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Signalling pathway-dependent differences in antagonist affinities at the adenosine-A_1 receptor

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Classic receptor theory states that the affinity of an antagonist for a given receptor should be independent of the method by which it is measured, the signalling-pathway monitored or the agonist used (Baker and Hill, 2007a). More recently, the demonstration of ligand bias at G protein-coupled receptors, suggests that ligand affinities and efficacies may differ depending on the signalling pathway which is measured (Baker et al., 2003b, Lefkowitz et al., 2010). This may therefore depend on which receptor conformation is predominant in a particular assay. Here we show that the measured affinities of two antagonists at the human adenosine-A_1 receptor (A_1R) differ depending on the signalling pathway used to determine them.

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) were used. Binding affinities of the antagonists DPCPX and XAC and the agonist NECA were determined in a whole cell competition binding assay using [^{3}H]DPCPX (1-2nM) as radioligand, and incubating with competing ligand for 2h at 37°C. Affinities were also determined from functional assays by classic Gaddum analysis using NECA as agonist in the presence of a fixed concentration of XAC or DPCPX. For inhibition of forskolin-stimulated [^{3}H]cAMP accumulation, cells were incubated with antagonist (90min, 37°C) before incubation with NECA for 70min in the presence of 3 μM forskolin. For receptor internalisation assays cells were pre-incubated with antagonist (90min, 37°C) before stimulation with NECA (90min 37°C). Internalisation was then quantified by granularity analysis using an Image Express Ultra (Chilvers et al., 2011). Statistical analysis was by one-way ANOVA followed by Tukey's post-hoc test, with 'n' referring to the number of independent experiments performed

Saturation radioligand binding assays in CHO-A_1GFP cells using [^{3}H]DPCPX indicated an expression level of 67.0±4.4fmol/mg protein and pK_D=8.74±0.01 (mean±s.e.m, n=5). Competition binding experiments gave pK_i values for NECA, XAC and DPCPX of 6.13±0.04, 7.44±0.13 and 8.78±0.03, respectively (n=3-5). Quantification of receptor internalisation by means of a granularity analysis revealed substantial, concentration-dependent intracellular accumulation of the A_1R following NECA stimulation (pEC_{50}=5.95±0.08, n=27). This response was competitively antagonised by the antagonists DPCPX (10nM) and XAC (10nM) with affinities (pK_B) of 8.83±0.20 (n=9) and 8.64±0.12 (n=4) respectively; indicating XAC to have a significantly higher affinity in this assay compared to its pK_D value from competition binding ($P<0.05$). NECA also caused a concentration-dependent inhibition of forskolin-mediated [^{3}H]cAMP formation (pEC_{50}=8.83±0.06, n=6). This was also antagonised competitively by DPCPX and XAC (pK_B=7.72±0.06 and 7.93±0.06, n=3, respectively); indicating that in this assay DPCPX showed a lower affinity than observed using radioligand binding ($P<0.05$).

Taken together these results provide initial evidence for signalling pathway-dependent differences in the affinities of the antagonists XAC and DPCPX. Further investigation will reveal whether this is due to sampling of different receptor conformations present in the functional and binding assays used.

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