SIGNAL TRANSDUCTION ASSAY FOR ARACHIDONIC ACID RELEASE FROM CHOM1 CELLS USING CYTOSTAR-T™ SCINTILLATING MICROPLATES*

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Introduction

Muscarinic acetylcholine receptors (M1-M5) play important roles in numerous physiological functions such as the control of heart rate, motor and sensory control, as well as higher cognitive processes. The receptors mediate their diverse intracellular signals via coupling to G-proteins. Depending on the receptor subtype, stimulation can lead to activation of phospholipases A2, C and D, tyrosine kinase, or inhibition of adenylate cyclase[1].

The muscarininic receptor-mediated activation of phospholipase A2 catalyzes the hydrolysis of phosphatidylcholine to generate arachidonic acid, which is released from the cell in the free form, or following metabolic conversion to bioactive eicosanoids such as prostaglandins and leukotrienes[2]. Arachidonic acid release can be used to measure the extent of receptor stimulation[3,4].

An established assay for measuring arachidonic acid release involves the pre-labelling of receptor-expressing cells with [^{3}H]arachidonic acid, which is metabolically incorporated into phospholipids. The agonist-induced stimulation of [^{3}H]arachidonic acid release into the medium is then measured by sampling the medium, and scintillation counting. We have developed an alternative, homogeneous assay using [^{14}C]arachidonic acid and Cytostar-T scintillating microplates. The assay uses CHO cells stably transfected with the muscarinic M1 receptor (CHOM1), with parental CHO cells as a control.

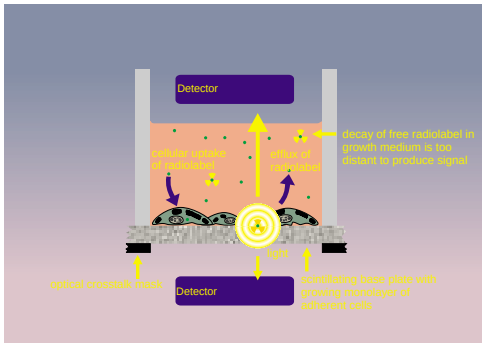

Figure 1. *Cytostar-T scintillating microplate principle*

Cytostar-T scintillating microplates are standard format, 96 well tissue culture treated microplates. The transparent base of each well is composed of a plastic and scintillant mixture, compatible with the cultivation and observation of adherent cell monolayers. Radioisotopes having suitable decay characteristics can be brought into proximity with the scintillant containing base by virtue of the biological processes within the cells, thereby generating light. The signal generated can be measured in real time, and is proportional to the amount of radioisotope within the cell (signal increase assay format). In contrast, the decay of free radiolabel in solution is too distant to produce a significant signal (**Figure 1**). In the alternative signal decrease assay format, which is utilized in this study, the loss of radiolabel from pre-labelled cells can be followed.

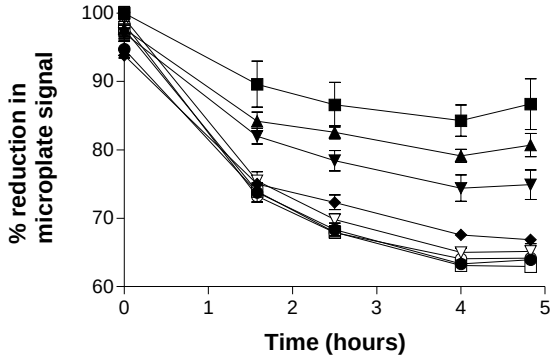

Figure 2. *Release of [^{14}C]arachidonic acid from CHOM1 cells incubated with: (■) 0; (▲) 0.1; (▼) 0.5; (◆) 1; (●) 2; (□) 5; (▽) 10; (○) 100µM McN-A343. Cells were seeded at 1 x10^5 (0.2ml/well) in medium with 0.5µCi/ml [^{14}C]aracidonic acid. Following overnight incubation, the microplate was counted using a Wallac MicroBeta™ counter. Approx. 30,000 microplate counts were measured per well, compared to a background for radiolabel in wells with no cells of <500 microplate counts. The estimated microplate counting efficiency was 30%.*

The medium was replaced with fresh medium containing agonist but no radiolabel. The microplate was counted immediately[1], then regularly for up to 5 hours. Between counts the microplate was returned to the incubator at 37°C. All results in this study are presented +/- SEM (n = 3).

[1] Microplate counts across the plate were reduced by about 10% following the wash step.

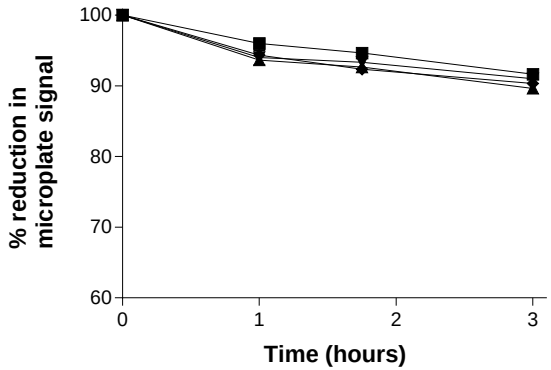

Figure 3. *Release of [^{14}C]arachidonic acid from CHO cells incubated with: (■) 0; (▲) 2; (▼) 10; and (◆) 100µM McN-A343.*

Results

Figure 2 shows the release of [^{14}C]arachidonic acid from CHOM1 cells incubated with the M1 agonist McN-A343. The microplate signal for cells in the absence of agonist was decreased by 10% after 1 hour of incubation, followed by no further reduction in counts. With McN-A343, the counts were further reduced by about 25% over the next 3 hours, with the maximum effect occuring at 2µM agonist and above. The signal reduction was accompanied by the appearance of radiolabel in the medium, as measured by liquid scintillation counting. This labelled material was identified as [^{14}C]arachidonic acid by TLC (results not shown).

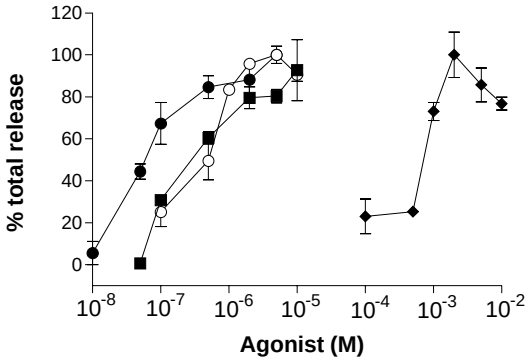

Figure 4. *Effect of agonists on [^{14}C]arachidonic acid release from CHOM1 cells, incubated with: (■) pilocarpine; (○) McN-A343; (●) muscarine chloride and (◆) acetylcholine. Results are calculated from cumulative microplate counts after 3 hours incubation.*

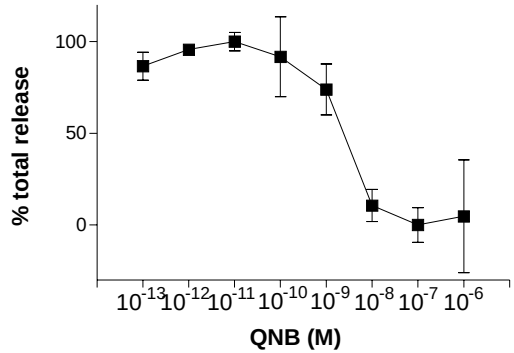

Figure 5. *Effect of quinuclidinyl benzilate (QNB) on release of [^{14}C]arachidonic acid from CHOM1 cells incubated with 2µM McN-A343.*

The incubation of parental CHO cells with up to 100µM McN-A343 caused no decrease in the microplate signal compared to untreated control cells (**Figure 3**). **Figure 4** shows the effect of several agonists on the release of [^{14}C]arachidonic acid from CHOM1 cells. From this data, EC_{50} values of approx. 0.08µM (muscarine chloride), 0.8µM (McN-A343), 0.3µM (pilocarpine) and 0.9mM (acetylcholine) can be estimated. The microplate assay was also used to measure the effect of the muscarinic receptor antagonists QNB (**Figure 5**) and atropine (**Figure 6**), which showed IC_{50} values in the 2 - 10nM range.

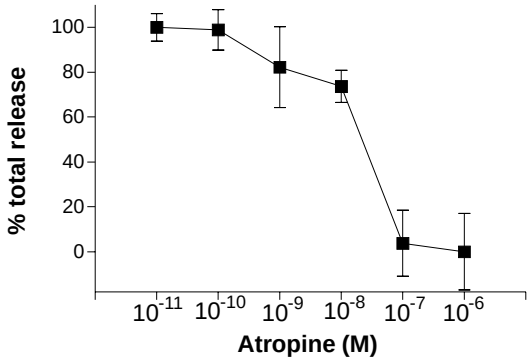

Figure 6. *Effect of atropine on release of [^{14}C]arachidonic acid from CHOM1 cells incubated with 2µM pilocarpine.*

Conclusions

The EC_{50} values obtained with the microplate assay for muscarinic agonists and antagonists are comparable with published results[3,4]. The non-invasive homogeneous assay configuration, ease of microplate counting and multiwell format allows rapid analysis of large numbers of samples. In addition to basic research applications, the Cytostar-T microplate assay may therefore play a useful role in screening for novel agonists or antagonists acting at receptors which signal via arachidonic acid release, or for compounds which act further downstream in the signal transduction process.

References

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