QUANTIFYING BINDING OF A FLUORESCENT AGONIST TO THE HUMAN ADENOSINE-A1 RECEPTOR IN SINGLE LIVING CELLS

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We have previously described the synthesis of a novel fluorescent agonist for the adneosine-A_1 receptor (A_1-AR), ABEA-X-BY630 (Briddon *et al*, 2002). This compound, based on the known A_1-AR agonist, *N*-ethyl carboxamidoadenosine (NECA), retains agonist activity and allows visualisation of membrane-bound ligand by confocal microscopy. Here, we have used fluorescence correlation spectroscopy (FCS) to quantify both the number and the diffusion characteristics of agonist-receptor complexes in small areas of the membrane of CHO cells expressing the human A_1-AR.

Functional responses to A_1-AR stimulation were assessed in CHO-A1 cells by labelling with either [^{3}H]adenine (cAMP accumulation) or [^{3}H]inositol (inositol phosphate turnover) as previously described (Cordeaux *et al.*, 2000). FCS measurements were performed on a modified Zeiss Confocor 2 at 22±2°C, as previously reported (Briddon *et al.*, 2004). Data are quoted as mean±s.e.mean for the number of experiments indicated. Statistical analysis was by unpaired Student's t-test, with $P<0.05$ taken to indicate statistical significance.

ABEA-X-BY630 retained potent agonist activity in CHO-A1 cells as assessed by both inhibition of forskolin-stimulated cAMP production (pEC_{50}=8.47±0.08; $E_{MAX=}$99±1%, n=4) and inositol phosphate production (pEC_{50}=7.42±0.04, n=3). In both cases, stimulation was through the A_1-AR, since it was antagonised in a competitive manner by the antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 100nM; apparent pK_D=7.85±0.01, n=4 and 8.14±0.16, n=3 for cAMP inhibition and inositol phosphate production, respectively). FCS analysis of ABEA-X-BY630 (50nM, in HEPES-buffered saline, Briddon *et al.* 2004) showed it had a diffusion time of τ_{D3}=71±2μs (n=4). FCS measurements were then performed on the upper membrane of CHO-A1 cells following incubation with ABEA-X-BY630 (5nM, 10 min, 22°C). In addition to the fast-diffusing free ligand component, two slower diffusing components were found (τ_{D2}=9.5±0.7ms and, τ_{D3}=267±27ms, n=47). These two species were receptor-ligand complexes, since pre-incubation with DPCPX (100nM, 30 min, 37°C) significantly reduced the quantity of both (τ_{D2}=8.5±0.9 vs 4.3±0.5, τ_{D3}=14.5±1.6 vs 8.2±0.9 receptors/μm^2, control and DPCPX, n=20 and 25, respectively, $P<0.01$). Diffusion times were not affected by exposure of the cells to DPCPX ($P>0.1$).

FCS therefore allows both the diffusion properties and number of agonist-receptor complexes to be measured in small areas of cell membrane. Further investigation of these complexes will reveal more of the membrane organisation of receptor binding and activation.

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