Developing Assays for Screening GPCRs with Transiently Transfected Cells Using the MaxCyte® STX™ System

Introduction

G protein coupled receptors (GPCRs) represent targets for at least one third of drugs that are currently marketed, and they are of central importance to ongoing drug discovery efforts throughout the pharmaceutical industry. GPCRs contain seven membrane spanning domains and are activated by ligand binding or through other external signaling mechanisms that induce conformational changes in the receptors and lead to activation of associated G proteins and downstream signaling pathways. The activity of GPCRs is often monitored by quantifying intracellular levels of second messengers, such as calcium or cyclic AMP (cAMP).

The most common approach to developing a GPCR screening assay involves generating a cell line that stably expresses a particular GPCR on the cell surface. This cell line may also be engineered to express a reporter molecule that allows GPCR signaling to be measured via luminescent or fluorescent detection systems. However, stable cell line production is a costly and labor-intensive process, requiring multiple rounds of selection and clonal isolation, which can delay screening efforts for at least several months.

The MaxCyte STX system offers an attractive alternative to stable cell line development for GPCR screening. Commonly used cell lines, such as CHO and HEK, can be transfected in bulk with GPCR expression constructs alone or in combination with reporter or accessory plasmids. The transfected cells are suitable for immediate use in cell-based assays or they can be aliquoted and cryopreserved for future applications. Up to 1 $x10^{10}$ cells can be transfected at one time in less than thirty minutes using MaxCyte's flow electroporation technology. Optimization of assay conditions is accomplished by performing a series of small scale, static electroporations with varying concentrations of DNA. The DNA concentration that yields optimal results in a small scale transfection can then be used to generate identical assay results with cells transfected via large scale, flow electroporation.

Here we describe an example of cell-based assay development using HEK 293 cells that were transiently transfected with a M1 muscarinic acetylcholine receptor expression plasmid and screened using the FLIPR$^{TETRA®}$ system. The M1 muscarinic receptor is one of five subtypes of muscarinic acetylcholine receptors that are expressed on the surfaces of neurons and other cell types. Muscarinic receptors are frequently used as targets in cell-based assays to identify drugs for treating disorders of the central nervous system. In this experiment, we used a calcium mobilization assay to demonstrate that transiently transfected cells respond robustly in a dose dependent manner to the ligand carbachol. We also show how assay sensitivity can be controlled by varying the concentration of the target expression plasmid that is added to the transfection reaction.

Materials

HEK 293 cells (ATCC CRL1573)
DMEM/F12 (Mediatech 10-092-CM)
Fetal Bovine Serum (FBS) (Mediatech 30-002-CI)
Penicillin-streptomycin (pen/strep) (Mediatech 30-002-CI)
Phosphate buffered saline (PBS)(Lonza 17-516F)
0.25% Trypsin (Mediatech 25-053-CI)
Tissue culture dishes (BD Falcon 353025)
Human M1 muscarinic cholinergic receptor expression plasmid (Origene SC126556)
Carbachol (Sigma, catalog # C4382)
Quest Fluo-8™ Calcium Assay Kit (ABD-Bioquest 2180)
DNase I (Sigma D4263)

Methods

Cell culture:
HEK 293 cells were plated in 150mm dishes and cultured at 37°C with 5% CO_2 in DMEM/F12 supplemented with 10 % FBS, 1% pen/strep. Cells were passaged every 2-3 days and maintained at 50-90% confluency. The cells were split one day

prior to electroporation to ensure that they were healthy and in log phase growth at the time of transfection.

Electroporation:
Cells were harvested for electroporation with trypsin. After neutralizing the trypsin with an equal volume of complete medium, cells were pelleted at 250 X g for 10 minutes, rinsed in MaxCyte electroporation buffer (2-5X final electroporation volume), pelleted again, and then suspended in MaxCyte buffer at a density of $1x10^8$ cells/mL. Cells were then mixed with plasmid DNA (dissolved in sterile, distilled water) and transferred to OC-400 processing assemblies (PAs). After electroporation using the preset "HEK 293" protocol, transfected cells were transferred from PAs into sterile 1.5 mL tubes and placed in a 37°C water bath for 20 minutes DNase I (200 U/mL) was added to the cells during the 20 minute incubation to remove DNA that did not completely enter the cells. Following the 20 minute incubation, cells were diluted into 20 mL of complete culture medium, counted with trypan blue and plated as described below.

Calcium Influx Assays:
Cells were monitored in a calcium influx assay using the FLIPR$^{TETRA®}$ (MDS Analytical Technologies) both 24 and 48 hours post-transfection. For the 24 hour post-transfection assay, cells were plated immediately after counting at 20,000 viable cells per well in a 384-well plate. For the 48 hour post transfection assay, cells were plated in 100 mm dishes, cultured for 24 hours, then harvested and plated in 384 well plates as described above.

The calcium influx FLIPR assay was performed with the Fluo-8 Calcium Assay Kit (ABD-Bioquest) as follows:

1. Media were removed from the wells and replaced with 12.5 µl of Fluo-8 dye. Cells were incubated for 30 minutes at 37ºC.
2. A stock solution of carbachol (1 mM) was prepared in HBPS (HEPES-buffered physiological saline).
3. A dilution series was prepared to create 5X concentrated carbachol solutions (µM): 150, 50, 15, 5, 1.5, and 0.5
4. After the 30 minutes dye incubation, 12.5 µl of HBPS was added to each well.
5. 6.5 µl of 5X carbachol solution was then added immediately to give final test concentrations of (in µM): 30, 10, 3, 1, 0.3, and 0.1.

FLIPR$^{TETRA®}$ Settings:
- Excitation wavelength: 470-495 nm; Emission wavelength: 515-575 nm
- Camera gain: 90
- Exposure time: 0.8 s
- Excitation intensity: 90%
- Dispense speed: 8 µl/s
- Dispense height: 24 µl
- First read interval: 1s, 75 reads, 15 reads before dispense; Second read interval: 5s, 36 reads

Results

HEK 293H cells were transfected with increasing concentrations of M1 muscarinic receptor plasmid DNA, treated with carbachol, exposed to calcium sensitive dye and assayed on the FLIPR$^{TETRA®}$ system. Figure 1A shows that the intensity of the cells' response to carbachol directly correlated with the concentration of plasmid DNA that was added to the transfection reaction. When cells were exposed to increasing concentrations of carbachol, they responded in a dose-dependent manner (Figure 1B). Note that untransfected HEK 293 cells also exhibit an endogenous response to carbachol. However, by increasing the concentration of plasmid DNA in the transfection reaction, the response to carbachol that was mediated through the transiently expressed M1 receptor can be distinguished clearly from the endogenous response.

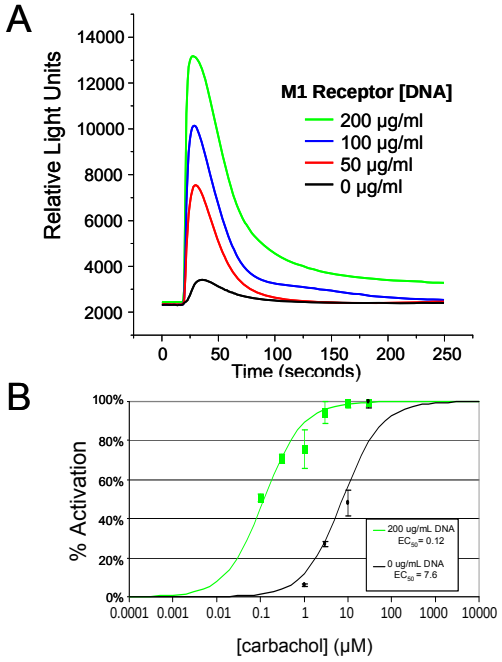

Figure 1. Carbachol-induced calcium mobilization in HEK 293H cells transiently transfected with M1 muscarinic receptor plasmid DNA. A. Untransfected control cells and cells transfected with three different concentra-

tions of M1 muscarinic receptor DNA were treated with 0.1 µM carbachol and assayed on the FLIPR$^{TETRA®}$ as described in METHODS. B. Cells transfected with 200 µg/mL of M1 muscarinic receptor plasmid were treated with increasing concentrations of carbachol and assayed along with untransfected control cells. Error bars indicate the standard error of the mean derived from measurements in eight replicate wells. The EC_{50} value of the transfected cells (0.12 µM) is considerably lower than that of the control cells (7.6 µM), indicating a clear distinction between M1 muscarinic receptor activity and endogenous responses to carbachol.

Summary

- ✓ The MaxCyte STX transient transfection system enables rapid development of cell-based assays to screen GPCRs and other target molecules.
- ✓ Cells transfected with the MaxCyte STX system are suitable for screening with a variety of automated assay technologies, such as the FLIPR$^{TETRA®}$.
- ✓ MaxCyte STX users can exert tight control over assay sensitivity and dynamic range by varying the concentration of plasmid DNA in the electroporation reaction.

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