Quantitative Assessment of Agonist-Stimulated Intracellular Accumulation of the Adenosine-A_1 Receptor by High Content Screening

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Considerable debate surrounds the extent to which the adenosine-A_1 receptor (A_1R) internalises in response to agonist (Ferguson *et al*., 2000, Ramkumar *et al.,* 1991). Additional interest in the internalisation mechanism also arises from the differential rates at which the A_1R and closely related A_3R internalise (Palmer *et al.,* 1996). Here, we use automated quantitative analysis of confocal images, to investigate further the extent and pharmacology of agonist-mediated internalisation of the A_1R.

Chinese Hamster Ovary (CHO) cells stably expressing the human A_1R tagged on its C-terminus with green fluorescent protein (GFP; CHO-A_1GFP cells; expression level ~750 fmol/mg protein) were seeded on to black-sided Greiner 96-well view plates and cultured for 48 h to confluence. Cells were incubated with a range of antagonists (30 min, 37℃) before addition of agonist and a further incubation of 90 min at 37℃. For pertussis toxin (PTx) investigations, cells were pre-incubated with PTx (16 h, 100 ng/ml) prior to the addition of agonist. Cells were then washed, fixed and images (4 sites/well) were obtained using an ImageXpress Ultra confocal plate reader. The accumulation of intracellular receptor was quantified using granularity analysis which determined the number and intensity of cellular granules (6-9µm) and normalised these to the nuclear count. Data were fitted by non-linear regression (GraphPad Prism v5.03) and antagonist affinity (pK_B) values estimated from single antagonist concentrations using Gaddum analysis. Timecourses were fitted to a one-site binding hyperbola to obtain $t_{1/2}$ values. All data are presented as mean ± s.e. mean, of *n* separate experiments.

Substantial and rapid accumulation of A_1R in peri-nuclear regions was seen in response to the agonists *N*-ethyl carboxamidoadenosine (NECA; $t_{1/2}$=20.4±1.6min), 2-chloro-*N*-cyclopentyl-2'-methyladenosine (2'-MeCCPA; $t_{1/2}$=18.6±0.3min) and (-)-N^6-(R-phenylisopropyl)adenosine ((R)-PIA; $t_{1/2}$=28.1±0.9min, each n=3). These responses were concentration-dependent, with pEC_{50} values of 5.95±0.08, 5.87±0.05 and 6.51±0.04 (n=27, 10 and 12), respectively and could be competitively antagonised by DPCPX, XAC and CGS15943 with affinities consistent with their action at the A_1R. PTx pre-incubation resulted in partial inhibition of the agonist-stimulated accumulation; producing maximal responses that were 36.1±4.3, 22.4±0.0 and 31.2±3.9% of the response in the absence of treatment for NECA, 2'-MeCCPA and (R)-PIA (n=4, 4 and 3) respectively. Confocal and TIRF microscopy demonstrated that this accumulation was not accompanied by significant receptor clustering or loss of receptor from the cell membrane.

These results demonstrate that the A_1R accumulates in peri-nuclear regions in an agonist-dependent manner, with typical A_1R pharmacology. In contrast to the A_3R however, this response is partially sensitive to PTx, and does not appear to involve substantial receptor clustering or loss from the plasma membrane. Future work will concentrate on determining the mechanism and nature of intracellular accumulation of the A_1R.

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