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Identification of an allosteric site on the adenosine A2B receptor with the fluorescent ligand ABEA-X-BY630

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The HEK293 GloSensor (HEK293G) cell line endogenously expresses adenosine -A2A and -A2B receptors, with the latter being the predominant subtype [Alcock et al, 2010a]. Recently, we have demonstrated that real-time cAMP responses (Promega GloSensor), to adenosine and N-ethylcarboxamidoadenosine (NECA) could not be fully antagonised by the A2B selective antagonist PSB603 (even in the presence of the A2A selective antagonist SCH58261;100nM). In contrast, cAMP responses to a fluorescent analogue of NECA (ABEA-X-BY630;ABEA; [Middleton et al., 2007]) were fully sensitive to antagonism by PSB603 [Alcock et al, 2010b]. These data suggest that PSB603 may have an allosteric mechanism of action. In the present study we have investigated the nature of the binding site of PSB603 on the adenosine A2B receptor using the fluorescent ligand ABEA.

HEK293G cells seeded at $1x10^5$ cells per 32 mm coverslip were left overnight prior to use. Fluorescence was measured on Zeiss LSM 510 laser scanning confocal microscope and HBSS (HEPES Buffered Saline Solution) delivered and removed from coverslip utilising a perfusion system that allows for conditions of infinite dilution as described in [May et al, 2010]. HBSS was delivered in the presence or absence of ABEA (30nM) with or without the addition of PSB603 (1-10µM), the non-selective XAC (10µM), the A2A-selective ZM241385 (10µM), SCH58261 (1µM) or NECA (10-100µM), at 37°C for 10 minutes at a flow rate of at least 4ml/min which reflects approximately 10 complete fluid exchanges per minute. The rate of association (k_{onobs}) and dissociation (k_{off}) of ABEA binding to the HEK293G cell line were best described by two rate constants (fast and slow); $k_{onobs\text{-}fast}$ 3.37±0.73 min^{-1} (17%±3.03), $k_{onobs\text{-}slow}$ 0.47±0.07 min^{-1} (n=48); $k_{off\text{-}fast}$ 1.77±0.34 min^{-1} (28.10%±2.69), $k_{off\text{-}slow}$ 0.21±0.04 min^{-1} (n=14). Following a 3 minute ABEA association, 3µM PSB603 (in the presence of 30nM ABEA) was able to displace ABEA binding, fitting also to a 2-phase dissociation; $k_{off\text{-}fast}$ 2.44±0.88 min^{-1} (23.07%±5.35), $k_{off\text{-}slow}$ 0.37±0.05 min^{-1} (n=5). Under conditions of infinite dilution addition of PSB603 (1-10µM) did not affect the ABEA dissociation kinetics. In contrast following a 3 minute ABEA association XAC, ZM241385, SCH58261 and NECA were unable to significantly displace ABEA binding, however, both XAC and ZM24185 increased the ABEA dissociation kinetics under conditions of infinite dilution; XAC $k_{off\text{-}slow}$ 0.36±0.03 min^{-1} (n=3); ZM241385; $k_{off\text{-}slow}$ 0.40±0.05 min^{-1} (n=4).

The ability of PSB603 but not NECA, XAC or ZM241385 to displace the binding of ABEA suggests that ABEA and PSB603 share an allosteric binding site on the adenosine A2B receptor that is separate from the orthosteric site targeted by NECA, XAC and ZM241385. The influence of orthosteric ligands on the dissociation kinetics of ABEA binding is consistent with this suggestion.

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