The pharmacological characterisation of a GPCR using pH sensitive cyanine dyes on the LEADseeker Cell Analysis System.

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INTRODUCTION

G-protein coupled receptors (GPCR) have been implicated in a number of disease states, and are one of the major targets for drug discovery. In recent years a large amount of effort has been invested in designing assays which will allow the identification of novel ligands at these receptors. Here we describe a novel assay utilising the GPCR's inherent ability to be internalised into the cell's endosomal pathway on agonist stimulation. Using a derivative of our Cy™ dyes which is sensitive to environmental changes in pH, we have labelled the GPCR and monitored agonist-mediated receptor internalisation into the cell, in a homogeneous assay format, using the LEADseeker™ Cell Analysis System.

RESULTS

A novel red-excited pH-sensitive Cy dye has been designed and synthesised. This dye is non-fluorescent at pH 7.4 and is fluorescent at acidic pH, when it is protonated. Figure 1a describes the structure of the dye and its site of protonation, the indolenine nitrogen. Protonation stabilises the cyanine dye (fluorescent) form of the probe (λ_{abs}650nm, λ_{em} 665nm). The basic (deprotonated) form of the probe is non-fluorescent (λ_{abs} 480nm).

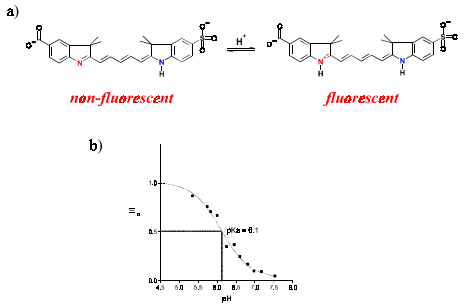

Figure 1. a) The chemical structure of the pH sensitive Cydye™ in neutral and acidic conditions. b) A graph demonstrating the pKa of the pH sensitive Cydye.

Agonist activation of GPCRs almost invariably results in the internalisation of the receptor from the plasma membrane to the endosomal pathway within the cell. By N-terminally tagging a GPCR with an epitope, and labelling an antibody to that epitope with a pH sensitive dye, the internalisation of a GPCR can be monitored within the cell. In this case two cell lines (each expressing a different receptor and epitope) were prepared - CHO (Chinese Hamster Ovary) cells stably expressing a *c-myc*-tagged δ-opioid receptor (Bmax=3709 fmoles bound/mg cell homogenate; Kd=1.5nM) and CHO cells stably expressing a VSV-tagged TRHR (Thyrotropin Releasing Hormone Receptor) – Bmax = 2297 fmoles bound/mg cell homogenate, apparent Kd=49.2nM. The literature apparent Kd value for ^{3}H-TRH binding was 38.0 nM (Drmota *et al* 1998). (Figure 2)

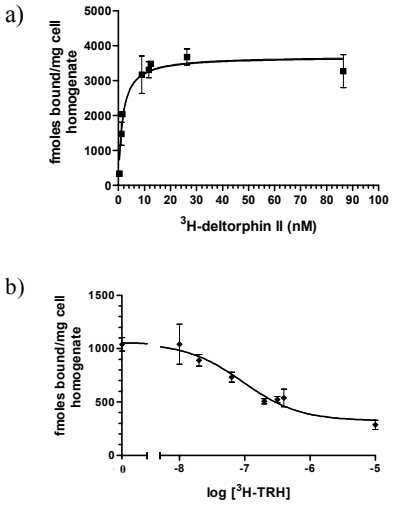

Figure 2 Radioligand binding analysis of CHO cell clones expressing a) c-myc-δ-opioid receptor and b) VSV-TRHR. Binding studies were performed using a) ^{3}H-deltorphin II and b) ^{3}H-TRH. 20µg aliquots of whole cell homogenate were incubated with a) increasing amounts of ^{3}H-deltorphin II +/- 300µM naloxone for 30mins at 30°C and b) 41nM ^{3}H-TRH +/- increasing amounts of TRH (0-10µM) for 60 mins at 30°C. Samples were then harvested using a Skatron Cell Harvester and the filters soaked in Optiphase were counted for ^{3}H. Scatchard analysis was applied to a) and deBlasi analysis (deBlasi et al, 1989) was applied to b) to determine Kd and Bmax values.

Cells expressing the *c-myc*-δ-opioid receptor were preincubated with 0.01mg/ml pH Cy dye-labelled anti-*c-myc* antibody (clone 9E10). This incubation can be performed at either 37°C or ambient temperature. In this case cells were incubated with antibody for 10 mins at room temperature. The agonist, DADLE (D-Ala,D-Leu enkephalin), was then added for 20mins at 37°C and the fluorescence at 633nm excitation was measured, using the LEADseeker Cell Analysis System (figure 3a). A marked increase in intracellular, perinuclear, deep red fluorescence was observed after agonist treatment, and was absent in the absence of agonist. The data were quantified using a proprietary algorithm, which will directly measure the presence and intensity of large perinuclear granules (figure 3b).

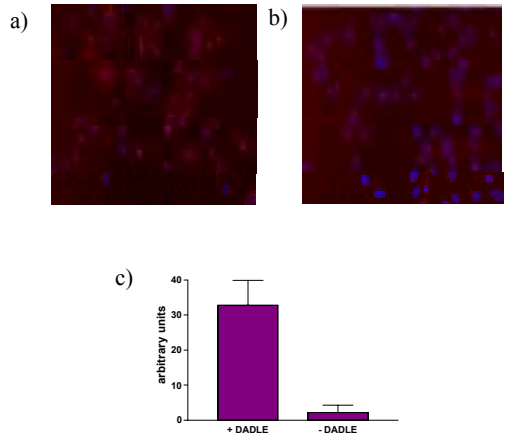

Figure 3 a) c-myc-δ-opioid receptor expressing CHO cells, preincubated with pH Cydye labelled anti-c-myc antibody (red) and 10µM Hoechst nuclear stain (blue), and then treated with a) 100nM DADLE b) no DADLE for 20mins at 37°C. b) Quantification of the pH Cydye (δ-opioid receptor) internalisation.

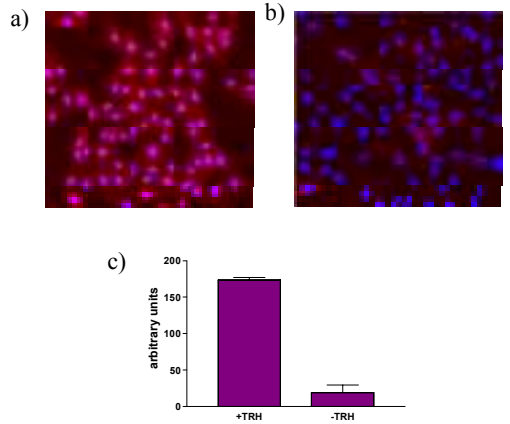

Figure 4 a)VSV-TRHR expressing CHO cells preincubated with pH Cydye labelled anti VSV-G antibody (red) and 10µM Hoechst nulcear stain (blue), and then treated with a)10µM TRH b) no TRH for 30 mins at 37°C. b) Quantification of the pH Cydye (VSV-TRH receptor) internalisation.

Cells expressing the VSV-TRHR were preincubated with 0.01mg/ml pH Cydye-labelled anti-VSV-G antibody (clone P5D4). 10µM TRH was added and these cells were then incubated for 30mins at 37°C. A very marked translocation to the perinuclear recycling endosomes was observed, as well as a large signal increase (figure 4). In another set of experiments the cells were treated with various concentrations of TRH (0-10µM), after they had been preincubated with pH Cydye-labelled antibody. The data were analysed using a proprietary algorithm, and a sigmoidal dose response curve with an EC_{50} value of 0.52nM was observed (Figure 5).

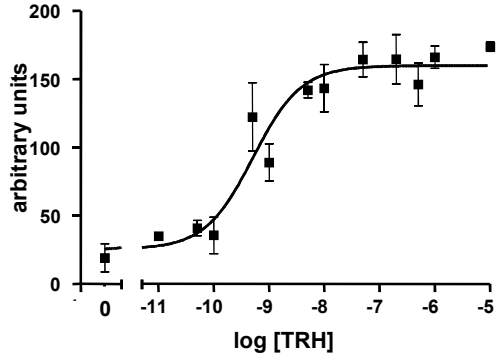

Figure 5 The measurement of pH dye labelled anti-VSV-G antibody internalisation in response to increasing concentrations of TRH (0-10µM), in CHO cells expressing the VSV-TRHR. The experiments were performed as for figure 4. The data were quantified using a proprietary algorithm. The EC_{50} value for this experiment was 0.52nM. A further three identical experiments produced similar results.

References

Drmota T, Gould G W, Milligan G (1998) J Biol Chem 24000-24008
DeBlasi A, O'Reilly K, Motulsky H J (1989) TIPS 10:227-229

CONCLUSIONS

- ***A novel pH sensitive Cydye was conjugated to the extracellular portion of GPCRs via a labelled antibody, and was shown to be an excellent monitor of agonist activation.***
- ***The properties of the pH sensitive Cydye ensure that there is a maximal S:N – the dye is dark at the cell surface and fluoresces in the acidic endosomes.***
- ***The deep red excitation of these dyes make them excellent partners for multiplexing with GFP translocation assays, in high content cellular screening assays.***

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