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Role of the C terminus in neuropeptide Y Y_1 receptor desensitization and internalization[†]

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ABBREVIATIONS: 7TM, 7 transmembrane domain; BSA, bovine serum albumin; C1, Colony 1; CHO, Chinese hamster ovary; GRK, G protein coupled receptor kinase; HEK293, human embryonic kidney 293 cells; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; GTPγS, guanosine-5'-*O*-(3-thio) triphosphate; HA, haemagglutinin; HAY1S* and HAY1T*, HA-tagged Y1 receptor truncated at serine 352 and threonine 361 respectively; NPY, neuropeptide Y; PYY, peptide YY.

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ABSTRACT

We have studied truncation mutants of the rat neuropeptide Y (NPY) Y_1 receptor lacking four (Thr361stop, Y1T361*), or eight (Ser352stop, Y1S352*) potential Ser / Thr C terminal phosphorylation sites. NPY-stimulated haemagglutinin (HA)-tagged Y1, Y1T361* and Y1S352* receptors all efficiently activated G proteins in Chinese hamster ovary (CHO) cell membranes, but desensitization following NPY pre-treatment was only prevented in the HAY1S352* clone. In transfected colonic carcinoma epithelial layers, functional Y1 and Y1T361* peptide YY responses became more transient as the agonist concentration increased, while those mediated by the Y1S352* receptor remained sustained. NPY-stimulated HAY1 receptor phosphorylation was increased by transient overexpression of G protein coupled receptor kinase 2, and only Ser352stop truncation abolished this response in CHO or human embryonic kidney (HEK) 293 cells. Rapid internalization of cell surface HAY1 receptors in HEK293 cells was observed in response to agonist, resulting in partial colocalization with transferrin, a marker for clathrin-mediated endocytosis and recycling. Surprisingly both truncated receptors were constitutively internalized, predominantly in transferrin-positive compartments. NPY increased cell surface localization of HAY1S352* receptors, while the distribution of both mutants was unaltered by BIBO3304. Recruitment of green fluorescent protein (GFP)-tagged β-arrestin2 to punctate endosomes was observed only for HAY1 and HAY1T361* receptors, and solely under NPY-stimulated conditions. Thus the key C terminal sequence between Ser352 and Lys360 is a major site for Y_1 receptor phosphorylation, is critical for its desensitization and contributes to the association between the receptor and β-arrestin proteins. However, additional β-arrestin independent mechanisms control Y_1 receptor trafficking under basal conditions.

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Neuropeptide Y (NPY) related peptides comprise both a neuromodulator (NPY) and hormones (peptide YY (PYY) and pancreatic polypeptide) whose widespread distribution contributes to a diversity of physiological functions. They act in man through four cloned Y receptor subtypes (Y_1, Y_2, Y_4 and Y_5), members of the class A seven transmembrane domain (7TM) receptor family that signal through pertussis-toxin sensitive G proteins (Michel et al., 1998). Of these, the Y_1 receptor was the first to be cloned (Krause et al., 1992) and remains the subtype for which the greatest range of selective agonists (Michel et al., 1998), antagonists (e.g. BIBO3304; Wieland et al., 1998b) and knock-out models (Pedrazzini et al., 1998) are available. These multiple approaches have defined important roles for central Y_1 receptors in the feeding response to NPY released from hypothalamic arcuate neurones (Pedrazzini et al., 1998; Wieland et al., 1998b), and NPY-induced anxiolysis (Gibbs et al., 2004). In the periphery this subtype contributes to sympathetic vasoconstriction (Pedrazzini et al., 1998) and to the inhibition of gastrointestinal secretion (Cox and Tough, 2002) by both neuronal NPY and PYY released from colonic endocrine cells. Y_1 receptor activation also has long term consequences, mediating the neuroproliferation of olfactory progenitors (Hansel et al., 2001) and regulating the growth of intestinal epithelial cells (Mannon, 2002).

Although Y receptors activate a very similar repertoire of $G_{i/o}$ linked second messenger and ion channel responses, the regulatory mechanisms defining the extent of this signalling may differ substantially between the subtypes (Michel et al., 1998). For example, Y_1 receptor responses rapidly wane after prolonged agonist exposure whether endogenously expressed (Michel, 1994) or transfected (Gicquiaux et al., 2002; Holliday and Cox, 2003), a process known as desensitization. The Y_1 receptor is also readily sequestered inside the cell when occupied by different ligands (Gicquiaux et al., 2002; Parker et al., 2001; Pheng et al., 2003). In contrast the Y_2 receptor, which also binds NPY and PYY with high affinity, undergoes relatively less

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desensitization and endocytosis (Gicquiaux et al., 2002; Parker et al., 2001). Recently Berglund *et al.* (2003) have provided one explanation for these differences, by demonstrating that agonist-occupied Y_1 receptors interact with the protein β-arrestin2 much more efficiently than the Y_2 subtype. By preventing contact between the stimulated receptor and G protein, the recruitment of β-arrestin family members mediates the desensitization of many 7TM receptors (Gurevich and Gurevich, 2004; Shenoy and Lefkowitz, 2003). β-arrestins also guide receptor internalization via coated pits (through additional binding sites for phosphoinositides, clathrin and accessory proteins such as AP-2), and they participate as molecular scaffolds for protein kinase cascades signalling to the cytoplasm and nucleus (Shenoy and Lefkowitz, 2003). They are the best known of an expanding array of partners that bind 7TM receptors directly, other than heterotrimeric G proteins (Brzostowski and Kimmel, 2001). Understanding how different 7TM receptors regulate these additional interactions is key to defining the full array of signalling pathways available to them, and how the nature of their trafficking influences responses over time.

Since the pioneering studies on the β_2-adrenoceptor (Bouvier et al., 1988), the C terminal region has emerged as a crucial domain governing the binding of many 7TM receptors to β-arrestins (Braun et al., 2003; Kisselev et al., 2004; Neuschafer-Rube et al., 2004) and other regulatory proteins (e.g. Xiang et al., 2002). However, little is known about the molecular determinants that dictate the desensitization and trafficking of different Y receptor subtypes. Here we address the regulatory role of the Y_1 receptor C terminal domain by comparing the full length receptor with two truncated mutants. We identify a key phosphorylated motif that is essential for its desensitization in biochemical and functional assays, and for β-arrestin2 binding. Our findings also reveal regulation of constitutive Y_1 receptor internalization by an arrestin-independent mechanism.

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Experimental Procedures

Materials. cDNAs for the rat Y_1 receptor and β-arrestin2 tagged with green fluorescent protein (GFP) at the N terminus were kindly provided by Professor T. Schwartz (Laboratory for Molecular Pharmacology, Copenhagen, Denmark); G protein coupled receptor kinase 2 (GRK2) and GRK2(K220R) cDNAs were gifts from Professor S. Nahorski (University of Leicester, UK). Molecular biology reagents were purchased from New England Biolabs (Hitchin, UK), Roche Molecular Biochemicals (Lewes, UK) or Qiagen (Crawley, UK), with the exception of vectors pCruz-HA (Santa Cruz Biotechnology, Santa Cruz, CA) and pGEM-T (Promega, Southampton, UK). The rat monoclonal antibody against the hemaggluttin (HA) epitope (clone 3f10) was obtained from Roche, while transferrin-Texas Red and fluorophore conjugated secondary antibodies were from Molecular Probes Invitrogen (Paisley, UK). Cell culture reagents were sourced as follows: Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen, Paisley, UK), DMEM: Ham's F12, G418 sulfate and L-glutamine (Sigma-Alrich, Poole, UK), fetal bovine serum of Australian origin (Invitrogen), trypsin (Lorne Laboratories, Reading, UK) and other antibiotics (MP Biomedicals, Oxford, UK). [^{125}I]PYY (2200 Ci/mmol) and [^{35}S]guanosine-5'-*O*-(3-thio) triphosphate (GTPγ[^{35}S], 1250 Ci/mmol) were supplied by Perkin Elmer Life Sciences (Boston, MA), and both were stored in single use aqueous aliquots at –20°C; $H_3{}^{32}PO_4$ (10 mCi/ml) was from Amersham Biosciences (Little Chalfont, UK). Peptides, stored as frozen aliquots of aqueous solution, were from Bachem (Merseyside, UK); UK14,304 (5-Bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine, Sigma) was prepared as a 10 mM stock solution in dimethylsulfoxide. BIBO3304 ((*R*)- N^2-(diphenylacetyl)-*N*-[(4-(aminocarbonylaminomethyl)phenyl)methyl]-argininamide) and piretanide were gifts from Boehringer Ingelheim Pharma KG (Biberach, Germany) and Hoechst Marion Roussel (Swindon,

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UK) respectively. Calphostin C was purchased from Calbiochem (Nottingham, UK) and other reagents were from Sigma or VWR International (Poole, UK).

Truncated Y_1 receptor constructs. Site directed mutagenesis was performed with the Transformer mutagenesis kit (Clontech, Palo Alto, CA), using the rat Y_1 receptor cDNA cloned into pGEM-T as a template (Holliday and Cox, 2003) and the following primers (with bold mutated nucleotides and underlined diagnostic restriction sites as indicated) to introduce stop codons in place of Ser352 (5'-GACTATAGCCATGT**G**<u>**A**A**G**C**T**T</u>GCATACGGACGTG-3', *Hind III*) and Thr361 (5'-CGGACGTGTCCAAG**T**<u>**AA**T**A**TT</u>TGAAGCAGGCAAGCCCG-3', *Ssp I*). The *BstX1 / KpnI* fragment containing the substitutions was excised and ligated into mammalian expression vectors pTEJ8-rY_1 (which places the native Y_1 receptor cDNA under the control of the ubiquitin C promoter), and pCruz-HAY_1, in which the rat Y_1 receptor cDNA is modified to include an N terminal HA epitope (YPYDYPDVA), as described (Holliday and Cox, 2003). These cDNA constructs were each verified by double stranded sequencing.

Cell culture. Cells were maintained in DMEM containing 10 % fetal bovine serum (HEK293 and HCA-7 Colony 1 adenocarcinoma cells, a gift of Dr. S. Kirkland, Imperial College London, UK; Marsh et al., 1993) or DMEM: F12 supplemented with L-glutamine (200 mM) and 10 % heat-inactivated fetal bovine serum (Chinese hamster ovary (CHO) K1 cells, kindly provided by Professor S. Hill, University of Nottingham, UK) as described previously (Holliday and Cox, 2003). Transfections were performed by calcium phosphate co-precipitation and glycerol (15 %) shock, followed by selection, as appropriate, in 0.8 mg/ml G418 sulfate for 7 – 10 days. Individual stable Colony 1 clones (transfected with untagged Y_1 receptors) were isolated directly and screened for Y_1 receptor function in I_{SC} measurements. Single CHO and HEK293 clones (HA-tagged receptor constructs) with similar levels of [^{125}I]PYY binding were generated by limiting dilution.

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[^{125}I]PYY and GTPγ[^{35}S] binding. These assays are described in detail in Holliday and Cox (2003). Briefly, competition [^{125}I]PYY binding experiments were performed by incubating fresh membrane preparations (120 min, 21°C) in buffer (pH7.4) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 8 mM, KCl 4 mM, $NaHCO_3$ 20 mM, $MgSO_4$ 1.0 mM, K_3PO_4 1.0 mM, $CaCl_2$ 2.0 mM, bovine serum albumin (BSA) 0.2 % (HEK293, CHO) or 0.4 % w/v (Colony 1), and bacitracin (0.1 mg/ml). Radioligand concentration was 10 pM (CHO) or 25 pM (Colony 1), and bound [^{125}I]PYY was separated by filtration. Saturation assays in duplicate (CHO, HEK293 clones) measured [^{125}I]PYY binding at a range of concentrations (0.01 – 5 nM, diluting the specific activity 20 fold with unlabelled PYY), determining non-specific binding in the presence of 1 μM BIBO3304.

Measurements of GTPγ[^{35}S] binding to CHO membrane aliquots (15 – 30 μg protein) were performed in triplicate. In desensitization experiments cells were incubated with or without 100 nM NPY in DMEM for 10 min at 37°C, and washed extensively (2x DMEM, 3x PBS) prior to membrane preparation. The optimized assay was performed in 10 mM HEPES buffer (pH7.4) containing 50 or 100 mM NaCl, 10 mM $MgCl_2$, guanosine diphosphate (GDP, 1 or 10 μM), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 % BSA and 0.1 mg/ml bacitracin. Following preincubation (90 min, 21°C) with or without agonist and / or antagonist, GTPγ[^{35}S] (200 pM) was added for 20 min, before termination of the reactions by filtration. Filters were dried before addition of scintillant (Ultima Gold MV, Packard Instruments, Berkshire, UK), and after 24 h the retained radioactivity was determined in a beta counter.

Measurement of anion secretion in epithelial layers. Epithelial layers of Colony 1 transfected clones grown on permeable filters (area 0.2 cm^2) were mounted in Ussing chambers, bathed in oxygenated (95% O_2 / 5% CO_2) Krebs-Henseleit solution (composition in mM: NaCl 118,

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KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5 and glucose 11.1; pH 7.4) at 37ºC and voltage clamped at 0 mV (WP Instruments, Florida, USA) as described (Holliday and Cox, 2003). Cell responses to vasoactive intestinal polypeptide (VIP), PYY and other agents were measured continuously as changes in short-circuit current I_{SC}, equivalent to the net electrogenic ion transport across the epithelial barrier.

Detection of phosphorylated receptors. For phosphorylation analysis CHO or HEK293 clones were seeded in 6 well plates, and where appropriate they were also transiently transfected with GRK2 or GRK2(K220R) cDNAs (10 µg / well) the next day, 48 h prior to the experiment. On reaching 80 % confluence, cells were loaded with 50 µCi H_3PO_4 in phosphate-free Krebs buffer for 1 h at 37°C. Calphostin C (0.5 µM) was included in the buffer for the final 30 min of the loading period where necessary. Peptides and BIBO3304 were subsequently added for the times specified in the text. After two washes in ice-cold phosphate buffered saline, cells were disrupted in immunoprecipitation buffer (RIPA; 50 mM Tris, 100 mM NaCl, 50 mM NaF, 10 mM $Na_4P_2O_7$, 5 mM EDTA, 1 % Nonidet P40, 0.5 % sodium deoxycholate, 0.1 % SDS, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, 200 µM activated Na_3VO_4, 100 nM okadaic acid, 10 µg/ml leupeptin and aprotinin; pH 8.0) by repeated passage through a 21 G needle. The solubilized extracts were rotated end over end for 2 h at 4°C, clarified by centrifugation (20,000 g, 15 min, 4°C) and equalized for protein content (BCA protein assay, Pierce, Cheshire, UK). Immunoprecipitations were carried out overnight at 4°C by addition of directly-conjugated anti-HA agarose (clone 3f10), and then the precipitates were washed (4°C, 15 min, rotating) twice with RIPA buffer, twice with high salt wash buffer (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.1 % Nonidet P40, 0.05 % deoxycholate; pH 8.0) and once with low salt wash buffer (50 mM Tris, 5 mM EDTA, 0.1 % Nonidet P40, 0.05 % deoxycholate; pH 8.0). Samples containing equal numbers of receptors were denatured in Laemmli loading buffer (80°C, 3 min), and resolved by

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SDS-polyacrylamide gel electrophoresis (PAGE; 10 % Tris-HCl Ready Gels; Biorad, Hemel Hempstead, UK). The dried gels were exposed to pre-flashed Amersham Hyperfilm MP for 24 – 72 h at –70°C to detect [^{32}P] labelled proteins. Proteins immunoprecipitated under the same conditions were also probed on Western blots with Y_1 antibody CT/2 (a gift of Prof. A. Beck-Sickinger, University of Leipzig, Germany; Wieland et al., 1998a) or Anti HA (both detected by horseradish peroxidase conjugated secondary antibodies and enhanced chemiluminescence) to ensure equal receptor loading. The relative density of bands on scanned Western blots or autogradiographs was analysed by Scion Image (version 4.0, Scion Corporation, Frederick, MD).

Immunofluorescence microscopy. HEK293 clones were first grown to 50 – 70 % confluence on poly-L-lysine coated glass coverslips. Coverslips were transferred to humidified 35 mm Petri dishes and treated for 1 h in serum-free DMEM at 37°C, before live labelling with anti-HA antibody (8 µg/ml, 30 min at 37°C) in serum-free DMEM /1 % BSA) in the presence of concanavalin A (0.3 mg/ml) or BIBO3304 (1 µM) as appropriate. Y_1 receptor ligands (with or without transferrin-Texas Red, 1:250) were then added for 5 – 30 min at 37°C. Two washes with ice-cold phosphate buffered saline (PBS) terminated trafficking and cells were subsequently fixed (2 % paraformaldehyde in PBS, 15 min; quenched by PBS-25 mM glycine; 2 x 5 min) and permeabilized (0.075 % Triton X in PBS, 5 min). The primary antibody was visualized with goat anti-rat IgG-Alexa 488 (1:200 in PBS / 1% BSA; 30 min at 21°C). Cells were postfixed in 2 % paraformaldehyde, nuclear DNA stained with 4',6-diamidino-2-phenylindole and coverslips mounted in Mowiol 40-88 (Calbiochem). For studies of β-arrestin2 translocation, HEK293 clones were transiently transfected with GFP-β-arrestin2 cDNA (10 µg / 25 cm^2 flask) 24 h before splitting onto coverslips. After 48 h, cells were treated with 1 µM NPY for 5 – 30 min at 37°C, fixed and mounted as described above.

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Immunofluorescent analysis was performed as described in detail previously (Sunyach et al., 2003). Briefly, a vertical stack of 25 – 30 fluorescent images was acquired digitally on a Zeiss Axiovert 100 microscope (63x oil objective; Omega Optical excitation and emission filter sets), using Openlab 2.0 (Improvision) to direct a piezo *z*-axis drive in 0.2 μm steps. The central 15 images (or 10 for studies of GFP-tagged β-arrestin2 localization) for each fluorophore were then deconvolved to remove out-of-focus light (Openlab) and reconstructed in three dimensions (3.0 μm *z*-section) in Volocity (Improvision).

Analysis. Displacement [^{125}I]PYY binding curves and concentration-response curves in GTPγ[^{35}S] binding studies were analysed as combined data groups from individual triplicate experiments using the program Graphpad Prism v. 3.0 (Graphpad software, San Diego, USA), to yield pIC_{50} or pEC_{50} values ± 1 standard error of the mean (s.e.m.). Total [^{125}I]PYY binding sites (B_{max}) estimates were obtained from single site saturation curves (CHO, HEK293 clones), or from PYY displacements in Colony 1 clones, where B_{max} = TSB x IC_{50} / [L] (TSB is the total specific binding in the absence of agonist and [L] is the free radioligand concentration). Concentration-response curves in electrophysiological studies were constructed from responses to single additions of the desired agonist, and fitted by non-linear regression (sigmoidal dose response of variable slope; Graphpad Prism). Unpaired Student's t tests (two data groups) or one way analysis of variance followed by Bonferroni's post test (multiple comparisons) assessed statistical significance.

Results

The distal Y_1 receptor C terminus does not influence G protein signalling. We constructed two truncation mutants of the HA-tagged rat Y1 receptor, in which Ser352 (HAY1S*) and Thr361 (HAY1T*) were replaced by stop codons (Fig. 1). When stably

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expressed in CHO cells at 4 – 5 pmol/mg, HAY1, HAY1S* and HAY1T* receptors exhibited similar affinities for PYY, NPY and the Y_1 antagonist BIBO3304 in [^{125}I]PYY competition experiments (Table 1). [^{125}I]PYY binding to both wild type and mutant receptors was significantly disrupted by a maximum concentration of 1 μM GTPγS (45 – 60 %; Table 1), a measure of the extent to which high affinity agonist binding is promoted by G proteins (Holliday and Cox, 2003). We also assessed Y_1 receptor-G protein coupling directly using the GTPγ[^{35}S] binding assay. Under buffer conditions (100 mM NaCl, 10 μM GDP) that maximized agonist-induced activation (Fig. 2A-C), basal binding was 77.9 ± 7.3 fmol/mg (n = 6) in CHO-HAY1, 81.6 ± 10.6 fmol/mg (n = 7) in CHO-HAY1S* and 68.9 ± 5.1 fmol/mg (n = 9) in CHO-HAY1T* membranes. NPY concentration-response curves were similar in potency for HAY1 (pEC_{50}: 8.33 ± 0.07, n = 4), HAY1T* (pEC_{50}: 7.66 ± 0.06, n = 4) and HAY1S* receptors (pEC_{50}: 7.98 ± 0.05, n = 4), while in all cases PYY exhibited lower efficacy than NPY, particularly in CHO-HAY1S* membranes. BIBO3304 prevented 1 μM NPY responses in each of the three clones (Fig. 2A - C), and also acted as a weak negative agonist. For example, in buffer containing 50 mM NaCl and 1 μM GDP, BIBO3304 attenuated basal GTPγ[^{35}S] binding to a similar extent in membranes from CHO-HAY1 (pEC_{50}: 8.21 ± 0.41; 100 nM maximum 12.1 ± 2.9 %, n = 3) and CHO-HAY1S352* clones (pEC_{50}: 9.04 ± 0.38; 100 nM maximum 9.0 ± 1.7 %, n = 3).

HAY1S* receptors are resistant to desensitization. We also investigated GTPγ[^{35}S] responses in membranes from cells that had been subjected to a short desensitizing pre-treatment with NPY (100 nM, 10 min at 37°C). When compared with controls that had received vehicle alone, pretreated CHO-HAY1 membranes exhibited a substantial reduction in the maximal 1 μM NPY response (Fig. 2D), with only a small change in the pEC_{50} value (Control 8.71 ± 0.05 vs pretreated 8.36 ± 0.11, n = 5) in buffer containing 50 mM NaCl and 1 μM GDP. The absence of

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an increase in basal GTPγ[^{35}S] binding (Control 216.4 ± 15.6 fmol/mg vs NPY treated 227.4 ± 10.0 fmol/mg, *n* = 5) or an enhanced sensitivity of this binding to BIBO3304 (control 3.8 ± 2.8 % inhibition vs pretreated 3.9 ± 3.7 %, *n* = 5) confirmed that the first agonist addition had been adequately removed. The attenuated NPY responses in pre-exposed membranes were therefore a consequence of the loss of functionally coupled HAY1 receptors. While an identical reduction in maximal NPY response was observed in pretreated CHO-HAY1T* membranes, agonist stimulation mediated by the HAY1S* receptor was much less affected (Fig. 2E and F). Thus these experiments implicated the C terminal region between Ser352 and Thr361 as a critical sequence governing Y_1 receptor desensitization.

Expression of truncated Y_1 receptors in epithelial cells. Three stably transfected clones of the HCA-7 Colony 1 cell line were generated that expressed the wild type Y_1 (C1Y1), Y_1T361* (C1Y1T*) and Y_1S352* receptors (C1Y1S*) at moderate levels (PYY pIC_{50}: 8.16 – 8.33 and B_{max} (in fmol/mg) of 340 ± 120 (C1Y1; *n* = 6), 255 (C1Y1T*; *n* = 2) and 135 (C1Y1S*; *n* = 2) from [^{125}I]PYY competition experiments). As confluent epithelial layers, these cells secrete chloride ions after elevations in intracellular cAMP and Ca^{2+}. Responses are followed in real time by I_{SC} techniques, allowing Y receptor function to be studied at a signalling endpoint (Holliday and Cox, 2003).

Basal parameters varied between clones and the parent Colony 1 cell line (mean resistance range of 39.1 – 66.5 Ω cm^2, initial I_{SC} of 6.5 – 22.7 μA/cm^2; *n* = 72 – 531), as we have previously observed for stably transfected subpopulations (Holliday and Cox, 2003). Each clone also responded to VIP, an agonist which elevates cAMP, with similar pEC_{50} values (8.12 – 8.51; *n* = 3 – 69). Peak responses to VIP (30 nM) were sensitive to the loop diuretic piretanide, indicating that much of the I_{SC} response was generated by electrogenic Cl^- secretion (Table 2 and

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Fig. 3). In contrast to host Colony 1 epithelial layers, in which functional PYY responses are absent (Table 2), basolateral addition of PYY inhibited VIP-stimulated I_{SC} in C1Y1, C1Y1T* and C1Y1S* clones (Fig. 3). PYY pEC_{50} values (derived from pooled single addition concentration response relationships) were 7.70 ± 0.07 (C1Y1; n = 5 – 8), 7.53 ± 0.30 (C1Y1S*; n = 3 – 5) and 7.64 ± 0.33 (C1Y1T*; n = 3 – 8). The maximal responses to 100 nM PYY (Table 2) were significantly lower in both truncated Y_1 receptor clones compared to C1Y1 epithelial layers, as were maximal I_{SC} decreases to 1 μM UK14,304 (an agonist acting at endogenous α_2-adrenoceptors, pEC_{50} range of 6.95 - 7.25 between clones; n = 3 – 5; Table 2). Thus these differences reflected more general variation between the size of G_i-mediated antisecretory responses of individual clones, rather than a specific effect of Y_1 receptor truncation.

A selective analog with high affinity for Y_1 receptors, [Leu^{31}, Pro^{34}]PYY (100 nM; Michel et al., 1998), also decreased VIP-stimulated I_{SC} (e.g. in C1Y1 cells: -11.3 ± 0.9 μA/cm^2, n = 3), while 10 min preincubation with BIBO3304 (300 nM) abolished subsequent 100 nM PYY responses in each clone (n = 3, data not shown). Significant responses to PYY (100 nM) were only obtained when peptide was added to the basolateral reservoir. The small apical responses observed (e.g. in C1Y1: -1.5 ± 0.3 μA/cm^2 and in C1Y1S*: –1.2 ± 0.2 μA/cm^2; each n = 3) did decrease the subsequent sensitivity to a basolateral application (e.g. basolateral 100 nM PYY 10 min after apical in C1Y1S* –2.0 ± 0.6 μA/cm^2 (n = 3); $P < 0.05$ vs control –5.4 ± 0.9 μA/cm^2; n = 5), suggesting that they arose from the stimulation of basolateral receptors after leakage of peptide through the epithelial barrier.

S352* truncation generates a sustained functional Y_1 receptor phenotype.

Increasing concentrations of PYY initiated markedly more transient reductions in VIP-stimulated I_{SC} in C1Y1 cells (Fig. 4A). An identical change to a short-lived PYY time-course was also

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apparent in the C1Y1T* clone (Fig. 4B), but in contrast responses mediated by the Y_1(S352*) receptor remained sustained at the highest PYY concentration investigated (300 nM) and over at least a 10 min period (Fig. 4C). When normalized as a percentage of the peak response, significant differences between C1Y1S* and either C1Y1 or C1Y1T* 100 nM PYY time-profiles were evident within 4 min of agonist addition (following peak reductions at 2 - 3 (C1Y1, C1Y1T*) or 4 min; C1Y1S*) and were maintained thereafter (Fig. 4D). In contrast to the more sustained phenotype of transfected Y_1(S352*) receptors, the time-courses of 1 µM UK14,304 responses in the C1Y1S* clone were essentially identical to those in C1Y1 and C1Y1T* cells, decaying to approximately 50 % of the peak response 10 min after agonist application (Fig. 4E).

HAY1 receptor phosphorylation is prevented by S352 truncation. We next assessed the incorporation of $^{32}P_i$ into HAY1 receptors immunoprecipitated with Anti-HA from solubilized extracts of CHO-HAY1 cells (Table 1) or a stable HEK293 clone (HEK-HAY1; [^{125}I]PYY B_{max} 1.5 ± 0.2 pmol/mg, n = 3). Both host cell types yielded similar results, and only those from HEK293 cells are illustrated.

No phosphorylated proteins were identified in immunoprecipitates from non-transfected HEK293 cells and only a faint specific band of 71 – 100 kD (n = 4) was observed for the HEK-HAY1 clone under basal conditions. This corresponded to the major mature receptor protein detected by Western blotting with either Anti HA or Anti CT/2 (a polyclonal antibody directed against the Y_1 receptor C terminus; Wieland et al., 1998a). When the cells were stimulated by 1 µM NPY before solubilization and immunoprecipitation the labelling intensity of this band was substantially increased (Fig. 5), a response which was rapid (maximal for 1 min agonist treatments) and maintained for longer incubation periods (up to 15 min, data not shown). HA-Y_1 receptors were also phosphorylated after PYY addition (1 µM response at 1 min: 2.0 fold over basal, compared to 3.6 fold for 1 µM NPY in the same 2 experiments), and agonist-induced

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phosphorylation was prevented by 10 min preincubation with 1 μM BIBO3304 (*n* = 2, data not shown).

We also observed agonist-dependent $^{32}P_i$ labelling of HAY1T* receptors in the HEK-HAY1T* clone ([^{125}I]PYY B_{max} 2.0 ± 0.2 pmol/mg; *n* = 3), with 1 min NPY treatment resulting in a predominant band of slightly lower molecular weight than in HEK-HAY1 cell extracts (70 – 89 kD; *n* = 3). However under the same stimulated conditions HAY1S* receptors were not significantly phosphorylated, either in the HEK-HAY1S* clone ([^{125}I]PYY B_{max} 1.2 ± 0.2 pmol/mg; *n* = 3; Fig. 5A) or in CHO-HAY1S* cells (*n* = 2, data not shown).

To investigate the kinases involved in phosphorylating the Y_1 receptor, we transiently transfected cDNAs for GRK2 or its inactive mutant K220R into HEK HAY1 cells. Overexpression of GRK2 substantially enhanced NPY-induced phosphorylation, but expression of GRK2(K220R) failed to attenuate the response (*n* = 2; Fig. 5B). Furthermore the protein kinase C inhibitor calphostin C (0.5 μM) was unable to reduce agonist-stimulated Y_1 receptor phosphorylation (*n* = 2; Fig. 5B).

Constitutive internalization of truncated HA-Y_1 receptors. HAY1 receptor trafficking was investigated in the stable HEK293 clones, which provide a favourable cell type for the study of intracellular GPCR localization by immunofluorescence microscopy. In these experiments (performed at least 3 times independently) we identified only the receptor population that resided initially at the cell surface, by labelling cells with Anti HA (8 μg/ml) prior to agonist treatment, fixation and detection. Using this method we observed no staining in non-transfected HEK293 cells. Addition of transferrin -Texas Red during agonist treatment identified the clathrin-coated internalization and subsequent endosomal pathways employed by its constitutively recycling receptor.

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Under basal conditions HA-Y_1 receptors were located predominantly at the plasma membrane in HEK-HAY1 clone (Fig. 6), although we observed a relatively small degree of constitutive internalization of the receptor attached antibody in some cells. A rapid redistribution to punctate intracellular endosomes occurred on stimulation with NPY or PYY; significant HAY1 receptor internalization was observed at 10 nM agonist, and for short incubation times of 5 min (1 μM NPY; see Supplemental Fig. S1). Intracellular structures labelled with anti HA antibody were distributed throughout the cytoplasm, but became more concentrated close to the nucleus as agonist concentration or treatment time increased. Substantial, although not complete, colocalization with Transferrin-Texas Red labelling was observed as illustrated in the example cell treated with 100 nM NPY in Fig. 6. 1 μM BIBO3304 did not alter HAY1 trafficking alone, but pre-treatment with the antagonist blocked all internalization in response to NPY (Fig. 6). Pre-incubation with concanavalin A (0.3 mg/ml) prevented HAY1 receptor endocytosis and significantly restricted the cycling of the transferrin receptor, with punctate transferrin-Texas Red labelling predominantly at or close to the plasma membrane (data not shown).

In contrast to the cell surface distribution of unstimulated HAY1 receptors, live labelling of either HEK-HAY1T* or HEK-HAY1S* cells with Anti HA resulted in substantial internalization of the antibody under basal conditions (Fig. 6); in each case the pattern of intracellular localization and transferrin colocalization resembled that for HAY1 receptors stimulated by NPY, but was unaffected by 1 μM BIBO3304 (Fig. 6). In the continuous presence of concanavalin A, HAY1T* and HAY1S* receptors labelled with anti HA were exclusively located at the plasma membrane (data not shown). Surprisingly we found that the presence of agonist revealed differences in the behaviour of HAY1T* and HAY1S* receptors. When stimulated with 100 nM NPY for the final 30 min of the HA antibody incubation, the

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predominantly intracellular location of HAY1T* receptors did not change. However significantly more HAY1S* receptors were observed at the cell surface, and this redistribution was prevented by co-incubation with 1 μM BIBO3304 (Fig. 6).

Recruitment of GFP-β-arrestin2 by HAY1 receptors. We transiently expressed GFP-tagged β-arrestin2 in the stable HEK-HAY1, HAY1T* and HAY1S* cells to assess its distribution in unstimulated cells, or those treated with NPY for different periods. Under control conditions, diffuse GFP fluorescence was observed throughout the cytoplasm in all three clones (Fig. 7). 1 μM NPY stimulation for 5 min or 15 min resulted in the appearance of punctate endosomes intensely labelled with GFP in several HEK-HAY1 cells, but after 30 min agonist incubation these structures were less prominent (Fig. 7A). A similar, though less pronounced, change in distribution was observed after NPY activation of the HAY1T* receptor (Fig. 7B). However, no GFP-βarrestin2 translocation could be observed in HAY1S* cells under the same conditions (Fig. 7C).

Discussion

Here we investigated the role of Y_1 receptor C terminus in regulating its G protein signalling, functional responses and intracellular trafficking. We observed that truncation at Thr361 (HAY1T*) or Ser352 (HAY1S*) did not alter HA-tagged Y_1 receptor-G protein coupling, in contrast to a profound reduction in receptor efficacy after mutation of the more proximal palmitoylation site Cys337 (Holliday and Cox, 2003). However desensitization of HAY1S* mutant was specifically inhibited, when compared to either HAY1 or HAY1T* receptors, using two complementary approaches. Only HAY1S* responses (measured as NPY-stimulated GTPγ[^{35}S] binding in CHO clones) were substantially maintained after NPY pre-treatment. In addition stable expression of wild type and truncated Y_1 receptors in Colony 1

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epithelial layers enabled I_{SC} measurement of their antisecretory responses in real-time. Both full length and truncated Y_1 receptors exhibited an expected pharmacology, and loss of the distal C terminus did not cause apical misdirection of the Y_1 receptor, in contrast to truncated lutenising hormone and thyrotropin receptors (Beau et al., 2004). From our functional observations of sided responses we cannot exclude an additional intracellular accumulation of poorly targeted S352* receptors, as observed for the truncated mGluR7a subtype (McCarthy et al., 2001). Strikingly C1Y1S* PYY responses, as an inhibition of VIP-stimulated I_{SC}, remained sustained in the presence of maximal PYY concentrations, in contrast to the transient nature of those from C1Y1 and C1Y1T* clones. We therefore confirmed the effects of S352* truncation on desensitization in an assay where the sequential processes of activation and inactivation can be followed after a single agonist addition (Holliday and Cox, 2003). Furthermore these functional responses represent the culmination of a signalling cascade involving G_i-mediated decreases in cAMP production and a reduction in protein kinase A sensitive basolateral K^+ and apical Cl^- conductances (MacVinish et al., 1993), for which desensitizing mechanisms may exist at several stages (e.g. stimulation of $G_i\alpha$ GTPase activity by regulators of G protein signalling proteins (Roy et al., 2003). Our data clearly demonstrate that, as the first step of this pathway, Y_1 receptor inactivation is key in determining the pattern and duration of the emerging functional response.

We have demonstrated for the first time that Y_1 receptors are phosphorylated in response to agonist, and have shown that S352* but not T361* truncation inhibited this effect. Although Y_1 receptor signalling can lead to protein kinase C activation, a specific inhibitor (calphostin C) did not inhibit its phosphorylation. Moreover the protein kinase C recognition sites in the Y_1 receptor C terminus (S362; Fig. 1) and third intracellular loop (T258) lie outside the critical phosphorylated sequence of four Ser / Thr residues between Ser352 and Lys360 (Fig. 1),

suggesting that the role of this kinase is limited in homologous Y_1 receptor desensitization. On the other hand our results clearly demonstrate that the 7TM receptor kinase GRK2 can phosphorylate the Y_1 receptor. Given that the GRK2 K220R mutant did not prevent agonist-induced responses, an additional contribution of other GRK family members (particularly those that are constitutively membrane associated; Willets et al., 2003) is also possible.

Prior phosphorylation of agonist-occupied receptors by GRKs is a requirement for β-arrestin recruitment, whose specificity is generated by dual domains that recognise the active conformation and phosphorylated sequences (Gurevich and Gurevich, 2004). Several investigations have implicated a short phosphorylated Ser/Thr rich stretch of the 7TM receptor C terminus in controlling β-arrestin binding and desensitization, recent examples including rhodopsin (Kisselev et al., 2004), EP4 prostaglandin (Neuschafer-Rube et al., 2004) and complement C5a receptors (Braun et al., 2003). Despite the relatively promiscuous nature of the β–arrestin recognition domain (Gurevich and Gurevich, 2004), our results also suggest that phosphorylation of the short region between Ser352 and Lys 360 is a minimum requirement for subsequent Y_1 receptor desensitization. However they do not exclude the possibility of sites in the more distal C terminus, or those in the intracellular loops for which earlier phosphorylation of this region is required (Ohguro et al., 1993).

The involvement of β-arrestin2 in Y_1 receptor desensitization was originally suggested by Berglund *et al*. (2003), who measured the interaction between the two proteins on NPY stimulation in bioluminescence resonance energy transfer experiments (association $t_{1/2}$ of 3 – 5 min). We also observed that activated HAY1 and HAY1T*, but not HAY1S* receptors caused the translocation of cytoplasmic GFP-β-arrestin2 to intracellular endosomes. This provides a

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consistent mechanism (inhibition of phosphorylation and β-arrestin recruitment) for the specific effect of S352* truncation on desensitization in CHO and epithelial cells.

The internalization of NPY-stimulated HAY1 receptors in HEK293 cells is in accordance with previous studies using GFP-tagged Y_1 receptors (Gicquiaux et al., 2002), or radiolabelled agonists (Parker et al., 2001; Pheng et al., 2003). As before, the speed of Y_1 receptor endocytosis in response to NPY or PYY, colocalized with labelled intracellular transferrin receptors, indicated a mechanism dependent on clathrin coated pits and the subsequent sorting of receptors to recycling endosomes. However BIBO3304 did not alter Y_1 receptor distribution (predominantly cell surface) in these experiments, beyond its effective blockade of NPY-induced endocytosis. The Y_1 subtype is one of the few reported 7TM receptors to undergo antagonist-induced sequestration (Pheng et al., 2003), but it appears that this may either be specific for one compound (GR231118), or it reflects the very modest basal internalization that we observed.

Surprisingly unstimulated HAY1S* and HAY1T* receptors underwent substantially enhanced constitutive endocytosis, at least partly to transferrin positive recycling compartments. Constitutive internalization of 7TM receptors can occur in the absence of agonist-independent activation (α1a-adrenoceptor; Morris et al., 2004), or as a consequence of it (e.g. CB1 and US28 receptors (Fraile-Ramos et al., 2003; Leterrier et al., 2004). Although basal activitation and endocytosis of a truncated sst2 somatostatin receptor has also been reported (Schwartkop et al., 1999), our observations indicate that Y_1 receptor truncation mutants are not constitutively active. In contrast to the findings of Schwartkop et al. (1999), HAY1S* and HAY1T* receptors did not exhibit increased agonist affinities when expressed in any cell lines, or a markedly enhanced sensitivity of agonist binding to GTPγS. Neither truncation mutant elevated basal G protein activation in the GTPγ[^{35}S] assay, and the slight negative agonist activity of BIBO3304 was

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similar in both HAY1 and HAY1S* membranes. In addition basal internalization of the truncated HAY1 receptors was prevented by concanavalin A but not BIBO3304, and was βarrestin-independent, suggesting a mechanism that does not require a spontaneously active phenotype.

Direct interaction between the HAY1T* and HAY1S* receptors and the AP-2 complex, which may underlie constitutive US28 receptor endocytosis in cells deficient in β-arrestins1 and 2 (Fraile-Ramos et al., 2003), would provide a feasible alternative for their recruitment to clathrin coated pits. YXXϕ motifs recognised by the μ2-adaptin subunit of AP-2 are present in both the third intracellular loop and the C terminus of the Y_1 receptor (Fig. 1), and would be retained after S352* truncation. An equivalent C terminal sequence has been implicated in the tonic internalization of the thomboxane A_2 β receptor (Parent et al., 2001) and in the thrombin activated endocytosis of protease activated receptor PAR1 (Paing et al., 2004) both of which are β-arrestin independent. Interestingly Paing *et al*. (2004) also observed that a PAR1 truncation just distal to the critical YXXϕ motif (as for the HAY1S* receptor) generated constitutive endocytosis. A similar consequence for HAY1S* and HAY1T* receptors may result from exposure of this motif, and / or the loss of more distal inhibitory elements (e.g. those that may bind PDZ motif proteins; Fig. 1; Xiang et al., 2002). The contribution of this mechanism to tonic internalization of the full length protein (which was low in HEK293 cells) could also be enhanced in cell types containing a high proportion of intracellular endogenous Y_1 receptors (Zhang et al., 1999). This would generate a receptor pool protected from agonist exposure and would potentially allow more rapid resensitization (Parent et al., 2001).

We found that the absence of β-arrestin2 binding to HAY1S* receptors did alter its trafficking, as substantial redistribution to the plasma membrane in the presence of agonist

distinguished the behaviour of these receptors from the continued internalization of the HAY1T* mutant. Constitutive endocytosis of HAY1S* receptors may be retarded on NPY stimulation by interaction of the active conformation with other plasma membrane proteins, perhaps those that organise G protein signalling complexes (e.g. A kinase anchoring protein (Tao et al., 2003) or ezrin; Sitaraman et al., 2002). In this case one role of β-arrestin binding would be to dissociate such interactions (as required for desensitization), allowing endocytosis of agonist-occupied HAY1 and HAY1T* receptors to proceed.

In summary we have identified a C terminal motif of the Y_1 receptor that is the minimal requirement for its phosphorylation, and is necessary for β-arrestin2 binding and its rapid desensitization. Complexity in the trafficking of HAY1S* and HAY1T* receptors suggest the contribution of additional β-arrestin-independent mechanisms to the control of internalization, and future investigations to this end will shed new light on the additional protein interactions that regulate Y_1 receptor signalling.

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Footnotes

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Fig. 1. The rat Y_1 receptor C terminus. The amino acid sequence is shown from the conserved NPXXY motif in transmembrane (TM) domain VII, indicating the position of the Thr361Stop and Ser352Stop truncations. Potential Ser / Thr phosphorylation sites are highlighted in white on black, while the YXXϕ consensus site for the AP-2 μ2-adaptin subunit and the class III PDZ binding motif at the extreme C terminus are underlined.

Fig. 2. Y_1 receptor-stimulated GTPγ[^{35}S] responses in CHO membranes. NPY and PYY concentration-response curves were performed using CHO-HAY1 (*A*) CHO-HAY1T* (*B*) and CHO-HAY1S* (*C*) clones in incubation buffer containing 100 mM NaCl and 10 μM GDP. The combined data from triplicate experiments ($n = 4 – 5$) is expressed as a percentage of basal binding (values were similar in each clone and are given in the text), and the effect of 1 μM BIBO3304 (alone, or in conjunction with 1 μM NPY) is indicated for each receptor. In a separate series of experiments (*D, E, F*), NPY stimulated-GTPγ[^{35}S] binding (in 50 mM NaCl, 1 μM GDP) was measured in membranes from cells that had previously been exposed to vehicle, or NPY (100 nM) for 10 min at 37°C. Basal binding and the effect of 1 μM BIBO3304 were monitored to ensure that the pretreating agonist had been removed during the wash stage, as described in the text. Pooled concentration-response relationships ($n = 4$) yielded similar control and pretreated NPY pEC_{50} values, respectively 8.71 ± 0.05 and 8.36 ± 0.11 in CHO-HAY1 (*D*), 8.84 ± 0.13 and 8.41 ± 0.14 in CHO-HAY1T* (*E*) and 8.33 ± 0.05 and 8.24 ± 0.18 in CHO-HAY1S* membranes (*F*). Significant differences between the maximal control and pretreated 1 μM NPY response are indicated by *** $p < 0.001$.

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Fig. 3. Functional Y_1 receptor responses in Colony 1 epithelial layers. Representative I_{SC} recordings (0.2 cm^2 area) are illustrated for C1Y1, C1Y1T* and C1Y1S* Colony 1 clones stable transfected with the native Y_1, Y_1(T361*) or Y_1(S352*) truncated receptors. Basolateral additions of VIP (30 nM), PYY (300 nM), UK14,304 (UK, 1 µM) and piretanide (Piret, 200 µM) were made at the times indicated. The initial basal I_{SC} (in µA) is given to the left of each trace.

Fig. 4. PYY response profiles. Epithelial clones were stimulated for VIP (30 nM) for 20 min, followed by the addition of PYY (at time $t = 0$). The mean (± s.e.m.) reductions in I_{SC} of subsequent pooled responses ($n = 3 – 8$) to 3 nM (○), 10 nM (●), 30 nM (□), 100 nM (■) and 300 nM (Δ) agonist are illustrated for C1Y1 (A), C1Y1T* (B) and C1Y1S* (C) epithelial layers for the following 10 min. C1Y1 time-courses are plotted on an increased vertical scale. In (D) and (E), individual 100 nM PYY and following 1 µM UK14,304 responses were normalized as a percentage of the peak I_{SC} and pooled data ($n = 5 – 8$) plotted for C1Y1 (○), C1Y1T* (□) and C1Y1S* (Δ) clones. Significant differences (* $p < 0.05$) between the size of C1Y1S* and C1Y1 responses are indicated at the appropriate time point.

Fig. 5. Phosphorylation of HAY1 receptors and truncation mutants. Native HEK293 cells (HEK) or clones expressing HAY1, HAY1T* or HAY1S* receptors were loaded for 1 h with 50 µCi inorganic phosphate in phosphate-free Krebs buffer, before treatment with vehicle (-) or 1 µM NPY (+) for 1 min at 37°C. Cell proteins were solubilized and immunoprecipated with anti-HA agarose, after which equal numbers of native and mutant Y_1 receptors (calculated according to saturation binding experiments and confirmed by Western blotting) were resolved by SDS-PAGE. In (A) basal and NPY-stimulated phosphorylation of HAY1, HAY1T* and HAY1S*

receptors are compared. To identify the mechanism of Y_1 receptor phosphorylation (B), HEK-HAY1 cells received no treatment (control), were transiently transfected (48 h earlier) with cDNAs for GRK2 or its dominant negative (DN) K220R mutant, or were incubated with the protein kinase C inhibitor calphostin C (Cal C) for the final 30 min of the loading period. Representative autoradiographs (48 h exposure) of fixed and dried gels are illustrated from 3 similar experiments in (A), and 2 in (B). The relative positions of molecular weight markers (in kD) are indicated in each case. To the right, the mean relative density of each major NPY-stimulated band (71 – 100 kD; shaded bars) is expressed as a fold increase over basal levels (open bars), for each treatment and receptor clone. * $p < 0.05$ HEK-HAY1S* compared to HEK-HAY1.

Fig. 6. Constitutive and agonist-stimulated internalization of HAY1 and truncated receptors. Stably transfected HEK-HAY1 (A), HEK-HAY1T* (B) and HEK-HAY1S* (C) cells were incubated with anti HA antibody for 30 min at 37°C to initially label cell surface receptors, in the presence of 1 μM BIBO3304 as appropriate (for BIBO, and BIBO and NPY). Cells were subsequently treated for 30 min with vehicle (Control, BIBO) or 100 nM NPY (NPY, BIBO and NPY), including Texas Red conjugated transferrin (Red) to highlight the clathrin dependent endocytic and recycling pathways. After fixation and permeabilization the receptor attached anti-HA was detected with an Alexa Fluor488 conjugated secondary antibody (Green). Single representative cells illustrate example experiments for each clone (repeated at least 3 times). A vertical stack of 15 fluorescent images (acquired on a Zeiss Axiovert 100 microscope) was deconvolved and reconstructed in Volocity (see Materials and Methods), to obtain the 3.0 μM *z*-section shown as a view from above. Scale Bar = 10 μm.

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Fig. 7. Translocation of GFP β-arrestin2 in response to NPY. Stable HEK-HAY1 (A), HAY1T* (B) or HAY1S* (C) clones were transiently transfected with 10 µg GFP-tagged β-arrestin2 cDNA before passaging onto coverslips. At 50 – 70 % confluence cells were treated with vehicle (control) or 1 µM NPY for the indicated times, before fixation and viewing on a Zeiss Axiovert 100 microscope. To ensure equivalent moderate expression levels of GFP-tagged β-arrestin2 were compared, camera exposure and deconvolution settings were kept constant in the capture of these example cells from a single experiment (repeated at least once). Images were reconstructed from 10 deconvolved vertical sections (at 0.2 µm *z*-steps) in Volocity (see Materials and Methods). The illustrated clear punctate pattern of GFP-fluorescence was observed for 10 – 20 % of NPY-stimulated HAY1 and HAY1T* cells that also expressed β-arrestin2. Scale bar = 10 µm.

Fig. S1. Internalization of HAY1 receptors. HEK-HAY1 cells were live-labelled with Anti-HA and treated with 1 µM NPY for 5, 15 or 30 min (A), or for 30 min with NPY or PYY (B) at the indicated concentrations. Labelled receptors were detected with an AlexaFluor488 conjugated secondary antibody (Green), and their localization compared to that of transferrin (Red), added at the same time as the agonist. Images of representative cells (n = 3 or more) were acquired and analysed as described in 'Materials and Methods'. Scale bar = 10 µm.

TABLE 1

[^{125}I]PYY saturation and competition binding in stably transfected CHO HAY1, HAY1T* and HAY1S* clones.

pK_d and B_{max} values were calculated from the direct fit to saturation experiments performed with 0.05 – 5.0 nM [^{125}I]PYY, in which non-specific binding was assessed with 1μM BIBO3304. PYY, NPY and BIBO3304 pIC_{50} values are given for competition assays performed with 10 pM radioligand; 1 μM GTPγS attenuated total [^{125}I]PYY binding (expressed as percentage inhibition of total binding) without altering the pIC_{50} values for competing PYY significantly (range of 9.22 - 9.57). Data are expressed as mean ± 1 s.e.m., from the number of experiments indicated in brackets.

	[^{125}I]PYY saturation		[^{125}I]PYY competition			
	pK_d	B_{max} pmol / mg	PYY pIC_{50}	NPY pIC_{50}	BIBO3304 pIC_{50}	Inhibition by 1 μM GTPγS (%)
CHO-HAY1	9.52 ± 0.12	5.4 ± 1.6	9.52 ± 0.05	9.61 ± 0.09	9.32 ± 0.08	45.2 ± 4.1
	(5)	(5)	(7)	(4)	(4)	(3)
CHO-HAY1S*	9.32 ± 0.07	5.5 ± 1.1	9.39 ± 0.04	9.80 ± 0.07	9.41 ± 0.07	49.8 ± 3.0
	(4)	(4)	(8)	(5)	(4)	(3)
CHO-HAY1T*	9.48 ± 0.10	3.9 ± 1.1	9.85 ± 0.03	9.58 ± 0.05	9.48 ± 0.04	59.8 ± 0.9
	(5)	(5)	(6)	(4)	(3)	(3)

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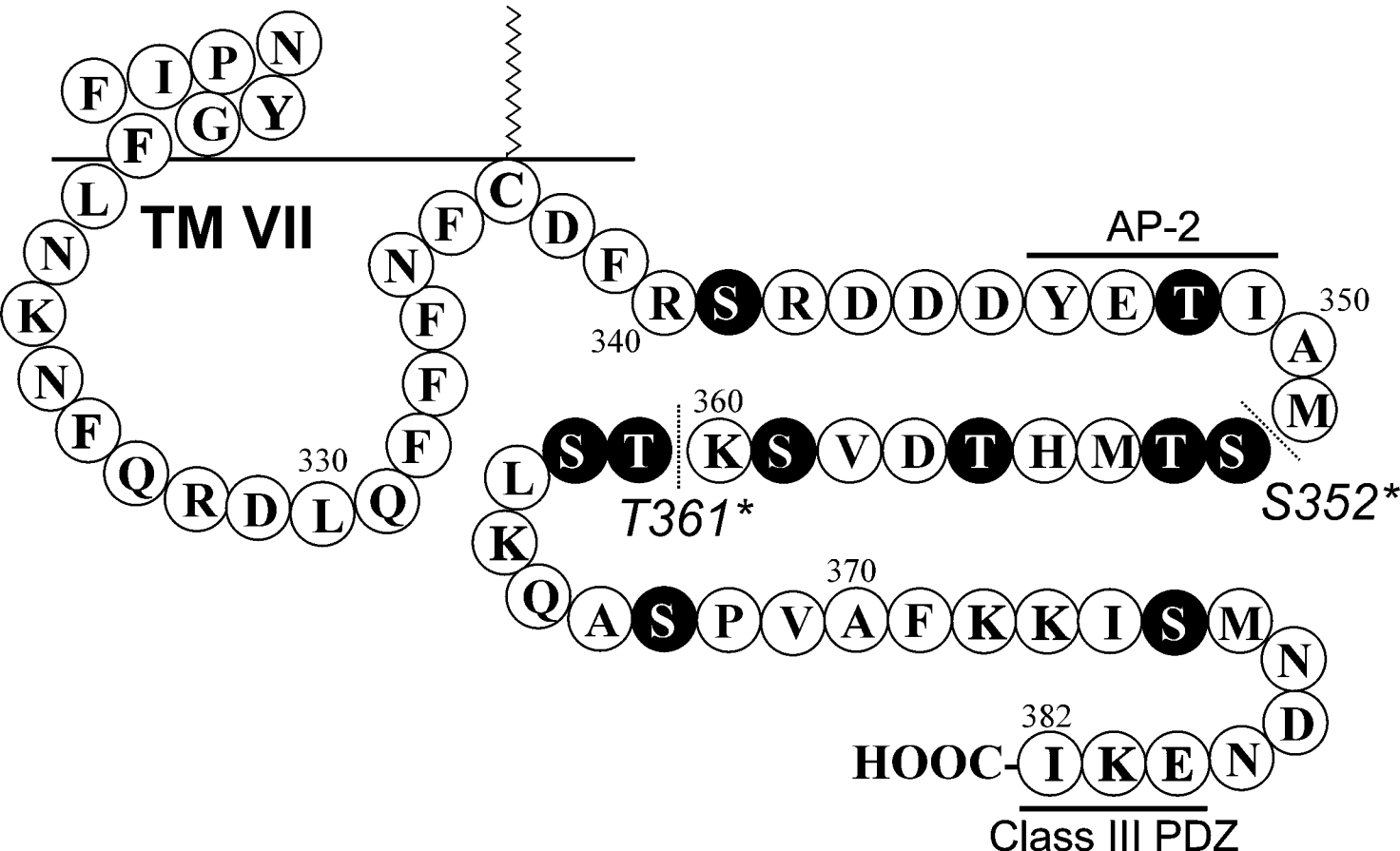
TABLE 2

Summary of functional responses in Colony 1 epithelial clones.

The peak changes in I_{SC} in response to VIP, PYY and the α_2-adrenoceptor agonist UK14,304 are indicated for non-transfected Colony 1 epithelial layers and the three clones expressing Y_1, Y_1(S352*) and Y_1(T361*) receptors. Data are expressed as mean ± 1 s.e.m., with *n* values indicated in brackets.

	VIP 30 nM $\mu A/cm^2$	PYY 100 nM $\mu A/cm^2$	UK14,304 1 µM $\mu A/cm^2$
Colony 1	+29.6 ± 1.2 (200)	0.0 ± 0.0 (21)	-10.4 ± 0.9 (4)
C1Y1	+34.9 ± 1.8 (69)	-9.4 ± 1.2 (8)	-26.0 ± 6.0 (4)
C1Y1S*	+26.4 ± 1.8 (54)	-5.4 ± 0.9 (5)	-8.5 ± 2.1 (4)
C1Y1T*	+48.5 ± 2.3 (53)	-5.2 ± 0.8 (8)	-8.0 ± 1.8 (3)

Figure 1



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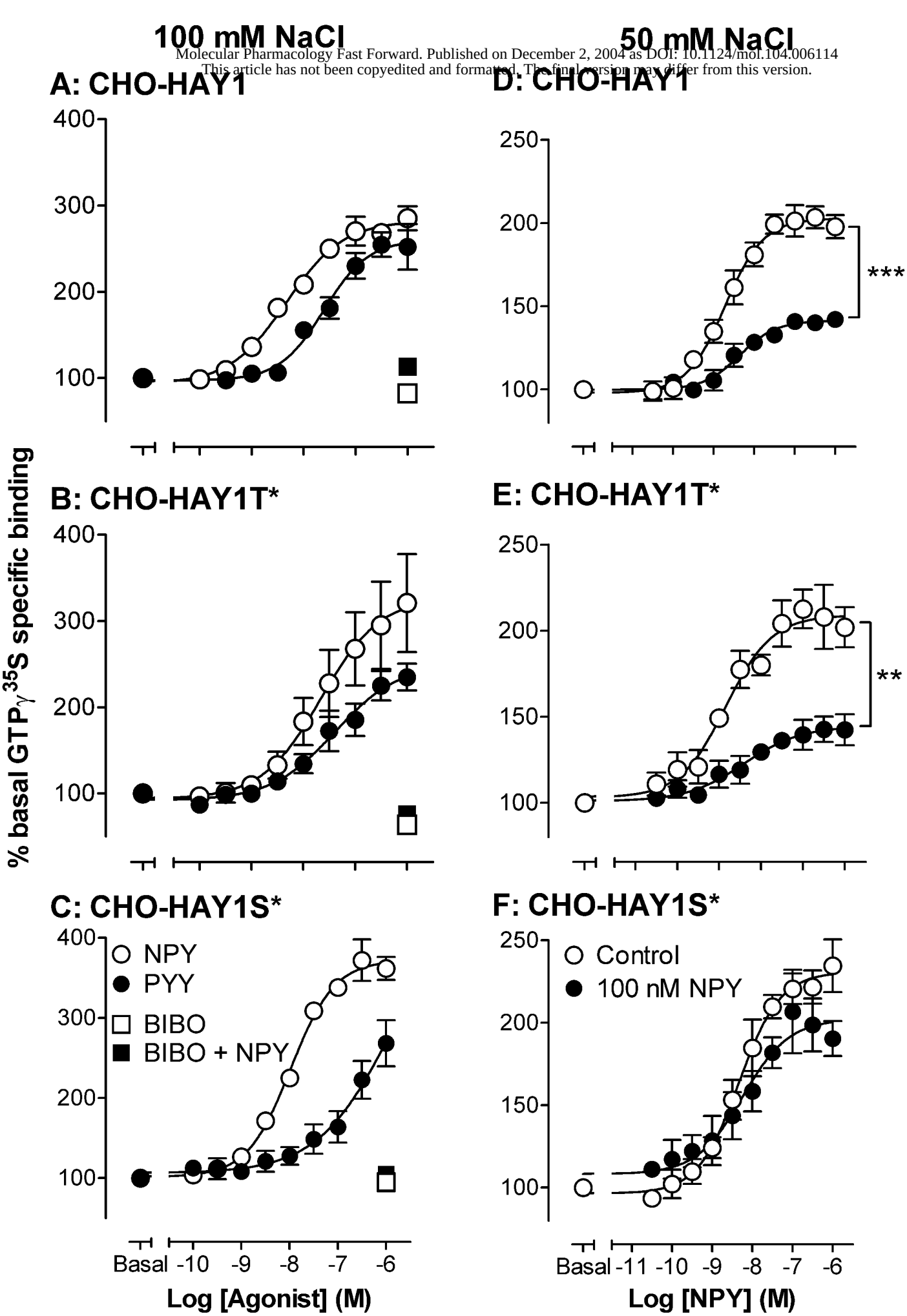
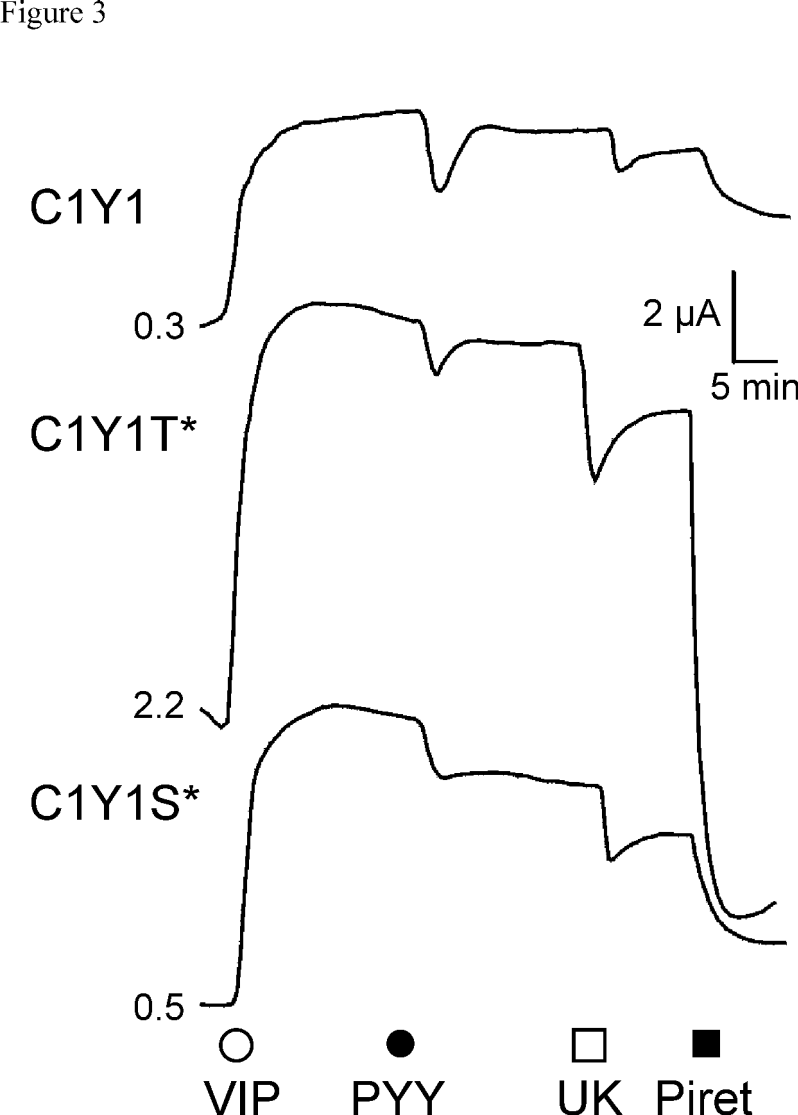


Figure 2

Figure 3



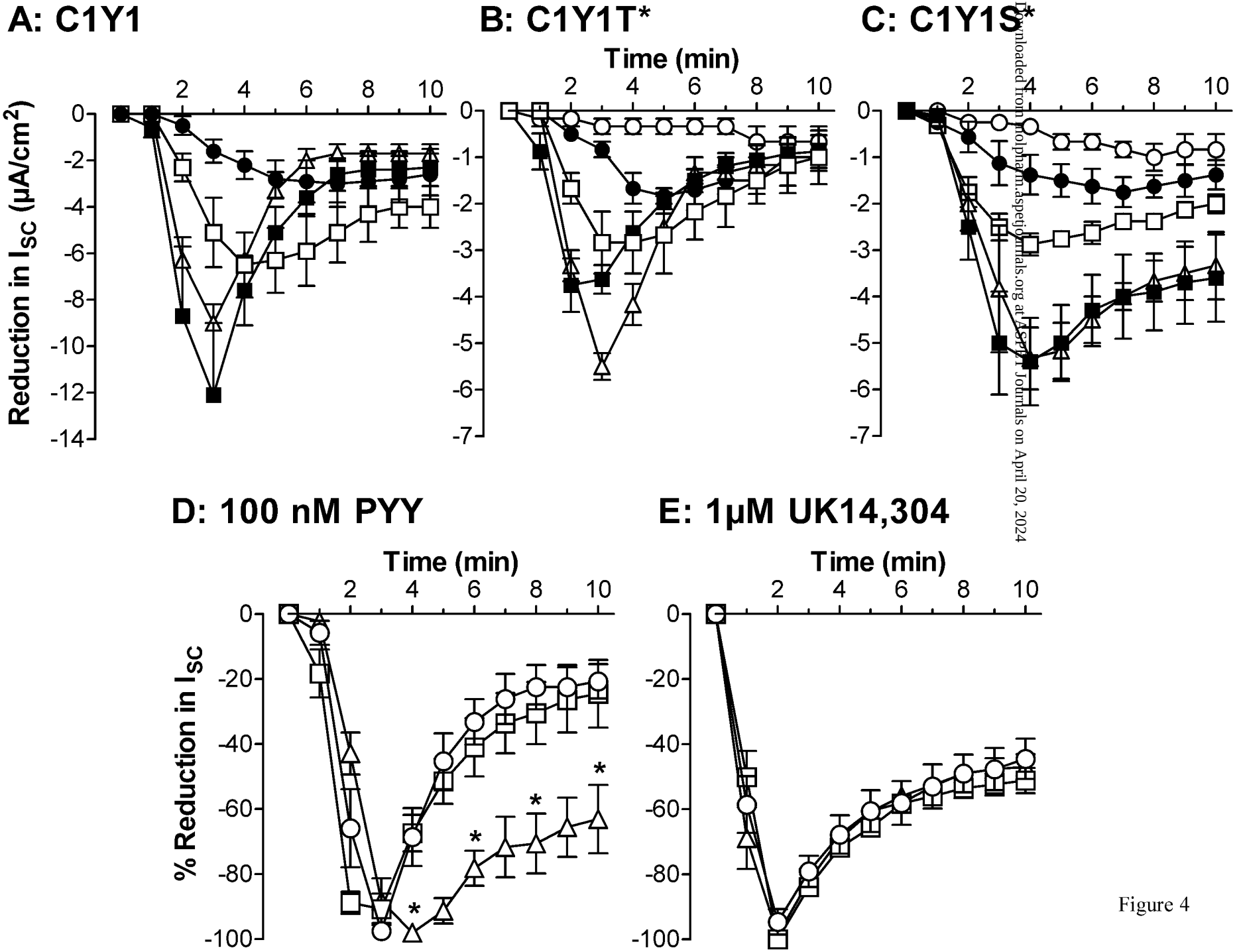
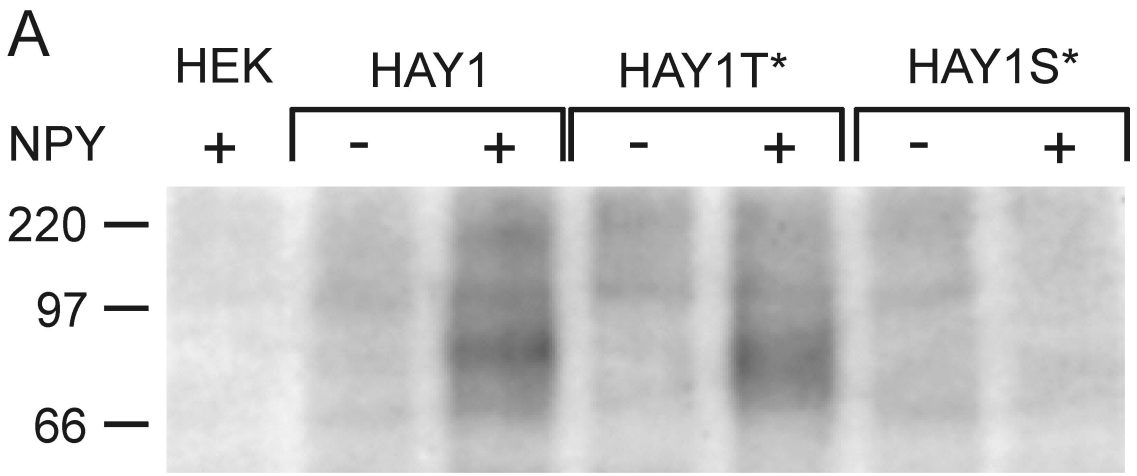
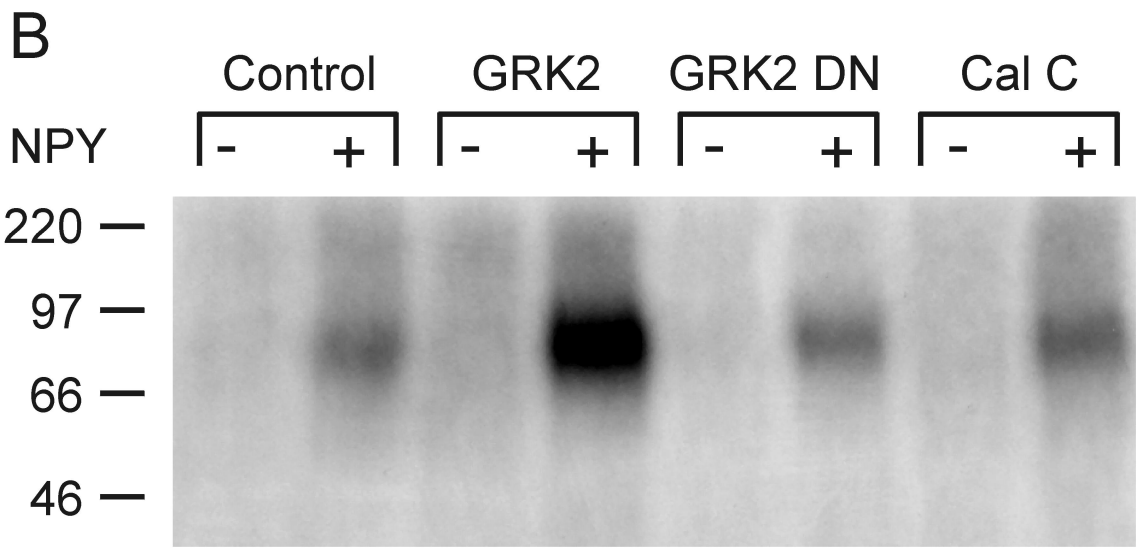
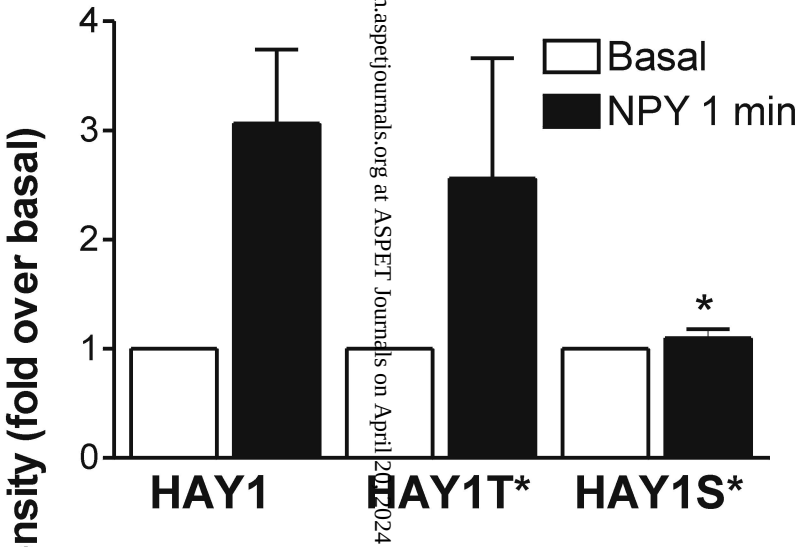


Figure 4

Figure 5







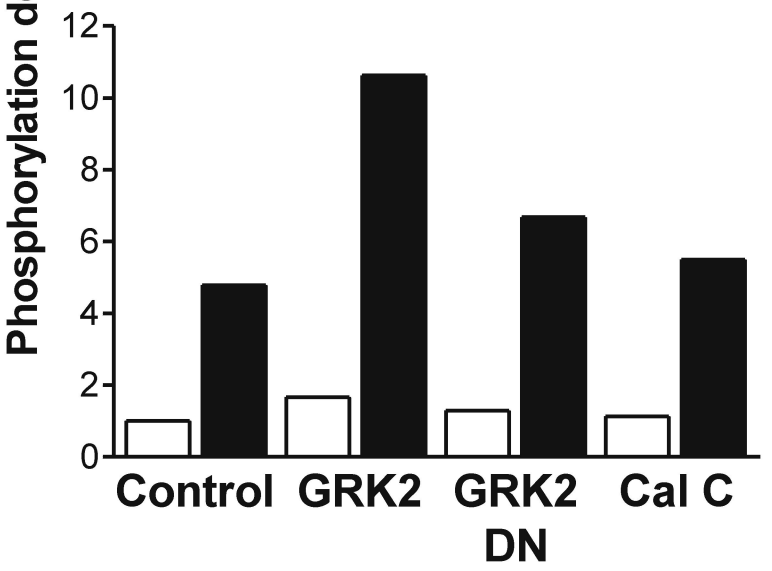


Figure 6

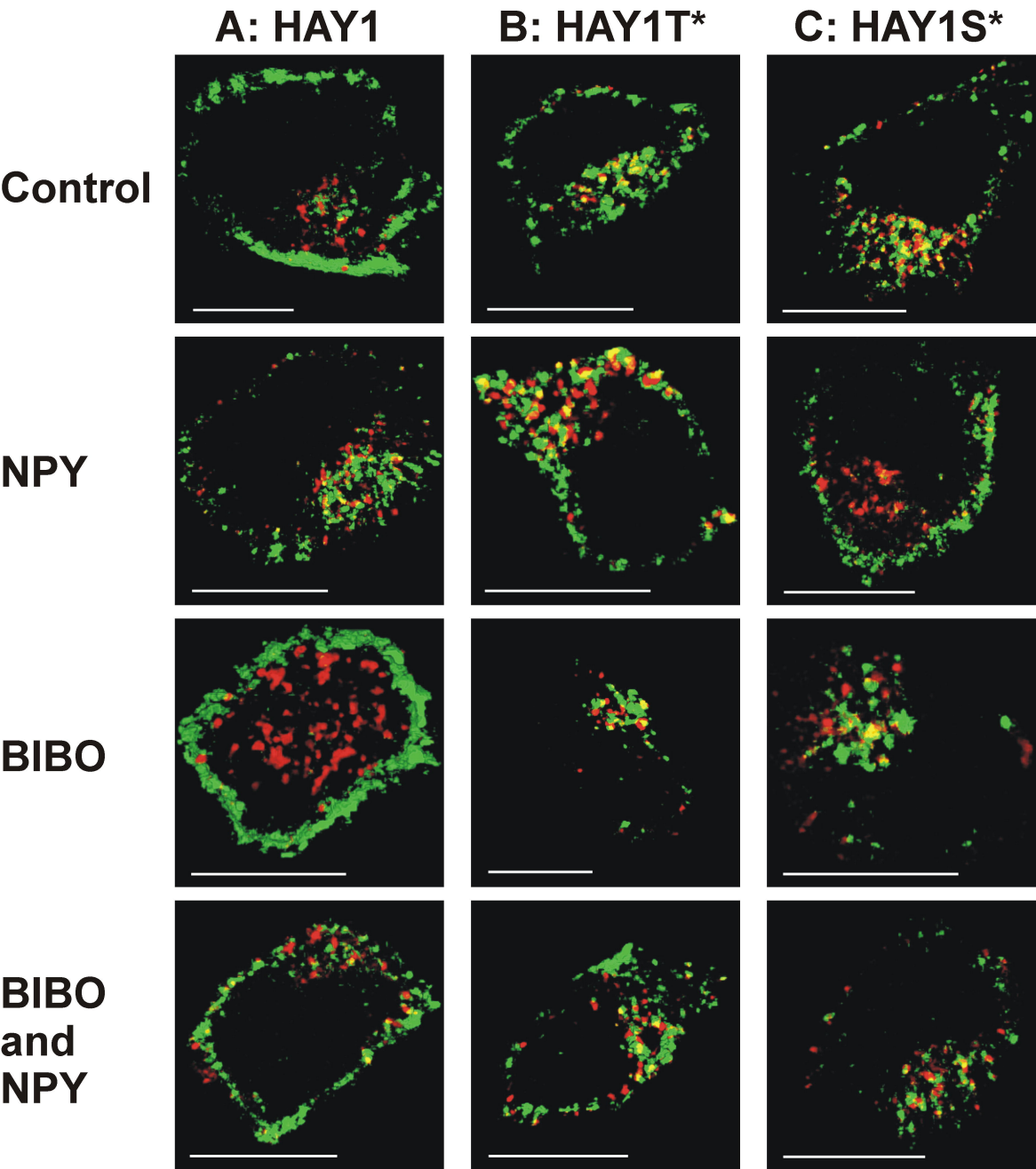


Figure 7