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Purification of 5-hydroxytryptamine$_3$ receptors from porcine brain

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1 We demonstrate, for the first time, the purification of the 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor from a native tissue source, pig cerebral cortex.

2 From a range of detergents, the non-ionic detergent Triton X-100 was demonstrated to exhibit the least inhibition of [^{3}H]-(S)-zacopride binding to membrane bound 5-HT$_3$ receptors from pig cerebral cortex at concentrations above its critical micellular concentration (CMC). This detergent was therefore selected to solubilize 5-HT$_3$ binding sites from homogenates of pig cerebral cortex. Maximum yield ($43.8 \pm 3.7\%$, mean $\pm$ s.e.mean, $n=13$) was obtained with Triton X-100 at 0.4% ($22.1 \times$ CMC). Radioligand binding studies with [^{3}H]-(**S**)-zacopride indicated that the solubilized 5-HT$_3$ receptor displayed near identical pharmacology to the membrane bound receptor (the correlation coefficient (r) between the pK_i values of structurally unrelated compounds competing for [^{3}H]-(**S**)-zacopride binding in the membrane bound and solubilized 5-HT$_3$ receptor preparations was 0.99, $B_{max} = 20.7 \pm 4.2$ fmol mg^{-1} protein, $K_d = 1.57 \pm 0.53$ nM, mean $\pm$ s.e.mean, $n=6$).

3 Solubilized (0.4% Triton X-100) 5-HT$_3$ receptors were affinity purified using Affi-Gel 15 coupled to the high affinity 5-HT$_3$ receptor ligand GR119566X. Radioligand binding studies indicated that the pharmacological profile of the affinity purified 5-HT$_3$ receptor, assessed using ligands with a range of affinities spanning 3 orders of magnitude, was similar to that in both crude homogenates ($r=0.85$) and solubilized 5-HT$_3$ receptor sites ($r=0.85$) from pig brain. The specific activity for the purified 5-HT$_3$ receptor overlapped the theoretical specific activity of the receptor ($B_{max} = 3.27 \pm 1.41$ and 5.35 ± 2.33 nmol mg^{-1} protein, assessed by saturation and competition studies respectively, mean $\pm$ s.e.mean, $n=3-4$), which indicated a 60 000–100 000 fold purification of the membrane bound receptor.

4 Under non-reducing conditions, samples of the affinity purified protein failed to enter a 10% separating gel in SDS–PAGE analysis, indicating a molecular mass for the receptor complex of >200 kDa. Further investigation of the non-reduced purified protein with a 7.5% separating gel gave a mass for the complex of $\sim$279 kDa. Under reducing conditions, SDS–PAGE analysis of the affinity purified 5-HT$_3$ receptor resulted in 3–6 silver stained bands at apparent molecular masses of 37, 44–50, 52, 57–61, 63 and 65–71 kDa ($n=12$). Unlike protein bands at 45, 50, 60 and 66 kDa, the bands corresponding to proteins of 52, 57, 63 and 71 kDa consistently gave no reaction with an antiserum specific for the cloned A subunit of the 5-HT$_3$ receptor in both a modified dot blot procedure and a Western blot procedure ($n=2-5$).

5 We conclude that we have purified the 5-HT$_3$ receptor from pig brain to homogeneity and suggest this may contain non-5-HT$_3$-A receptor subunit(s).

Keywords: 5-Hydroxytryptamine; 5-HT$_3$ receptor; solubilization; purification; pig brain; receptor subunits

Introduction

Unlike all other known receptors for 5-hydroxytryptamine (5-HT), which are predicted to be 7 transmembrane domain macromolecules coupled to G proteins (Hoyer *et al*., 1994; Boess & Martin, 1994), the 5-HT$_3$ receptor is a ligand gated ion channel (Derkach *et al*., 1989; Maricq *et al*., 1991). It is likely to be composed of multiple subunits in common with other members of this superfamily (e.g. Cockcroft *et al*., 1990) with which the 5-HT$_3$ receptor subunit displays some sequence homology (e.g. between 20–30% homology with subunits of the nicotinic, γ-aminobutyric acid (GABA)$_A$ and glycine receptors; Maricq *et al*., 1991). Given the relatively long period of time which has elapsed since the disclosure of the cDNA for the 5-HT$_3$-A receptor subunit (Maricq *et al*., 1991), it may be significant that only closely related additional subunit sequences have been described – a rat homologue of the 5-HT$_3$-A subunit (Johnson & Heinneman, 1992; Isenberg *et al*., 1993) and human homologues of a murine subunit designated 5-HT$_3$-A$_S$, which represents an alternatively spliced variant (Hope *et al*., 1993; Belelli *et al*., 1995; Miyake *et al*., 1995). The predicted amino acid sequence homology between the identified species variants of the 5-HT$_3$-A receptor subunit ranges between 82–98%, and may account for some of the marked inter-species pharmacological (for reviews see Kilpatrick & Tyers, 1992; Bentley & Barnes, 1995) and electrophysiological (for review see Peters *et al*., 1992) differences in the 5-HT$_3$ receptor. However, given the precedence of other ligand gated ion channels, the presence of other 5-HT$_3$ receptor subunits remains attractive. Furthermore, compelling evidence for the presence of additional 5-HT$_3$ receptor subunits stems from direct comparison of the murine recombinant 5-HT$_3$-A receptor with the 5-HT$_3$ receptor expressed in murine superior cervical ganglion. Whilst the pharmacological profile of 5-HT$_3$ receptors in these two preparations was very similar, they displayed marked electrophysiological differences (e.g. 0.4–0.6 vs 10 pS conductance for the recombinant vs native receptor, respectively; Hussy *et al*., 1994).

Previous studies have described the purification of the 5-HT$_3$ receptor from several cell lines (NG108-15 cells, Boess *et al*., 1992; N1E-115 cells, Lummis & Martin, 1991; NCB20 cells, McKernan *et al*., 1990), which express relatively high densities of 5-HT$_3$ receptor, but display relatively low conductances comparable to the conductance of recombinant homomeric 5-HT$_3$-A/A$_s$ receptors (Peters *et al*.,

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1992). In the present study we have taken the biochemical approach of purifying the 5-HT$_3$ receptor expressed in pig cerebral cortex as a first important step in an attempt to identify additional subunits. Preliminary accounts of the data have been presented to the British Pharmacological Society (Fletcher & Barnes, 1995; 1996a; 1997).

Methods

Preparation of brain homogenate for radioligand binding assays

Pig brain tissue was obtained from a local abattoir within 30 min of death and transported over ice. Tissues were dissected and frozen at -80°C within 2 h of death. To prepare the radioligand binding homogenate, brain tissues were gently thawed and homogenized (Polytron blender, full power; 10 s) in ice-cold Tris/Krebs buffer (mM: Tris 50.0, NaCl 118.0, KCl 4.75, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.0; pH 7.4). The homogenate was washed twice by centrifugation (25 000 $\times g$, 10 min, 4°C) and subsequent resuspension of the pellet in Tris/Krebs buffer, and finally resuspended at a concentration of 500 mg original wet weight ml^{-1}.

Radioligand binding assays

For radioligand binding assays, test tubes (in triplicate) contained one volume of competing drug or vehicle (radioligand binding buffer; total binding) and two volumes of [^{3}H]-(**S**)-zacopride (for saturation studies a range of final concentrations was used between 0.2–32.2 nM; for competition studies, the final concentration was between 0.4–2.2 nM). For assays involving membrane homogenates, one volume = 50 μl and the radioligand binding buffer was Tris/Krebs; for solubilized receptor preparations, one volume = 150 μl and the buffer was Tris; for purified receptor preparations, one volume = 100 μl and the buffer was Tris. One volume of receptor preparation (membrane homogenates 2.9–5.6 mg ml^{-1}, solubilized receptor 2.5–3.8 mg ml^{-1}, purified receptor 25–135 ng ml^{-1}) was added to initiate binding, which was allowed to proceed at 37°C for 60 min before termination by rapid filtration (Brandel cell-harvester) under vacuum through pre-wet (at least 3 h in 0.3% v/v polyethyleneimine in binding buffer) Whatman GF/B filters followed by washing with ice-cold binding buffer (wash time 2 × 4 s; wash volume 2 × 5 ml). Bound radioactivity remaining on the filters was assayed in 4.5 ml of Ecoscint A (National Diagnostics) by liquid scintillation spectroscopy at an efficiency of approximately 47%. To prevent oxidation of 5-HT, pargyline (10 μM) was included in the radioligand binding buffer for assays involving this compound (pargyline at this concentration did not interfere with [^{3}H]-(**S**)-zacopride binding; data not shown).

Receptor solubilization

5-HT$_3$ receptor binding sites were solubilized from homogenates of pig cerebral cortex (1 g original wet weight ml^{-1} in 25 mM Tris, pH 7.4) by addition of an equal volume of solubilization buffer (25 mM Tris, 2 mM EDTA, 0.1 mM PMSF, 10 μg ml^{-1} bacitracin, 10 μg ml^{-1} sodium azide, 10 μg ml^{-1} soybean trypsin inhibitor, pH 7.4) containing 0–2% Triton X-100 (final concentrations, N.B. soybean trypsin inhibitor was omitted from the solubilization buffer in the purification protocol to prevent addition of a non-5-HT$_3$ receptor protein). The preparation was gently mixed in a rotating shaker for 60 min at 4°C, before being centrifuged at 100 000 $\times g$ for 60 min at 4°C. The supernatant was removed and used immediately for radioligand binding studies, and the pellet gently resuspended in an equal volume of solubilization buffer (containing Triton X-100) before residual 5-HT$_3$ receptor levels were assessed by radioligand binding.

Synthesis of the affinity resin

A GR119566X-affinity column was prepared in a similar manner to that previously described (Boess *et al*., 1992) which has been used successfully to purify the 5-HT$_3$ receptor from NG108-15 cells. Briefly, Affi-Gel 15 (Bio-Rad) was washed with three volumes of isopropanol. To 25 ml of Affi-Gel (equivalent to 375 μmol of active *N*-hydroxysuccinimide ester sites), 12.5 mg of GR119566X (37.5 μmol, equivalent to 10% of the active sites on the resin) dissolved in 25 ml of isopropanol was added. The mixture was incubated for 16 h at room temperature with gentle stirring, and the loss of GR119566X in the supernatant measured by u.v. spectroscopy at 320 nm (90% decrease). To block uncoupled reactive sites on the Affi-Gel, 40 mM of ethanolamine was added ($\sim 5 \times$ excess) and the Affi-Gel incubated for a further 6 h at room temperature. The affinity resin was washed with 4 volumes of isopropanol, one volume of methanol and 5 volumes of distilled water, and packed into a column (1.5 × 15 cm) at 4°C. Before use, the column was washed with 10 volumes of 3 M urea, 3 M NaCl, 1% Triton X-100 in 25 mM Tris (pH 7.4) and 10 volumes of solubilization buffer containing 0.4% Triton X-100 and 500 mM NaCl. After use the column was washed with 4 volumes of regeneration buffer (3 M urea, 3 M NaCl, 1% Triton X-100 in 25 mM Tris, pH 7.4), and stored in 0.02% sodium azide solution.

Affinity purification

Solubilized 5-HT$_3$ receptor (450 ± 77 ml; 2.7–3.8 mg protein ml^{-1}) was applied to the affinity column at a rate of 20 ml h^{-1}. To remove non-specifically bound protein, the column was subjected to the following washes: solubilization buffer containing 0.4% Triton X-100 and 500 mM NaCl (wash buffer 1, 10 volumes), and solubilization buffer containing 0.4% Triton X-100 and 250 mM NaCl (wash buffer 2, 30 volumes). 5-HT$_3$ receptor bound to the affinity column was eluted specifically with 5 volumes of granisetron (4 purifications 0.1 mM, 1 purification 0.01 mM) in wash buffer 2, circulated in 3 aliquots. The eluents were concentrated with either Centriprep 100 concentrators (Amicon) or negative pressure dialysis. To remove the granisetron, samples of purified 5-HT$_3$ receptor were applied to pre-equilibrated PD10 columns which retained the granisetron following elution of the purified 5-HT$_3$ receptor by solubilization buffer containing 0.4% Triton X-100.

SDS-polyacrylamide gel electrophoresis

Purified 5-HT$_3$ receptor protein (13–42 ng) was precipitated with acetone, and solubilized in 30 μl of either reducing or non-reducing SDS sample buffer (final concentrations; 2% SDS, 125 mM Tris, 10% sucrose, 0.01% bromophenol blue, containing 5% 2-β-mercaptoethanol for the reducing buffer, pH 8.0), heated at 90–95°C for 30 min and applied to the gel. Electrophoresis was performed with 1 mm slab gels (5% stacking gel, 10% separating gel). Gels were stained with both Coomassie Blue R-250 and a silver stain procedure (Silver Stain Plus, Bio-Rad). Several different molecular weight standards were used (β-amylase (200 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (25 kDa), the heavy and light chains of both human and rabbit IgG (50 and 25 kDa) and prestained molecular weight markers (BioRad, range 7–211 kDa)) to estimate the molecular masses of separated proteins.

Dot-blot procedure

In a dot-blot procedure, up to 4 μg of purified 5-HT$_3$ receptor preparation was applied onto a nitro-cellulose membrane (0.45 mm, Schleicher & Schuell) in 2 μl aliquots and allowed to dry at 37°C for 1 h. The membrane was incubated in blocking

buffer (PBS/0.1% Tween/2% powdered skimmed milk (Sainsburys), PBS mM: NaCl 136.8, Na_2HPO_4 10.1, KH_2PO_4 1.5 and KCl 0.3) overnight at 4°C. The membrane was subjected to 5 washes in PBS/Tween, placed in a solution of either pre-immune serum or an antiserum specific for the 5-HT$_3$-A receptor (Turton *et al*., 1993) in PBS/Tween for an hour, washed 5× in PBS/Tween, incubated in conjugated secondary antibody solution (sheep anti-rabbit IgG-peroxidase) in blocking buffer, and washed 3× in PBS/Tween and 5× in PBS buffer. Dot blots were also performed with another antiserum specific for the cloned subunit of the 5-HT$_3$ receptor (Morales *et al*., 1996), by use of the procedure described by Morales *et al*. (1996). Immunoreactive proteins were visualized using an ECL detection system (Amersham).

Modified dot-blot procedure

Gels were polymerized using N,N′-diallyl-tartardiamide (DATD) as a crosslinker (0.08% w/v stacking gel, 0.3% w/v separating gel), rather than bisacrylamide. Silver-stained SDS–PAGE separated proteins were excised from the gels using a scalpel, dissolved in 5 volumes of 2% periodic acid at room temperature for 2 h, dotted (2–5 μl) onto nitrocellulose membrane and analysed as described above.

Western blot procedure

Following SDS–PAGE, protein was transferred from the gel to PVDF membrane (Bio-Rad) by use of a Semi-Dry Transfer Cell (Bio-Rad), and analysed for immunoreactivity as described above. Protein transferred to the membrane was visualized using the Enhanced Colloidal Gold Total Protein Detection Kit (Bio-Rad), following removal of any antibodies bound to the membrane by incubating at 50°C in stripping buffer (100 mM 2-β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7).

Protein determination

Protein was assayed in membrane and solubilized preparations by the Bio-Rad Coomassie Brilliant Blue method (Bradford, 1976) with bovine serum albumin as the standard. For purified receptor preparations, protein was assayed on silver stained SDS-polyacrylamide gels by quantitative densitometry with an image analysis system (MCID, Imaging Research Inc.). A standard curve was constructed by densitometry of bovine serum albumin over the range 10–1000 ng which was linear (data not shown).

Data analysis

Saturation and competition data were analysed by computer assisted iterative curve fitting according to the equation; $b = (B_{max}[L]^n/([L]^n{}_H + (K)^n{}_H)$, where b = bound radioligand; B_{max} = maximum binding at equilibrium; for saturation studies K = molar equilibrium dissociation constant or for competition studies K = molar concentration of competing compound to reduce the specific binding by 50%; for saturation studies L = free molar concentration of radioligand or for competition studies L = molar concentration of competing compound; n_H = Hill coefficient. K_i values were obtained from IC_{50} values by use of the Cheng-Prusoff equation $K_i = IC_{50}/(1 + ([RL]/K_d))$, where IC_{50} = molar concentration of competing compound to reduce the specific binding by 50%, [RL] = molar concentration of radioligand, and K_d = molar equilibrium dissociation constant.

Statistics

The significance of the difference between Hill values and unity was assessed by means of both a 2 tailed t test and by calculating the 95% confidence interval ($P < 0.05$). Both tests gave the same results.

Materials

5-Carboxyamidotryptamine (maleate; 5-CT; RBI), GR113808A ([1-[2-methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3-carboxylate; Glaxo), GR1-19566X (1,2,3,9-tetrahydro-3-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-(3-aminopropyl)-4*H*-carbazol-4-one; Glaxo), granisetron (HCl; SmithKline Beecham), 5-HT (bimaleate; Sigma), *meta*-chlorophenylbiguanide (HCl; mCPBG; RBI), methiothepin (mesylate; RBI), ondansetron (hydrochloride dihydrate; Glaxo), paroxetine (HCl; SmithKline Beecham), phenylbiguanide (PBG; Aldrich), tropisetron (ICS 205-930; (1 αH, 3α, 5α*H*-tropan-3-yl)1-*H*-indole-3-carboxylic acid ester; Sandoz) and (**S**)-zacopride (HCl; Delalande) were dissolved in a minimum quantity of distilled water and diluted with buffer. [^{3}H]-(**S**)-zacopride (78 Ci mmol^{-1}, Amersham) was supplied in ethanol and diluted in buffer. GENAPOL (polyoxyethylene (10) dodecyl ether) was obtained from Calbiochem-Novabiochem, and Triton X-100 (t-octylphenoxypolyethoxyethanol), CHAPS (3-[3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate), octylglucoside (n-octyl b-D-glucopyranoside) and deoxycholate (5β-cholan-24-oic acid-3α,12α-diol, sodium salt) from Sigma. All drugs and reagents were used as received.

Results

*Inhibition of [^{3}H]-(**S**)-zacopride binding to 5-HT$_3$ binding sites by detergents*

Figure 1 shows the binding of [^{3}H]-(**S**)-zacopride to membrane bound 5-HT$_3$ receptors in the presence of different concentrations of 5 detergents. The detergents with high critical micellular concentration (CMC) values (>1 mM; CHAPS, 3–5 mM; octyl glucoside, 19–25 mM; deoxycholate 1–4 mM; CMC values from Neugebauer, 1992) markedly inhibited [^{3}H]-(**S**)-zacopride binding at concentrations below and around their CMC. The non-ionic detergent Triton X-100 (CMC = ~0.25 mM) exhibited the least inhibition of [^{3}H]-(**S**)-zacopride binding above its CMC. Dilution of the solubilized receptor preparation in the binding assay was found to reverse

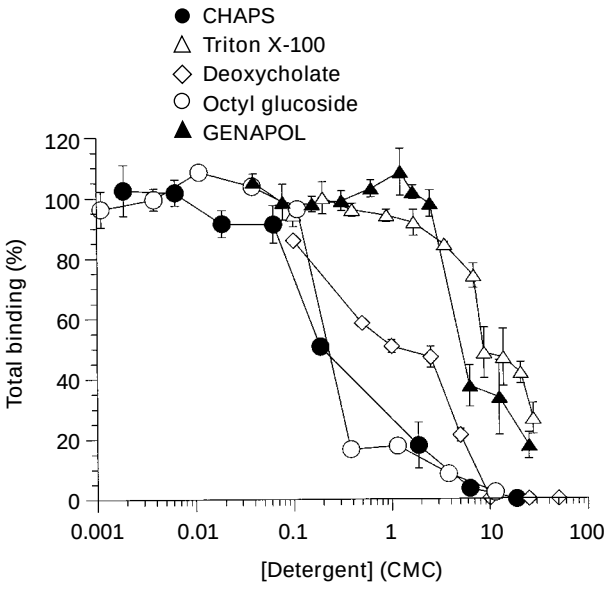

Figure 1 Inhibition of [^{3}H]-(**S**)-zacopride binding to membrane bound 5-HT$_3$ receptors originating from pig cerebral cortex by various detergents; CHAPS, Triton X-100, deoxycholate, octyl glucoside and GENAPOL. The final protein concentration (0.95–1.44 mg ml^{-1}) was constant for each experiment. Data represent mean and vertical lines show s.e.mean, $n = 3$.

some of this inhibition of binding (e.g. a dilution of 1:4 or greater was found to reverse up to 66% of the inhibition of binding induced by 0.4% Triton X-100, data not shown).

Solubilization of 5-HT$_3$ receptor binding sites

Figure 2 shows the effect of Triton X-100 concentration on the yield of 5-HT$_3$ receptors and total protein. As the concentration of detergent was increased, the amount of protein solubilized also increased, but above 0.4% Triton X-100 (22.1 × CMC) there was no further increase in receptor yield. Maximum yield (43.8 ± 3.7%, mean ± s.e.mean, $n = 13$) was obtained with Triton X-100 at 0.4% (22.1 × CMC, detergent:protein (w/w) ratio ~ 1.2:1). The presence of different concentrations of NaCl (0.25–2 M) in the solubilization buffer was investigated and found to have no significant effect on the yield of receptor, although at higher concentrations (1.5–2 M) there was a significant decrease in the specific binding (approximately 50% of specific binding in the absence of salt, data not shown). Ammonium sulphate (200–800 mM), glycerol (5–20%) and phosphatidylcholine (0.15–1%) were all found to inhibit [^{3}H]-(**S**)-zacopride binding, and did not improve 5-HT$_3$ receptor yield (data not shown).

Affinity purification

5-HT$_3$ receptors solubilized by 0.4% Triton X-100 were purified with Affi-Gel 15 coupled to the high affinity 5-HT$_3$ receptor ligand GR119566X. Most of the specific 5-HT$_3$ receptor binding in the solubilized 5-HT$_3$ receptor preparation was retained by the affinity column (Table 1, Figure 3). Total protein concentration in the soluble extract after it had been passed through the affinity column was not significantly different from that before it had been passed through the column (3.26 ± 0.28 compared to 3.16 ± 0.24 mg ml^{-1}, mean ± s.e.mean, $n = 4$), i.e. very little protein was retained by the column. 5-HT$_3$ receptor bound to the column was eluted with 0.1 mM ($n = 4$) or 0.01 mM ($n = 1$) granisetron. Less receptor was eluted by 0.01 mM granisetron compared to the fractions eluted by 0.1 mM granisetron (2.1% yield compared to 6.5 ± 0.8% yield (mean ± s.e.mean, $n = 4$), however, the pharmacology of the fractions was similar.

Saturation binding

[^{3}H]-(**S**)-Zacopride labelled an apparently homogeneous, saturable population of specific binding sites with nanomolar affinity in both the solubilized and purified receptor preparations (Table 2). These values are very similar to that previously determined for the 5-HT$_3$ receptor in a membrane preparation derived from pig cerebral cortex (Table 2). The Hill coefficients in all three preparations were not significantly different from unity (Table 2). The B_{max} values for the purified receptor preparation (Table 2) indicated an ~60 000 fold purification of the membrane bound receptor.

Competition binding

Both the solubilized and purified 5-HT$_3$ receptor preparations displayed a pharmacological profile similar to that of the receptor sites in crude homogenates (Table 3). The correlation coefficients between the pK_i values of these (structurally un-

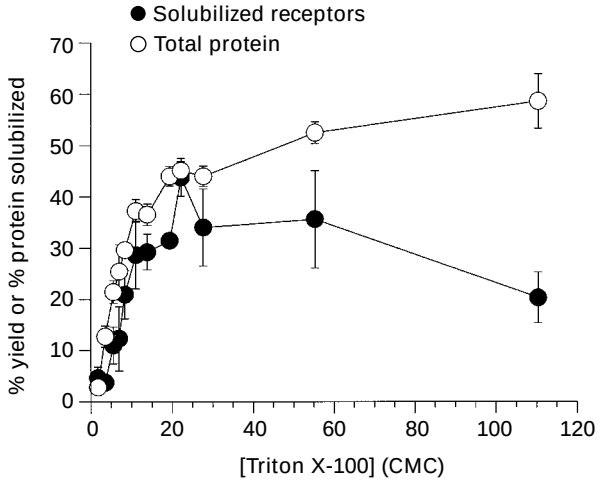

Figure 2 Effect of Triton X-100 concentration on the yield of solubilized 5-HT$_3$ receptors and total protein from pig cerebral cortex membranes. The final protein concentration (2.66–3.81 mg ml^{-1}) was constant for each solubilization. Data represent the mean and vertical lines show s.e.mean, $n = 3–13$.

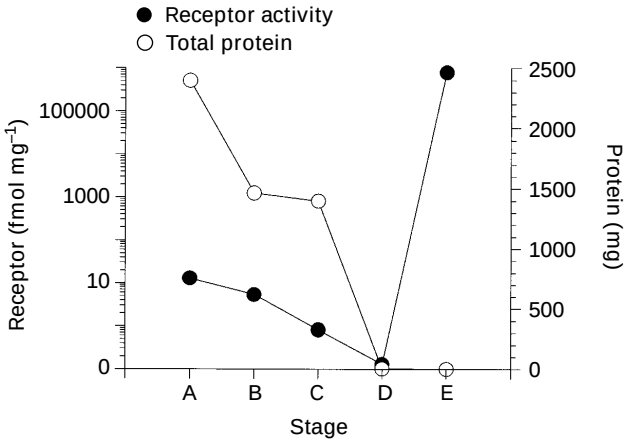

Figure 3 Receptor activity (fmol mg^{-1}) and total protein (mg) at various stages of the purification procedure. Stages are: A, membrane homogenates; B, solubilized receptor; C, protein not retained by the affinity column; D, protein eluted in column washes; E, protein eluted by 0.1 mM granisetron. The data represent the mean of 4 purifications.

Table 1 Purification of the 5-HT$_3$ receptor with a GR119566X-affinity column

Fraction	*Receptor* (fmol mg^{-1})[a]	*%yield*	*Protein* (mg)
Membranes	12.89 ± 0.51	100	2395 ± 538
Solubilized receptor	5.39 ± 0.86	42.0 ± 7.5	1463 ± 307
Protein not retained by affinity column	0.82 ± 0.31	5.6 ± 1.8	1396 ± 238
Protein eluted in column washes	0.13 ± 0.13	2.1 ± 1.4	2.4 ± 2.3
Protein eluted by 0.1 mM granisetron	792341 ± 93174	6.5 ± 0.8	0.00275 ± 0.00048

5-HT$_3$ receptors were solubilized from 168 ± 34 g wet weight pig brain (mean ± s.e.mean, $n = 4$) in 0.4% Triton X-100, and eluted from the GR119566X-affinity column by granisetron (0.1 mM) in 0.4% Triton X-100. Percentage values are mean ± s.e.mean from 4 experiments. [a]Obtained by use of [^{3}H]-(**S**)-zacopride at 1.0–2.5 nM.

Table 2 Saturation data obtained with [^{3}H]-(**S**)-zacopride binding to membrane bound, solubilized (Triton X-100, 0.4%) and affinity purified (GR19566X/Affi-Gel) 5-HT$_3$ receptor preparations originating from pig cerebral cortex

5-HT$_3$ receptor	*B_{max}*	K_d (nM)	*Hill coefficient*
Membrane bound[a]	54±6 fmol mg^{-1}	1.62±0.35	1.06±0.12
Solubilized	20.7±4.2 fmol mg^{-1}	1.57±0.53	1.05±0.14
Purified	3.27±1.41fmol mg^{-1}	1.70±0.35	1.21±0.15

Solubilized 5-HT$_3$ receptor (450±77 ml, 7274±931 fmol receptor, 1463±307 mg protein, derived from 168±34 g wet weight pig brain, mean±s.e.mean, $n=4$) was applied to the affinity column. The values represent the mean±s.e.mean, $n=4-6$. [a]Data from Fletcher & Barnes (1996b).

Table 3 The affinities of various compounds to compete for specific [^{3}H]-(**S**)-zacopride binding to 5-HT$_3$ receptor sites in membrane homogenates, solubilized (Triton X-100, 0.4%) and affinity purified (GR119566X/Affi-Gel) preparations originating from pig cerebral cortex

	Membrane homogenates[b]		*Solubilized 5-HT$_3$ receptor*		*Purified 5-HT$_3$ receptor*	
Drug	*pK_i*	*Hill*	*pK_i*	*Hill*	*pK_i*	*Hill*
Granisetron	9.24±0.03	1.27±0.07	9.66±0.06	1.28±0.10	8.22±0.34	0.86±0.21
(**S**)-Zacopride	9.08±0.06	0.77±0.13	9.10±0.11	1.20±0.24	8.61±0.15	1.73±0.16
GR119566X	8.21±0.06	0.91±0.09	8.53±0.11	1.08±0.08	8.80±0.31	0.64±0.06[a]
mCPBG	8.14±0.07	0.93±0.08	8.16±0.05	1.13±0.22	7.92±0.10	1.49±0.22
Ondansetron	7.71±0.16	1.07±0.13	7.84±0.06	0.88±0.28	8.49±0.20	1.14±0.19
Tropisetron	7.44±0.05	0.90±0.03	7.52±0.18	0.91±0.17	7.93±0.06	1.49±0.29
5-HT	6.28±0.02	0.92±0.05	6.43±0.12	0.95±0.09	6.00±0.12	1.39±0.33
PBG	6.17±0.02	1.00±0.03	6.29±0.04	1.13±0.28	6.03±0.21	1.82±0.34

Data represents the mean±s.e.mean, $n=3-6$. [a]Mean±range ($n=2$). [b]Data from Fletcher & Barnes (1996b).

related) compounds competing for [^{3}H]-(**S**)-zacopride binding in the membrane bound and solubilized 5-HT$_3$ receptor preparations, and membrane bound and purified 5-HT$_3$ receptor preparations were $r=0.99$ and $r=0.85$, respectively. The Hill coefficients of competing compounds for the membrane bound and solubilized receptor binding were not different from unity. However, the Hill coefficients generated by the agonists 5-HT, mCPBG, and PBG, and the antagonists tropisetron and (**S**)-zacopride competing for [^{3}H]-(**S**)-zacopride binding to purified 5-HT$_3$ receptor were greater than unity, but only that for (**S**)-zacopride reached statistical significance. Methiothepin (1 μM, 5-carboxamidotryptamine (1 μM), paroxetine (1 μM) and GR113808A (100 nM) reduced the specific binding of [^{3}H]-(**S**)-zacopride to the purified receptor by less than 3.2% ($n=2$). B_{max} values derived from the displacement of a single concentration of [^{3}H]-(**S**)-zacopride (1.90–2.21 nM) by 'cold' (**S**)-zacopride gave a density of radiolabelled 5-HT$_3$ receptors of 5.35±2.33 nmol mg^{-1} for the purified protein fraction (mean±s.e.mean, $n=3$).

SDS–PAGE analysis

SDS–PAGE of the affinity purified protein in reducing buffer resulted in 3–6 silver stained bands at apparent molecular masses of 37, 44–50, 52, 57–61, 63 and 65–71 kDa ($n=12$, Figure 5). Corresponding samples in non-reducing buffer failed to enter the separating gel, indicating a molecular mass for the receptor complex of >200 kDa. Further investigation of the non-reduced purified protein by use of a 7.5% separating gel gave a band at approximately 279 kDa.

Dot and Western blots

In a dot blot procedure, the purified receptor preparation gave a positive reaction with two antisera specific for the cloned A subunit of the 5-HT$_3$ receptor (Turton *et al.*, 1993; Morales *et al.*, 1996). No reaction was detected with pre-immune serum (data not shown). By use of one of these antisera (Turton *et al.*, 1993) in the modified dot blot procedure, several bands excised from DATD gels gave a positive signal with the 5-HT$_3$ receptor antiserum (e.g. 45, 60 and 66 kDa) but a band corresponding to a protein of 52 kDa gave no reaction ($n=3$, Figure 4). In Western blots, bands at 45, 50, 60 and 66 kDa gave a positive

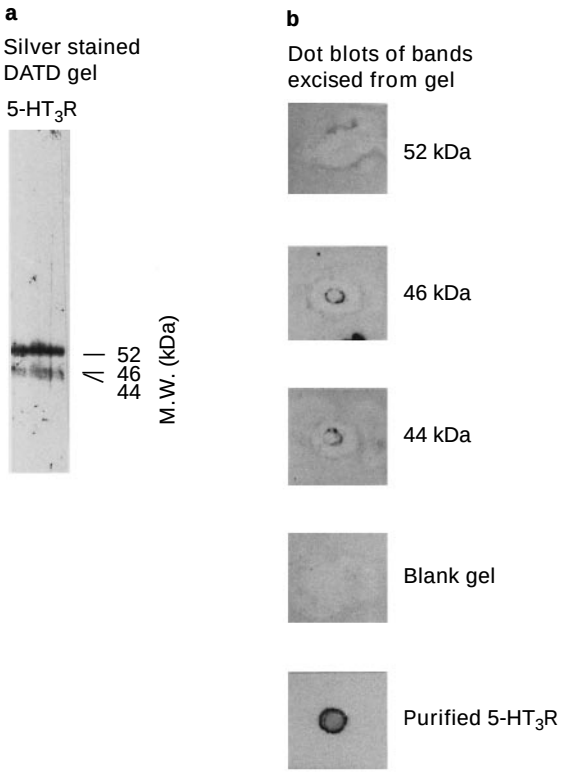

Figure 4 An example of a modified dot blot. (a) Purified 5-HT$_3$ receptor protein analysed by SDS–PAGE; 28 ng of protein was loaded onto the gel. Only 3 bands were visible by silver staining on this particular gel. (b) Dot blots of bands excised from the gel and probed with an antiserum specific for the cloned A subunit of the 5-HT$_3$ receptor.

reaction with the 5-HT$_3$-A antiserum, however, bands at 52, 57, 63 and 71 kDa gave no reaction ($n=2-3$, Figure 5). All bands had transferred from the gel to the membrane, as shown by gold staining (Figure 5). No reaction was detected with pre-immune serum (data not shown).

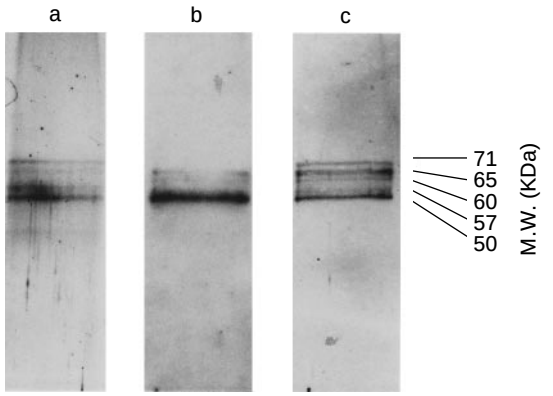

Figure 5 Purified 5-HT$_3$ receptor protein analysed by (a) SDS–PAGE, (b) transferred to PVDF membrane following SDS–PAGE and probed with an antiserum specific for the cloned A subunit of the 5-HT$_3$ receptor, and (c) stained for protein with a gold stain after transfer to PVDF membrane; 28 ng of protein was loaded onto the gel. Bands at 50, 60 and 65 kDa gave a positive reaction with the 5-HT$_3$-A antiserum in this particular experiment, whereas bands at 57 and 71 kDa gave no reaction (lane b). All five bands were visible following total protein staining (lane c). Although a band of lower molecular weight ($\sim$39 kDa) was seen on the original gel (lane a), this did not transfer to the membrane, and therefore was not visualized by either the 5-HT$_3$-A antiserum or the total protein stain (lanes b, c).

Discussion

Previous studies have described the purification of the 5-HT$_3$ receptor from several cell lines (NG108-15 cells, Boess *et al*., 1992; N1E-115 cells, Lummis & Martin, 1991; NCB20 cells, McKernan *et al*., 1990). In this paper we demonstrate, for the first time, the purification of the 5-HT$_3$ receptor from a native tissue source, pig cerebral cortex. Several receptors have been successfully purified from pig brain and spinal cord, including the galanin receptor (Chen *et al*., 1993) and the glycine receptor (Graham *et al*., 1985); this latter receptor represents another member of the ligand gated ion channel superfamily. This preparation offers several advantages for the purification of the central 5-HT$_3$ receptors in that it is available in large quantities and expresses a relatively high level of the 5-HT$_3$ receptor (Fletcher & Barnes, 1996b).

From a range of detergents, the non-ionic detergent Triton X-100 was demonstrated to exhibit the least inhibition of binding of the selective 5-HT$_3$ receptor ligand [^{3}H]-(**S**)-zacopride at concentrations above its CMC. This detergent was therefore selected to solubilize 5-HT$_3$ binding sites from homogenates of pig cerebral cortex. Triton X-100 has been used previously for the solubilization of other receptors, including the 5-HT$_3$ receptor expressed in NG108-15 cells (Boess *et al*., 1992). As the concentration of detergent was increased, the amount of protein solubilized also increased, but above 0.4% Triton X-100 (22.1 $\times$ CMC) there was no further increase in receptor yield. This may reflect an increase in irreversible inhibition of [^{3}H]-(**S**)-zacopride binding. We found that the presence of salt (0.25–2 M) had no significant effect on the yield of receptor solubilized by the non-ionic detergent Triton X-100. This contrasts with data for this detergent solubilizing the 5-HT$_3$ receptor from a cell line (Boess *et al*., 1992) and may reflect a difference in receptor environment.

5-HT$_3$ receptors solubilized by 0.4% Triton X-100 were purified with Affi-Gel 15 coupled to the high affinity 5-HT$_3$ receptor ligand GR119566X, an analogue of the high affinity 5-HT$_3$ antagonist GR67330X (Kilpatrick *et al*., 1990). Such a column has been used previously for the purification of 5-HT$_3$ receptors from a cell line (Boess *et al*., 1992). The affinity of the receptor for GR119566X did not appreciably change on either solubilization or purification (Table 3). In saturation studies, the affinity of [^{3}H]-(**S**)-zacopride for 5-HT$_3$ binding sites in both solubilized and purified receptor preparations was comparable and similar to that for the receptor expressed in pig brain homogenates. Competition studies demonstrated that the pharmacological profile of solubilized and purified 5-HT$_3$ receptors (assessed by use of a range of structurally unrelated 5-HT$_3$ receptor ligands, which covered a range of affinities over 3 orders of magnitude) was essentially the same as that of the membrane bound receptor.

The specific activity for the purified 5-HT$_3$ receptor in this study was 3.27 ± 1.41 nmol mg^{-1} protein assessed by saturation studies, and 5.35 ± 2.33 nmol mg^{-1} protein calculated from (**S**)-zacopride competition for [^{3}H]-(**S**)-zacopride binding. This represents $\sim$60 000–100 000 fold purification of the membrane bound receptor. Given the error involved in protein quantification, and the possibility that gel filtration may have resulted in a loss of binding activity due to the instability of the receptor in the absence of antagonist or phospholipid, this is remarkably close to the theoretical specific activity of the receptor (3.6 nmol mg^{-1}), assuming a molecular mass for the receptor complex of $\sim$280 kDa, and one binding site per complex. The density of the 5-HT$_3$ receptor we have purified from a native source is comparable to that obtained for the 5-HT$_3$ receptor purified from cell lines (e.g. values of 3.1 and 3.2 nmol mg^{-1}; McKernan *et al*., 1990; Boess *et al*., 1992).

Samples of the affinity purified 5-HT$_3$ protein in non-reducing buffer failed to enter a 10% separating gel in SDS–PAGE analysis, indicating a molecular mass for the receptor complex of $>$200 kDa. Further investigation of the non-reduced purified protein with a 7.5% separating gel gave a mass for the complex of $\sim$279 kDa. This is similar to values obtained for other ligand gated ion channel receptor complexes (Strange, 1988) and to values previously calculated for the 5-HT$_3$ receptor (e.g. 370 and 250 kDa for purified 5-HT$_3$ receptor in the presence and absence of detergent respectively, McKernan *et al*., 1990; Boess *et al*., 1992).

SDS–PAGE of the affinity purified protein in reducing buffer resulted in between 3–6 silver stained bands at apparent molecular masses of 37, 44–50, 52, 57–61, 63 and 65–71 kDa. This pattern was similar to that observed for the 5-HT$_3$ receptor purified from NG108-15 cells by Boess *et al*. (1992), where broad bands were observed at 36, 40, 50 and 76 kDa. In contrast Lummis & Martin (1991) observed only a single distinct band of 55 kDa for the 5-HT$_3$ receptor purified from N1E-115 cells, whilst McKernan *et al*. (1990) showed that the 5-HT$_3$ receptor purified from NCB20 cells gave broad bands with apparent molecular masses of 38 and 54 kDa. They suggested that the broad, indistinct bands may be composed of more than one species arising from heterogeneity in post-translational processing or distinct subunits migrating closely.

The 5-HT$_3$ receptor purified from pig brain bound weakly to nitrocellulose, resulting in analysis by Western blotting being difficult. Two approaches were adopted to overcome this problem; the use of a modified dot blot technique, where bands were excised from DATD gels and solubilized with 2% periodic acid, and blotting onto PVDF membrane. In this modified dot blot procedure, several bands excised from DATD gels gave a positive signal with the 5-HT$_3$-A receptor antiserum (e.g. 45, 60 and 66 kDa) but the band corresponding to a protein of 52 kDa gave no reaction (Figure 4). In Western blots, bands at 45, 50, 60 and 66 kDa gave a positive reaction with the 5-HT$_3$-A antiserum. However, bands at 52, 57, 63 and 71 kDa gave no reaction (Figure 5). The theoretical molecular mass of the cloned 5-HT$_3$-A (ligand binding) subunit is 56 kDa (Maricq *et al*., 1991). Turton *et al*. (1993) observed a broad band at $\sim$54 kDa on a Western blot of the 5-HT$_3$ receptor purified from NCB20 cells, but no reaction from a weak band at 38 kDa. Mukerji *et al*. (1996) transfected COS cells with the 5-HT$_3$ receptor and observed a broad band at $\sim$61 kDa in a Western blot. Broad bands could arise from closely migrating glycosylated isoforms of a protein. Green *et al*. (1995) deglycosylated the recombinant 5-HT$_3$ receptor expressed in a baculovirus with endoglycosidase H and demonstrated a shift in apparent molecular mass from $\sim$56 to $\sim$49 kDa in the

labelled band on a Western blot. Different glycosylation states of the 5-HT$_3$ receptor could account for the bands seen at ~45, 50, 60 and 66 kDa on our gels, which gave a positive reaction in a Western blot procedure and in a modified dot blot procedure using an antiserum recognising the 5-HT$_3$-A receptor subunit was used (Turton *et al*., 1993).

Bands at 52, 57, 63 and 71 kDa gave no reaction in either a modified dot blot or a Western blot procedure with an antiserum specific for the A subunit of the 5-HT$_3$ receptor (Figures 4,5), despite the fact that all these bands transferred to the membrane, as shown by gold staining (Figure 5). These bands might represent additional subunits of the 5-HT$_3$ receptor. This would help explain some of the considerable evidence for the presence of intra-species subtypes of 5-HT$_3$ receptor. The most compelling evidence for the presence of additional 5-HT$_3$ receptor subunits stems from direct comparison of the murine recombinant 5-HT$_3$-A receptor with the 5-HT$_3$ receptor expressed in murine superior cervical ganglion. Whilst the pharmacological profile of 5-HT$_3$ receptors in these two preparations is very similar, they displayed marked electrophysiological differences (e.g. 0.4–0.6 vs 10 pS conductance for the recombinant vs native receptor, respectively; Hussy *et al*., 1994). The potential non-A subunit of our purified 5-HT$_3$ receptor may not influence the pharmacology of the ligand gated ion channel, but may affect its conductance. This would also help explain the apparent failure to detect intra-species pharmacologically different 5-HT$_3$ receptors (e.g. Butler *et al*., 1990; Ito *et al*., 1995; Perren *et al*., 1995; Akuzawa *et al*., 1996; but see Bonhaus *et al*., 1993). Another possibility is that the bands may represent a protein co-purifying with the 5-HT$_3$ receptor, for example an accessory protein serving a similar function to the 43 kDa protein of the nicotinic acetylcholine receptor which is involved in receptor clustering (Froehner *et al*., 1990; Philips *et al*., 1991; Brennan *et al*., 1992) or the 93 kDa protein gephyrin, which is thought to be involved in the anchoring of the glycine receptor to microtubules in the postsynaptic membrane (Kirsch *et al*., 1991), or an endogenous protein tyrosine kinase such as that which co-purifies with, and modulates the function of, the *N*-methyl-D-aspartate receptor (Yu *et al*., 1997).

In conclusion, we have purified to apparent homogeneity the 5-HT$_3$ receptor complex expressed by pig cerebral cortex. We suggest that the 5-HT$_3$ receptor complex purified from pig cerebral cortex may contain a non-5-HT$_3$-A receptor protein. Amino acid micro-sequencing and cloning studies are expected to expand these findings.

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