I}]\text{GVIA}\) for 60 min at room temperature. After incubation, the plate was centrifuged and then counted in a PerkinElmer TopCount. Nonspecific binding was determined in the presence of 100 nM unlabelled GVIA. The nonspecific binding was about 15% of total binding. The data were analyzed with GraphPad PRISM.

**RESULTS**

**Effect of incubation time, SPA bead, and membrane protein concentrations on the binding**

The association of \[^{125}\text{I}]\text{GVIA}\) with rat brain membranes at room temperature was rapid and reached near maximal levels by approximately 1 h, with relatively minor incremental increases over the ensuing 19 h (Fig. 1). Therefore, we chose 1 h as the incubation time in subsequent studies. Binding increased with increasing concentrations of SPA beads or membrane protein (Fig. 2A, B). The nonspecific binding for SPA beads and membranes was not significantly changed with increasing SPA beads or membranes within the range tested. A total of 2.5 µg/well membrane protein was used in the following experiment.

**Characterization of N-type calcium channels in rat brain membranes**

To characterize the N-type calcium channels in rat brain membranes, we examined the binding ability of the N-type
calcium channel–specific radioligand [125I]GVIA using SPA. As shown in Figure 3, [125I]GVIA bound to rat brain membranes in a saturable manner, reaching half-maximal binding at about 13 pM and reaching saturable binding at about 25 pM.

Analysis of the saturation data for [125I]GVIA showed that the binding was best fitted using a 1-site model, with $K_d$ and $B_{max}$ values of 13 pM and 225 fmol/mg protein, respectively.

A number of calcium channel blockers were tested for their ability to inhibit [125I]GVIA binding. It is well known that GVIA and MVIIA are specific blockers of the N-type calcium channel, whereas $\omega$-agatoxin IV A is a specific P/Q-type calcium channel blocker, and MVIIC blocks both N-type and P/Q-type calcium channels.30-33 Inhibition of [125I]GVIA binding to rat brain membranes by these calcium blockers was in the order GVIA > MVIIA > MVIIC >> agatoxin (Fig. 4). GVIA, MVIIA, and MVIIC binding data were best fit by 1-site models.

**SPA for HTS of N-type calcium channels**

Library compounds were stored as 1 mM stock solutions containing 30% DMSO. To test the effect of DMSO on binding, varying DMSO concentrations were used. Concentrations of DMSO up to 16% did not significantly affect the assay (data not shown).

To determine well-to-well variance, 48 wells for total binding and 48 wells for nonspecific binding were tested in a 96-well plate. As shown in Figure 5A, the total binding was about 1600 cpm and the nonspecific binding was about 260 cpm, the ratio of total binding to nonspecific binding was about 6, and the calculated $Z'$ factor34 was about 0.75. HTS for N-type
N-Type Calcium Channel Blockers

FIG. 4. Effect of calcium channel blockers on the binding of \(^{125}\text{I}\)GVIA to rat brain membranes. Varying concentrations of calcium channel blockers were incubated with scintillation proximity assay beads (1 mg/well) precoupled with rat brain membranes (2.5 µg/well) and \(^{125}\text{I}\)GVIA (15 pM). Inhibition curves for GVIA, MVIIA, and MVIIC were best fitted by a 1-site binding model. Data are means ± standard deviation (n = 3).

calcium channel blockers was conducted at room temperature for 60 min in a 96-well plate containing 200 µl of 50 mM Hepes-Tris (pH 7.4), 0.05% BSA, 1 mg/well SPA beads, 2.5 µg/well membrane protein, 10 µM compound, and 15 pM \(^{125}\text{I}\)GVIA. The mapping of each plate was as follows: columns 1 to 11 contained compounds and column 12 contained controls including total binding in wells A and B, nonspecific binding in wells C to F, and a 70% inhibition control in wells G and H. Buffer, compounds, and \(^{125}\text{I}\)GVIA were added to plates using Caliper’s Allegro automation system. Rat brain membranes were added by a MultiDrop-384 (Thermo Electron Corporation, Franklin, MA) to start the reaction. After incubating at room temperature for 60 min, the plates were centrifuged and counted using a PerkinElmer TopCount. The data from 5 plates were analyzed and shown in Figure 5B (Z factor = 0.66).³⁴

Using this automation method, we screened approximately 150,000 compounds at a rate of more than 10,000 compounds per day. Most (89.3%) of the compounds did not significantly affect \(^{125}\text{I}\)GVIA binding (<10% inhibition), whereas 10.5% of the compounds inhibited the binding between 10% and 40%. The hit rate (40% inhibition of \(^{125}\text{I}\)GVIA binding to rat brain membranes) was about 0.22%, according to the frequency distribution shown in Figure 6.

DISCUSSION

Because GVIA binds selectively and potently to N-type calcium channels, \(^{125}\text{I}\)GVIA has been the ligand of choice for decades.³⁵ Filtration assays with \(^{125}\text{I}\)GVIA have been used to characterize N-type calcium channels in various tissues and cell lines. There are several disadvantages to using filtration assays for large-scale drug screening. Filtration assays require large amounts of wash buffer, and generate large quantities of radioactive liquid waste, and very few reliable high-throughput automation systems are able to perform filtration steps. This assay, the 1st to combine \(^{125}\text{I}\)GVIA with SPA beads in an automated assay for N-type calcium channel blockers, was simple and rapid.

This assay characterized N-type calcium channels in rat brain membranes and showed that \(^{125}\text{I}\)GVIA selectively bound to high-affinity sites with a \(K_d\) value of 13 pM and a \(B_{max}\) value of 225 fmol/mg. Currently, the most comparable technology with SPA is filtration assay; to date, there are no publications using filtration technology for HTS of N-type calcium channels. Although many N-type calcium channel publications use filtration assays with \(^{125}\text{I}\)GVIA as the ligand, the data obtained are not consistent. The \(K_d\) values range from 0.6 to 215 pM. Consistent with our result (\(K_d\) = 13 pM), \(K_d\) values average about 20 pM. Similarly, published \(B_{max}\) values range from 540 fmol/mg to 1029 pmol/mg, with most \(B_{max}\) values about 1000 fmol/mg, which is higher than the 225 fmol/mg from the present study. The lower \(B_{max}\) in this study is likely due to the incomplete attachment of membranes to the scintillation beads. \(^{125}\text{I}\)GVIA bound to free membranes does not produce light signals, resulting in a lower \(B_{max}\) value.

N-type calcium channel blockers such as GVIA and MVIIA completely and potently inhibited binding. Binding was inhibited by the P/Q-type calcium channel blocker MVIIC but with relatively lower potency. In contrast, agatoxinIVA, another P/Q calcium channel blocker, did not reduce binding to rat brain membranes up to 100 nM. These results, consistent with those of previous reports,¹⁶,¹⁷,³⁰ support that \(^{125}\text{I}\)GVIA potently blocks N-type calcium channels but not other calcium channels.

The function of calcium channels may also be tested using other methods, such as a calcium flux assay, a fluorescence-based assay, or patch clamp electrophysiological assay, which are all cell-based. Patch clamping is the gold standard for determining calcium channel function, providing high-quality physiologic and functional data at a single-cell and single-channel level. However, electrophysiological study is limited by its low throughput.³⁶⁻³⁸ Fluorescence-based assays such as the fluorometric imaging plate reader (FLIPR) are widely used for drug discovery.³⁹ FLIPR assays generally provide robust and homogenous cell population measurements and are relatively easy to set up in a high-throughput manner. The disadvantage of this approach is that the compounds identified may not be selective for the desired target. In the case of the N-type calcium channel,
its activity can be modulated by its α2δ subunit, its β subunit, or by G-protein subunits that could provide alternate binding sites for the compound. In addition, the N-type channel pore is similar to other calcium channel pores, such as L-type and P/Q type; therefore, the compounds could inhibit any of these channels nonselectively. Because GVIA specifically binds to N-type calcium channels, the hit compounds identified with a [125I]GVIA binding assay are likely to be selective for the N-type calcium channel. However, as in a filtration assay, a radioactive ligand is used in the present assay. Ligand displacement assays are advantageous for identifying selective hits for this channel. A limitation of binding assays, however, is that they may not detect modulators for this channel acting at other binding sites or via other binding modes. Therefore, the sequential combination of FLIPR and [125I]GVIA binding assays, as primary and secondary screens, respectively, could provide an efficient way to identify and characterize selective N-type calcium channel blockers.

To our knowledge, this is the 1st successfully developed HTS assay for N-type calcium channel blockers using an SPA. The assay was automated with an Allegro system using a 96-well format. More than 150,000 compounds were screened at 10 μM, and about 340 compounds were identified as hits, exhibiting at least 40% inhibition of [125I]GVIA binding. The hit rate was about 0.22%. These results demonstrate that [125I]-labeled peptides can be used with SPA beads to provide a binding assay for the evaluation of the N-type calcium channel. This binding assay provides an additional useful tool for analgesic drug discovery.

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