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The properties of 5-HT_3 receptors in clonal cell lines studied by patch-clamp techniques

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1 The characteristics of transmembrane currents evoked by 5-hydroxytryptamine (5-HT) in the neuroblastoma × Chinese hamster brain cell line NCB-20 and neuroblastoma clonal cell line N1E-115 have been studied under voltage-clamp conditions by the whole-cell recording and outside-out membrane patch modes of the patch-clamp technique.

2 In 73% of NCB-20 cells examined ($n = 221$), and all N1E-115 cells studied ($n = 80$), 5-HT (10 μM) elicited a transient inward current at negative holding potentials, this being associated with an increase in membrane conductance. In both cell lines responses to 5-HT reversed in sign at a potential of approximately −2 mV and demonstrated inward rectification.

3 The reversal potential of 5-HT-induced currents ($E_{5\text{-HT}}$) recorded from either NCB-20 or N1E-115 cells was unaffected by total replacement of internal K^+ by Cs^+. In N1E-115 cells, reducing internal K^+ concentration from 140 to 20 mM produced a positive shift in $E_{5\text{-HT}}$ of approximately 28 mV, whereas reducing external Na^+ from 143 to 20 mM was associated with a negative shift in $E_{5\text{-HT}}$ of about 37 mV. A large reduction in internal Cl^- concentration (from 144 to 6 mM) had little effect on $E_{5\text{-HT}}$.

4 5-HT-induced currents of NCB-20 cells were unaffected by methysergide (1 μM) or ketanserin (1 μM), but were reversibly antagonized by GR38032F (0.1–1.0 nM) with an IC_{50} of 0.25 nM. GR 38032F (0.3 nM) reduced 5-HT-induced currents in N1E-115 cells to approximately 26% of their control value.

5 On outside-out membrane patches excised from both NCB-20 and N1E-115 cells, 5-HT induced small inward currents which could not be clearly resolved into discrete single channel events. Such responses were: (i) reversibly antagonized by GR 38032F (1 nM) (ii) reversed in sign at 0 mV, and (iii) subject to desensitization.

6 Fluctuation analysis of inward currents evoked by 5-HT (1 μM) in N1E-115 cells suggests that 5-HT gates a channel with a conductance of approximately 310 fS. Such a relatively small conductance could readily explain why the response of outside-out membrane patches to 5-HT cannot at present be resolved into clear single channel events.

Introduction

It has been proposed that vertebrate 5-hydroxytryptamine (5-HT) receptors exist as three distinct subtypes termed '5-HT_1-like', 5-HT_2 and 5-HT_3 (Bradley *et al.*, 1986). 5-HT_3 receptors are present on various neurones of the peripheral nervous system, mediating membrane depolarization and neurotransmitter release (Wallis, 1981; Fozard, 1984a; Richardson & Engel, 1986). The action of 5-HT at these sites is potently and selectively antagonized by several recently synthesized compounds, including MDL72222 (1αH, 3α, 5αH-tropan-3-yl-3,5-dichlorobenzoate), ICS 205-930 [(3α-tropanyl)-1H-indole-3-carboxylic acid ester] and GR 38032F (1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl) methyl]-4H-carbazol-4-one) (Fozard, 1984b; Richardson *et al.*, 1985; Butler *et al.*, 1988). Such

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compounds are behaviourally active, possessing potentially anxiolytic (Tyers *et al.*, 1987; Jones *et al.*, 1988) and antipsychotic (Costall *et al.*, 1987) properties. Together with the recent demonstration of a binding site within brain tissue that displays a pharmacological profile consistent with that of a 5-HT_3 receptor (Kilpatrick *et al.*, 1987; Peroutka & Hamik, 1988; Barnes *et al.*, 1988), the behavioural data suggest that functional 5-HT_3 receptors may occur in the CNS. Consistent with this suggestion, 5-HT_3 receptor activation has recently been reported to elicit inward currents from a population of voltage-clamped rat hippocampal neurones in culture (Yakel & Jackson, 1988) and also to modulate the release of dopamine from rat striatal slices (Blandina *et al.*, 1988).

Current knowledge of the molecular events that occur at 5-HT_3 receptors is limited. Electrophysiological studies have shown that in sympathetic and visceral primary afferent neurones, 5-HT_3 receptor activation elicits a rapidly desensitizing membrane depolarization which is the result of a simultaneous increase in membrane permeability to sodium and potassium ions (Wallis & North, 1978; Nigashi & Nishi, 1982). Similar depolarizing responses to 5-HT have been observed in several neuronal clonal cell lines which are amenable to advanced electrophysiological techniques. In murine N1E-115 neuroblastoma cells, 5-HT increases membrane conductance to monovalent cations, generating a depolarizing response under current-clamp conditions (Guharay & Usherwood, 1981; Peters & Usherwood, 1983), and an inward current when the membrane is voltage-clamped at negative holding potentials ((Neijt *et al.*, 1986). Both the 5-HT-induced depolarization and inward current are antagonized by the selective 5-HT_3 receptor antagonists MDL 72222 and ICS 205-930, but are unaffected by antagonists acting at '5-HT_1-like' or 5-HT_2 sites (Neijt *et al.*, 1988). A binding site corresponding to the 5-HT_3 receptor has been identified in N1E-115 cells (Hoyer & Neijt, 1988) and the neuroblastoma × glioma clone NG 108-15 (Hoyer & Neijt, 1987). Collectively, these observations suggest that certain neuronal clonal cell lines may be appropriate model systems in which to study 5-HT_3 receptors and their associated ion channels.

In the present study, membrane currents elicited by 5-HT in N1E-115 cells and the clonal neuroblastoma × Chinese hamster brain cell line NCB-20 were examined under voltage-clamp conditions, by the patch-clamp technique (Hamill *et al.*, 1981). 5-HT has previously been shown to stimulate the formation of cyclic AMP in the latter system (Berry-Kravis & Dawson, 1983) and also to evoke a membrane depolarization, the two effects occurring via pharmacologically distinct subtypes of 5-HT receptor (MacDermot *et al.*, 1979). The present work demonstrates that the electrical response in NCB-20 cells is, like that of the N1E-115 clone, mediated by 5-HT_3 receptors. Additionally we have examined the ionic selectivity of 5-HT_3 receptor gated ion channels and present evidence from fluctuation analysis of 5-HT-induced currents, recorded from whole cells and excised outside-out membrane patches, which suggests that such channels are of unusually low conductance. Preliminary accounts of some of this work have appeared in abstract form (Hales *et al.*, 1988a,b).

Methods

Cell culture

Cells of the clonal lines NCB-20 (Minna *et al.*, 1975) and N1E-115 (Amano *et al.*, 1972) were grown essentially as described by MacDermot *et al.* (1979) and Khimi *et al.* (1976) respectively. Briefly, NCB-20 cells were cultured in 25 cm^2 'Nunclon' tissue culture flasks (Gibco) containing 10 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with 5% (vol/vol) foetal calf serum (FCS), hypoxanthine (13.6 $mg\,l^{-1}$), aminopterin (0.178 $mg\,l^{-1}$), thymidine (3.88 $mg\,l^{-1}$), streptomycin (50 $mg\,l^{-1}$) and penicillin (5×10^4 $iu\,l^{-1}$). Cells were grown to confluency over a period of 7 days, from an initial innoculum of 2×10^5 cells per flask and collected by mechanical agitation and subsequent centrifugation (100 *g* for 5 min). N1E-115 cells were grown and harvested in a similar manner, but in a growth medium consisting only of DMEM supplemented with 10% (vol/vol) FCS and streptomycin and penicillin at the concentrations stated above. For electrophysiological experiments, approximately 10^4 NCB-20 or N1E-115 cells were plated into 35 mm diameter 'Nunclon' petri dishes (Gibco) containing 2 ml of their respective growth media. Prior to use in electrophysiological experiments, NCB-20 cells were exposed to 1 mM N^6, O^{12}-dibutyryl adenosine 3':5'-cyclic monophosphoric acid for 2 to 7 days to initiate cell differentiation. N1E-115 cells did not receive this treatment. The cell lines were incubated at 37°C in an atmosphere of 90% air/10% CO_2 at 100% relative humidity. A number of experiments utilized cultures of adult bovine adrenomedullary chromaffin cells. These were prepared following an established methodology (Cottrell *et al.*, 1987).

Electrical recordings

Agonist-activated currents from 'whole-cells' and outside-out membrane patches were recorded by

standard patch-clamp techniques (Hamill *et al.*, 1981) and a List Electronics L/M EPC7 converter headstage and amplifier. The extracellular recording medium used in the majority of experiments consisted of (in mM): NaCl 140, KCl 2.8, $MgCl_2$ 2.0, $CaCl_2$ 1.0 and HEPES 10 (pH 7.2). Cells were continually superfused with this solution at a rate of 3–5 ml min^{-1}. The pipette solution usually employed to dialyse the cell interior comprised (in mM): CsCl 140, $MgCl_2$ 2.0, HEPES 10, $CaCl_2$ 0.1, EGTA 1.1, (free $[Ca^{2+}] = 10^{-8}$ M at pH 7.2). Caesium was chosen as the predominant internal cation in order to suppress membrane potassium conductances. In experiments designed to evaluate the ionic-dependence of 5-HT-evoked currents, modified extracellular and intracellular media were employed. Pipette solutions containing potassium, rather than caesium, as the predominant internal cation were prepared by total replacement of CsCl by KCl or K gluconate. In experiments where the internal concentration of potassium was reduced, KCl was partially replaced by tetraethylammonium Cl. External sodium ion concentration was reduced to either 75 or 20 mM by partial substitution of L-glucosamine HCl for NaCl. All solutions were titrated to pH 7.0–7.2 with 1 M NaOH; Na ions so introduced were included in the calculation of external and internal sodium ion concentrations.

To minimize changes in reference electrode potential during the bath application of the modified recording media to the cells, the bath was held at virtual ground via a salt bridge containing 3 M KCl in 4% (w/v) agar. When the pipette and bath contained salines of differing ionic composition, a liquid junction potential developed at the tip of the patch pipette prior to 'giga-seal' formation. Such potentials, which were estimated by the method of Fenwick *et al.* (1982) to range between 2 and 10 mV (pipette negative), were taken into account when setting the holding potential in voltage-clamp recordings. Experiments started approximately 10 min after establishing the whole-cell recording and in all cases were conducted at room temperature (17–21°C).

In most experiments, 5-HT was locally applied to cells either by pressure ejection (1.4×10^5 Pa, 30–300 ms) from modified patch pipettes filled with 5-HT (10 μM) in recording medium or by ionophoresis. Ionophoretic pipettes contained 20 mM 5-HT in twice distilled deionized water (pH 3.5–4.0) and had resistances > 80 MΩ. A constant current pump based on the design of Dreyer & Peper (1974) was used to supply ejection and retaining currents to the pipette. Antagonist compounds were introduced to the bath via the superfusion system. In experiments where the unitary conductance of 5-HT-activated ion channels was to be estimated from fluctuation analysis of whole-cell currents, a relatively slowly rising response to 5-HT was desired. For this purpose, 5-HT (1 μM in recording medium) was applied to cells by diffusion from coarse-tipped (4–10 μm diameter) micropipettes. All quantitative data are reported as the arithmetic mean ± standard error of the mean (s.e.mean).

Fluctuation analysis of 5-HT-induced whole-cell currents

Estimates of single channel conductance were made by analysis of the random fluctuations in the 5-HT-induced whole cell membrane currents. Current fluctuations were a.c. coupled (high pass filter 2.0 Hz) and recorded on a separate tape channel after extra amplification.

Continuous records of mean d.c. current and current fluctuations, starting before the application of 5-HT (1 μM) and continuing throughout (1–3 min), were played back, digitised at a rate of 1 kHz using a Data Translation DT2801A or a Cambridge Electronic Design 502 laboratory interface and stored on an Amstrad PC1640 personal computer or a PDP 11 73 minicomputer respectively. Anti-aliasing filtration of the current fluctuation signal was performed using a Frequency Devices 901, 8 pole Butterworth low pass filter (500 Hz cut-off) and a Neurolog NL 125 high-pass filter (2 Hz cut-off).

The variance analysis method was used to estimate the single channel conductance (Marty *et al.*, 1984; Cull-Candy *et al.*, 1988; Ascher *et al.*, 1988). The record was split into a series of 0.5 s blocks (512 samples) with variance and mean current calculated for each block from the equation:

$$\sigma^2 = \sum_{J=1}^{n} \frac{(I_J - I_{av})^2}{(n-1)} \qquad (1)$$

where σ_2 is the variance, I_{av} the mean current, n the number of samples in the block, and I_J current sample J. σ^2 has a parabolic relationship to I_{av}, rising from zero to a maximum (at $P = 0.5$) and falling to zero again as the open channel probability, P, is varied from 0–1. For small values of P (≤ 0.2), the relation is approximately linear and the value of the unitary current can be calculated from the slope of the fitted line.

σ^2/I_{av} plots were obtained from blocks recorded during the onset of the 5-HT current, with P increasing as the 5-HT concentration increased. Unless specified otherwise, background variance contributed by sources other than the 5-HT-activated ion channels was removed by subtracting the mean value of 32–64 blocks recorded before 5-HT application. The σ^2/I_{av} plots were well fitted by straight lines using linear regression (correlation coefficient 0.71–

0.97), suggesting that P was indeed small. The slope was therefore used as an estimate of the unitary current.

Reagents

All constituents of cell culture media were obtained from Gibco Europe. The following compounds were used in experiments: γ-aminobutyric acid (GABA), 5-hydroxytryptamine creatinine sulphate complex (both from Sigma), citalopram hydrogen bromide (Lundbeck), GR 38032F (1,2,3,9-tetra-hydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one hydrochloride dihydrate) (Glaxo), ICS 205-930 ([3α-tropanyl]1H-indole-3-carboxylate) (Research Biochemicals), ketanserin tartrate (Janssen) and methysergide hydrogen maleate (Sandoz). All drugs were freshly prepared as concentrates in twice distilled deionised water and diluted into recording medium as required.

Results

5-HT-induced responses

Local application of 5-HT (100 μM) to NCB-20 cells elicited a transient membrane depolarization under current-clamp conditions. Such responses were accompanied by an apparent increase in membrane conductance, this being reflected in a decrease in the amplitude of the anelectrotonic potential to current injection during a 5-HT-evoked depolarization (Figure 1a). The inward current which underlies the 5-HT-induced depolarization was recorded directly in cells voltage-clamped at −60 mV. Of 221 NCB-20 cells challenged with pressure applied 5-HT (10 μM), 161 responded with an inward current which ranged in amplitude between several pA and in excess of −1 nA. The remaining 27% of cells sampled had no discernible response to 5-HT. In comparison, all voltage-clamped N1E-115 cells tested responded to 5-HT (10 μM) with an inward current ($n = 80$). Figure 1b shows a typical response to 5-HT recorded under voltage-clamp from an NCB-20 cell. The large increase in clamp-current required to drive the membrane potential transiently between −60 and −80 mV during the action of 5-HT confirms that such responses result from an increase in membrane conductance. Temporally close applications of 5-HT resulted in a progressive decline of the amplitude of the 5-HT-induced current. Such desensitization could normally be avoided by applying 5-HT at intervals ≥90 s (data not illustrated).

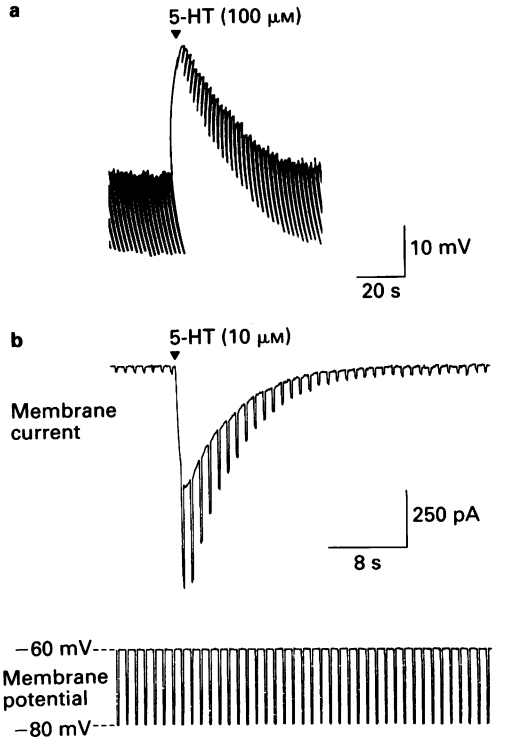

Figure 1 Responses of NCB-20 cells to 5-hydroxytryptamine (5-HT) in current- and voltage-clamp recording modes. (a) Current-clamp record illustrating a depolarizing response to microperfused 5-HT (100 μM). The downward deflections in the trace are anelectrotonic potentials to square pulses of current (−0.1 nA, 200 ms, 0.6 Hz) injected into the cell to monitor membrane conductance. The reduction in the size of the anelectrotonic potential during the 5-HT-evoked response indicates an apparent increase in conductance. (b) Voltage-clamp record showing an inward current (upper trace) to pressure applied 5-HT (10 μM). Membrane potential (lower trace) was held at −60 mV and transiently stepped to −80 mV (200 ms, 1.0 Hz) to assess membrane conductance. The increase in clamp current required to drive the membrane potential between −60 and −80 mV during the action of 5-HT indicates that the response is due to an increase in membrane conductance (of approximately 20 nS in this example). Voltage and current recordings were low-pass filtered at 1.0 kHz.

Current-voltage relationships and the ionic basis of the 5-HT-induced response

Figure 2 illustrates the results of an experiment in which the influence of holding potential upon the 5-HT-induced current was examined in an NCB-20 cell dialysed with a Cs^+-containing pipette solution.

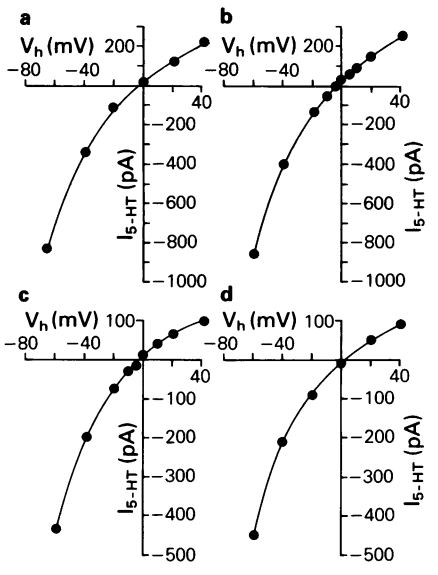

Figure 2 The relationship between the amplitude of the 5-hydroxytryptamine (5-HT)-induced current ($I_{5\text{-HT}}$) and holding potential (V_h) in NCB-20 and N1E-115 cells dialysed with various pipette solutions. The standard extracellular recording medium, with a composition detailed under Methods, was used in all experiments. (a and b) Current-voltage relationship in an NCB-20 cell (a) and N1E-115 cell (b) dialysed with a pipette solution containing Cs^+ as the predominant internal cation ($[Cs^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). Note that in both cell types the 5-HT-induced response displays inward rectification and reverses in sign at a potential of approximately −2 mV. (c) Current-voltage relationship in an N1E-115 cell dialysed with a solution in which Cs^+ was completely replaced with K^+ ($[K^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). Replacement of Cs^+ by K^+ has no effect upon the reversal potential of the response (−2 mV) and does not conspicuously alter the pattern of inward rectification. (d) Current-voltage relationship obtained from an N1E-115 cell dialysed with a Cl^--deficient solution ($[Cl^-]_i = 6$ mM; $[K^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). The reduction in $[Cl^-]_i$ has little effect upon the reversal potential of the response (+1.0 mV) and inward rectification is still apparent. In (a) 5-HT (10 μM) was locally applied to the cell by pressure ejection from a modified patch pipette, whereas in (b), (c) and (d) 5-HT was ionophoretically applied with ejection charges of 1.24 to 1.6 nC.

The amplitude of the response to locally applied 5-HT (10 μM) decreased with membrane depolarization and reversed in sign, in this example, at a holding potential of −3.0 mV (Figure 2a). From similar experiments performed on 7 NCB-20 cells dialysed with Cs^+-based pipette solution, the reversal potential of the 5-HT-induced current ($E_{5\text{-HT}}$) was estimated by interpolation to be -1.4 ± 1.5 mV. Under identical recording conditions, $E_{5\text{-HT}}$ in N1E-115 cells was determined to be -2.2 ± 0.9 mV ($n = 5$). In both NCB-20 (Figure 2a) and N1E-115 cells (Figure 2b), the response to 5-HT demonstrated inward rectification. Such an effect is unlikely to result from the use of Cs^+ as the predominant internal cation because a qualitatively similar rectification was observed in recordings performed on NCB-20 and N1E-115 cells employing a KCl-based pipette solution (Figure 2c). The reversal potential of the 5-HT-induced current under the latter recording conditions was estimated to be -2.0 ± 2.6 mV ($n = 3$) in NCB-20 cells and -2.1 ± 1.0 mV ($n = 7$) in N1E-115 cells. However, these values may be subject to slight errors, as the activation of voltage-dependent K currents at depolarized potentials made the measurement of 5-HT-induced currents close to their reversal potential difficult.

In an attempt to determine the ionic basis of the 5-HT-evoked current, the influence of a number of cation and anion substitutions upon $E_{5\text{-HT}}$ was examined using N1E-115 cells and ionophoretic application of 5-HT. The effect of complete replacement of KCl by K-gluconate in the pipette solution, such that internal chloride ion concentration was reduced from 144 to 6 mM, was studied in 4 cells. With the Cl^--deficient solution, $E_{5\text{-HT}}$ was estimated to be $+1.2 \pm 1.3$ mV (Figure 2d), a value close to that of −2.1 mV found under control conditions (Figure 2c). Thus it is unlikely that 5-HT elicits an appreciable increase in the Cl^- permeability of the cell membrane. In contrast, partial replacement of extracellular Na^+ by glucosamine had marked effects upon the amplitude and reversal potential of 5-HT-induced currents recorded with pipettes containing a KCl-based intracellular solution. Reducing the external concentration of sodium ($[Na^+]_o$) from its control value of 143 mM to 75 mM depressed the amplitude of the 5-HT-induced current, recorded at a holding potential −60 mV, to $42.5 \pm 10.5\%$ ($n = 3$) of its control value and produced a negative shift in $E_{5\text{-HT}}$ to -18.0 ± 2.8 mV ($n = 3$; Figure 3a). When $[Na^+]_o$ was further reduced to 20 mM, the 5-HT-induced current was reduced to $6.9 \pm 1.2\%$ ($n = 3$) of control and its reversal potential shifted to -39.0 ± 2.6 mV ($n = 3$; Figure 3a). The effects of reduced $[Na^+]_o$ upon the amplitude and reversal potential of the 5-HT-induced current were reversed upon returning to control solution (not illustrated). Collectively, these observations suggest that the current evoked by 5-HT is at least partially mediated by an influx of Na^+. To test whether K^+ may also contribute to the response, the internal concentration of K^+ was reduced from its standard value of 140 mM to 20 mM by partial replacement of KCl with

tetraethylammonium Cl. Under such conditions, $E_{5\text{-HT}}$ was determined to be $+25.9 \pm 0.5$ mV ($n = 4$; Figure 3b), a value considerably more positive than that of -2.1 mV found in control, suggesting that an efflux of K^+ from the cell also contributes to the 5-HT-evoked response.

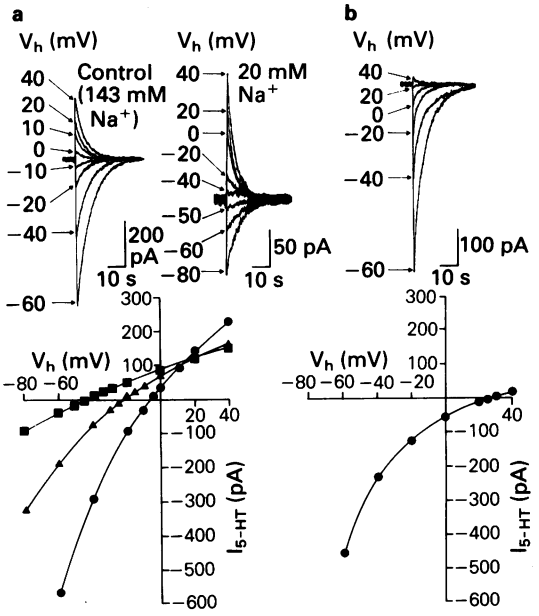

Figure 3 5-Hydroxytryptamine (5-HT)-induced currents are associated with an increase in membrane conductance to Na and K ions. (a) Influence of extracellular Na^+ concentration upon 5-HT-induced currents and their reversal potential in an N1E-115 cell. The traces illustrate transmembrane currents, recorded at various holding potentials (V_h) in response to iontophoretically applied 5-HT (3.0 nC, 0.01 Hz). The traces shown were obtained in standard extracellular medium containing 143 mM Na^+ (left) and in a solution in which $[Na^+]_o$ was reduced to 20 mM by partial replacement of NaCl with glucosamine HCl (right). Note that leakage currents have been subtracted. The graphical representation of the data shows the relationship between the amplitude of the 5-HT-induced current and holding potential with $[Na^+]_o$ equal to 143 mM (●), 75 mM (▲) and 20 mM (■). The reduction in $[Na^+]_o$ is accompanied by a hyperpolarizing shift in the reversal potential of the 5-HT-induced current. All data were obtained from the same cell which was dialysed with K^+-based pipette solution ($[K^+]_i = 140$ mM; $[Na^+]_i = 6$ mM). (b) 5-HT-induced currents recorded from an N1E-115 cell dialysed with a pipette solution which reduced $[K^+]_i$ to 20 mM. The experiment was performed with standard extracellular solution in the bath and ionophoretic applications of 5-HT (4.5 nC, 0.01 Hz). Note that in comparison to cells dialysed with a pipette solution containing 140 mM K^+ (e.g. Figures 2c and 3a), the reversal potential of the 5-HT-evoked response in this cell is displaced to a more positive potential (+25 mV).

Pharmacological categorization of the 5-HT receptor

Membrane currents evoked on NCB-20 cells by locally applied 5-HT (10 μM) were suppressed by bath applied GR 38032F (0.1–1.0 nM), a selective 5-HT_3 receptor antagonist. Such antagonism was concentration-dependent, as illustrated in the inset to Figure 4, and was readily reversed upon washout (Figure 5a). From pooled data obtained from several

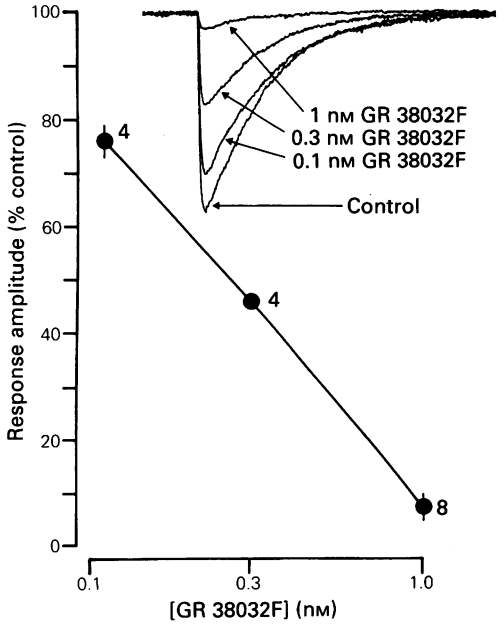

Figure 4 Concentration-dependent blockade of 5-hydroxytryptamine (5-HT)-induced inward currents in NCB-20 cells by GR 38032F. The inset depicts the results of an experiment on one cell where currents elicited by locally applied 5-HT (10 μM) were recorded in control and in the presence of a range of concentrations (0.1–1.0 nM) of bath-applied GR 38032F. Each current trace shown is the computer-generated average of 4 responses to 5-HT. Although not illustrated, antagonism by all concentrations of GR 38032F was completely reversible. In the graph, the amplitude of the 5-HT-evoked current, as a percentage of its control value, is plotted against the logarithm of the concentration of GR 38032F in the medium. From this plot, the IC_{50} for GR 38032F was estimated to be 0.25 nM. Each data point represents the mean value calculated from experiments performed on 4 to 8 cells and vertical lines indicate s.e.mean. All responses were recorded at a holding potential of -60 mV and low-pass filtered at 500 Hz.

cells, the IC_{50} for blockade by GR 38032F was estimated to be 0.25 nM (Figure 4). A similar antagonism of 5-HT-induced currents by GR 38032F (0.3 nM) was observed in N1E-115 cells, with responses being reduced to 26.0 ± 3.6% ($n = 4$) of their control value in the presence of the drug. In contrast to the potent blockade observed with GR 38032F, neither the mixed '5-HT$_1$-like'/5-HT$_2$ receptor antagonist methysergide (1 μM), nor the selective 5-HT$_2$ receptor antagonist ketanserin (1 μM), had any effect upon 5-HT-evoked currents in NCB-20 cells (Figure 5b,c). In preliminary experiments, the 5-HT$_3$ receptor antagonist ICS 205-930 had no effect upon the amplitude of 5-HT-evoked currents in NCB-20 cells when bath applied at a concentration of 10 pM. Virtually complete blockade could be demonstrated with 300 pM ICS 205-930, but recovery upon washout was incomplete (Figure 5d). Intermediate concentrations of ICS 205-930 (30–100 pM) produced a very slowly developing antagonism of the response, such that equilibrium was difficult to establish with certainty within the time that stable recordings could be maintained. In view of these practical difficulties, no attempt was made to determine the IC_{50} for the blocking action of ICS 205-930.

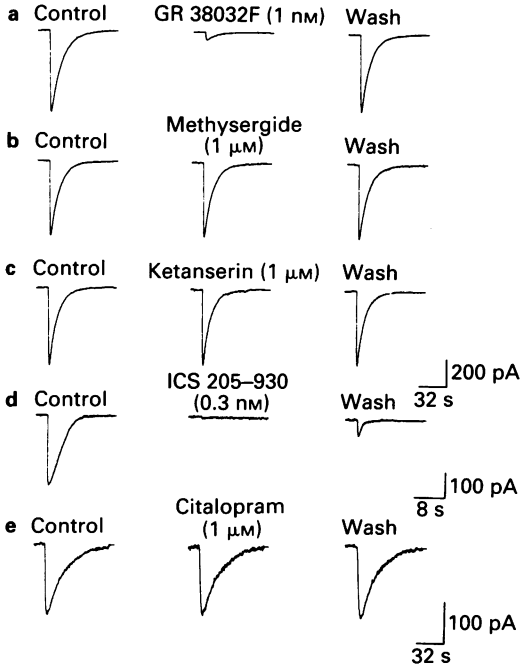

Figure 5 The pharmacology of 5-hydroxytryptamine (5-HT)-induced currents in NCB-20 cells. (a) Reversible antagonism of the 5-HT-evoked response by the 5-HT$_3$ receptor antagonist GR 38032F (1 nM). In contrast, methysergide (b) and ketanserin (c), each at a concentration of 1 μM, have no effect upon 5-HT-induced currents. (d) ICS 205-930 (0.3 nM) blocks the response to 5-HT, but reversal of the antagonism is incomplete. (e) The 5-HT uptake inhibitor citalopram (1 μM) does not modify responses to 5-HT. Traces depicted in (a), (b) and (c) were obtained from experiments performed on the same NCB-20 cell. Responses in (d) and (e) are the results from 2 other cells. All currents illustrated are the computer-generated average of 4 responses to pressure applied 5-HT (10 μM) recorded at a holding potential of −60 mV. Recordings were low-pass filtered at 500 Hz.

In the rat superior cervical ganglion, the presence of a 5-HT uptake system has been shown to influence estimates of antagonist potency when 5-HT is applied by superfusion (Ireland *et al.*, 1987). In the present investigation, the 5-HT uptake inhibitor citalopram (1 μM) had no effect on the amplitude of responses to 5-HT recorded from NCB-20 cells (Figure 5e). Similarly, citalopram does not potentiate the response of the rabbit superior cervical ganglion to bolus injections of 5-HT (Round & Wallis, 1986). The lack of effect of citalopram in the present work might suggest that uptake of 5-HT does not occur in NCB-20 cells, or that uptake does not exert a significant effect upon the rapid and transient increase in 5-HT concentration that is achieved with local pressure application of 5-HT.

5-HT-evoked membrane currents on outside-out membrane patches

In an effort to study the single channel events which underlie the 5-HT-induced current in NCB-20 and N1E-115 cells, 5-HT was locally applied to the extracellular face of outside-out membrane patches, either by pressure, or by ionophoresis. In a sample of 11 patches, all taken from 5-HT-sensitive NCB-20 cells, 5 had no detectable response to pressure applied 5-HT (10 μM), whereas the remainder responded with an inward current at negative holding potentials (−50 to −60 mV). Such currents, which appeared as relatively smoothly rising and decaying signals (Figure 6a), varied in amplitude between patches (range −1 to −6.5 pA). Despite the resolution of the recordings, discrete single channel events uniquely associated with the presence of 5-HT were not apparent during these inward currents. To illustrate this feature, Figure 6a compares a 5-HT-evoked current observed on an NCB-20 cell patch with single channel currents of similar amplitude, evoked by GABA (1 μM), on an outside-out patch excised from a bovine adrenomedullary chromaffin cell (Cottrell *et al.*, 1985). Responses qualitatively similar to those obtained on NCB-20 cell membranes were observed when 5-HT was ionophoretically applied to outside-out membrane patches taken from N1E-115 cells ($n = 4$; Figure 6c). This suggests that the unusual response to 5-HT is not a peculiarity of

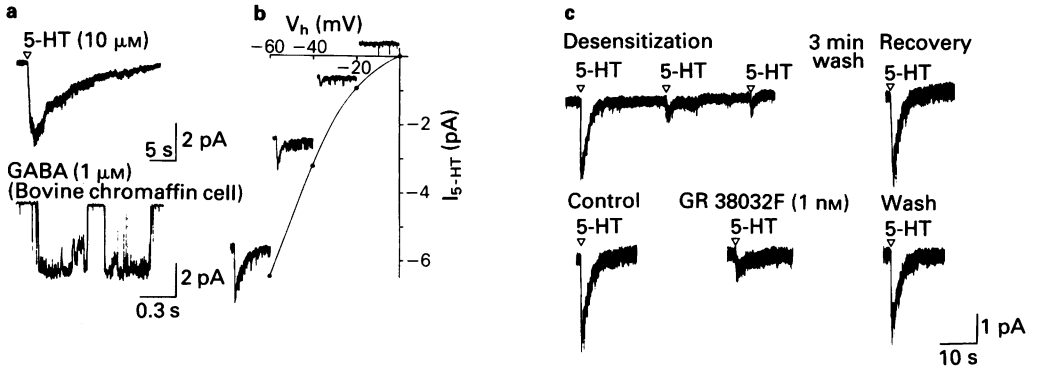

Figure 6 5-Hydroxytryptamine (5-HT)-evoked currents on outside-out membrane patches excised from NCB-20 and N1E-115 cells. (a) Upper trace: inward current evoked by pressure applied 5-HT (10 μM, 1s, 1.4×10^5 Pa) on an outside-out patch taken from an NCB-20 cell. The patch was voltage-clamped at a holding potential of −60 mV (inside negative) and the 5-HT response low-pass filtered at 500 Hz. Lower trace: single channel currents recorded on an outside-out patch excised from a bovine chromaffin cell in response to bath applied γ-aminobutyric acid (GABA, 1 μM). Currents were recorded at a holding potential of −100 mV and low-pass filtered at 1 kHz. The two traces have been deliberately selected to compare 5-HT- and GABA-evoked currents of similar amplitude. Note that unlike the response to GABA, the 5-HT-induced current is not resolved into discrete single channel events. (b) Influence of holding potential upon responses to pressure applied 5-HT (10 μM) recorded from an outside-out patch excised from an NCB-20 cell. Currents recorded at holding potentials of −60, −40, −20 and 0 mV are illustrated. Note that the single channel events superimposed upon the currents are unrelated to the action of 5-HT. Such single channel currents appeared as uncharacterized 'spontaneous' activity in some recordings. In common with the current-voltage relationship found in 'whole-cell' recordings (Figure 2), the relationship between holding potential and 5-HT-induced current amplitude on the outside-out patch shows inward rectification and a reversal potential close to 0 mV. (c) Inward currents elicited by ionophoretically applied 5-HT (40 nA, 200 mS) on an outside out patch excised from an N1E-115 cell. Currents were recorded at a holding potential of −50 mV. Repeated applications of 5-HT induce a reversible desensitization of the response. The introduction of GR 38032F (1 nM) to the bath reversibly suppressed the response to 5-HT. All currents illustrated in (b) and (c) were low-pass filtered at 500 Hz.

the NCB-20 cell line, or a consequence of the pressure application of 5-HT. It is conceivable that the inward current represents the summed activity of many small conductance 5-HT-gated ion channels (see below).

The response to 5-HT recorded on membrane patches from either NCB-20 or N1E-115 cells demonstrated properties similar to those found under 'whole-cell' recording conditions. Firstly, the amplitude of the response decreased with membrane depolarization and was nullified at a potential close to 0 mV (Figure 6b). Secondly, repeated applications of 5-HT produced a reversible desensitization of the response (Figure 6c). Finally, inward currents evoked by 5-HT on outside-out membrane patches were reversibly antagonized by 1 nM GR 38032F (Figure 6c), strongly suggesting that 5-HT_3 receptor activation underlies such responses.

Fluctuation analysis of 5-HT-induced currents

Figure 7 illustrates the results of a typical experiment in which 5-HT was applied by microperfusion to an N1E-115 cell voltage-clamped at a holding potential of −60 mV. Microperfusion was chosen as the method of agonist application since with careful positioning of the drug pipette it was possible to obtain relatively slowly rising inward currents in response to a low concentration (1 μM) of 5-HT, which were suited to the analysis of 5-HT-induced membrane noise. The onset of the response (Figure 7a) was in all cases accompanied by a modest, but clearly discernible, increase in membrane noise as revealed by high-gain a.c.-coupled recordings of the current elicited by 5-HT (e.g. Figure 7b). Fluctuation analysis of such records indicated that the noise variance (σ^2) was linearly related to the mean amplitude (I_{av}) of the 5-HT-evoked inward current during its rising phase. Assuming that the increase in σ^2 arises from the opening of an homogeneous class of channels (see Discussion), the slope of the relationship between σ^2 and I_{av} allows an estimate of the current (i) flowing through one 5-HT-activated channel to be made. In the exemplar cell, i was determined to be 21.6 fA at a holding potential (Vh) of −60 mV. Using this value, in conjunction with the reversal potential of the 5-HT-induced whole cell current determined under identical ionic conditions

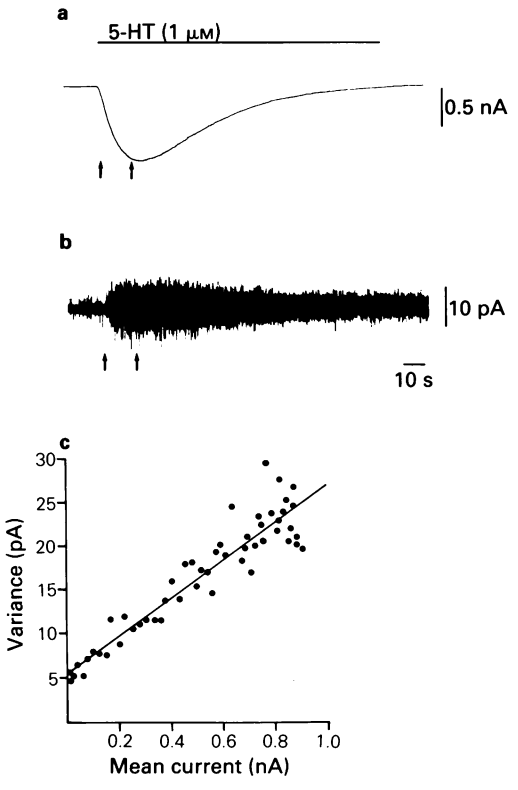

Figure 7 Fluctuation analysis of an inward current evoked by 5-hydroxytryptamine (5-HT) in an N1E-115 cell. (a) d.c.-coupled inward current, recorded at a holding potential of −60 mV, in response to 5-HT (1 μM) applied by microperfusion. Note the relatively slow rise of the current and its subsequent decline during the continued presence of the agonist, indicating the onset of some form of desensitization. (b) High-gain, a.c.-coupled, record of the response elicited by 5-HT. The record illustrates the clear increase in current fluctuation which parallels the development of the inward current evoked by 5-HT. High- and low-pass filtering was performed with cut-off frequencies of 2.0 and 500 Hz respectively. (c) Plot of membrane current variance against mean inward current during the rising phase of the 5-HT-induced response. The analysis was conducted over the bandwidth 2.0–500 Hz and restricted to the data segment contained within the arrows illustrated in (a). The slope of the line fitted to the data points by least squares regression analysis ($r = 0.97$), gives an estimate of the value of the elementary current (i) flowing through a single 5-HT-activated channel. In this example, i was estimated to be 21.6 fA, corresponding to an elementary conductance of 360 fS at a holding potential of −60 mV (see text for further details). In this instance, membrane current variance in the absence of agonist was not subtracted from the total variance observed during the response to 5-HT. The intercept of the fitted line with the ordinate thus yields the 'background' current variance in this recording.

Table 1 Estimates from fluctuation analysis of the current (i) flowing through a single 5-HT-activated ion channel and its conductance (γ) at a holding potential of −60 mV in N1E-115 cells

Cell	*i*(fA)	γ(fS)	*r*
1	19.2	320	0.92
2	9.6	160	0.77
3	11.4	190	0.80
4	15.6	260	0.71
5	27.0	450	0.75
6	19.8	330	0.87
7	22.2	370	0.93
8	21.0	350	0.79
9	21.6	360	0.97
mean ± s.e.	18.6 ± 1.9	310 ± 32	—

i was estimated from the slope of a straight line fitted to the relationship between mean agonist-induced current and current variance. *r* is the correlation coefficient associated with the fit. γ was calculated according to equation 2.

(i.e. −2.1 mV, Figure 2b), allows an estimation of the elementary conductance (γ) of the 5-HT-activated channel to be made since:

$$i = \gamma(V_h - E_{5\text{-HT}}) \quad (2)$$

For the example illustrated in Figure 7, γ was estimated to be 360 fS at a holding potential of −60 mV. Estimates of i and γ obtained from experiments performed on 8 other cells are listed in Table 1. As expected, on 3 N1E-115 cells voltage-clamped at $E_{5\text{-HT}}$, the application of 5-HT was not associated with any detectable increase in current variance.

Discussion

The results of the present study indicate that 5-HT-induced currents in NCB-20 cells are mediated exclusively by the 5-HT$_3$ subtype of receptor. This conclusion is based on the potent blockade of the response observed with GR 38032F and ICS 205-930, and the lack of effect of either ketanserin or methysergide. In particular, blockade of the 5-HT-induced current by GR 38032F occurred over a concentration-range (0.1 to 1.0 nM; $IC_{50} = 0.25$ nM) at which this compound exerts no antagonist action at either '5-HT$_1$-like' or 5-HT$_2$ receptors. Such concentrations are however similar to those at which GR 38032F acts as an antagonist of 5-HT$_3$ receptor-mediated depolarization in rabbit vagus nerve ($pA_2 = 9.4$; Butler *et al.*, 1988).

Adenylate cyclase activity is stimulated through the activation of '5-HT$_1$-like' receptors in NCB-20 cells (Berry-Kravis & Dawson, 1983), but several

lines of evidence suggest that this effect is distinct from the electrical response to 5-HT. Firstly, the enhancement of adenylate cyclase activity shows no decline in the continued presence of 5-HT, whereas the depolarization and inward current elicited by 5-HT desensitize rapidly (MacDermot *et al.*, 1979). Secondly, both D-lysergic acid diethylamide and methysergide act as partial agonists at '5-HT_1-like' receptors in NCB-20 cells, but neither compound affects the electrical response to 5-HT (MacDermot *et al.*, 1979 and present study). Cells of the phaeochromocytoma cell line PC-12, and neuroblastoma clone N-2a accumulate 5-HT from the external medium by diffusion and high affinity transport systems (Yoffe & Borchardt, 1982a,b). If the latter process also occurs in NCB-20 cells, it is unlikely to influence the present results because the selective 5-HT uptake blocker citalopram had no effect on 5-HT-induced currents.

No attempt was made to characterize in detail the 5-HT receptor mediating the inward current response to 5-HT in N1E-115 cells, since there is already substantial evidence that this effect is due to the activation of the 5-HT_3 receptor subtype (Neijt *et al.*, 1986; 1988). The blockade by GR 38032F of 5-HT-induced currents described here adds to this evidence.

5-HT_3 receptor activation is thought to result in the opening of an associated ion channel which conducts monovalent cations. Wallis & Woodward (1975) found that the extracellularly recorded depolarizing response to 5-HT in the rabbit superior cervical ganglion was diminished by the removal of Na^+ from the bathing solution, but was not influenced by replacing Cl^- with an impermeant anion. In a subsequent intracellular study (Wallis & North, 1978), a simultaneous increase in membrane conductance to Na^+ and K^+ was proposed to underlie the 5-HT-induced depolarization. Firm evidence for this suggestion was provided by Higashi & Nishi (1982) in a voltage-clamp study of 5-HT-induced currents in the nodose ganglion of the rabbit. Consistent with these reports, it has been proposed that a simultaneous increase in membrane conductance to Na^+ and K^+ mediates both the 5-HT-evoked depolarization and dopamine-induced inward current in N1E-115 cells (Peters & Usherwood, 1983; Kato & Narahashi, 1982). The latter response has recently been attributed to 5-HT_3 rather than dopamine receptor activation (Peters & Usherwood, 1984; Neijt *et al.*, 1986).

The present study provides evidence obtained under voltage-clamp, with known intra- and extracellular ion concentrations, that 5-HT induces a simultaneous increase in membrane conductance to both Na^+ and K^+. Chloride ions are unlikely to contribute to the response since a large reduction in internal Cl^- concentration had no significant effect upon $E_{5\text{-HT}}$. In a previous study (Peters *et al.*, 1988), varying the extracellular concentration of either Ca^{2+} or Mg^{2+} within the range 0.1 to 3.0 mM did not change $E_{5\text{-HT}}$ in N1E-115 cells, although these divalent cations were observed to modulate both the amplitude and duration of the current. The insensitivity of $E_{5\text{-HT}}$ to $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ might suggest that these ions do not permeate the channel to a significant extent. However, the range of concentrations of Ca^{2+} and Mg^{2+} examined was restricted, and the absence of an observable effect upon $E_{5\text{-HT}}$ does not necessarily exclude the possibility that these ions possess a finite permeability. Assuming that the 5-HT-induced current is carried exclusively by Na^+ and K^+ in physiological solutions, the permeability of $Na^+(P_{Na})$ relative to $K^+(P_K)$ may be calculated from the Goldman-Hodgkin-Katz voltage equation (e.g. Hille, 1984)

$$E_{5\text{-HT}} = \frac{RT}{zF} \quad \text{In} \quad \frac{P_{Na}[Na^+]_o + P_K[K^+]_o}{P_{Na}[Na^+]_i + P_K[K^+]_i} \quad (3)$$

where z, F, R and T have their usual meaning. Employing the known intra- and extra-cellular concentrations of the permeant ion species, and the value of $E_{5\text{-HT}}$ determined with the KCl based pipette solution (−2.1 mV), P_{Na}/P_K was estimated to be 0.92. However, it should be noted that such an estimate does not quantitatively account for the shifts in $E_{5\text{-HT}}$ observed with Na^+- and K^+-deficient media. Reducing $[K^+]_i$ from 140 to 20 mM would, on the basis of equation 3 and a P_{Na}/P_K ratio of 0.92, be expected to shift $E_{5\text{-HT}}$ to +41 mV rather than +25.9 mV as observed. Similarly, $E_{5\text{-HT}}$ found with $[Na^+]_o$ equal to 20 mM (i.e. −39.0 mV) is less negative than that expected (−48.7 mV), whereas the empirically determined and theoretically predicted values of $E_{5\text{-HT}}$ with $[Na^+]_o$ equal to 75 mM correspond perfectly, both being approximately −18.0 mV. The departure of the experimental data from ideal behaviour may be due to several factors (see Hille, 1984), one possibility being that the cations used to replace Na^+ and K^+ are themselves slightly permeant. Further experiments with a variety of replacement cations are required to clarify this issue.

The relationship between holding potential and the amplitude of 5-HT-induced currents in both NCB-20 and N1E-115 cells indicates that the response is inwardly rectifying (e.g. Figures 2 and 3). In NCB-20 and N1E-115 cells, such rectification was not conspicuously altered by replacing Cs^+ in the pipette solution with K^+. This suggests that voltage-dependent blockade of the 5-HT-gated ion channel by internal Cs^+ is unlikely to account for the non-linearity of the current-voltage relationship. Indeed, in both NCB-20 and N1E-115 cells, $E_{5\text{-HT}}$ was found

to be -1 to -2 mV with either Cs^+- or K^+-based pipette solutions, suggesting that these two ions permeate the 5-HT-activated channel equally well. Inward rectification has also been reported for dopamine-induced currents in N1E-115 cells (Kato & Narahashi, 1982) and 5-HT$_3$ receptor mediated membrane currents recorded from clonal NG108-15 hybrid cells and murine striatal and hippocampal neurones in cell culture (Yakel *et al.*, 1988; Yakel & Jackson, 1988). In contrast, the current-voltage relationship obtained with 5-HT in the nodose ganglion of the rabbit is essentially linear (Higashi & Nishi, 1982). We are at present performing voltage-jump experiments in an effort to elucidate the origin of the rectification in the clonal cell lines.

In an attempt to investigate the single channel correlate of 5-HT-induced whole-cell currents, 5-HT was applied locally to outside-out membrane patches excised from NCB-20 and N1E-115 cells. Although 5-HT clearly evoked an inward current in a proportion of the patches examined, such responses could not be resolved into discrete single channel events. Instead, the currents appeared as relatively smooth rising and decaying signals which varied in amplitude from patch to patch. Membrane currents induced by 5-HT on outside-out patches were reversibly antagonized by GR 38032F (1 nM), diminished in amplitude by membrane depolarization and nullified at 0 mV and reversibly desensitized by repeated agonist applications. Collectively, these features strongly suggest that the response is 5-HT$_3$ receptor-mediated.

One interpretation of the data obtained on outside-out patches is that 5-HT gates an ion channel with a conductance too small to allow the direct visualization of individual channel openings, despite the relatively high resolution afforded by this recording mode. The simultaneous activation of many such channels could conceivably produce responses with the characteristics illustrated in Figure 6. Such a mechanism is not unprecedented. Glutamate, or kainate can activate cation-conducting channels with a conductance as low as 140 fS in cerebellar granule cells and membrane patches excised from them (Cull-Candy & Ogden, 1985; Cull-Candy *et al.*, 1988). Additionally, in rat spinal neurones kainate can elicit currents which appear only as an increase in the membrane noise of outside-out patches (Ascher and Nowak, 1988). The results we have obtained using the technique of fluctuation analysis provide considerable support for the view that 5-HT activates a channel of small conductance. Analysis of high-gain a.c.-coupled records of the membrane noise of both NCB-20 and N1E-115 cells, in the absence and presence of 5-HT, indicates that 5-HT-evoked whole cell currents are accompanied by only a modest increase in current variance, which appears to correspond to the activation of single channels with a conductance of approximately 310 fS. However, the results obtained with fluctuation analysis require cautious interpretation. An underestimate of single channel conductance could occur if either the probability of an individual channel being in the open state was not low, or if the bandwidth over which the analysis was performed excluded significant frequency components (Marty *et al.*, 1984). The whole cell currents used in fluctuation analysis were evoked with agonist applied by microperfusion from pipettes containing 1 μM 5-HT and it is likely, that due to diffusion, the actual concentration of 5-HT at the receptor plane is somewhat lower than this value. Such concentrations constitute the foot of the dose-effect relationship for 5-HT in N1E-115 cells (Neijt *et al.*, 1988), suggesting that the probability of a single channel being open would indeed be low. The linear relationship between the variance of 5-HT-induced current fluctuations and mean current (e.g. Figure 7) is consistent with this suggestion. In a number of experiments the bandwidth over which current fluctuations were analysed was extended, either by reducing the high-pass filtering to 1.0 Hz, or by increasing the low-pass filtering to 1 kHz cut-off points. Neither of these changes had any significant effect upon the estimated single channel conductance.

It should be noted that our estimates of single channel conductance were derived under non-steady-state conditions during the period of time that agonist concentration was increasing from zero towards 1 μM. We have assumed that single channel conductance is independent of agonist concentration, and also that the desensitization which is likely to occur during the relatively slow rise of the 5-HT-induced current (e.g. Figure 7a) has not influenced our results. Recent studies have established that for several other agonist-activated cation selective channels, the concept of just two conductance states of the channel, open and closed, is an over-simplification. Instead it is now clear that in addition to a most frequently observed main conductance state, such channels may open to states of higher or lower conductance (e.g. Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987). Fluctuation analysis is likely to provide a weighted-mean estimate of channel conductance that is influenced by the relative frequency of all conducting states. Clearly, the present results may also suffer from this limitation, and although we have interpreted our data in terms of a 5-HT gated channel of unitary conductance, the possibility that 5-HT activates multiple conductance states certainly cannot be excluded. Indeed, on one outside-out membrane patch voltage-clamped at -60 mV and challenged with microperfused 5-HT (1 μM), distinct channel openings of small amplitude

(~0.5 pA) were clearly superimposed upon a relatively smooth inward current of the form described above. Such conductance states associated with the application of 5-HT have been observed on but one occasion, and a far more extensive study is required to establish the reproducibility of this effect. Despite these potential problems, the value of 310 fS (Table 1) thus far suggested for the elementary conductance of the 5-HT-evoked channel is entirely consistent with the lack of observable single channel activity during 5-HT-evoked currents on the majority of outside-out membrane patches. For example, at a holding potential of −60 mV and assuming a reversal potential of −2 mV, a single 5-HT-gated channel of 310 fS conductance would pass a current of only 19 fA, a value far too small to stand out from background noise as a discrete event.

A previous study (Guharay *et al.*, 1985) has shown that 5-HT activates a relatively large conductance (140 pS) ion channel on cell attached, inside-out, and outside-out mebrane patches from N1E-115 cells. On no occasion did we observe single channel currents with this characteristic, neither on whole cells, nor isolated membrane patches. At the present time, a satisfactory explanation for these disparate findings is not available. However, it should be noted that in contrast to the present study, the recordings of Guharay *et al.*, (1985) were conducted on N1E-115 cells differentiated by the inclusion of 2% (vol/vol) dimethylsulphoxide in the cell culture medium, a treatment known to interfere with nucleic acid metabolism (Friend & Freedman, 1978). Additionally, in the latter study membrane patches were bathed with a constant concentration of 5-HT (100 nM), rather than transiently exposed to the agonist as in the present work. We chose a transient method of agonist application in an attempt to minimize the desensitization which even low concentrations of 5-HT are known to induce in N1E-115 cells (Neijt *et al.*, 1988). Finally a degree of genetic instability is inherent to clonal cell systems, including N1E-115 cells, as even amongst cells of the same clone large variations in chromosome number may occur (Amano *et al.*, 1972). Although the results obtained from the two clones examined in the present study are consistent, it is nevertheless desirable to substantiate such observations with results obtained from untransformed tissue. We are presently investigating whether 5-HT induces currents similar to those reported here in rodent hippocampal neurones which have recently been reported to express 5-HT_3 receptors (Yakel & Jackson, 1988).

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