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Solubilization, Purification, and Functional Reconstitution of 5-Hydroxytryptamine$_3$ Receptors from N1E-115 Neuroblastoma Cells

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SUMMARY

5-Hydroxytryptamine$_3$ (5-HT$_3$) receptors from N1E-115 neuroblastoma cells were solubilized using 1.1% *n*-octylglucoside; five other detergents were less effective. Purification was achieved by affinity chromatography using immobilized GR119566X and biospecific elution with quipazine. Saturation analyses with [^{3}H]GR67330 binding revealed high affinity binding to homogeneous populations of sites in both the solubilized (K_d = 0.05 ± 0.02 nM) and purified (K_d = 0.10 ± 0.04 nM) preparations. Competition experiments indicated that the solubilized and purified receptor preparations retained the characteristics observed in N1E-115 cells *in vivo*. Polyacrylamide gel electrophoresis of the purified receptor revealed a single protein band of 54.7 ± 1.3 kDa. The purified receptor was incorporated into liposomes, and the functional integrity of the protein was demonstrated by measurement of *m*-chlorophenylbiguanide-stimulated ^{22}Na uptake. Saturation analysis of the reconstituted preparation revealed a K_d of 0.24 ± 0.07 nM and suggested that 0.2% of 5-HT$_3$ receptors present in the original membrane preparation had been incorporated into liposomes.

5-HT, like many other neurotransmitters, is found to interact with several pharmacologically distinct receptor types located in neuronal membranes (1). Many of these 5-HT receptors mediate their effects through GTP-binding proteins, but 5-HT$_3$ receptors differ in that their activation causes rapid depolarization as a result of an increased cell membrane conductance to sodium and potassium ions (2); these receptors, therefore, appear to be members of the family of ligand-gated ion channels. There has recently been considerable interest in the 5-HT$_3$ receptor, with increasing evidence that its antagonists are effective against chemotherapy- and radiotherapy-induced emesis and have potential in the treatment of several psychiatric disorders (see Ref. 3 for review). In order to explore the properties of this receptor at the molecular level, it is necessary to isolate and biochemically characterize the receptor protein. The relatively low concentration of the 5-HT$_3$ receptor in the mammalian central nervous system (4) compromises this work. However, the receptor has been identified and characterized on a number of immortal cell lines (5, 6), and we have shown that the density of the 5-HT$_3$ receptor in N1E-115 cells is high, 200,000 sites/cell (7). This enriched source is, therefore, ideal for isolation of the receptor protein, and the fact that the receptor possesses an integral ion channel provides a means of examining its functional integrity after insertion of the purified material into liposomes.

Experimental Procedures

Materials

[^{3}H]GR65630 (85 Ci/mmol) was obtained from Amersham International. GR119566X was synthesized by Dr. Paul Beswick, Glaxo Group Research; [^{3}H]GR67330 (85 Ci/mmol), GR65630 maleate, GR38032F, BRL 43694, and 2-methyl-5-HT hydrochloride were obtained from Glaxo Group Research, MDL 72222 and ICS 205-930 from RBI Inc., and quipazine maleate from Miles Laboratories.

Receptor Solubilization

Solubilization. N1E-115 mouse neuroblastoma cells were cultured as described previously (7). When confluent, cells were either solubilized directly with detergent in inhibition buffer (1 mM EDTA, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM Tris-citrate, pH 7.5) or removed from plates in inhibition buffer, homogenized, and centrifuged at 40,000 × *g* for 20 min, before use or storage at −20°. Solubilization was achieved by

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; mCPBG, *m*-chlorophenylbiguanide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; GABA, γ-aminobutyric acid.

gentle shaking for 60 min at 4°. Soluble receptors were harvested from the supernatant after centrifugation at 100,000 × *g* for 1 hr.

Assay. Aliquots of solubilized receptor or nonsolubilized control membrane samples were incubated for 60 min at 0° with 0.5 nM [^{3}H]GR65630 or 0.1 nM [^{3}H]GR67330 (unless otherwise stated) and ligands being tested in 10 mM HEPES buffer, pH 7.5. Nonspecific binding was determined using 2-methyl 5-HT (100 μM) or mCPBG (10 μM). Incubations were terminated by filtration, followed by three 2-ml ice-cold buffer washes, through GF/B filters that had been presoaked for at least 3 hr at 4° in 0.3% polyethyleneimine. Saturation and competition experiments were routinely conducted with eight ligand concentrations; for the former, these ranged from 0.005 to 2.0 nM. Association and dissociation experiments were performed as described previously (7). Data were analyzed using the computer programs EBDA, LIGAND, and KINETIC (8). Significance levels were determined using Student's *t* test.

Receptor Purification

Affinity column preparation. Packed Affi-Gel 15 (Bio-Rad) was washed in 3 volumes of isopropanol. GR119566X [1,2,3,9-tetrahydro-9-(3-aminopropyl)-3-[(5-methyl-1*H*-immidazol-4-yl)methyl]-4*H*-carbazol-4-one], equivalent to 10 mol % of the available *N*-hydroxysuccinimide groups on the Affi-Gel 15, was dissolved in 2 volumes of isopropanol and added to the Affi-Gel 15. The suspension was mixed on a rotator for at least 8 hr at room temperature; the loss of GR119566X was monitored using UV spectroscopy. The remaining sites were blocked by incubation with excess ethanolamine for 30 min. After the reaction, the gel was washed with 10 volumes of isopropanol, 5 volumes of methanol, and 20 volumes of distilled water. The gel was then transferred to a cold room at 4°, where all subsequent procedures were carried out. Before use the gel was washed with 3 volumes of high salt buffer (see below). After use the gel was washed with 3 volumes of regeneration buffer (3 M urea, 0.5% Triton X-100, 3 M KCl, 10 mM Tris-citrate, pH 7.5), followed by 10 volumes of inhibition buffer.

Receptor purification. Receptor solubilized in 1.1% *n*-octylglucoside was added to 4 volumes of inhibition buffer containing, in addition, CHAPS and KCl, to give final concentrations of 0.6% and 300 mM, respectively. This solution was recirculated on the affinity column (flow rate, 1 ml/min) for at least 12 hr. The column was then washed with 5 volumes of inhibition buffer containing 0.22% *n*-octylglucoside, 0.6% CHAPS, and 300 mM KCl (wash 1). It was subsequently washed with 20 volumes of high-salt buffer (0.22% *n*-octylglucoside, 0.6% CHAPS, 300 mM KCl, 1 mM EDTA, 10 mM Tris-citrate, pH 7.5) (wash 2), followed by 5 volumes of low-salt buffer (0.22% *n*-octylglucoside, 0.6% CHAPS, 150 mM KCl, 1 mM EDTA, 10 mM Tris-citrate, pH 7.5) (wash 3). The purified receptor was then eluted with 4 × 1.3 volumes of 1 mM quipazine in low-salt buffer. Quipazine was removed from the eluate by overnight dialysis against 0.05% Lubrol, 10 mM Tris-citrate, pH 7.5, followed by G-25 column chromatography; 2.5 ml of dialysate were applied to prepacked G25 columns (PD10; Pharmacia) pre-equilibrated with 0.05% Lubrol, 10 mM Tris-citrate, pH 7.5. The receptor was eluted in 3.5 ml of 0.05% Lubrol, 10 mM Tris-citrate, pH 7.5. Purified receptor was assayed as described above.

SDS-PAGE. Samples of purified concentrated receptor preparations, prepared using Microsep filtration units (100-kDa cut-off), were precipitated using a chloroform/methanol procedure (10). SDS-PAGE was carried out on 0.5-mm slab gels of 10–12.5% polyacrylamide (5% stacking gel), as previously described (9). Protein bands were visualized using silver staining (11).

Receptor Reconstitution

Liposome preparation. Liposomes were prepared using gel filtration at room temperature. Lipids (72 mg of asolectin, 8 mg of cholesterol hemisuccinate) were dissolved in chloroform and stored overnight in a dessicator after evaporation of the chloroform under nitrogen. They were then resuspended in 2 ml of choline flux buffer (145 mM choline chloride, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5) containing 0.22% *n*-octylglucoside and 0.6% CHAPS. After this, 2 ml of concentrated purified receptor preparation were added. The mixture was applied to a Sephadex G25 column (24 × 1.5 cm, preequilibrated with 100 ml of choline flux buffer) at 5 ml/hr. The liposomes eluted in the void volume (20–28-ml fraction) and were used immediately in the [^{3}H]GR67330 binding assay as described previously or in the ^{22}Na flux assay described below.

Measurement of ^{22}Na influx. Aliquots of the liposome preparation were incubated at room temperature in a final assay volume of 400 μl of choline flux buffer (145 mM choline chloride, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5) containing 0.2 μCi of ^{22}Na (200 μCi/ml; Amersham), in the presence or absence of 5×10^{-4} M mCPBG. Some samples were also incubated in the presence of 10^{-5} M quipazine. The incubation was continued for 30 sec before rapid dilution to 3.4 ml with ice-cold sodium flux buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.5) and filtration under vacuum through Whatman GF/B filters, which were then washed with 3 ml of sodium flux buffer. Radioactivity was determined by scintillation counting. Significance levels were determined using Student's *t* test.

Results

Receptor solubilization. A variety of detergents were screened in preliminary experiments to determine which most effectively solubilized 5-HT$_3$ receptors from N1E-115 cells (Table 1); 1% *n*-octylglucoside solubilized the highest percentage of receptors (19.7 ± 3.5%, mean ± standard error, three experiments). More detailed studies with this detergent revealed that the highest percentage of receptors were solubilized at a concentration of 1.1% *n*-octylglucoside in the presence of 0.15% (w/v) soybean phospholipids (22.3 ± 3.5%, mean ± standard error, 17 experiments). At higher *n*-octylglucoside concentrations, fewer 5-HT$_3$ receptors were solubilized. The soybean lipids were retained in the protocol during characterization of the soluble material but omitted for the subsequent purification studies. The percentage of total protein solubilized by the *n*-octylglucoside-lipid mixture could not be accurately determined because of the large proportion of soluble proteins released from the intact cells. In control preparations (containing no detergent), [^{3}H]GR65630 binding was only detected in the 100,000 × *g* pellet.

Saturation analysis of equilibrium binding to solubilized receptor preparations demonstrated a single population of high affinity sites. The K_d determined from these data (0.19 ± 0.04 nM) was not significantly different from that in the membrane pellet from control preparations (0.19 ± 0.02 nM). B_{max} values were 176.9 ± 29.2 fmol/culture plate in soluble preparations and 838.1 ± 86.7 fmol/culture plate in control preparations

TABLE 1

Effects of various detergents on solubilization of 5-HT$_3$ receptors, expressed as percentage of specific [^{3}H]GR65630 binding sites, compared with control preparations

Each value is the mean ± standard error of at least three experiments.

Detergent	Solubilized receptor
	%
1% *n*-Octylglucoside	19.7 ± 3.5
1% CHAPS	12.3 ± 2.5
1% Triton X-100	7.8 ± 1.7
1% Cholate	3.9 ± 2.2
1% *n*-Octyltrioxyethylene	3.5 ± 1.1
1% Deoxycholate	ND[a]

[a] ND, not detected.

(mean ± standard error, four experiments). Association and dissociation data were best fit by single-exponential curves, giving a k_{-1} of 0.78 ± 0.11 min^{-1} and a k_{obs} of 4.51 ± 1.31 min^{-1} (mean ± standard error, four experiments). Using the equation $k_1 = (k_{obs} - k_{-1})/[L]$, $k_1 = 7.46 \times 10^9$ min^{-1} M^{-1}, giving a calculated K_d (k_{-1}/k_1) of 0.105 nM.

The ability of a range of ligands to compete with specific [^{3}H]GR65630 binding in soluble receptor preparations is shown in Table 2. A plot of pK_i of these ligands for soluble receptor preparations versus intact N1E-115 cells revealed a correlation coefficient of 0.84; a similar plot versus rat brain membrane data revealed a correlation coefficient of 0.89. Hill coefficients for the antagonists GR65630, GR38032F, quipazine, metoclopramide, ICS 205-930, MDL 72222, and BRL 43694 and the agonist 2-methyl-5-HT were not significantly different from unity (Table 2). The Hill coefficient for 5-HT, however, was 1.62 ± 0.26 (significantly different from 1, $p < 0.1$, four experiments). Hill coefficients determined in parallel for intact N1E-115 cells were 1.59 ± 0.21 (significantly different from 1, $p < 0.1$, four experiments) and 1.36 ± 0.30 (three experiments) for 5-HT and 2-methyl-5-HT, respectively.

Receptor purification. The profile of specific [^{3}H]GR67330 binding during purification is shown in Fig. 1. Because these data were obtained using a single ligand concentration, they must be interpreted with caution, because the K_d for [^{3}H]GR67330 was found to vary during the procedure (data not shown). It can, however, be clearly seen that 5-HT_3 receptors were retained by the affinity column and specifically eluted by the antagonist quipazine. BRL 43694 and GR65630 were found to be similarly effective (data not shown). Binding parameters obtained from saturation analyses of [^{3}H]GR67330 binding data of membrane-bound, soluble, and purified receptor preparations are shown in Table 3. Scatchard transformations were all monophasic, suggesting a single population of binding sites. Despite the changes in affinity during the procedure, the K_d values for the purified and soluble preparations were not significantly different from that of the membrane-bound preparation ($p < 0.05$), and the B_{max} values revealed 12.1% yield of pure receptor from the original membrane preparation.

The ligand recognition profile of the purified receptor is shown in Table 4 and is similar to those of the soluble and membrane-bound 5-HT_3 receptor preparations. A plot of pK_i of these ligands versus soluble and membrane-bound data revealed correlation coefficients for both of 0.92. Hill coefficients for all the antagonists and for 2-methyl-5-HT were not significantly different from unity; the Hill coefficient for 5-HT was 1.59 ± 0.22 (significantly different from unity, $p < 0.1$, four experiments; Table 4).

SDS-PAGE of a sample of purified receptor preparation, after silver staining, is shown in Fig. 2. The amount of protein applied was estimated from the binding data, assuming a molecular mass of 50,000, and was routinely 50–200 ng. There is a single discrete band of apparent molecular mass 54.7 ± 1.3 kDa (four experiments). Occasionally, a broad band in the region of 21–22 kDa was observed; this may have been due to degradation of the protein or to the presence of trypsin inhibitor, which was used in the solubilization procedure.

Receptor reconstitution. A typical experiment showing the stimulation of ^{22}Na influx into reconstituted receptor preparations in the presence of the 5-HT_3 agonist mCPBG, and the antagonism of the stimulation by quipazine, is shown in Fig. 3. Some ^{22}Na uptake was observed into the liposome preparation in the absence of agonist; this is shown as background influx. Taking background influx as 100%, mCBPG (5×10^{-4} M) was able to stimulate ^{22}Na influx into liposomes to 162.2 ± 16.7% (significantly different from 100%, $p < 0.05$, mean ± standard error, five experiments). In the presence of agonist and 10^{-5} M quipazine, ^{22}Na influx was reduced to 114.5 ± 2.1% (mean ± standard error, four experiments).

Saturation analysis of [^{3}H]GR67330 binding to aliquots of the liposome preparation indicated a homogeneous population

TABLE 2

Competition of various ligands against [^{3}H]GR65630 binding in the solubilized receptor preparation

$K_i = IC_{50}/(1 + [L]/K_d)$. IC_{50} is the concentration that inhibits 50% of specific binding. Each value is the mean ± standard error of at least three experiments.

Compound	K_i	n_H
	M	
BRL 43694	$1.8 \pm 0.5 \times 10^{-10}$	0.86 ± 0.05
ICS 205-930	$2.7 \pm 0.6 \times 10^{-10}$	1.02 ± 0.03
Quipazine	$6.2 \pm 1.7 \times 10^{-10}$	1.05 ± 0.18
GR65630	$8.5 \pm 0.9 \times 10^{-10}$	1.14 ± 0.09
GR38032F	$2.4 \pm 0.4 \times 10^{-9}$	0.78 ± 0.18
MDL 72222	$1.1 \pm 0.4 \times 10^{-8}$	1.02 ± 0.25
5-HT	$7.4 \pm 1.6 \times 10^{-8}$	1.62 ± 0.26[a]
2-Methyl-5-HT	$2.2 \pm 0.2 \times 10^{-7}$	1.01 ± 0.14

[a] Significantly different from unity, $p < 0.1$.

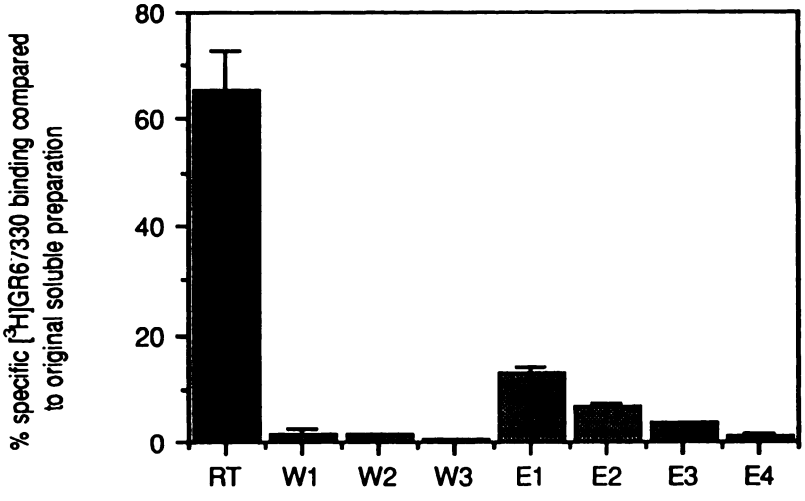

Fig. 1. Affinity purification of the 5-HT_3 receptor. Data represent percentage of specific [^{3}H]GR67330 binding at 0.1 nM, compared with that in the original soluble preparation. Original material, 100%; *RT*, run through, 65.2 ± 7.7%; *W1*, wash 1, 1.4 ± 0.3%; *W2*, wash 2, 1.7 ± 1.0%; *W3*, wash 3, 0.3 ± 0.1%; *E1*, eluate 1, 12.8 ± 1.3%; *E2*, eluate 2, 6.9 ± 0.6%; *E3*, eluate 3, 3.4 ± 0.4%; *E4*, eluate 4, 1.2 ± 0.2%. Values are mean ± standard error of nine experiments.

TABLE 3

[^{3}H]GR67330 binding parameters

Data were obtained as described in Experimental Procedures. Each value is the mean ± standard error of three to five experiments.

	K_d	B_{max}	Volume	Yield
	nM	fmol/ml	ml	%
Membranes	0.11 ± 0.04	922 ± 123	30	100
Soluble	0.05 ± 0.02	98.0 ± 34.5	60	21.3
Purified	0.10 ± 0.04	167 ± 61	20	12.1
Reconstituted	0.24 ± 0.07	15.2 ± 2.8	4	0.2

TABLE 4

Competition studies of various ligands against [^{3}H]GR67330 binding in the purified receptor preparation

K_i is as described in Table 2. Each value is the mean ± standard error of at least three experiments.

Compound	K_i	n_H
	M	
BRL 43694	$6.3 \pm 0.3 \times 10^{-10}$	0.97 ± 0.08
Quipazine	$1.7 \pm 0.8 \times 10^{-9}$	1.05 ± 0.12
GR38032F	$2.5 \pm 1.4 \times 10^{-9}$	1.09 ± 0.07
mCPBG	$1.2 \pm 0.2 \times 10^{-8}$	0.89 ± 0.16
MDL72222	$1.3 \pm 0.2 \times 10^{-8}$	1.01 ± 0.11
5-HT	$3.9 \pm 0.6 \times 10^{-8}$	1.59 ± 0.22[a]
2-Methyl-5-HT	$4.6 \pm 1.2 \times 10^{-7}$	1.16 ± 0.10

[a] Significantly different from unity, $p < 0.1$.

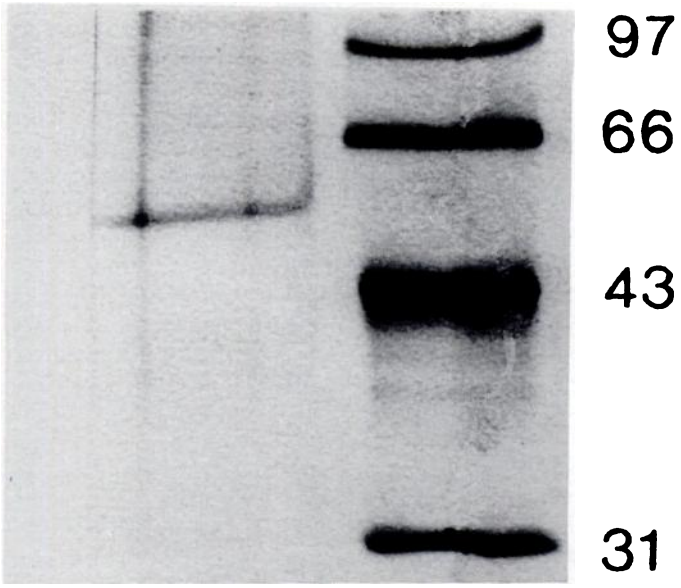

Fig. 2. SDS-PAGE of a sample of the affinity-purified 5-HT$_3$ receptor from N1E-115 cells, prepared and silver-stained as described in Experimental Procedures and run on a 12.5% gel. Standards, with molecular masses in kDa, are also shown.

of sites, with a K_d not significantly different from that in membrane preparations (Table 3). B_{max} values revealed that 0.2% of the original membrane preparation had been incorporated into the liposomes.

Discussion

Functional reconstitution of a solubilized, affinity-purified, receptor protein provides the most convincing evidence of the integrity of the purified material. The present study demonstrates that the 5-HT$_3$ receptor protein, the most recently

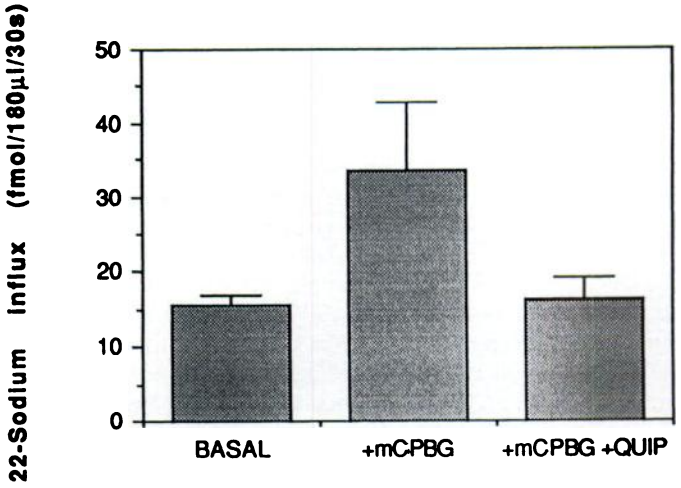

Fig. 3. ^{22}Na uptake into liposomes incorporated with a sample of the purified 5-HT$_3$ receptor. *BASAL*, ^{22}Na uptake into liposomes in the absence of agonist. mCPBG concentration was 5×10^{-4} M; quipazine (*QUIP*) concentration was 10^{-5} M. Data shown are from one experiment (four determinations) and are typical of four similar experiments.

TABLE 5

Comparisons of K_i values (see Table 2 legend) in intact cells and soluble and purified N1E-115 preparations

Values are mean of at least three experiments.

Compound	K_i		
	Cells[a,b]	Soluble[b]	Purified[c]
	nM		
BRL43694	0.18	0.18	0.63
Quipazine	0.77	0.62	1.7
GR38032F	5.7	2.4	2.5
MDL72222	12	11	13
5-HT	560	74	39
2-Methyl-5-HT	1550	224	460

[a] Data from Lummis *et al.* (7).
[b] [^{3}H]GR65630.
[c] [^{3}H]GR67330.

identified member of the family of ligand-gated ion channel proteins, can be successfully solubilized and purified, to yield a single protein band on SDS-PAGE. The ligand recognition profiles of the soluble and purified preparations are the same as those in intact N1E-115 cells (Table 5), and the purified protein, when inserted into liposomes, retains its functional activity, as demonstrated by 5-HT$_3$ agonist-stimulated ^{22}Na influx (Fig. 3).

To purify a protein, a rich source of material is desirable. 5-HT$_3$ receptors are present at relatively low density in mammalian brain but are expressed at high concentrations in certain neuroblastoma cell lines, including N1E-115 murine neuroblastoma cells, NG108-15 murine neuroblastoma × rat glioma cells, and NCB 20 murine neuroblastoma × Chinese hamster cells (6). We have previously characterized 5-HT$_3$ receptors on N1E-115 cells, using the specific 5-HT$_3$ ligand GR65630, and revealed that these cells possess close to 200,000 sites/cell (7). We, therefore, selected these cells for our study. The receptors appeared to be optimally solubilized using 1.1% *n*-octylglucoside, a nonionic detergent that has been successfully used to solubilize a number of neurotransmitter receptor proteins; results expressed as percentage of specific [^{3}H]GR65630 binding/culture plate show that 22.3% of 5-HT$_3$ receptors were solubilized under these conditions. At higher detergent concentra-

tions, soluble 5-HT_3 receptors may be unstable; nonsolubilized [^{3}H]GR65630 binding decreased linearly for *n*-octylglucoside concentrations between 0.4 and 1.4% (correlation coefficient = 0.86), whereas solubilized [^{3}H]GR65630 binding reached a maximum at 1.1% *n*-octylglucoside and then decreased. The detergents CHAPS, cholate, *n*-octyltrioxyethylene, deoxycholate, and Triton X-100 were less effective in solubilizing 5-HT_3 receptors from N1E-115 cells, although this may be due to increased receptor instability in these detergents (Table 1).

The characteristics of the soluble receptors, as determined by competition with [^{3}H]GR65630, revealed that they had binding properties very similar to those of membrane-bound receptors. Specific [^{3}H]GR65630 binding was saturable, and the data were best fit to a single population of sites with a K_d of 0.19 nM, similar to the K_d values observed in intact N1E-115 cells (0.69 nM) (7) and in rat brain membranes (0.35 nM) (4) with the same ligand. The ligand recognition profile of the soluble receptors also was similar to that of intact cells and rat brain membrane preparations; the relationship between the pK_i values of soluble versus intact N1E-115 cells and rat brain membrane preparations for eight ligands revealed correlation coefficients of 0.84 and 0.89, respectively.

Solubilized 5-HT_3 receptors were effectively retained by an affinity gel constructed from GR119566X, an analogue of GR67330, and could be specifically eluted by the 5-HT_3 antagonist quipazine. More specific 5-HT_3 ligands also eluted the purified material (GR65630 and BRL 43694), but quipazine was routinely used because of its high water solubility. Ligand binding data obtained from equilibrium binding studies on the purified receptor preparation using [^{3}H]GR67330 showed that 12.1% of the receptors in the original membrane preparation were recovered in the purified form (Table 3). Both [^{3}H]GR67330 and [^{3}H]GR65630 have similar binding characteristics in N1E-115 preparations (Table 3),[2] and the two can be used interchangeably. The affinity of the purified receptor preparation does not change significantly during the purification procedure; the K_d of the purified material (0.1 nM) is similar to those observed in soluble and membrane-bound N1E-115 preparations (Table 3) and in rat brain membranes (0.04 nM) (12) assayed with [^{3}H]GR67330. Examination of a range of 5-HT_3-selective ligands reveals that the purified receptor has the pharmacological properties expected for a 5-HT_3 receptor. Similar results have been obtained for 5-HT_3 receptors purified from NCB 20 cells, using an affinity column made with a derivative of ICS 205-930 (13).

An interesting property of all the N1E-115 preparations is the high Hill coefficient obtained from 5-HT displacement curves, which is retained throughout the purification procedure. Hill coefficients greater than unity have been previously reported for agonist displacement curves of certain radiolabeled 5-HT_3 antagonists, including [^{3}H]GR65630, [^{3}H]GR67330, and [^{3}H]ICS 205-930; it has been suggested that they represent positive cooperativity (4, 5, 12). However, Hill slopes close to unity for agonists have been reported in studies using ^{3}[H] quaternized ICS 205-930 (NCB 20 cells) (13) and [^{3}H]zacopride (NG108-15 cells) (14), suggesting that high Hill coefficients are only distinguished with certain radioligands. There is also evidence that steep Hill slopes are only observed in certain tissues; high Hill coefficients for agonist displacement of [^{3}H] ICS 205-930 in N1E-115 membrane preparations (5) contrast with values not significantly different from unity in membrane preparations from the related neuroblastoma × glioma cell line NG108-15 (15), and recently we have observed similar results with [^{3}H]GR65630 in the two cell lines (7).[2] Electrophysiological experiments reveal steep Hill slopes for 5-HT in both N1E-115 cells (16) and NG108-15 cells (17). Thus, it appears that 5-HT_3 receptors are unusual among ligand-gated ion channels, in that the apparent positive cooperativity observed in functional assays is retained in radioligand binding experiments in at least some tissues. The significance of this is yet to emerge.

Analysis of the protein subunit composition after SDS-PAGE reveals a single protein band of molecular size 54.7 ± 1.3 kDa (mean ± standard error, four experiments). This value is similar to the molecular size of one of the two major protein bands, 38 and 54 kDa, reported for 5-HT_3 receptors purified from NCB 20 cells (13), although lower subunit sizes have been reported for rat brain 5-HT_3 receptors, 49 kDa (18) and 35 kDa (19) estimated from crude membrane preparations using radiation inactivation. The same technique has also revealed a larger target size, 99 kDa, for N1E-115 5-HT_3 receptors, which may represent a dimer (18). Estimations of the total molecular size of the receptor include 250 kDa (20), 600 kDa (19), and between 443 and 669 kDa (21). Thus, it appears that the 5-HT_3 receptor, like other members of the ligand-gated ion channel family, has a multimeric structure. The particular subunit composition may determine the characteristics of the channels, as has been previously demonstrated for the GABA receptor (22). Electrophysiological data have shown that the characteristics of 5-HT_3 receptor activation in N1E-115 cells differ from those in other systems; for example, guinea pig submucous plexus 5-HT_3 receptors have single-channel conductances of 9 and 15 pS (2), whereas in N1E-115 cells the single-channel conductance has been estimated at 310 fS (23). This apparent discrepancy may reflect different subunit compositions of the two receptor proteins, although as yet there is no evidence to substantiate this proposal.

The direct measurement of ligand ion channel function using the uptake of radiolabeled ions into liposomes is a useful assay to examine the integrity of purified ligand-gated ion channels. In this study the purified receptor material was introduced into liposomes using techniques that have proved successful for the GABA receptor (9). Radioligand binding studies revealed that the reconstituted receptors had a similar affinity as those in intact N1E-115 cells (Table 3) and showed that 0.2% of the receptors in the original membrane preparation had been incorporated into liposomes, a value similar to that reported for the purified GABA receptor (0.7%) (9). 5-HT_3 receptor function was studied using ^{22}Na uptake assays; ^{22}Na uptake was stimulated by the specific 5-HT_3 agonist mCPBG, and the stimulation could be blocked by the 5-HT_3 antagonist quipazine. The uptake assays were rather fickle; mCPBG stimulated ^{22}Na flux in only five of nine experiments, but in all these experiments the agonist-stimulated uptake was blocked by antagonist. There was also a large variation in the amount of influx observed in those experiments that were successful. These variations may have been due to the amount of purified receptor preparation incorporated into the different liposome preparations and/or the amount of receptor that had been incorporated in the correct orientation for activation by the externally applied agonist.

In summary, we have demonstrated that 5-HT_3 receptors can be solubilized and purified from N1E-115 cells in a form that

[2]Unpublished observations.

retains the pharmacological characteristics of the membrane-bound receptors. The purified receptor material appears to consist of one or more subunits of 55 kDa and can be functionally reconstituted into liposomes. These advances in 5-HT$_3$ receptor methodology should allow rapid advances in the detailed molecular characterization of this receptor protein.

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