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PSAB-OFP, a selective α7 nicotinic receptor agonist, is also a potent agonist of the 5-HT_3 receptor

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Abstract

5-Hydroxytryptamine 3 (5-HT_3) and α7 nicotinic receptors share high sequence homology and pharmacological cross-reactivity. An assessment of the potential role of α7 receptors in many neurophysiological processes, and hence their therapeutic value, requires the development of selective α7 receptor agonists. We used a recently reported selective α7 receptor agonist, (*R*)-(−)-5′Phenylspiro[1-azabicyclo[2.2.2] octane-3,2′-(3′*H*)furo[2,3-*b*]pyridine (PSAB-OFP) and confirmed its activity on human recombinant α7 receptors. However, PSAB-OFP also displayed high affinity binding to 5-HT_3 receptors. To assess the functional activity of PSAB-OFP on 5-HT_3 receptors we studied recombinant human 5-HT_3 receptors expressed in *Xenopus* oocytes, as well as native mouse 5-HT_3 receptors expressed in N1E-115 neuroblastoma cells, using whole-cell patch clamp and Ca^{2+} imaging. Our results show that PSAB-OFP is an equipotent, partial agonist of both α7 and 5-HT_3 receptors. We conclude that it will be necessary to identify the determinant of this overlapping pharmacology in order to develop more selective α7 receptor ligands.

Keywords: α7 Nicotinic receptor; 5-HT_3 receptor; Cross-reactivity; Spiroazabicyclic

1. Introduction

5-Hydroxytryptamine 3 (5-HT_3) and nicotinic α7 receptors are both members of the superfamily of ligand-gated ion channels. These two receptor subtypes share the greatest similarity within the family displaying ~30% sequence homology (Maricq et al., 1991). Studies on chimeric receptors composed of α7 receptor amino-terminal sequences linked to carboxy-terminal portions of the 5-HT_3 receptor have demonstrated that the agonist recognition site is encoded by the amino terminus (Eiselé et al., 1993). Specific regions within this N-terminal domain have also been identified (Changeux et al., 1992; Karlin and Akabas, 1995; Arias, 2000). Homologies, as well as clear differences, have been identified within the ligand-recognition regions for the 5-HT_3 and α7 nicotinic acetylcholine receptors (Steward et al., 2000; Spier and Lummis, 2000; Yan et al., 1999; Boess et al., 1997) and may help to explain the overlapping, but clearly distinct, pharmacology described for these two receptor subtypes.

Cross-reactivity of various α7 and 5-HT_3 receptor ligands has been reported. For example, serotonin is reported to be an antagonist at wild type α7 receptors (Palma et al., 1996; Fucile et al., 2002), whilst nicotine and acetylcholine, among other nicotinic acetylcholine receptor agonists, are competitive 5-HT_3 receptor antagonists (Gurley and Lanthorn, 1998). Interestingly, 5-HT is an agonist of the L247T mutant α7 receptor (Palma et al., 1996, 1997; Fucile et al., 2002), whilst acetylcholine is an agonist at a F107N mutant 5-HT_3 receptor (Steward et al., 2000). Moreover, the 5-HT_3 receptor antagonist tropisetron (ICS 205-930) has been shown to be a potent, selective partial agonist at α7 nicotinic acetylcholine receptors (Macor et al., 2001), while the α7 receptor selective agonist 3-(2,4)-dimethoxybenzylidene-anabeseine (DMXBA; GTS-21) has been shown to antagonise 5-HT_3 receptors (Gurley and Lanthorn, 1998; Machu et al., 2001). Interestingly some

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of the closely related hydroxy-benzylidene metabolites of DMXBA display partial agonism at 5-HT_3 receptors (Machu et al., 2001). 3-(2-Hydroxy, 4-methoxybenzylidene)-anabeseine (2-OH-MBA) and 3-(4-hydroxy, 2-methoxybenzylidene)-anabeseine (HMBA; 4OH GTS-21) in particular were identified as partial agonists at both α7 and 5-HT_3 receptors (Machu et al., 2001; Kem et al., 1996). A further crossover is observed with the ability of 5-hydroxyindole (which lacks the amine group of the 5-HT molecule) to act as a potentiator of both 5-HT_3 (Van Hooft et al., 1997) and α7 nicotinic acetylcholine receptors (Gurley et al., 2000; Zwart et al., 2002). Once again mutation at L247T in the α7 receptor changes the properties of the receptor such that 5-hydroxyindole displays agonist properties (Gurley and Lanthorn, 2000).

In order to gain more insights into this overlapping pharmacology, we assessed α7 receptor selective agonists for binding to and function on 5-HT_3 receptors. Binding was performed on recombinant human 5-HT_3 receptors using [^{3}H]GR65630 as a radioligand. Functional activity was assessed using human recombinant 5-HT_3 receptors expressed in oocytes, and mouse native 5-HT_3 receptors expressed by N1E-115 neuroblastoma cells, using patch clamp and Ca^{2+} imaging techniques. Using this approach we have identified a selective α7 receptor agonist which displayed high affinity for the 5-HT_3 receptor. This compound, (*R*)-(−)-5′Phenylspiro[1-azabicyclo[2.2.2]octane-3,2′-(3′*H*)furo[2,3-*b*]pyridine (PSAB-OFP), was found to be an equipotent, partial agonist at 5-HT_3 and human α7 nicotinic acetylcholine receptors.

2. Materials and methods

2.1. Radioligand binding assay

Membranes with recombinant human 5-HT_3 receptors (RB-HS3M) were from Receptor Biology (Perkin-Elmer Life Sciences, Cambridge, UK). Assays were performed at room temperature in a final volume of 200 μl, containing 20 μl tissue (approx. 3 μg protein), 20 μl [^{3}H]GR65630 (3.4 nM), 140 μl assay buffer (mM): Tris/HCl, 50; EDTA, 1; $MgCl_2$, 5; pH 7.5 at 4 °C) and 20 μl of displacing ligand. These were incubated together for 1 h. Non-specific binding was determined in the presence of 100 μM tropanyl 3,5-dichlorobenzoate (MDL 72222). Separation of bound and free [^{3}H]GR65630 was achieved by vacuum filtration through pre-soaked (0.1% polyethylenimine/0.9% saline) Whatman GF/B filter paper, using a 96-well Brandel cell harvester. Filters were washed three times with wash buffer (0.9% saline at 4 °C) to remove excess radioligand and subsequently dried (for 90 s in a microwave) prior to MeltiLex® treatment (Perkin-Elmer). Bound tritium was counted using a β-plate counter. Average specific binding was >90% using this protocol.

2.2. Cell culture

N1E-115 mouse neuroblastoma cells were cultured in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 4 mM glutamine. Cells were harvested weekly using trypsin and seeded at a dilution of 1:4–1:10. For experiments cells were plated onto poly-L-lysine-coated (50 μg/ml) glass coverslips and used 1–3 days after plating.

HEK-293 cells stably expressing human α3β2, α4β2, α2β4, α3β4, α4β4 nicotinic acetylcholine receptors, were cultured in Dulbecco's modified Eagles Medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 μg/ml geneticin. GH4 cells stably expressing human α7 nicotinic acetylcholine receptors (GH4α7) were cultured in F-10 nutrient mixture supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 50 μg/ml geneticin. For experiments using a fluorescence imaging plate reader (FLIPR), cell lines were plated overnight in black-walled, transparent bottomed, poly-D-lysine coated 96-well plates at a density of 0.5×10^6 cells/ml, except for GH4α7 cells, which were plated at 1.0×10^6 cells/ml. All cells were grown in a humidified incubator maintained at 37 °C, with 95% air, 5% CO_2.

2.3. Whole-cell patch clamp

The patch-clamp technique in the tight-seal whole-cell configuration was used to record 5-HT_3-mediated currents in N1E-115 cells (Hamill et al., 1981). Recordings were made in an extracellular solution containing (mM): NaCl, 125; KCl, 5.5; $CaCl_2$, 1.8; $MgCl_2$, 0.8; HEPES, 20; glucose, 24; sucrose, 37, pH 7.3–7.4, osmolarity 315 mosM kg^{-1}. The intracellular solution consisted of (mM): K-glutamate, 100; HEPES, 20; sucrose, 120, pH 7.2, osmolarity 285–290 mosM kg^{-1}. Recordings were made at room temperature (21–24 °C) and drugs were applied focally for 2 s via an eight-channel superfusion system (APS, Bad Homburg, Germany). Electrodes were fabricated from borosilicate glass, 1.5 mm outer diameter (Harvard Apparatus, Kent, UK) on a Sutter programmable horizontal puller. The electrodes had resistances of 2–3 MΩ when filled with standard recording solutions. Currents were recorded using an Axopatch 200 A amplifier (Axon Instruments, Foster City, USA) and pClamp 7.0 software. A holding potential of −80 mV was maintained throughout experiments. Whole-cell capacitance transients were neutralised and series resistance compensation (70–80%) was applied to all cells. The recordings were filtered at 1 kHz and sampled at 2 kHz. pClamp 7.0 and SigmaPlot 5 were used for off-line analysis.

2.4. Ca^{2+} imaging

N1E-115 cells were loaded with 2 μM Fura-2 acetoxymethyl ester diluted in growth medium, at 37 °C for 30

min. Cells were then washed and incubated for at least a further 30 min at room temperature in a HEPES-buffered saline solution (HBSS) containing (mM): NaCl, 135: KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; Glucose, 10; HEPES, 10, pH 7.3. After this time cells on coverslips were mounted in a chamber onto the stage of an inverted epifluorescence microscope (Axiovert100TV, Zeiss, Germany) and viewed using a ×10 (air) or ×40 (oil immersion) fluorescence objective. Cells were alternately excited by light of 340- and 380-nm wavelength, provided by a polychrome II from T.I.L.L. photonics (Planegg, Germany) housing a Xenon lamp and monochromator. Emitted light was captured by a SensiCam cooled CCD camera (PCO CCD Imaging, Kelheim, Germany) after passage through a dichroic mirror (400 nm) and high pass barrier filter (480 nm). Digitised images were stored and processed using Axon Imaging Workbench software (Version 2.2 and 4.0, Axon Instruments) and Origin 6.1 software (OriginLab, MA, USA).

2.5. FLIPR experiments

The growth medium was removed from cells plated in 96-well plates, before addition of HBSS containing 10 μM Fluo-3 acetoxymethyl ester/0.05% pluoronic F-127 using an automated multidrop dispensor (Lab Systems, Helsinki, Finland). Cells were incubated with the dye for 1 h at room temperature before the medium was removed and replaced with HBSS in the absence of Fluo-3. The plates were then transferred to a FLIPR^{384} system (Molecular Devices, Winnersh, UK) for experiments (Schroeder and Neagle, 1996). Cells were excited by light of 488-nm wavelength from a 4-W Argon-ion laser and the emitted fluorescence passed through a 510- to 570-nm bandpass interference filter before detection with a cooled CCD camera (Princeton Instruments). Image data was transferred to a Dell Optiplex GX110 computer and stored for offline analysis using FLIPR^{384} system software and Origin 6.1 software (OriginLab). Drug dilutions were prepared in a separate 96-well plate using a Biomek 2000 (Beckman Instruments, Fullerton, CA, USA). Parameters for drug addition to the cell plate were programmed on the computer and delivery was automated through a 96-tip head pipettor. For each nicotinic acetylcholine receptor subtype, results are expressed normalised to maximal responses obtained to a non-selective nicotinic acetylcholine receptor agonist epibatidine (1 μM). Unless otherwise stated, all data are presented as mean ± standard error of the mean (S.E.M.).

2.6. Oocyte recordings

Xenopus oocytes were defolliculated manually after treatment with collagenase type I (4 mg/ml Ca^{2+}-free Barth's solution) for 1.5 h at room temperature. Plasmids containing the human α4, α7, β2 and β4 nicotinic acetylcholine receptors and the human 5-HT_3 receptor coding sequences were suspended in distilled water and injected into the nuclei of stages V and VI oocytes within 4 h after harvesting, using a Drummond (Broomall, PA, USA) variable volume microinjector. For homooligomeric α7 nicotinic acetylcholine receptors and 5-HT_3 receptors approximately 2 ng of cDNA was injected, whereas for the heteromeric α4β2 and α4β4 nicotinic acetylcholine receptors 1 ng of α4 and 1 ng of either β2 or β4 subunits was injected. The total injection volume was 18.4 nl/oocyte. After injection the oocytes were incubated at 18 °C in a modified Barth's solution containing (mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $Ca(NO_3)_2$, 0.3; $CaCl_2$, 0.41; $MgSO_4$, 0.82; HEPES, 15, and 50 mg/l neomycin (pH 7.6 with NaOH; osmolarity 235 mosM). Experiments were performed after 3–5 days of incubation.

Oocytes were placed in a recording chamber (internal diameter, 3 mm) and continuously perfused at a rate of ~ 10 ml/min with a saline solution composed of (mM): 115 NaCl, 2.5 KCl, 1.8 $BaCl_2$, 10 HEPES, pH 7.3 (NaOH), 235 mOsm. $BaCl_2$ was used instead of $CaCl_2$ in order to minimise the possible contribution of secondary Ca^{2+}-activated Cl^- currents. Dilutions of agonists in external saline were prepared immediately before the experiments and applied by switching between control and drug-containing saline using an eight-channel bath perfusion system (ALA Scientific, Westbury, NY, USA). Agonist applications were separated by 5 min (2 min for α7 receptors) of superfusion with agonist-free saline to allow the receptors to recover from desensitisation.

Oocytes were impaled by two microelectrodes filled with 3 M KCl (0.5–2.5 MΩ) and voltage clamped using a Geneclamp 500B amplifier (Axon Instruments) according to the method described by Stühmer (1996). The external saline was clamped at ground potential by means of a virtual ground circuit using an Ag/AgCl reference electrode and a Pt/Ir current passing electrode. The membrane potential was held at −60 mV. Membrane currents were low-pass filtered (four-pole low pass Bessel filter, −3 dB at 0.3 kHz), digitised (1000 samples/s), and stored on disk for off-line computer analysis. All experiments were performed at room temperature.

Ion current amplitudes were measured and normalised to the amplitude of control responses induced by the near-maximum effective concentration of 1 mM acetylcholine for nicotinic acetylcholine receptors and 100 μM 5-HT for 5-HT_3 receptors. Control responses were evoked alternately, in order to adjust for small variations in response amplitude over time. Concentration–response curves were fitted to the data obtained in separate experiments and mean ± standard deviation of estimated parameters were calculated for n oocytes. Agonist curves were fitted according to the equation:

$$i/i_{max} = 1/\{1 + (EC_{50}/[agonist])^{nH}\} \quad (1)$$

Curve fitting was performed using Jandel Sigmaplot 5.0 software (SPSS, Chicago, USA).

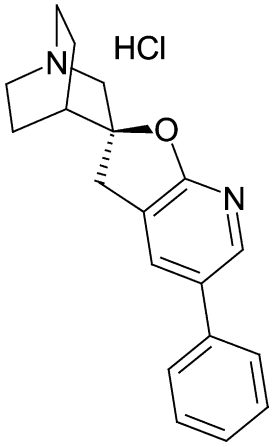

Fig. 1. Structure of (*R*)-(−)-5′Phenylspiro[1-azabicyclo[2.2.2]octane-3,2′-(3′*H*)furo[2,3-*b*]pyridine (PSAB-OFP).

2.7. Materials

Adult female *Xenopus laevis* frogs were obtained from Blades Biological (Edenbridge, UK) and housed at Oxford Brookes University (Dr. I. Bermudez, Oxford, UK). cDNAs encoding the human nicotinic acetylcholine receptor subunits were obtained from Merck Research Laboratories (La Jolla, CA, USA) and ligated into the pcDNA3 vector (Invitrogen, Carlsbad, CA, USA). N1E-115 cells were obtained from ECACC (Ref. No. 88112303) and all stable nicotinic acetylcholine receptor cell lines were from Merck Research Laboratories. Becton Dickinson 96 well black-walled, poly-D-lysine coated, transparent bottomed FLIPR plates were supplied by Marathon Laboratory Supplies (London, UK).

PSAB-OFP was synthesised by the Lilly chemistry laboratories at Windlesham (Surrey, UK). Acetylcholine, 5-HT, 5-hydroxyindole, and dihydro-β-erthroidine hydrobromide (DHβE) were purchased from Sigma-RBI (St. Louis, MO, USA). Methyllycaconitine, MDL 72222, m-chlorophenylbiguanide (mCBPG) and *N*-(1-azabicyclo[2.2.2]oct-3-yl)-6-chloro-4-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-8-carboxamide (Y25130) were from Tocris Cookson (Bristol, UK). Fura-2 acetoxymethyl ester, Fluo-3 acetoxymethyl ester and pluronic F-127 were from Molecular Probes (Leiden, The Netherlands). All cell culture reagents were from Gibco-BRL (Paisley, UK), except the fetal calf serum, which was obtained from Invitrogen.

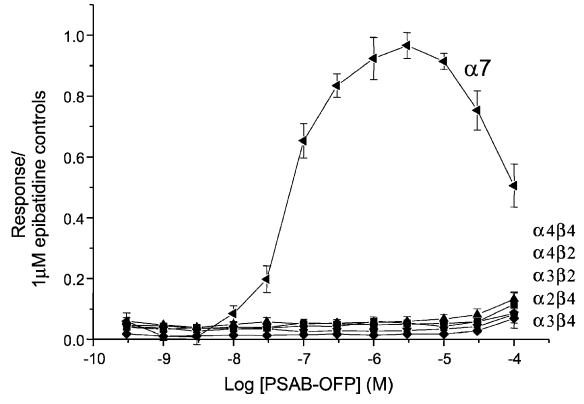

Fig. 2. PSAB-OFP is a selective agonist at α7 nicotinic acetylcholine receptors expressed in mammalian cells. Concentration–response curves of PSAB-OFP obtained from recombinant human nicotinic acetylcholine receptors (α2β4, α3β4, α4β4, α3β2, α4β2, α7) stably expressed in mammalian cells. Responses are expressed relative to maximal (1 μM) epibatidine control responses. Traces represent mean ± S.E.M. of 6–8 experiments for each nicotinic acetylcholine receptor subtype.

3. Results

Using mammalian cell lines stably expressing human α2β4, α3β4, α4β4, α3β2, α4β2 or α7 recombinant nicotinic acetylcholine receptors we confirmed that the reported α7 receptor selective agonist PSAB-OFP (Fig. 1) was selective for α7 over other nicotinic acetylcholine receptor subtypes (Fig. 2). Due to the high homology and reported cross-reactivity between α7 nicotinic and 5-HT_3 receptors, PSAB-OFP was also assessed for activity in a binding assay using human recombinant 5-HT_3 receptors (see Materials and

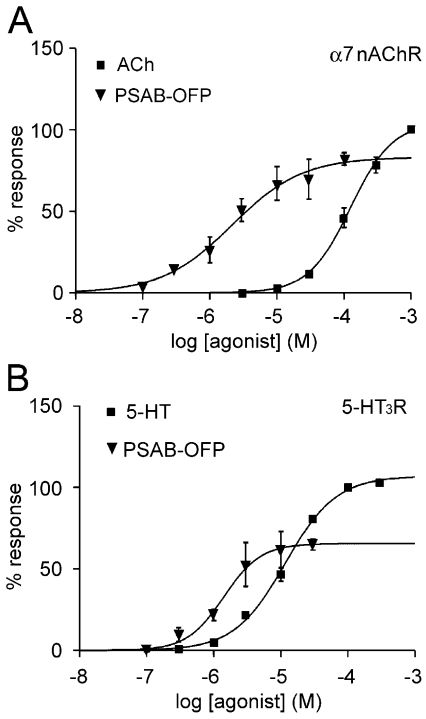

Fig. 3. PSAB-OFP is an agonist of recombinant human α7 nicotinic acetylcholine receptors and 5-HT_3 receptors expressed in *Xenopus* oocytes. (A) Concentration–response curves of acetylcholine and PSAB-OFP obtained from oocytes expressing human α7 nicotinic acetylcholine receptors. $EC_{50} = 141 \pm 74$ and 2.2 ± 0.6 μM, $E_{max} = 106 \pm 9\%$ and $83 \pm 6\%$, respectively. (B) Concentration–response curves of 5-HT and PSAB-OFP obtained from oocytes expressing human 5-HT_3 receptors. $EC_{50} = 12 \pm 2$ and 1.4 ± 0.4 μM, $E_{max} = 107 \pm 3\%$ and $66 \pm 6\%$, respectively.

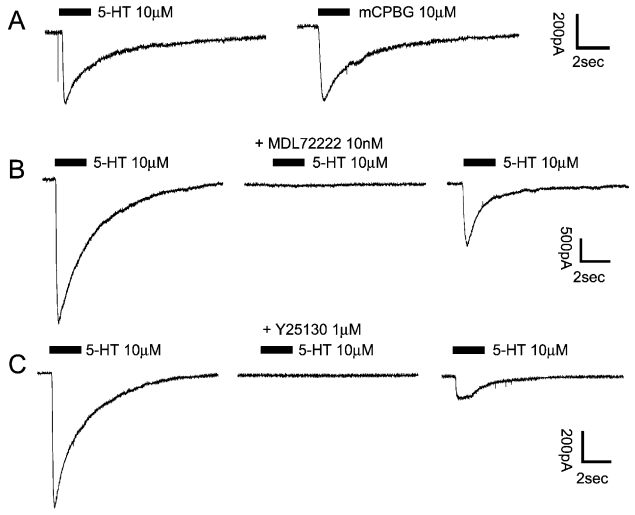

Fig. 4. Characterisation of 5-HT_3 receptor mediated inward currents in mouse N1E-115 neuroblastoma cells. (A) Rapid application of 5-HT (10 μM) or a 5-HT_3 receptor selective agonist, mCPBG (10 μM), induced inward currents in N1E-115 cells. Agonist responses were repeatable on multiple agonist additions. (B) Responses to 5-HT (10 μM) were blocked by a 2-min pre-treatment with the 5-HT_3 receptor selective antagonist MDL 72222 (10 nM). Block by MDL 72222 was partially reversible on wash out. (C) Responses to 5-HT (10 μM) were fully blocked by a 2-min pre-treatment with the 5-HT_3 receptor selective antagonist Y25130 (1 μM). Block by Y25130 was partially reversible on wash out.

methods). PSAB-OFP was found to display high affinity binding to 5-HT_3 receptors ($K_i = 51.25$ nM).

To establish whether PSAB-OFP was an agonist or antagonist at 5-HT_3 receptors, the compound was tested and activity compared on human recombinant α7 and 5-HT_3 receptors expressed in *Xenopus* oocytes.

Superfusion of voltage-clamped oocytes expressing human α7 nicotinic acetylcholine receptors with acetylcholine or with PSAB-OFP evoked concentration-dependent inward currents. Peak amplitudes of these currents, normalised to the peak amplitude of the 1 mM ACh-induced currents, are plotted against agonist concentration in Fig. 3A. Fitting concentration–response curves to the data yielded mean estimates for EC_{50}, E_{max} and nH of 141 ± 74 μM, $106 \pm 9\%$ and 1.8 ± 0.8 for acetylcholine ($n = 7$), and 2.2 ± 0.6 μM, $83 \pm 6\%$ and 0.8 ± 0.3 for PSAB-OFP ($n = 3$), respectively. PSAB-OFP up to 100 μM did not induce ion currents in oocytes expressing α4β2 or α4β4 nicotinic acetylcholine receptors (not shown), confirming its selectivity for α7 receptors.

In a similar manner, the superfusion of voltage clamped oocytes expressing human 5-HT_3 receptors with 5-HT, or PSAB-OFP, evoked concentration-dependent inward currents. Peak amplitudes of these currents, normalised to the peak amplitude of the 100 μM 5-HT-induced currents, are plotted against agonist concentration in Fig. 3B. Fitting concentration–response curves to the data yielded mean estimates for EC_{50}, E_{max} and nH of 12 ± 2 μM, $107 \pm 3\%$ and 1.2 ± 0.1 for 5-HT ($n = 3$), and 1.4 ± 0.4 μM, $66 \pm 6\%$ and 1.6 ± 0.7 for PSAB-OFP ($n = 3$), respectively. These results demonstrate that PSAB-OFP is also an agonist at human recombinant 5-HT_3 receptors.

We were particularly interested, at this stage, in evaluating if this cross-reactivity of PSAB-OFP on α7 and 5-HT_3

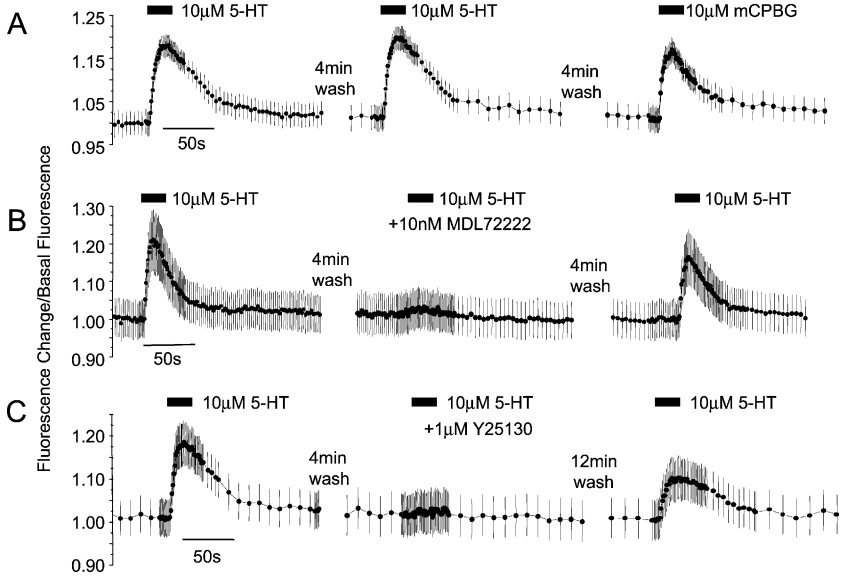

Fig. 5. Characterisation of 5-HT_3 receptor mediated Ca^{2+} responses in mouse N1E-115 neuroblastoma cells. (A) Thirty-second applications of 5-HT (10 μM) or a 5-HT_3 receptor selective agonist, mCPBG (10 μM), induced Ca^{2+} increases in Fura-2 loaded N1E-115 cells. Agonist responses were repeatable on multiple agonist additions. (B) Ca^{2+} responses to 5-HT (10 μM) were almost completely blocked by a 2-min pre-treatment with the 5-HT_3 receptor selective antagonist MDL 72222 (10 nM). Block by MDL 72222 was reversible on wash out, responses to 5-HT were largely recovered after a 4-min wash. (C) Ca^{2+} responses to 5-HT (10 μM) were fully blocked by a 2-min pre-treatment with the 5-HT_3 receptor selective antagonist Y25130 (1 μM). The block by Y25130 was reversible on washout of antagonist with full recovery taking >12 min. Traces show mean ± S.E.M. of 10–20 single cells from one experiment, representative of at least three similar experiments. Only 5-HT-responsive cells were analysed.

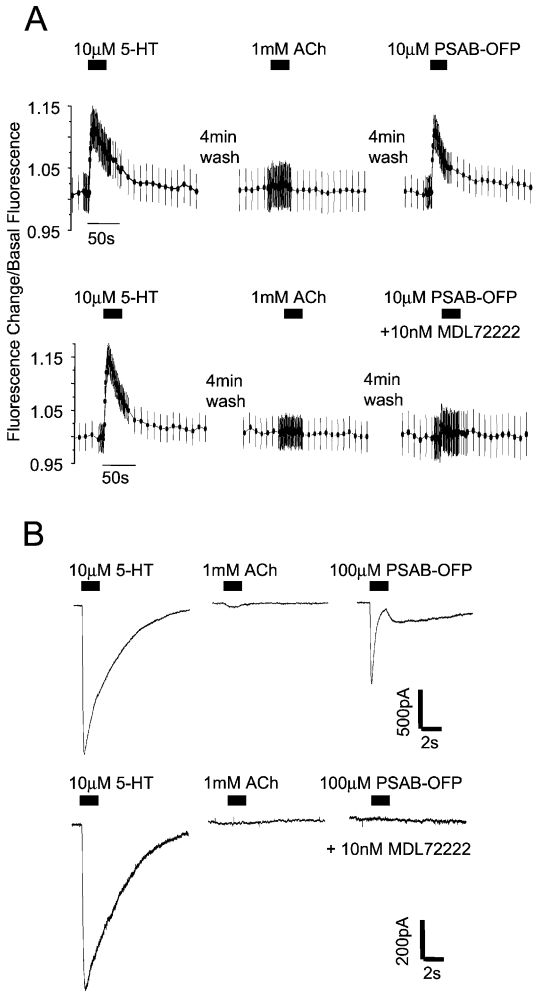

Fig. 6. PSAB-OFP evokes 5-HT_3 receptor mediated inward currents and Ca^{2+} responses in mouse N1E-115 neuroblastoma cells. Application of 5-HT (10 μM) or the α7-selective nicotinic acetylcholine receptor agonist PSAB-OFP (10 or 100 μM) induced Ca^{2+} increases (A) and inward currents (B). A 1 mM acetylcholine dose failed to induce a response. In 5-HT responsive cells, PSAB-OFP failed to induce a response after a 2 min pre-incubation with the $5HT_3$ receptor antagonist MDL 72222 (10 nM). Traces are representative of at least three similar experiments. Experiments were performed in the presence of 30 μM DHβE. Imaging traces show mean ± S.E.M. of single cell responses (>10 cells in each experiment). Only 5-HT responsive cells were analysed.

receptors was a finding unique to recombinant and heterologously expressed homomeric α7 and 5-HT_3 receptors, or if it was transferable to native neuronal receptors.

In order to obtain this information, mouse N1E-115 neuroblastoma cells, which are reported to possess native 5-HT_3 receptors, were studied (Neijt et al., 1988; Peters and Lambert, 1989). Focally applied 5-HT (10 μM) induced robust inward currents in N1E-115 cells (964 ± 258 pA, $n = 39$), as did the selective 5-HT_3 receptor agonist mCPBG (10 μM, 894 ± 347 pA, $n = 5$) (Fig. 4A). The 5-HT-evoked inward currents were fully blocked by two pharmacologically distinct 5-HT_3 receptor antagonists: MDL 72222 (10 nM) and Y25130 (1 μM) (Fig. 4B,C).

Activation of 5-HT_3 receptors in N1E-115 cells is also reported to increase intracellular Ca^{2+} (Hargreaves et al., 1994). Using N1E-115 cells loaded with Fura-2 we were able to confirm that 5-HT (10 μM) and mCPBG (10 μM) caused a significant rise in intracellular Ca^{2+} (Fig. 5A). These responses could also be fully blocked by with MDL 72222 (10 nM) and Y25130 (1 μM) (Fig. 5B,C).

The results from both techniques correlated well and confirmed the existence of native 5-HT_3 receptors in N1E-115 cells. Hence, N1E-115 cells were used as a model system to assess the functional effects of PSAB-OFP on native neuronal 5-HT_3 receptors.

In 5-HT responsive N1E-115 cells, application of PSAB-OFP (1–100 μM) induced both inwards currents and increases in intracellular Ca^{2+} (Fig. 6). Because N1E-115 cells are reported to contain α4β2 nicotinic acetylcholine receptors (Zwart et al., 1994) experiments were conducted in the presence of DHβE, at a concentration (30 μM) which fully blocks this subtype of nicotinic acetylcholine receptor (Chavez-Noriega et al., 2000). Hence, in Ca^{2+} imaging experiments, 5-HT responsive cells that displayed no response to acetylcholine (1 mM), subsequently responded to 1, 10 or 100 μM PSAB-OFP. Maximal responses were seen at a concentration of 10 μM PSAB-OFP, being 83 ± 7% of 5-HT-induced responses (Fig. 6A). Similarly in patch-clamp experiments, in cells which failed to respond to 1 mM ACh, PSAB-OFP (100 μM) subsequently induced inwards currents, which were ~70% of those evoked by 5-HT (10 μM) (Fig. 6B). Both current and Ca^{2+} responses evoked by PSAB-OFP were fully blocked by the 5-HT_3 receptor antagonist MDL 72222 (10 nM) (Fig. 6A,B). MDL 72222 (10 nM) had no effect on Ca^{2+} responses elicited on activation of recombinant α7 nicotinic acetylcholine receptors stably expressed in GH4

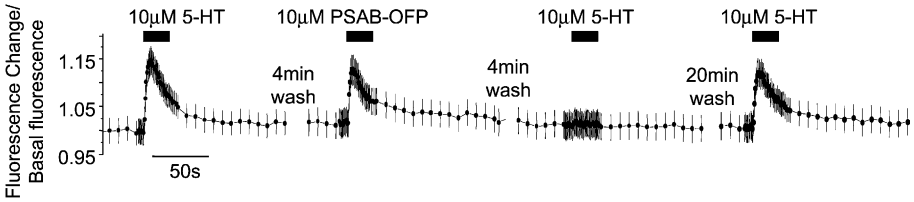

Fig. 7. PSAB-OFP causes long-term desensitisation of 5-HT_3 receptors. Ca^{2+} responses to 5-HT (10 μM) were fully blocked by prior exposure to PSAB-OFP (10 μM), full recovery of 5-HT responses took >20 min. Traces show mean ± S.E.M. of single cell responses (>10 cells in each experiment) and are representative of three similar experiments. Only 5-HT responsive cells were analysed.

cells. α7 receptor responses were 96.7 ± 11% of the control values measured in the absence of 10 nM MDL 72222 ($n = 3$, data not shown), confirming that blockade of PSAB-OFP-induced responses by this antagonist was through inhibition of 5-HT_3 receptors.

PSAB-OFP also induced a long lasting desensitisation of 5-HT_3 receptors (Fig. 7). Although multiple applications of 5-HT (with 4-min wash periods between applications) led to repeatable agonist responses (see Fig. 5), after application of PSAB-OFP, 5-HT failed to subsequently generate any agonist response. Responses to 5-HT were vastly reduced and did not recover fully for at least 20 min after washout.

4. Discussion

PSAB-OFP has recently been disclosed as a selective α7 nicotinic acetylcholine receptor agonist (Phillips et al., 1999; Astra Arcus USA, patent WO 99/03859). We confirmed the selectivity of this compound for α7 over other nicotinic receptor subtypes, however we found that PSAB-OFP is also a potent agonist of 5-HT_3 receptors. PSAB-OFP bound with high affinity to human recombinant 5-HT_3 receptors and displayed agonist activity at both human recombinant and native mouse 5-HT_3 receptors. In mouse N1E-115 neuroblastoma cells, the action of PSAB-OFP could be blocked with selective 5-HT_3 receptor antagonists and produced a long-lasting desensitisation of 5-HT_3 receptors, confirming that it was acting through the endogenous 5-HT_3 receptors. When comparing the activity of PSAB-OFP on human recombinant α7 nicotinic and 5-HT_3 receptors, the compound was found to act as an equipotent (2.2 ± 0.6 and 1.4 ± 0.4 μM) and partial (83 ± 6% and 66 ± 6%) agonist at both receptor types.

This finding raises several intriguing questions with pharmacological and therapeutic implications. Firstly, although pharmacological cross-reactivity between the α7 and 5-HT_3 receptor has previously been reported, most studies have found that agonist activity on one receptor translates to antagonist activity at the other receptor. For, example the endogenous ligands, serotonin and acetylcholine, act as antagonists on their counterpart (Palma et al., 1997; Gurley and Lanthorn, 1998). The nicotinic receptor agonists nicotine, choline, epibatidine, 1,1-dimethyl-4-phenyl-piperazinium (DMPP) and the α7 receptor selective partial agonist, DMXBA, are all competitive antagonists of mouse 5-HT_3 receptors (Gurley and Lanthorn, 1998; Machu et al., 2001). The 5-HT_3 receptor antagonist tropisetron, and quinuclidine analogues, but not ondansetron or LY278 584, showed partial agonist properties at α7 receptors (Macor et al., 2001). Non-specific antagonism has also been reported: for example, D-tubocurarine (Peters et al., 1990) and some of its analogues (Yan et al., 1998) inhibit both receptor types. Notably, the human 5-HT_3 receptor is much less sensitive to inhibition by D-tubocurarine than the mouse 5-HT_3 receptor, highlighting the importance of species differences (Hope et al., 1999).

To date, there is only one report of compounds that act as agonists of both α7 and 5-HT_3 receptors. Two primary metabolites of DMXBA, 2-OH-MBA and HMBA, are partial agonists at α7 receptors (Kem et al., 1996), with HMBA displaying 40% efficacy and an EC_{50} value of 26 μM on the human α7 receptor (Meyer et al., 1998). Machu et al. (2001) recently reported that these metabolites, 2-OH-MBA and HMBA, also act as partial agonists at mouse 5-HT_3 receptors, displaying 63% and 30% efficacy and EC_{50} values of 2 and 17 μM, respectively. These studies were conducted on mouse 5-HT_3 receptors, but as our present data on PSAB-OFP now show this feature of dual agonism can also extend to human 5-HT_3 receptors.

This is particularly relevant because of the current focus on α7 nicotinic acetylcholine receptors as possible therapeutic targets for a range of neurological disorders. Indeed, DMXBA is currently in clinical trials for the treatment of Alzheimer's disease (Kem, 2000). As discussed by Machu et al. (2001), the metabolites of DMXBA may be at high enough concentrations to affect peripheral 5-HT_3 receptors, but, due to their polar nature, these metabolites may not get into the brain at high enough concentrations to exert central effects. In contrast, PSAB-OFP displays significant blood–brain barrier penetration (data not shown) and any central effects of PSAB-OFP on 5-HT_3 and α7 receptors would be hard to separate. 5-HT_3 receptors have been implicated centrally in modulating neurotransmitter release (MacDermott et al., 1999; Barnes and Sharp, 1999; but see also Van Hooft and Vijverberg, 2000). Hence, effects on 5-HT_3 receptors may confound the use of these compounds as research tools and possibly as therapeutic agents. The most profound effect of 5-HT_3 receptor agonism may be seen peripherally with stimulation of 5-HT_3 receptors leading to cardiovascular and gastrointestinal side effects, 5-HT_3 receptor antagonists being widely used as anti-emetics in combination with chemotherapy (Boess and Kilpatrick, 1997; Yakel, 2000).

To date, the lack of selective and potent α7 nicotinic acetylcholine receptor agonists has prevented an accurate pharmacological mapping of the role of these receptors or their therapeutic value. The present finding that a compound, developed as a selective α7 nicotinic acetylcholine receptor agonist, can display such comparable potency and efficacy at human α7 and 5-HT_3 receptors serves to highlight a point, that as α7 receptor agonists are developed, their activity on α7 and 5-HT_3 receptors needs to be separated. The dramatic influence of small changes in ligand (Machu et al., 2001) or receptor structure (Steward et al., 2000) on functional activity at α7 and 5-HT_3 receptors, highlights the need to have a thorough understanding of the basis of this cross-reactivity to allow development of selective ligands.

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