Tracking G Protein-coupled Receptor Trafficking Using Odyssey® Imaging

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The cannabinoid receptor 1 (CB1) is a classic G protein-coupled receptor (GPCR), with 7 transmembrane domains, 3 intracellular and 3 extracellular loops as well as a long amino terminus and carboxy tail. While events leading to the internalization and recycling of the receptor have been worked out previously using confocal microscopy, many more regulatory elements of CB1 trafficking have yet to be determined. Recently, our lab has developed a novel way of quantifying the trafficking of CB1 to and from the cell membrane using IR fluorescence. With this technology, the detailed molecular mechanisms involved with GPCR trafficking can be elucidated.

INTRODUCTION

It has long been known that many G protein-coupled receptors will internalize and recycle following agonist activation. These trafficking processes may play an important role in cellular desensitization to the effects of specific agonists with repeated or prolonged exposure. The mechanisms underlying internalization and recycling of several types of GPCR have been elucidated. Receptors shown to internalize following specific agonist exposure include the CB1 cannabinoid receptor (Hseih et al., 1999; Jin et al., 1999), the β2-adrenergic receptor (Lefkowitz et al., 1997), the μ-opioid receptor (El-Kouhen, 2001) and the neurokinin 1 receptor (Garland, 1996) among many others (Claing, 2002). Laboratory techniques used to discern the molecular events involved with trafficking include biotinylation of surface proteins (Celis, 1994), flow cytometry (Janeway, 2001) and radioligand binding using impermeant ligands (Siegal, 1999). With the introduction of the LI-COR Odyssey system, it has become possible to perform a highly reproducible and sensitive assay with high throughput to follow receptor internalization. In this study, a HEK293 cell line stably expressing the cannabinoid receptor, CB1, was used to develop such an assay. One requirement for this procedure is that antibodies used for receptor detection be directed toward extracellular epitopes.

MATERIALS AND METHODS

Isolation of HA11rCB1/HEK293 cell line

HEK293 cells were transfected with pHrCB1/pcDNA3.0 using Lipofectamine2000 (Invitrogen, Carlsbad, CA) as per manufacturer's instructions. The CB1 construct contains a preprolactin signal sequence at its 5' end, followed by the HA11 epitope tag. These sequences are in frame with the rat CB1 sequence. Successfully transfected clones were selected using 1 mg/mL G418. Cell lines were confirmed using live cell staining with the monoclonal HA11 antibody (CRP, Berkely, CA) and a rabbit anti-hCB1 antibody produced in our lab.

Tissue Culture

Cells were grown to near 90% confluency on a 10 cm plate. These cells were washed in 1x PBS and trypsinized in 0.25% Trypsin/EDTA (Invitrogen, Carlsbad, CA). Cells were then washed and seeded into a poly-D lysine-coated 96 well plate at approximately

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20,000 cells/well. The cultures were allowed to grow over the next 24-48 hours until they reached a confluency of around 90-95%. A row of HEK293 cells was also included on the plate to provide a background signal for primary antibodies.

On Cell Western

Cells were washed with 1x PBS and covered with 200 µl HBS/BSA (in mM; 130 NaCl, 10 HEPES, 5.4 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 0.2 mg/mL BSA). Drugs used in each assay were diluted in HBS/BSA. Buffer was poured off and plates patted dry on paper towels prior to addition of drug. Samples were incubated at 37°C for allotted time. Media was poured off, plates were patted dry and were quickly placed on ice. Ice cold 4% paraformaldehyed was then added to each well and the plate was incubated at room temperature for 20 minutes. Cells were washed 5x5 minutes with 400 µL 1x PBS. They were then incubated in 150 µL LI-COR blocking solution for 90 minutes at room temperature with gentle rocking. Blocking buffer was removed and cells covered in 40 µL of primary antibody diluted 1:100 in LI-COR blocking buffer. Cultures were then incubated overnight at 4°C with gentle rocking. The next day, wells were washed 5x5 minutes with TBST and were then incubated with 40 µl of secondary antibody (Alexa Fluor® 680 GoatαMouse 1:200, Molecular Probes, Eugene, OR, and/or IRDye™ 800CW DonkeyαRabbit 1:800, Rockland, Gilbertsville, PA). Plates were kept under low light conditions as much as possible following addition of IR-conjugated antibodies. Following a 60 minute incubation at room temperature with gentle rocking, plates were again washed 5x5 minutes with TBST. They were then dried and imaged using the LI-COR Odyssey (169µ resolution, 5 sensitivity, 4.01235 mm offset, medium quality). Integrated intensities were analyzed using Excel (Microsoft Corp., Redmond, WA) and Prism (GraphPad Software, San Diego, CA) software.

RESULTS

Trafficking of G protein-coupled receptors to and from cell surfaces plays an important role in cell sensitivity to the presence of extracellular ligands. We are studying the mechanisms underlying trafficking events on a cellular level to better understand how these events contribute to the desensitization of cells following prolonged or repeated exposure to agonists. In our efforts to track the internalization and recycling of CB1 receptor, we analyzed an alternaltive In Cell Western approach that we call the "On Cell Western" (OCW). The main difference between the two assays is that the cell membranes are not permeablized prior to primary antibody incubation with OCWs.

Preliminary experiments were done to determine if the LI-COR Odyssey would provide enough sensitivity to efficiently detect the subset of receptors in our CB1 cell line located at the cell surface. In an effort to see if this approach would be feasible, antibodies targeted against the extracellular amino terminus of the receptor and the intracellular carboxy tail were used (Fig. 1 and 2).

Results from these experiments showed that OCW signal was typically 5 times higher than background for extracellular antibodies, but was only 2 fold higher for the intracellular L14 antibody (data not shown). Integrated intensity ratios of non-permeablized vs permeablized cells also showed that signal intensity for the intracellularly directed antibody was largely dependent on permeablization of the plasma membrane (Fig. 2).

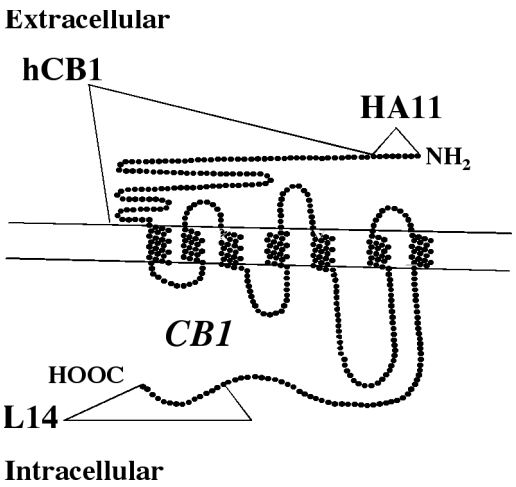

Fig. 1 Antibodies used were targeted against specific extracellular or intracellular domains of the CB1 receptor.

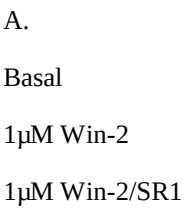

Fig. 2 OCW anti-hCB1 polyclonal antibody directed against the extracellular amino terminus of CB1 displayed signiÞ - cantly more signal than did the intracellular L14 antibody. Data is displayed as integrated intensity ratios of non-permeablized over permeablized signal multiplied by 100.

Studies using confocal microscopy have previously shown that CB1 internalizes following exposure to CB1-speciÞc agonists. In the current study, cells treated with 1 µM of the CB1 agonist,Win-2, show a signi Þcant drop in OCW signal (Fig 3). This effect is negated when the CB1-speciÞc antagonist, SR141716A (1 µM), is also present in the incubation medium (Fig. 3).

Assays were also done using the Odyssey to see if the detected time-course for internalization was consistant with that observed with confocal microscopy (Hseih, 1999). Graphs generated using the data from these experiments agree with previous data and show a similar pattern for internalization using both external antibodies (Figures 4a and 4b). Calculated half-lifes using these antibodies indicate that half of the receptors are internalized in approximately 5.5 minutes.

Finally, the ability of this assay to quantify not only internalization, but also recycling was shown in an experiment in which cells were pretreated for 2 hours with 10 µM cycloheximide to inhibit protein synthesis (Fig. 5). Cells treated in this manner and immediately Þxed showed no discernable drop in signal compared with untreated cells. When agonist was washed out of the cells, and the cells allowed to recover for 2 hours, a recovery of signal was observed with and without cycloheximide present. However, when agonist treatment was prolonged from 15 to 90 minutes, signal recovery was abolished in the cycloheximide-treated cells, indicating that new protein synthesis is required for recycling of the receptor following long-, but not short-term agonist exposure.

Fig. 3 A) Intensity levels were greatly reduced in wells treated with 1µM of the CB1-speciÞ c agonist, Win-2. Cells treated with Win-2 and the speciÞ c CB1 antagonist, SR1 displayed no reduction of signal with the treatment. B) Graph displaying results of three indipendent experiments done in quadruplicate.

DISCUSSION

GPCRs are important therapeutic targets due to the important roles they play in normal and pathological conditions. One potential drawback to using GPCR agonists in drug therapy is the development of tolerance. Tolerance is de Þned as resistance to the effects of a substance through prolonged or repeated exposure to it. GPCR inter-

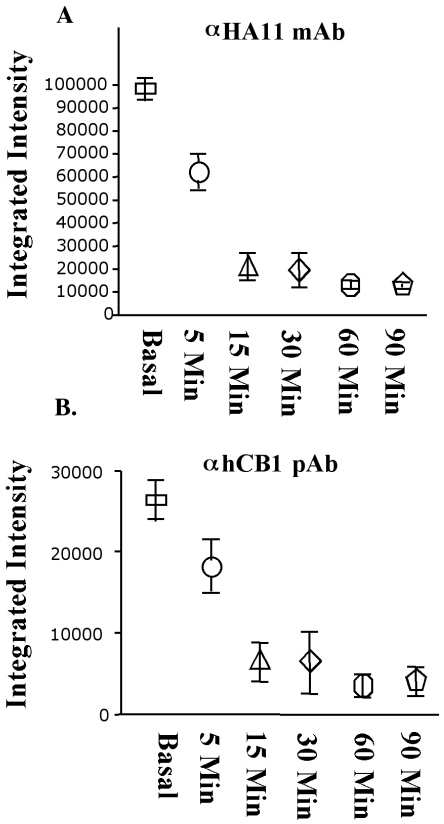

Fig. 4 Cells treated with 100nM Win-2 were probed with either the monoclonal anti-HA11 antibody (A), or the polyclonal anti-hCB1 (B) to generate very similar time courses.

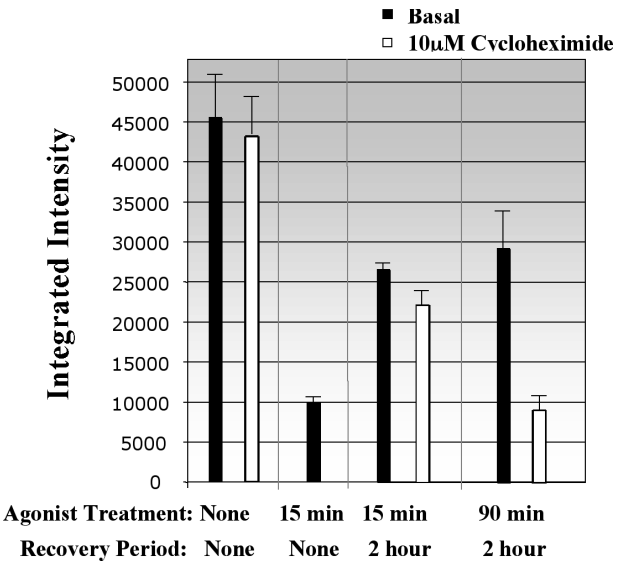

Fig. 5 Cells unable to synthesize protein (white bars) display OCW signal comparable to untreated cells (black bars). Cells treated with Win-2 for a long (90 min) vs a short (15 min) time period require the ability to synthesize protein in order to recover signal.

nalization from cell surfaces may be an important contributor to the development of and recovery from cellular desensitization and tolerance.

In this study, the internalization and recycling of the GPCR, CB1, was successfully detected using near IR dyes (NIR) and an On Cell Western protocol. The platform for these experiments was the Odyssey from LI-COR. This NIR scanner provides an extremely versatile platform from which to analyze many different types of data.

This work shows that OCW can reliably detect the loss of receptors from cell surfaces following agonist treatment. An important requirement to OCW is an antibody directed against extracellular epitopes. Common epitope tags may be readily added to extracellular regions of almost any GPCR using molecular biology techniques. Because antibodies directed against these tags are commercially available, it is relatively simple to produce the molecular tools needed for OCW. Antibodies directed against extracellur epitopes of CB1 displayed a 3-4 fold higher signal when compared to the intracellulary directed L14 antibody (Fig.1). This indicates that cell plasma membranes remain sufficiently intact to exclude the majority of primary antibody under the conditions used for the assay. The differences in signal were even higher between untreated vs agonist-treated cells (Fig. 3).

GPCR trafficking studies can be greatly facilitated using the Odyssey and OCW. Advantages include no radioactivity required, few steps between agonist treatment and signal detection, high sensitivity and a broad linear range. This platform can also be scaled up to a 384-well format for higher throughput making it a good system for deciphering the many molecular events that occur during internalization and recycling of G protein-coupled receptor proteins.

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