Proceedings of the British Pharmacological Society at http://www.pA2online.org/abstracts/Vol10Issue4abst053P.pdf.

Evaluating the Roles of Tyrosine 3.60 and the "DRY" Ionic Lock in β2 Adrenoceptor Internalisation

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Many studies support an "ionic lock" that stabilises the inactive conformation of class A GPCRs (Rovati *et al.*, 2007; Valentin-Hansen *et al.*, 2012). Thus in the β2-adrenoceptor (β2AR), Arg3.50 of the "DRY" motif, at the cytoplasmic end of transmembrane domain (TM) III, has been proposed to form a salt bridge with TM VI Glu 6.30 (Valentin-Hansen *et al.*, 2012). However these direct contacts between TM III and VI residues are not evident for most inactive GPCR crystal structures (Rasmussen *et al.*, 2007; Warne *et al.*, 2008). Instead adrenoceptor structures indicate another residue, Tyr3.60 in intracellular loop 2, might partner either Arg3.50 (β1AR, Warne *et al.*, 2008) or Glu6.30 (β2AR, Rasmussen *et al.*, 2007). Hence this study investigated effects of Tyr3.60, Arg3.50 and Glu6.30 mutants on agonist-stimulated β2AR internalisation, as one indicator of receptor activation.

SNAP-tagged β2AR cDNAs were constructed and stably expressed in HEK293 cells as described (2). Cells on 96 well plates were first labelled with SNAPsurface AF488 (0.1 μM, NEB) to identify β2AR initially at the cell surface (Valentin-Hansen *et al.*, 2012). Agonist treatments (45 min, 37°C) were in HBSS / 0.1% BSA and 5 μg/ml AF633-transferrin (Tf, Invitrogen). Following fixation, images were acquired using an IX Micro platereader (Molecular Devices) and automated image analysis (MetaXpress 2.0) quantified the intensity of labelled β2AR within Tf-identified internal compartments. Individual concentration response curves in triplicate were pooled to obtain pEC_{50} and R_{max} values (Graphpad Prism).

β2AR wild type (wt) and mutants were predominantly cell surface expressed under basal conditions and underwent isoprenaline-stimulated internalisation (10 μM responses (n = 2-6): 1.64±0.10 fold over basal (wt), 1.39±0.05 (Y3.60A), 1.57±0.11 (E6.30A) and 1.38 (R3.50A)). Salbutamol and salmeterol were partial agonists in stimulating β2AR wt internalisation, relative to isoprenaline (Table 1). E6.30A substitution resulted in significantly increased potency and relative R_{max} for all three agonists, and a modest increase in pEC_{50} values was also evident in the R3.50A mutant (Table 1). However compared to wt responses, isoprenaline and salbutamol were 3-7 less potent in stimulating β2AR Y3.60A endocytosis, while salmeterol was inactive (Table 1). Thus contrasting effects of Y3.60A and E6.30A in the internalisation assay support a role for Glu6.30 but not Tyr3.60 in constraining an inactive β2AR conformation. However Tyr3.60 may support active complexes (e.g. with arrestins) necessary for β2AR endocytosis.

Table 1 Summary of β2AR internalisation responses

Receptor	Isoprenaline		Salbutamol		Salmeterol	
	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)
WT	7.43±0.17	100	6.72±0.21	53.5±6.3	7.73±0.41	31.4±18.0
E6.30A	8.53±0.24**	100	7.63±0.20**	88.7±13.4	8.90±0.18*	106±17*
R3.50A	8.10	100	7.17	87.6	8.28	41.8
Y3.60A	6.90±0.11	100	5.90±0.49	28.9±12.7	N.D.	-1.7±10.1

Pooled data (3-6 expts, except R3.50A, n=2). R_{max} is expressed as maximum response relative to 10 µM Isoprenaline at the same receptor. N.D. not determined. *P<0.05, ** P<0.01 compared to wt (Student's t-test).

We thank Louise Valentin-Hansen and Prof Thue Schwartz (University of Copenhagen) for provision of cDNAs. Clare Ashley was an undergraduate Wellcome Trust Vacation Scholar.

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