FlashPlate® File #3

A Fast and Easy FlashPlate Assay for High Throughput Screening of Cloned G Protein-Coupled Receptors by [^{35}S]-GTPγS Binding

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Background

In the pharmaceutical industry, the drug discovery and development engine is fueled by directed screening of large compound collections and combinatorial libraries using rapid and high throughput assays that measure potency. Functional assays, which measure the efficacy of the compounds, are often of low throughput, and are usually reserved for those candidate compounds with the most desirable potency and selectivity profiles. This strategy, while generally economical, may have highly unfavorable consequences at a later stage in drug development. For example, subtle chemical changes in lead candidates can drastically alter their pharmacology, conceivably changing an agonist to an antagonist. Thus, the importance of using functional assays for screening has led to much research on assay miniaturization and development of systems amenable to high throughput methods.

In the present report, we used the versatile FlashPlate technology to study [^{35}S]-GTPγS binding, as a high throughput membrane-based functional assay for cloned G protein-coupled receptors (GPCRs). This is the first [^{35}S]-GTPγS binding assay ever described for testing the abilities of chemicals to affect GPCRs, that is robot compatible and allows truly high throughput. It is simple, rapid and applicable to a number of cloned GPCRs.

Materials

- 96-well Basic FlashPlate (SMP200)
- Fresh or frozen membranes from mammalian cell lines expressing human cloned GPCRs
- [^{35}S]-GTPγS, (NEG030H, S.A., 1250 Ci/mmol)
- GDP (Sigma Chemicals)
- Binding Buffer: 20 mM HEPES pH 7.4, 10 mM $MgCl_2$ and 100 mM NaCl
- DMSO (Dimethyl sulfoxide) (Aldrich)
- Test compounds (RBI, Tocris-Cookson)
- TopSeal™-S, Microplate Heat Sealing Film (Packard Instrument Co., Cat. #6005161)
- Robotic System Multiprobe 104DT (Packard Instrument Co.)
- Packard TopCount® Microplate Scintillation and Luminescence Counter (Packard Instrument Co.)

Methods

The FlashPlate assay for binding [^{35}S]-GTPγS to cloned human receptor membranes used a method modified from that described by Lazareno, *et al.*[1], for use with 96-well polystyrene microplates with plastic scintillant-coated wells. In addition, the assay volume was reduced to 200 µl for ease in robotic handling. Briefly, the robotic liquid handling system, the Multiprobe 104DT, was programmed to deliver 60 µl HEPES buffer, 20 µl GDP (1 µM) and 100 µl receptor membranes (10 µg of protein) in triplicate. All wells for assessing compounds received 20 µl or 40 µl HEPES and 20 µl test compound in DMSO (2%). Finally, the binding reaction was initiated by the addition of 20 µl [^{35}S]-GTPγS (0.1 nM) to all wells. The microplates were heat-sealed and shaken gently on a rotary shaker for 2 minutes. The incubation continued for an additional 28 minutes at room temperature. Immediately following incubation, the plates were centrifuged at 2,500 rpm for 5 minutes and then counted on a Packard TopCount for 1 minute.

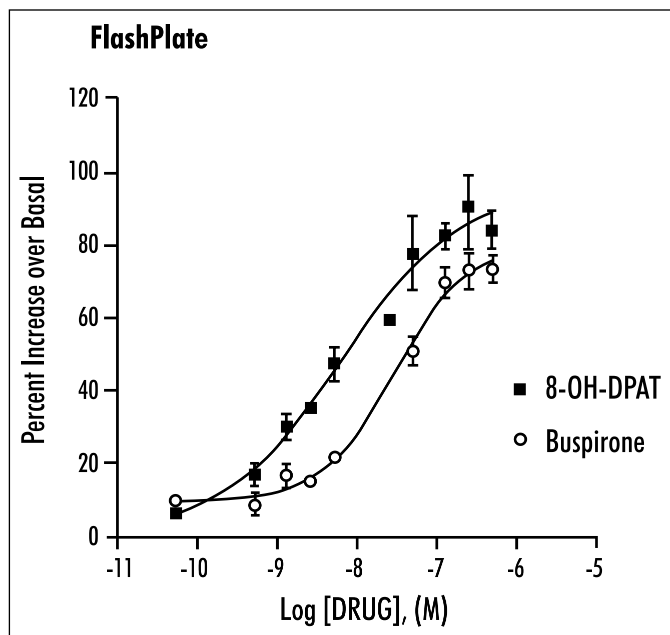
A filtration assay was conducted for comparison to the FlashPlate results, using the methods described by Lazareno, *et al.*[1], without modifications.

Results are shown in Figures 1 through 7.

Conclusions

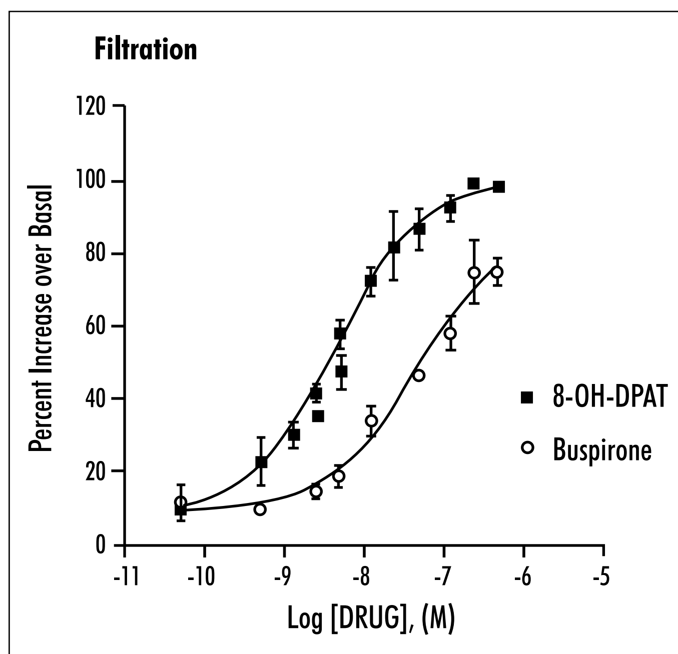
1. We have validated the use of the FlashPlate method to rigorously measure the pharmacology of GPCRs.
2. The FlashPlate method for [^{35}S]-GTPγS binding is a simple, cost-effective and rapid assay that can be used to evaluate cloned human GPCRs.
3. The FlashPlate method involves fewer steps and generates less radioactive waste than the conventional filtration assay.
4. The FlashPlate method can be readily formatted for performance by robotics, and is amenable to high throughput screening.

Figure 1



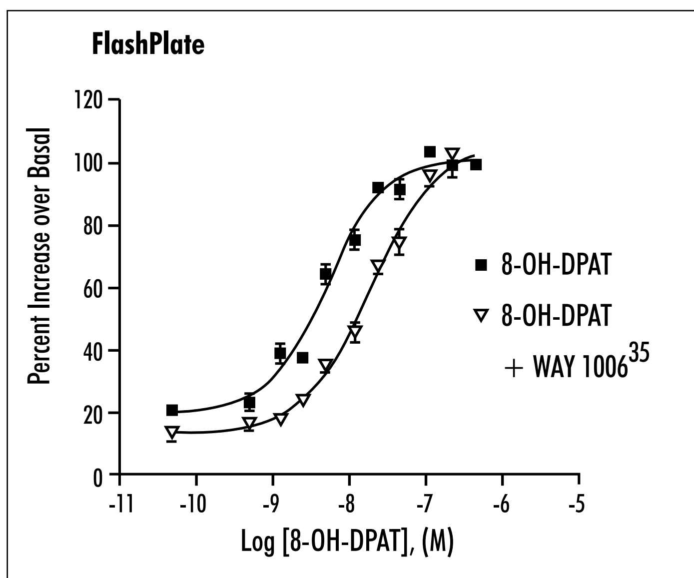
Stimulation of [^{35}S]-GTPγS binding to CHO cell membranes expressing human h5–HT_{1A} receptors by the full agonist 8-OH-DPAT and the partial agonist buspirone by the FlashPlate assay. The data are expressed as percentage increase over basal activity, each point representing the mean of duplicate observations. Buspirone maximized at about 72% the activity of the full agonist. The EC_{50} values for 8-OH-DPAT and buspirone were 6.1 x 10^{-9} M and 28.0 x 10^{-9} M, respectively.

Figure 2



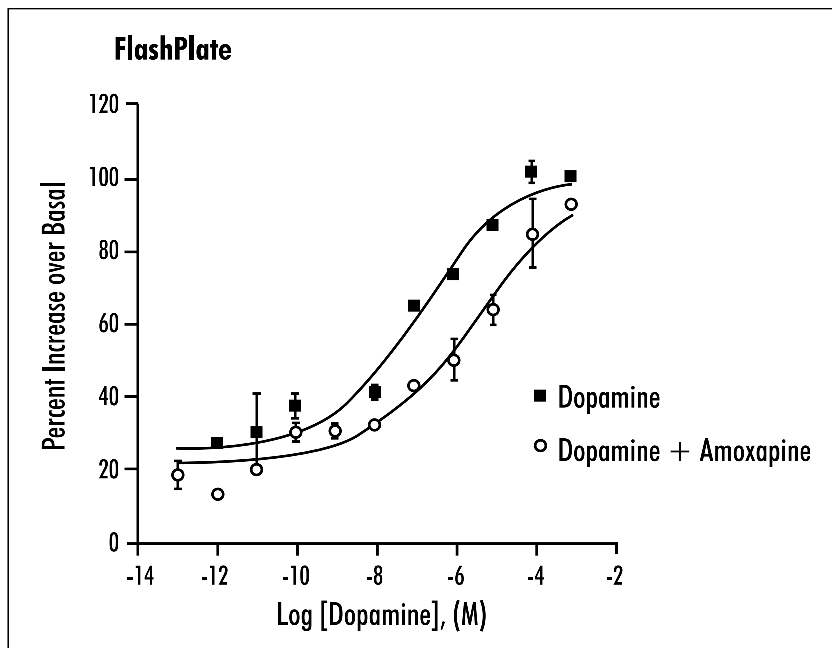
Stimulation of [35S]-GTPγS binding to CHO cell membranes expressing human h5-HT_{1A} receptors by the full agonist 8-OH-DPAT and the partial agonist buspirone by conventional filtration assay. The data are expressed as percentage increase over basal activity, each point representing the mean of duplicate observations. Buspirone maximized at about 76% the activity of the full agonist. The EC_{50} values for 8-OH-DPAT and buspirone were 5.1 x 10^{-9} M and 48.0 x 10^{-9} M, respectively.

Figure 3



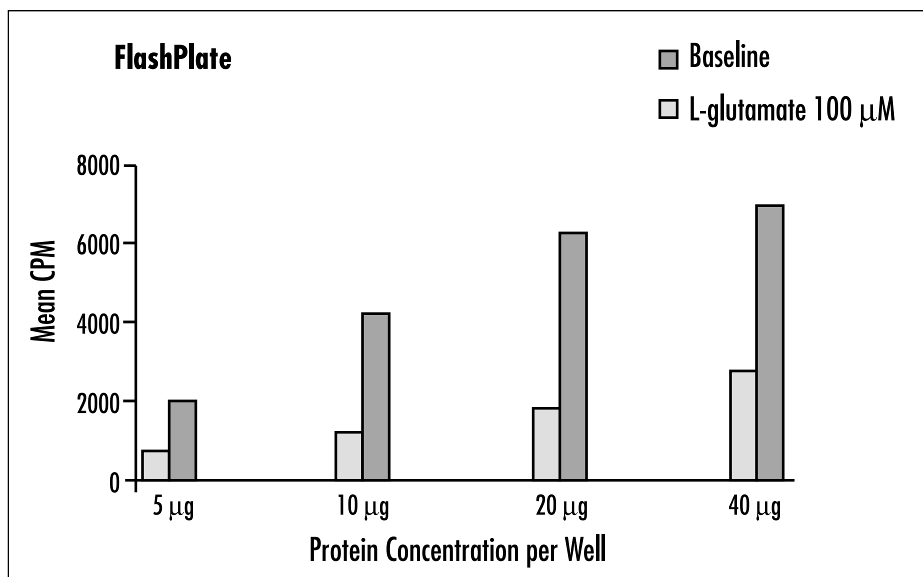
Stimulation of [35S]-GTPγS binding to CHO cell membranes expressing human h5–HT_{1A} receptors by the full agonist 8-OH-DPAT and the effect of a fixed concentration (1.0 nM) of the selective 5-HT_{1A} receptor antagonist WAY 1006[35]. The data are expressed as percentage increase over basal activity, each point representing the mean of duplicate observations. While the presence of the antagonist did not reduce the percent maximal stimulation in [35S]-GTPγS binding induced by 8-OH-DPAT, it caused a rightward shift of the dose-response curve, increasing the EC_{50} value for the full agonist from 5 x 10^{-9} M to 21 x 10^{-9} M.

Figure 4



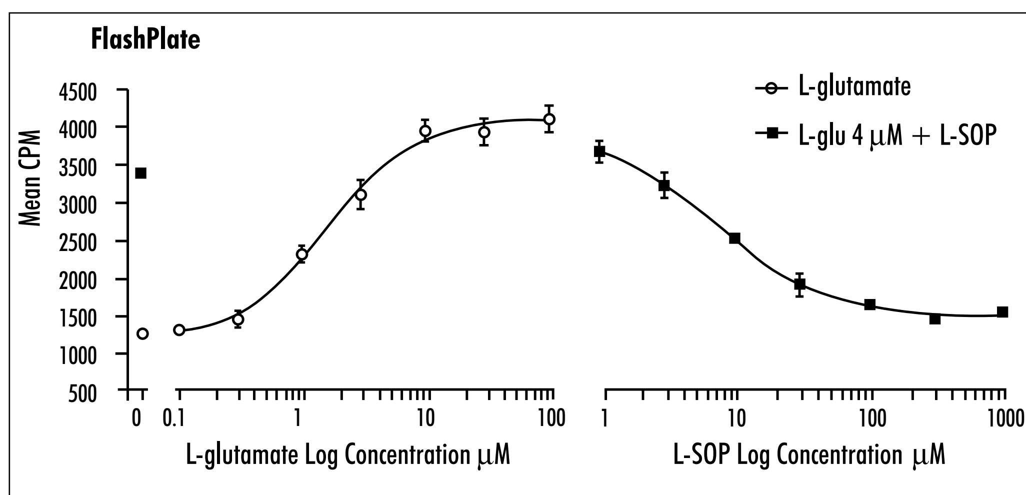
Stimulation of [^{35}S]-GTPγS binding to CHO cell membranes expressing human dopamine hD_2 short receptors by the natural ligand dopamine and the effect of a fixed concentration (10.0 nM) of a dopamine D_2 receptor antagonist amoxapine. The data are expressed as percentage increase over basal activity, each point representing the mean of duplicate observations. While the presence of amoxapine did not reduce the percent maximal stimulation in [^{35}S]-GTPγS binding induced by dopamine, it caused a rightward shift of the dose-response curve of dopamine, increasing its EC_{50} value from 0.1 x 10^{-6} M to 3 x 10^{-6} M.

Figure 5



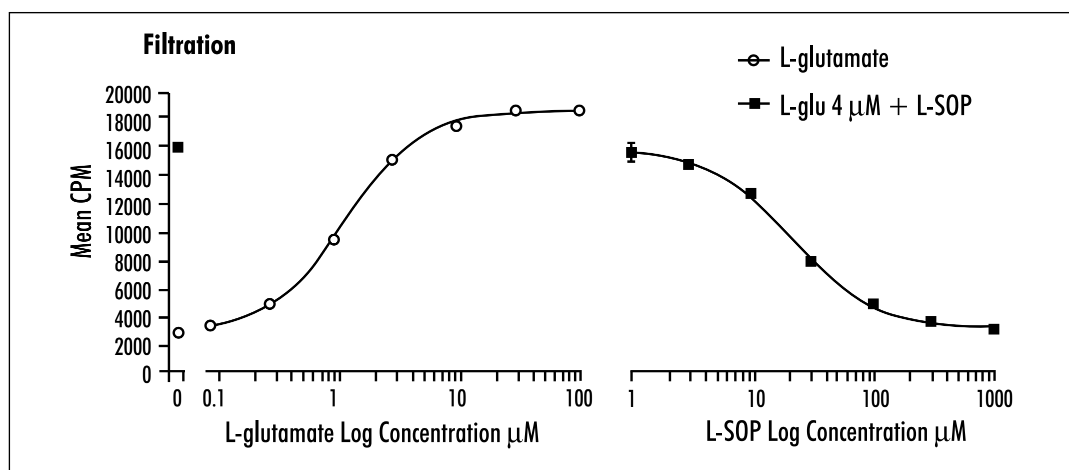
Effect of protein concentration on the stimulation of [^{35}S]-GTPγS binding to CHO cell membranes expressing human metabotropic glutamate receptor subtype 2 ($hmGluR_2$) by 100 µM of L-glutamate by the FlashPlate method. The data are expressed in counts per minute (CPM) and represent the means of triplicate observations. Maximal fold stimulation of [^{35}S]-GTPγS binding over baseline values was evident with as little as 10 µg of $hmGluR_2$ receptor protein, at 100 µM L-glutamate.

Figure 6



Log-dose response curve of L-glutamate (left panel) on the stimulation of [^{35}S]-GTPγS to CHO cell membranes expressing human metabotropic glutamate receptors subtype 2 (hmGluR$_2$) and inhibition (right panel) of the effect of a fixed L-glutamate concentration (4 µM) by L-SOP. The data are expressed in counts per minute (CPM), each point representing the mean of triplicate observations. Data analysis yielded pA2 value of 5.16 with a Schild slope of 0.97, suggestive of competitive inhibition by L-SOP.

Figure 7



Log-dose response curve of L-glutamate (left panel) on the stimulation of [^{35}S]-GTPγS binding to CHO cell membranes expressing human metabotropic glutamate receptors subtype 2 (hmGluR$_2$) and inhibition (right panel) of the effect of a fixed L-glutamate concentration (4 µM) by L-SOP. The data are expressed in counts per minute (CPM), each point representing the mean of triplicate observations. Data analysis yielded pA2 value of 5.16 with a Schild slope not different from unity (1.02).

Reference

1. Lazareno, S., T. Farries, and N. J. M. Birdsall. 1993. Pharmacological characterization of guanine nucleotide exchange reactions in membranes from CHO cells stably transfected with human muscarinic receptors M1 - M4. *Life Sci.* 52:449-456.

Notes

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