Communication

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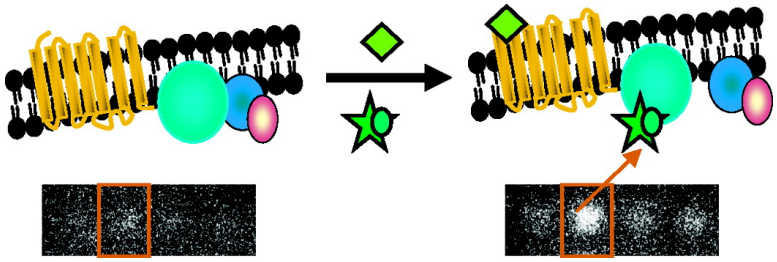

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This paper describes G-protein-coupled receptor (GPCR) microarrays on porous glass surfaces and functional assays to monitor their activation.

GPCRs, membrane spanning proteins whose activation influences nearly every aspect of cellular physiology, are the most ubiquitous class of drug targets.[1] "Functional" GPCR microarrays, if realized, could potentially enable the discovery of agonists, antagonists, or inverse agonists and their selectivity and safety profiles in the same assay.[2] Developing functional protein arrays is challenging. There has to be a sufficient fraction of GPCRs complexed to the G-protein upon immobilization, conformational changes in the immobilized receptor following activation must be enabled,[3] and both the extracellular ligand binding domain of the GPCR and the intracellular GTP binding domain of the GPCR−G-protein complex have to be accessible.

The first step in the activation of GPCRs is exchange of GDP for GTP at the G_α subunit of the trimeric G-protein ($G_{\alpha\beta\gamma}$) complexed to the receptor; this nucleotide exchange is followed by dissociation of GTP•G_α from the GPCR-$G_{\beta\gamma}$ complex and binding to downstream effectors, such as adenylate cyclase.[2] This activation is short-lived because the GTP bound to the G_α hydrolyzes back to GDP in a matter of seconds. Non-hydrolyzable analogues of GTP (GTP_{nh}) have been developed;[4] these analogues enable the convenient monitoring of GPCR activation by observation of bound fluorescent or isotope-labeled GTP_{nh}. We chose europium-labeled GTP_{nh} (Eu-GTP). Compounds labeled with europium chelates exhibit a large Stokes shift and have long fluorescence lifetimes, which can be used to effectively discard extraneous fluorescence and enable lower detection thresholds.[5] A moderate-power, CW argon laser emitting at 351 nm was employed to pump the Eu-GTP; the emitted fluorescence was captured on an intensified CCD detector time-gated to integrate the fluorescence beginning ∼100 μs after the pump pulse. The instrument was configured for reading arrays printed on slides at a resolution of ∼10 μm.

We have previously reported ligand binding assays on GPCR microarrays printed on flat glass substrates coated with γ-aminopropylsilane (GAPS).[6,7] The binding constants of ligands estimated using these assays were consistently similar to those reported in the literature. Since ligand affinity is effected by the extent of G-protein coupling to the receptor,[2] the results suggested that GPCR−G-protein complexes were conserved upon immobilization in the microarray. Functional assays using Eu-GTP on these flat surfaces were, however, unsuccessful. We rationalized that the simultaneous accessibility of the ligand to the N-terminal domain of the GPCR and the Eu-GTP to the G_α subunit of the GPCR−G-protein complex was hindered on the flat substrate. We hypothesized that porous, three-dimensional substrates may lead to supported membranes with access to both sides of membrane-bound protein complexes. We decided to test porous glass substrates[8] because of the ability to derivatize the glass surface with silane chemistries;[6] bare and derivatized glass have been extensively used as substrates for supported membranes.[9] The porous frit also offers very high surface area and, therefore, presents the capacity for greater amounts of immobilization relative to flat substrates.

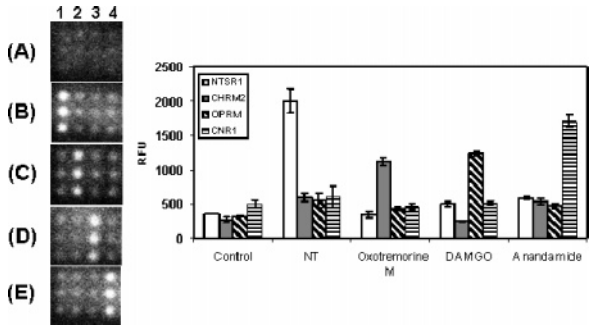

Figure 1. Images based on agonist-induced europium fluorescence that demonstrates the functional activation of GPCR microarrays. The microarrays (from *left* to *right*) consist of the NTSR1 (**1**), CHRM2 (**2**), OPRM (**3**), and CNR1 (**4**) receptors, printed in triplicate. (A) Fluorescence image of the microarray exposed to buffer (50 mM HEPES (pH 7.4) containing GDP (3 μM), $MgCl_2$ (5 mM), NaCl (100 mM), saponin (0.1 mg/mL), and Eu-GTP (10 nM)). (B) Image of the microarray exposed to buffer containing neurotensin (1 μM). (C) Image of the microarray exposed to buffer containing oxotremorine M (10 μM). (D and E) Images of the microarray exposed to buffer containing DAMGO (10 μM) and anandamide (1 μM), respectively. The histogram on the right shows the fold changes in fluorescence for the receptors upon exposure to the different agonists.

We fabricated porous glass slides by casting a frit consisting of crushed and milled borosilicate glass powder onto an impermeable calcium aluminosilicate glass slide followed by sintering at ∼700 °C.[8] The porous layers obtained were 15−20 μm thick with a mean pore size of ∼570 nm. Slides containing segregated porous patches were obtained by manual scraping of the porous frit down to the impermeable support using a blade. These slides were then coated with GAPS. Printing of the GPCR microarrays was accomplished using quill pins as described previously.[6]

We fabricated GPCR microarrays consisting of the neurotensin receptor 1 (NTSR1),[10] the cholinergic receptor muscarinic 2 (CHRM2),[11] the opioid receptor mu (OPRM),[12] and the cannabinoid receptor 1 (CNR1).[13] These receptors have important neurophysiological roles and have been implicated in a variety of disorders ranging from Parkinson's disease to addiction. The receptors are coupled through the G_α proteins, $G_{i\alpha}$ or $G_{q\alpha}$, which are well suited for GTP_{nh} assays.[14] The GPCR microarrays were incubated for 1 h in buffer containing GDP (3 μM) and Eu-GTP (10 nM), with or without an agonist. Excess GDP shifts the GDP−GTP equilibrium at the G_α subunit and helps reduce basal fluorescence. Figure 1 shows images of these arrays exposed to different ligands. Figure

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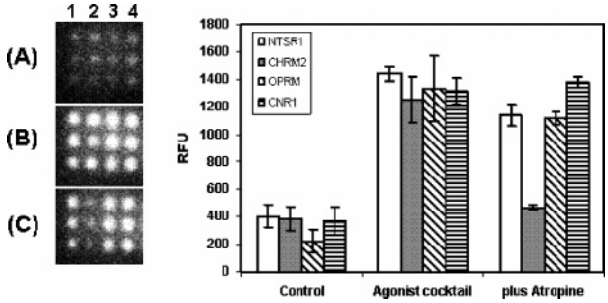

Figure 2. Fluorescence images of GPCR microarrays demonstrating the screening of antagonists using a cocktail of agonists. (A) Image of a microarray consisting of NTSR1 (**1**), CHRM2 (**2**), OPRM (**3**), and CNR1 (**4**) exposed to a solution containing buffer (50 mM HEPES (pH 7.4), GDP (10 μM), $MgCl_2$ (5 mM), and saponin (0.1 mg/mL)). (B) Image of the microarray exposed to the cocktail of agonists. The solution of agonists in buffer contained neurotensin (1 μM), oxotremorine M (10 μM), DAMGO (10 μM), and anandamide (10 μM), which are cognate agonists to NTSR1, CHRM2, OPRM, and CNR1, respectively. (C) Image of the microarray exposed to the agonist cocktail and atropine (10 μM); the selective inhibition of fluorescence for the CHRM2 receptor is observed (see histogram).

1B shows an image of the microarray incubated with neurotensin, the physiological agonist for NTSR1. A comparison of Figure 1Aand B shows a 5.7-fold increase in the fluorescence signal for NTSR1, demonstrating the selective activation of the receptor. Images C−E of Figure 1 show the selective activation of the CHRM2, OPRM, and CNR1 receptors by oxotremorine M,[15] DAMGO,[12] and anandamide,[13,16] respectively, which are known agonists for these receptors.

To demonstrate the use of these microarrays for detecting antagonists, we first tested whether a cocktail of the agonists above would lead to the activation of all the receptors. A comparison of Figure 2A and B shows the functional activation of all GPCRs by this cocktail. In Figure 2C, the microarray was incubated with the agonist cocktail and atropine; a selective decrease in fluorescence for CHRM2 is observed. Atropine is a known antagonist against muscarinic receptors,[17,18] but does not discriminate between the different subtypes (CHRM1−CHRM5). These results demonstrate the feasibility of multiplexed profiling of potential antagonists. Antagonist screening against a GPCR requires an agonist unless the GPCR is constitutively active; correspondingly, the use of microarrays of constitutively active receptors may enable ligand independent screening.[19]

The 3−6-fold activation observed for the receptors is encouraging and suggests the feasibility of titration experiments to estimate binding and inhibition constants. Figure 3A shows the increase in fluorescence for a CHRM2 array as a function of the concentration of oxotremorine M. From these data, we estimate $EC_{50} \sim 53$ nM, in agreement with the literature.[15] Figure 3B shows the decrease in fluorescence for CHRM2 as a function of atropine concentration at a fixed concentration of oxotremorine M. The estimated IC_{50} ($\sim$12 nM) is also in agreement with literature values.[17,18,20]

Multiplexed GPCR screening is important for the same reasons that make GPCRs highly "drugable"—their potent physiological roles and location on the cell surface. In traditional drug discovery, combinatorial libraries are only screened against the target GPCR, and information about the selectivity profile of the identified "hits" against other GPCRs is obtained much further downstream. Functional GPCR microarrays could potentially streamline drug discovery by helping integrate primary screening with selectivity and safety screening,[21] without compromising the essential functional information that is obtained using conventional cell-based assays.[22] The ability to reproduce biological signaling on an artificial substrate is fundamentally interesting and significantly increases the scope of functional assays realizable using protein arrays.[23,24]

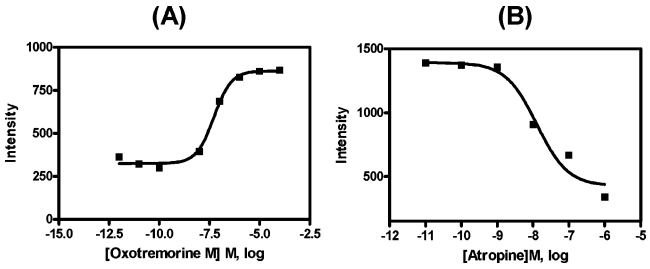

Figure 3. Estimations of EC_{50} and IC_{50} using GPCR microarrays. Microarrays of NTSR1, CHRM2, OPRM, and CNR1 were exposed to solutions containing different amounts of oxotremorine M, the cognate agonist for CHRM2. (A) Plot of the increase in fluorescence (at the CHRM2 receptor) as a function of the concentration of oxotremorine M; $EC_{50} \sim 53$ nM. (B) Plot of the decrease in fluorescence for CHRM2 with increasing concentrations of atropine, at a fixed concentration of oxotremorine M (10 μM); $IC_{50} \sim 12$ nM.

Note Added after ASAP Publication. The Amgen Inc. contribution was omitted in the version published ASAP on September 20, 2005. The complete list of authors was published ASAP on October 12, 2005; additionally, the GDP concentration was corrected in the fourth sentence of paragraph 6.

Supporting Information Available: Details of the imager, substrate and array fabrication, and assay protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Wise, A.; Gearing, K.; Rees, S. *Drug Discovery Today* **2002**, *7*, 235−246.
(2) Haga, T.; Berstein, G. *G Protein-Coupled Receptors*; CRC Press: Boca Raton, FL, 1999.
(3) Bissantz, C. *J. Recept. Signal Transduct. Res.* **2003**, *23*, 123−153.
(4) Valenzano, K. J.; Miller, W.; Kravitz, J. N.; Samama, P.; Fitzpatrick, D.; Seeley, K. *J. Biomol. Screen.* **2000**, *5*, 455−461.
(5) Diamandis, E. P.; Christopoulos, T. K.*Anal. Chem.* **1990**, *62*, 1149A−1157A.
(6) Fang, Y.; Frutos, A. G.; Lahiri, J. *J. Am. Chem. Soc.* **2002**, *124*, 2394−2395.
(7) Fang, Y.; Lahiri, J.; Picard, L. *Drug Discovery Today* **2003**, *8*, 755−761.
(8) Tanner, C. W.; Tepesch, P. D.; Wusirika, R. R.; Corning Incorporated: U.S. Patent 6,750,023 B2, 2003.
(9) Groves, J. T.; Boxer, S. G. *Acc. Chem. Res.* **2002**, *35*, 149−157.
(10) Kitabgi, P. *Curr. Opin. Drug Discovery Dev.* **2002**, *5*, 764−776.
(11) Eglen, R. M.; Choppin, A.; Dillon, M. P.; Hegde, S. *Curr. Opin. Chem. Biol.* **1999**, *3*, 426−432.
(12) Goldstein, A.; Naidu, A. *Mol. Pharmacol.* **1989**, *36*, 265−272.
(13) Buttarelli, F. R.; Patacchioli, F. R.; Palladini, G.; Brunetti, E.; Pontieri, F. E. *Curr. Neuropharmacol.* **2004**, *2*, 1−7.
(14) Harrison, C.; Traynor, J. R. *Life Sci.* **2003**, *74*, 489−508.
(15) DeLapp, N. W.; McKinzie, J. H.; Sawyer, B. D.; Vandergriff, A.; Falcone, J.; McClure, D.; Felder, C. C. *J. Pharmacol. Exp. Ther.* **1998**, *289*, 946−955.
(16) Khanolkar, A. D.; Makriyannis, A. *Life Sci.* **1999**, *65*, 607−616.
(17) Volz-Zang, C.; Waldhaeuser, T.; Schulte, B.; Palm, D. *Eur. J. Clin. Pharmacol.* **1995**, *49*, 45−49.
(18) Ford, A. P. D. W.; Levine, W. B.; Baxter, G. S.; Harris, G. C.; Eglen, R. M.; Whiting, R. L. *Mol. Neuropharmacol.* **1991**, *1*, 117−127.
(19) Chalmers, D. T.; Behan, D. P. *Nat. Rev. Drug Discovery* **2002**, *1*, 599−608.
(20) Daeffler, L.; Schmidlin, F.; Gies, J.-P.; Landry, Y. *Br. J. Pharmacol.* **1999**, *126*, 1246−1252.
(21) Klabunde, T.; Evers, A. *ChemBioChem* **2005**, *6*, 876−889.
(22) Cooper, M. A. *J. Mol. Recognit.* **2004**, *17*, 286−315.
(23) MacBeath, G.; Schreiber, S. L. *Science* **2000**, *289*, 1760−1761.
(24) Zhu, H.; Snyder, M. *Curr. Opin. Chem. Biol.* **2001**, *5*, 40−45.

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