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An electrophysiological investigation of the properties of a murine recombinant 5-HT_3 receptor stably expressed in HEK 293 cells

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1 The pharmacological and biophysical properties of a recombinant 5-HT_3 receptor have been studied by use of patch-clamp techniques applied to HEK 293 cells stably transfected with the murine 5-HT_3 R-A cDNA.

2 At a holding potential of −60 mV, 77% of cells investigated responded to ionophoretically applied 5-HT with an inward current. Such currents were unaffected by methysergide (1 μM), or ketanserin (1 μM), but were antagonized in a concentration-dependent and reversible manner by the selective 5-HT_3 receptor antagonist, ondansetron (IC_{50} = 440 pM) and the non-selective antagonists (+)-tubocurarine (IC_{50} = 1.8 nM) and metoclopramide (IC_{50} 50 nM).

3 The 5-HT-induced current reversed in sign ($E_{5\text{-}HT}$) at approximately −2 mV and exhibited inward rectification. The influence of extra- and intracellular ion substitutions upon $E_{5\text{-}HT}$ indicates the 5-HT-evoked current to be mainly mediated by a mixed monovalent cation conductance.

4 Calcium and magnesium (0.1-10 nM) produced a concentration-dependent, voltage-independent, inhibition of the 5-HT-induced response. Zinc (0.3-300 μM) exerted a biphasic effect with low concentrations enhancing, and high concentrations depressing, the 5-HT-evoked current.

5 Fluctuation analysis of inward currents evoked by a low (1 μM) concentration of 5-HT suggests the current to be mediated by the opening of channels with a conductance of 420 fS.

6 The pharmacological and biophysical properties of the 5-HT_3 R-A are similar to those previously described for 5-HT_3 receptors native to murine neuroblastoma cell lines, with the exception that the function of the recombinant receptor was enhanced by low concentrations of zinc. This observation suggests that the properties of the native receptor are not completely represented by the 5-HT_3 R-A subunit alone.

Keywords: 5-HT_3 receptor; recombinant 5-HT_3 receptor; 5-HT_3 receptor antagonists; 5-HT_3 single channels; 5-HT_3 receptor-evoked currents

Introduction

Electrophysiological studies have firmly established that, in contrast to other 5-HT receptors which mediate their effects through G-proteins, the 5-HT_3 subtype is a member of a ligand-gated ion channel family that includes nicotinic, $GABA_A$ and glycine receptors (Derkach *et al.*, 1989; Lambert *et al.*, 1989; Boess & Martin, 1994). Recently, a cDNA encoding a 5-HT_3 receptor subunit, termed 5-HT_3 R-A, was isolated from the murine hybridoma cell line NCB 20 (Maricq *et al.*, 1991). The predicted 5-HT_3 R-A protein shares many structural elements of the nicotinic acetylcholine receptor (Maricq *et al.*, 1991). Furthermore, the mouse 5-HT_3 R-A gene intron exon organisation is very similar to that of the neuronal α_7 nicotinic subunit of the chick (Uetz *et al.*, 1994). Expression of the 5-HT_3 R-A in *Xenopus laevis* oocytes results in the formation of functional, presumably homo-oligomeric, receptors which exhibit many of the physiological and pharmacological properties (Maricq *et al.*, 1991; Downie *et al.*, 1994) determined for the 5-HT_3 receptor native to NCB 20 cells (Lambert *et al.*, 1989).

The pharmacological properties of the 5-HT_3 receptor are species-dependent although, in contrast to other ligand-gated ion channels, there is no compelling evidence of pharmacological diversity within a species (Peters *et al.*, 1992). However, differences in biophysical properties have been noted. In particular, the 5-HT_3 receptor single channel conductance may vary within a species (Yang *et al.*, 1992; Hussy & Jones, 1993) which may suggest the presence of additional subunits. In common with other ligand-gated channels, the 5-HT_3 receptor is predicted to exist as a pentamer (Boess & Martin, 1994). Heterogeneity of these ligand-gated ion channels occurs through distinct subunit combinations, RNA editing and alternative splicing of receptor subunits (Wafford *et al.*, 1991; Sieghart, 1992; Seeburg, 1993). In mouse neuroblastoma cell lines and mouse brain, two forms of the 5-HT_3 R-A occur through the alternative use of two splice acceptor signals (Maricq *et al.*, 1991; Hope *et al.*, 1993; Uetz *et al.*, 1994) which delete six consecutive amino acid residues from the putative large intracellular loop between transmembrane regions M3 and M4. A comparison of the pharmacological profile of the homo-oligomeric receptors assembled in *Xenopus* oocytes from either 5-HT_3 R-A, or 5-HT_3 R-A_s subunits (the subscript 's' denotes the short form with the six amino acid deletion) revealed the maximal response elicited by the agonist 2-methyl-5-HT to be greatly reduced for the latter (Downie *et al.*, 1994). However, in all other aspects of pharmacology examined, the receptors were similar, both to each other, and to 5-HT_3 receptors native to murine neuronal cell lines.

Analysis of the biophysical properties of the 5-HT_3 R-A expressed in *Xenopus* oocytes has yielded variable results. In two studies, current responses to 5-HT were suppressed, in a voltage-dependent manner, by the presence of extracellular calcium and magnesium at physiological concentrations (Maricq *et al.*, 1991; Eiselé *et al.*, 1993). Such effects were not observed in a third study (Yakel *et al.*, 1993). Furthermore,

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although 5-HT$_3$ receptor-mediated currents recorded from murine cell lines (Peters *et al.*, 1988; Yang, 1990) are blocked by calcium and magnesium, this effect appears to be voltage-independent. It is unclear as to whether the oocyte expression system itself has any bearing on such differences. One potential limitation regarding the use of oocytes in the characterization of the 5-HT$_3$ R-A is the extremely low single channel conductance (0.3–0.6 pS) of the 5-HT$_3$ receptor native to cell lines that express this subunit (Lambert *et al.*, 1989; Yang, 1990; van Hooft *et al.*, 1994). If the recombinant homo-oligomer were to possess a comparable conductance, fluctuation analysis of whole-cell currents in oocytes would be problematic and the direct detection of current responses representing the simultaneous activation of 5-HT$_3$ R-A populations on excised outside-out membrane patches would require levels of expression far higher than those reported in the literature (Maricq *et al.*, 1991; Hope *et al.*, 1993; Yakel *et al.*, 1993; Downie *et al.*, 1994). Hence, in the present study, we have utilized the whole-cell recording mode of the patch-clamp technique to investigate the properties of the 5-HT$_3$ R-A (Maricq *et al.*, 1991) stably transfected into HEK 293 cells, which are more amenable to such studies. We report the pharmacological and biophysical properties of the 5-HT$_3$ R-A to be similar to those of the receptor native to murine neuronal cell lines, with the exception that 5-HT-mediated currents are enhanced by low concentrations of zinc (*cf.* Lovinger, 1991). Hence the 5-HT$_3$ R-A may not be completely representative of the 5-HT$_3$ receptor native to neuronal cell lines. A preliminary account of a part of this work has appeared in abstract form (Gill *et al.*, 1993).

Methods

Culture of HEK 293 cells

HEK 293 cells were stably transfected in the laboratory of Dr D. Julius (University of California, San Francisco) with the 5-HT$_3$ R-A cDNA contained within the expression vector pLNCX. Stable transformants were selected by the use of media supplemented with geneticin to which the expression vector confers resistance. Cells were grown to confluency in Dulbecco's Modified Eagle Medium (DMEM), supplemented with foetal calf serum (10% v/v), penicillin (1×10^3 iu ml^{-1}), streptomycin (100 μg ml^{-1}) and geneticin (200 μg ml^{-1}) in 25 cm^2 tissue culture flasks. The cells were harvested enzymatically with trypsin (0.5 mg ml^{-1}) and EDTA (0.2 mg ml^{-1}). Enzyme action was terminated by the addition of 10 ml of growth medium and the cells were dissociated further by gentle trituration, prior to centrifugation at 100 *g* for 2 min. For electrophysiological recordings, the cells were resuspended in growth medium and replated onto 35 mm Petri dishes at a density of 1×10^4 cells per dish. The culture medium was refreshed every three days and the cells were maintained at 37°C in an atmosphere of 90% air, 10% CO_2 at 100% relative humidity. Experiments were conducted 2 to 7 days after replating upon cells of passage numbers 5 to 35 inclusive.

Electrical recordings

Whole-cell recordings of agonist-evoked membrane currents were performed at room temperature (18–22°C) under voltage-clamp conditions with either a List Electronics L/M EPC-7 converter headstage and amplifier, or an Axopatch 1D amplifier, using standard patch-clamp techniques (Hamill *et al.*, 1981). Agonist-evoked currents were recorded at a holding potential of −60 mV, unless specified otherwise. Currents were low pass-filtered at a cut-off frequency of 3 KHz (two pole Bessel characteristic) and recorded onto video tape using a PCM-2 VCR adaptor (Medical Systems Corporation, Greenvale, NY, U.S.A.), or onto magnetic tape using a Racal Store 4DS F.M. tape recorder. The cells were continually superfused at a rate of 3–5 ml min^{-1} with an extracellular solution (E1) comprising (in mM): NaCl 140, KCl 2.8, $CaCl_2$ 1.0, $MgCl_2$ 2.0 and HEPES 10. Patch electrodes were fabricated from glass capillary tubing (Corning type 7052, Garner Glass Company, Claremont, CA, U.S.A.) and filled with a solution (I1) containing (in mM): CsCl 140, $MgCl_2$ 2.0, $CaCl_2$ 0.1, EGTA 1.1 and HEPES 10 (free $[Ca^{2+}] = 10^{-8}$ M at pH 7.2). Caesium was used as the predominant intracellular cation to reduce membrane potassium conductances. With the above solutions, electrode resistances were typically within the range 2 to 5 MΩ.

In experiments designed to investigate the ionic dependence of the agonist-evoked current, modified intracellular and extracellular salines were employed. To evaluate the potential contribution of chloride ions to the 5-HT-evoked response, NaCl in the extracellular medium was completely replaced by 140 mM sodium isethionate. The influence of sodium ions was assessed by reducing the extracellular concentration of this ion to either 75 or 20 mM by partial replacement with N-methyl-D-glucamine (NMDG). Additionally, the concentrations of $MgCl_2$ and $CaCl_2$ within the extracellular medium were reduced from 2 mM and 1 mM, respectively, to 0.1 mM each and KCl was totally replaced by NaCl. This simplified solution (E2) was also used in experiments where the internal solution was modified. In such experiments, the concentration of caesium ions was reduced to 20 mM by the partial replacement of CsCl with tetraethylammonium chloride (TEA; solution I2). With the exception of NMDG-containing extracellular solutions which were titrated to pH 7.2 with HCl, the pH of all external and internal solutions was adjusted to 7.2 with NaOH. Sodium ions so introduced were included in the calculation of external and internal sodium ion concentrations. During ion substitution experiments, changes in reference electrode potential due to the perfusion of modified external salines were minimized by using a salt bridge containing 3 M KCl in 4% (w/v) agar which coupled the recording chamber to an Ag/AgCl electrode. Liquid junction potentials at the tip of the patch pipette prior to gigaseal formation were estimated as previously described (Peters *et al.*, 1989) and the holding potential was corrected appropriately.

In the majority of experiments, 5-HT was applied focally to the cells by ionophoresis with an Intra 767 electrometer (World Precision Instruments). Ionophoretic electrodes had resistances > 30 MΩ when filled with a solution containing 20 mM 5-HT in twice distilled deionized water. Ejection currents of 30–120 nA and of 40–100 ms duration were superimposed upon a constant retaining current of approximately 4.0 nA. All antagonist compounds were applied by their inclusion within the superfusate. In experiments where the conductance of the 5-HT$_3$ R-A was assessed by fluctuation analysis of whole-cell currents, 5-HT (1 μM) was applied to cells by diffusion from a coarse-tipped (approximately 10 μm diameter) micropipette which was positioned to achieve a relatively slowly developing response to 5-HT. All quantitative data are expressed as the arithmetic mean ± the standard error of the mean (s.e. mean).

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

Random fluctuations in 5-HT-induced whole-cell currents were used to estimate the single channel conductance of the 5-HT$_3$ R-A. Signals were stored on magnetic tape for subsequent off-line analysis using the programme SPAN V3.0 (Dempster, 1993). For the analysis of whole-cell current noise, current fluctuations were a.c. coupled (high-pass filter, 1.0 Hz cut-off, Butterworth characteristic; Fylde Electronics, Preston, U.K.) and amplified. Anti-aliasing filtration of the current fluctuation signal was performed with a Fylde Electronics 8-pole Butterworth low-pass filter set to half the sampling frequency. Continuous records of mean d.c. current and current fluctuations in the presence and absence of 5-HT were replayed from video tape and digitized at a rate of

1 KHz using a Data Translation DT2801A laboratory interface and stored on a personal computer (Dell 'pentium' Dimension XPSP60). The digitized record was composed of blocks with a duration of $1/f_{res}$s, where f_{res} (Hz) was the desired resolution of the recording (usually 1 Hz). Blocks of data were edited visually and those containing obvious artifacts were excluded from further analysis. The variance method (Cull-Candy *et al.*, 1988; Lambert *et al.*, 1989; Dempster, 1993) was used to estimate the current, i, flowing through a single channel using the equation (Dempster, 1993):

$$\sigma^2 = \frac{1}{N-1} \sum_{j=1}^{N} (I_j - I_m)^2 \quad (1)$$

where σ^2 is the variance; I_m the mean current; I_j current sample j and N is the number A/D samples in the block. The relationship between σ^2 and I_m is parabolic rising from zero to a maximum (at an open channel probability, P, of 0.5) and falling to zero again as P is varied from 0 to 1, i.e.:

$$\sigma^2 = iI_m - \frac{I_m^2}{n} \quad (2)$$

where n is the number of channels within the cell. When P is small, (< 0.1) equation (2) simplifies to the linear function:

$$\sigma^2 = iI_m \quad (3)$$

σ^2 *vs.* I_m plots were obtained from blocks recorded during the development and desensitization of the inward current response to microperfused 5-HT (1 μM). Single channel current was estimated from parabolic or linear functions fitted to such plots by least-squares regression analysis. Background variance, attributable to sources other than 5-HT-activated

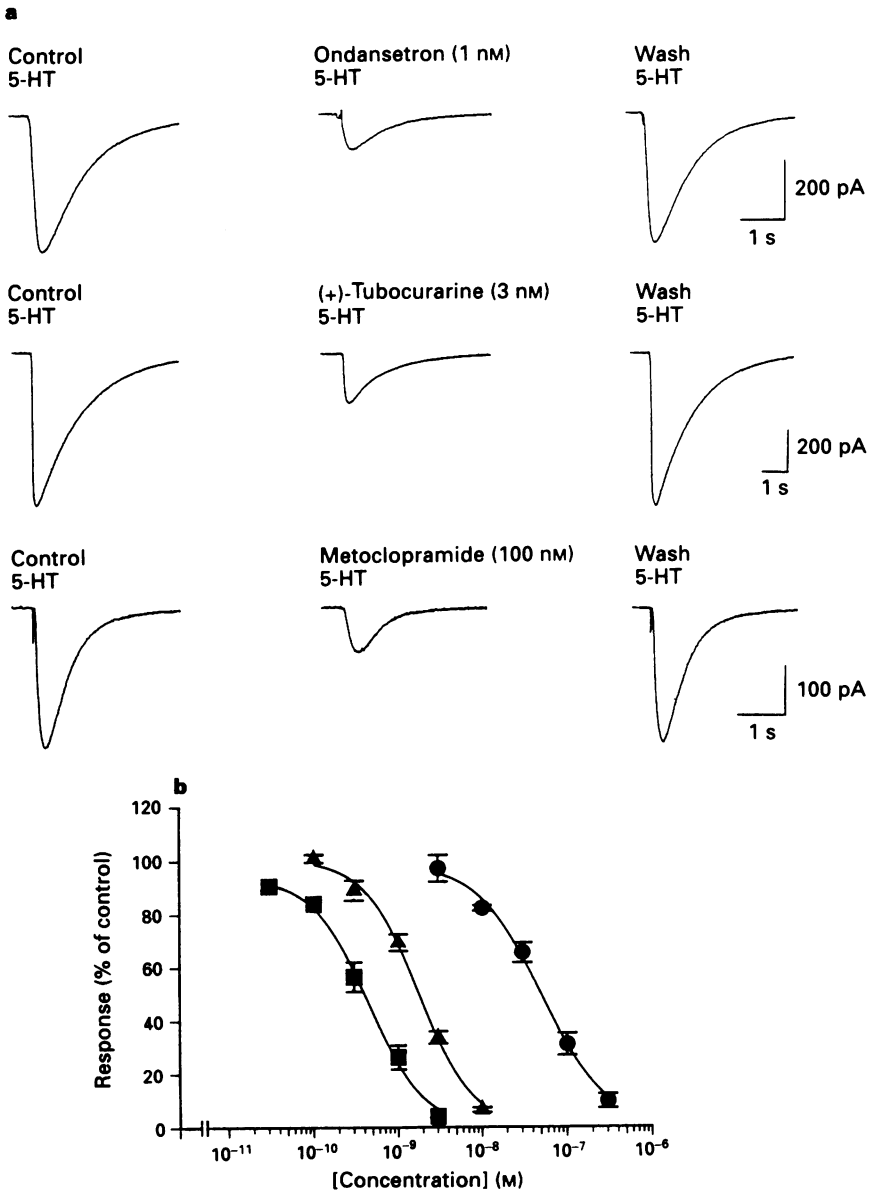

Figure 1 Inhibition of 5-hydroxytryptamine (5-HT)-induced currents by selective and non-selective 5-HT$_3$ receptor antagonists. (a) Inward current responses to ionophoretically applied 5-HT were reversibly inhibited by bath applied ondansetron (1 nM; top panel), (+)-tubocurarine (3 nM; middle panel) and metoclopramide (100 nM; bottom panel). All records are the computer generated average of four responses to 5-HT recorded at a holding potential of −60 mV. Each antagonist was tested on a different cell. (b) Graph depicting the concentration-dependent inhibition of 5-HT-induced currents by ondansetron (■), (+)-tubocurarine (▲) and metoclopramide (●). The amplitude of the 5-HT-induced inward current (expressed as a percentage of control; y axis) is plotted against the concentration of antagonist in the extracellular medium (log scale; x axis). Each point is the mean ± s.e.mean (vertical lines) of a minimum of four observations performed on separate cells. Curves were fitted to the data points by eye.

ion channels, was removed by subtracting the mean value of 16–32 blocks recorded prior to the application of the agonist.

Reagents

With the exception of geneticin (Sigma), all cell culture reagents were obtained from Gibco (UK). The experimental drugs used were: 5-hydroxytryptamine creatinine sulphate complex (5-HT), (+)-tubocurarine chloride, metoclopramide hydrochloride (all Sigma), ondansetron hydrochloride (Glaxo), methysergide hydrogen maleate (Sandoz) and ketanserin tartrate (Janssen). Compounds were freshly dissolved as concentrated stock solutions in either twice distilled deionised water, or extracellular saline.

Results

Pharmacological characterization

The ionophoretic application of 5-HT (20 mM) to solitary HEK 293 cells, voltage-clamped at −60 mV, induced an inward current on 77% (69 of 90) cells tested (range = 225–1080 pA, $n = 30$). Such responses were not observed in untransfected cells. The 5-HT-induced current was antagonised, in a concentration-dependent manner, by the selective 5-HT$_3$ receptor antagonist, ondansetron ($IC_{50} = 440 \pm 66$ pM; $n = 4$) and the non-selective antagonists (+)-tubocurarine ($IC_{50} = 1.8 \pm 0.2$ nM; $n = 4–5$) and metoclopramide (IC_{50} 50 ± 7 nM, $n = 4$). In all cases, the antagonism was readily reversed upon washout (Figure 1). The 5-HT$_2$ receptor

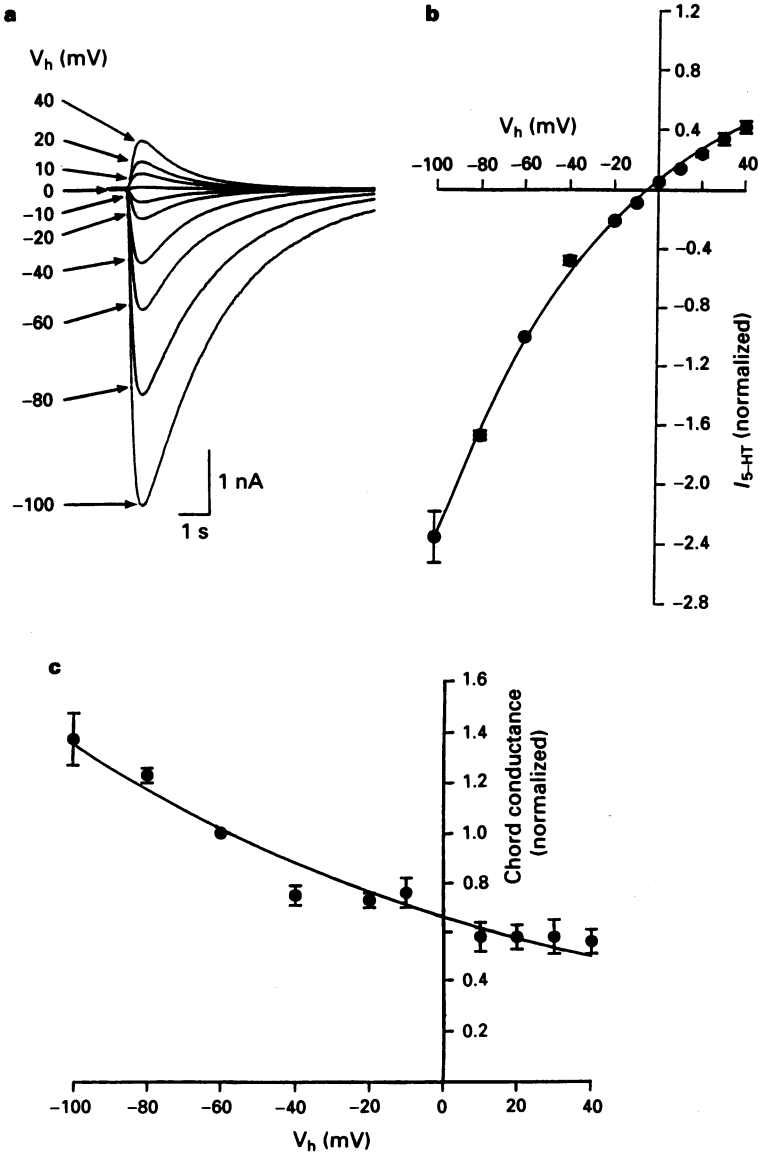

Figure 2 The current-voltage relationship for the 5-HT$_3$ R-A mediated electrical response in HEK 293 cells. (a) Current responses elicited by 5-HT applied ionophoretically to an HEK 293 cell voltage-clamped at the holding potentials (V_h) indicated adjacent to each trace. Each trace is the computer-generated average of 4 responses to 5-HT and leakage currents have been subtracted. (b) Graphical depiction of the 5-HT current-voltage relationship. In order to combine data obtained from several cells, the amplitude of the 5-HT induced current ($I_{5\text{-HT}}$) has been normalized by assigning a value of one to the response recorded at −60 mV and expressing current amplitudes at holding potentials (V_h) in the range −100 to 40 mV relative to that value. The current-voltage relationship demonstrates inward rectification and yields a reversal potential ($E_{5\text{-HT}}$) of −3 mV for the 5-HT$_3$ receptor-mediated response. Each data point is the mean ± s.e.mean of 7 to 10 observations performed on separate cells. The curve is fitted to the data points by eye. (c) The chord conductance of the 5-HT$_3$ R-A-mediated response (i.e. $I_{5\text{-HT}}/(V_h - E_{5\text{-HT}})$) calculated from the data illustrated in (b). The chord conductance increases e-fold for a membrane hyperpolarization of 151 mV. Data points are the mean ± s.e.mean of observations made from 7–10 cells.

antagonist, ketanserin and the mixed '5-HT$_1$-like' and 5-HT$_2$ receptor antagonist methysergide, each applied at a concentration of 1 μM had no effect upon the 5-HT-induced inward current ($n = 4$ for both compounds).

The ionic basis of the 5-HT-evoked response

Using the standard CsCl based pipette solution (I1) and the NaCl based bath saline (E1) described in the Methods, the amplitude of the 5-HT-evoked inward current decreased with membrane depolarization and reversed in sign at a potential ($E_{5\text{-HT}}$) of -2.2 ± 0.6 mV ($n = 15$). This value is similar to that previously found for the 5-HT$_3$ R-A expressed in *Xenopus* oocytes (Maricq *et al.*, 1991) and for 5-HT$_3$ receptor-mediated current responses of NCB-20 and N1E-115 cells (Lambert *et al.*, 1989). Inspection of the current-voltage relationship (Figure 2) reveals the 5-HT current to rectify inwardly. The rectification was quantified by calculating the chord conductance at a range of holding potentials (−100 to +40 mV). This analysis revealed that the 5-HT-induced chord conductance increases e-fold for a 151 mV hyperpolarization (Figure 2).

The total replacement of NaCl by sodium isethionate, to produce a solution in which the extracellular concentration of chloride ions was reduced from 148 mM to 8 mM had little influence upon $E_{5\text{-HT}}$. In the chloride ion deficient solution, $E_{5\text{-HT}}$ was estimated to be -1.0 ± 1.2 mV ($n = 5$), a value similar to that obtained with the standard extracellular medium. Thus as in other systems, the 5-HT$_3$ receptor-mediated current arises from the opening of channels that principally conduct cations. In some systems, permeability to Ca^{2+} and Mg^{2+} has also been clearly demonstrated (Yang, 1990; Yang *et al.*, 1992). In the present study, the contribution of extra- and intracellular monovalent cations to the 5-HT-induced current was examined by use of extracellular salines in which the concentration of both Ca^{2+} and Mg^{2+} was reduced to 0.1 mM to minimize their potential contribution to the response. Additionally, KCl was replaced by NaCl, so that Na was the sole monovalent cation present in the extracellular solution (E2). Under these conditions

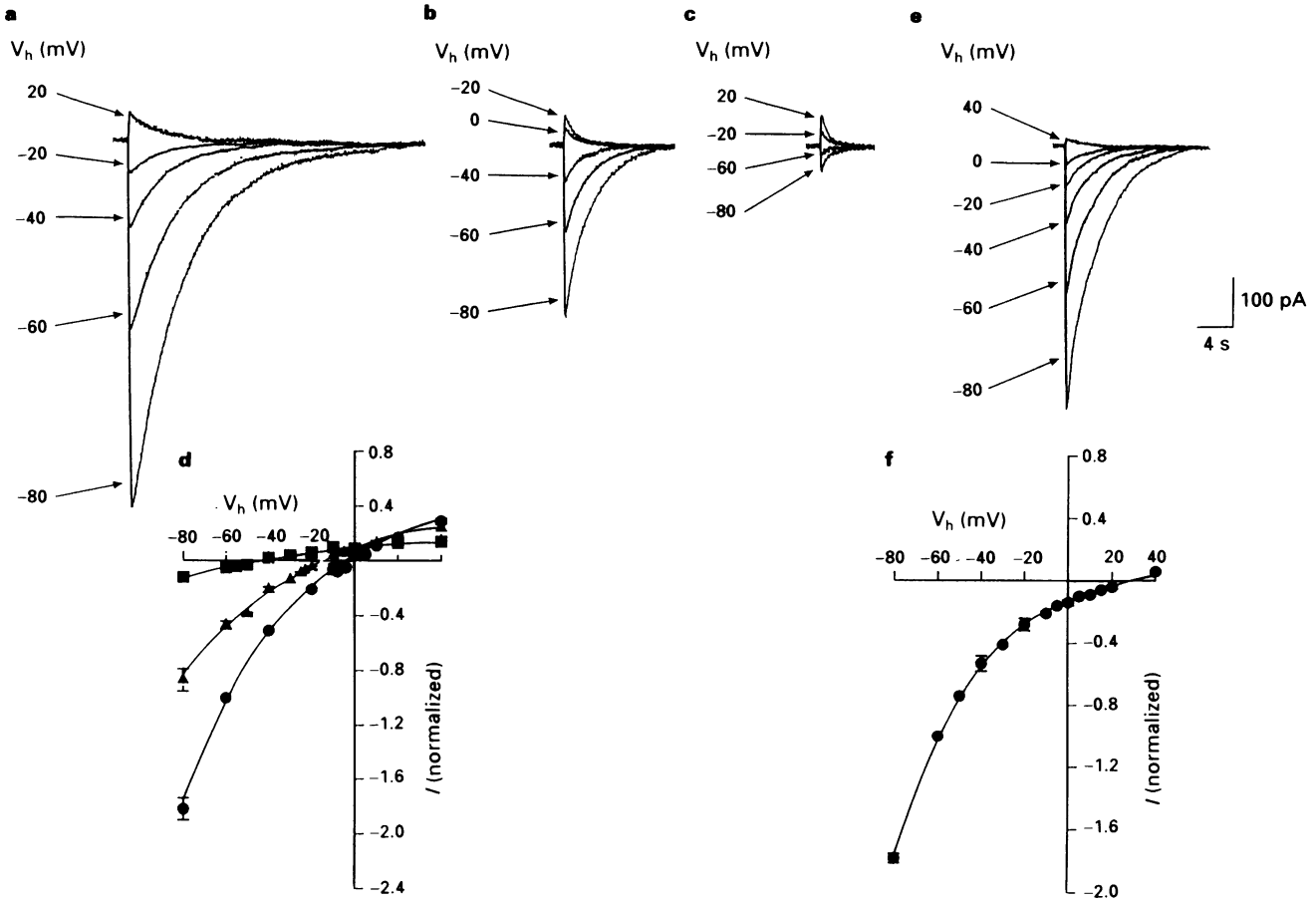

Figure 3 5-Hydroxytryptamine (5-HT)-induced currents are associated with an increase in membrane conductance to monovalent cations. (a-c) Traces illustrating transmembrane currents in response to ionophoretically applied 5-HT recorded at holding potentials (V_h) ranging between +20 and −80 mV. The currents were recorded in extracellular solutions containing 143 mM Na^+ (a), 75 mM Na^+ (b) and 20 mM Na^+ (c). The extracellular concentration of Na^+ ($[Na^+]_o$) was reduced by the partial replacement of NaCl with N-methyl-D-glucamine (see Methods). The records illustrated in (a), (b) and (c) were obtained from the same representative cell. (d) Current-voltage relationships for the 5-HT-evoked electrical response with $[Na^+]_o$ equal to 143 (●), 75 (▲) or 20 (■) mM. The amplitude of the 5-HT- induced current ($I_{5\text{-HT}}$), normalised as described in the legend to Figure 1, is plotted against the holding potential (V_h). The reversal potential ($E_{5\text{-HT}}$) was estimated to be −2.0, −14.5 and −43.0 mV in media containing 143, 75 and 20 mM Na^+ respectively. Data points are the means of 6 (▲) or 4 (●, ■) observations obtained from different cells. Curves were fitted to the data points by eye. Vertical lines, where they exceed the size of the symbol, indicate the standard error of the mean. (e) A family of 5-HT-evoked currents obtained from a representative cell bathed in an extracellular solution (E2) containing 143 mM Na^+ and dialysed with a pipette solution (I2) in which the concentration of Cs^+ was reduced from a standard value of 140 mM to one of 20 mM by partial replacement with the poorly permeant cation, tetraethylammonium chloride (TEA). (f) The amplitude of the normalised 5-HT-induced current ($I_{5\text{-HT}}$) as a function of holding potential (V_h) under the recording conditions described in (e). $E_{5\text{-HT}}$ was estimated to be +25 mV. Data points represent the mean of 3 observations obtained from different cells; the curve was fitted by eye. Vertical lines indicate s.e.mean. The traces illustrated in (a), (b), (c) and (e) are computer-generated average of four responses to 5-HT with leakage currents subtracted.

(Figure 3), $E_{5\text{-HT}}$ was found to be -2.0 ± 0.4 mV ($n = 4$), a value similar to that of -2.2 mV determined in the standard extracellular medium (E1). Reduction of the external concentration of NaCl to 75 and 20 mM by the iso-osmotic replacement of Na^+ with the poorly permeant monovalent cation N-methyl-D-glucamine (Yang, 1990; Malone *et al.*, 1994) produced a negative shift of $E_{5\text{-HT}}$ to values of -14.5 ± 0.4 mV ($n = 6$) and -43 ± 1 mV ($n = 4$) respectively (Figure 3). Reducing the internal concentration of Cs^+ from 140 to 20 mM, by partial substitution of CsCl with tetraethylammonium chloride (solution I2), produced a positive shift in $E_{5\text{-HT}}$ to $+25 \pm 0.8$ mV ($n = 3$; Figure 3). These observations suggest that under these ionic conditions the 5-HT-mediated current is carried mainly by Na^+ and Cs^+. Assuming the response to be mediated exclusively by a mixed Na^+ and Cs^+ cation conductance and utilizing the $E_{5\text{-HT}}$ value of -2 mV for salines I1 and E2, the ratio of Na and Cs permeabilities (P_{Na}/P_{Cs}) may be calculated from the Goldman-Hodgkin-Katz voltage equation (see Discussion) to be 0.94. It should be noted, however, that the computed P_{Na}/P_{Cs} ratio does not adequately account for the shifts in $E_{5\text{-HT}}$ observed with modified extra- and intra-cellular solutions. Possible reasons for this discrepancy are explored in the Discussion.

Modulation of the 5-HT-induced current by calcium, magnesium and zinc

The inhibitory effect of the divalent cations magnesium and calcium upon 5-HT$_3$ receptor-mediated currents is well documented (eg. Peters *et al.*, 1988; 1993; Yang, 1990). In the present study, reducing the extracellular concentrations of Ca^{2+} ($[Ca^{2+}]_o$) and Mg^{2+} ($[Mg^{2+}]_o$) to 0.1 mM, from their standard values of 1 mM and 2 mM respectively, enhanced the amplitude of the 5-HT-induced current, recorded at -60 mV, to $166 \pm 8\%$ of control ($n = 14$; Figure 4). In subsequent experiments, this divalent cation deficient solution was used as a control and the amplitude of the current evoked by 5-HT was expressed as a percentage of that obtained in this

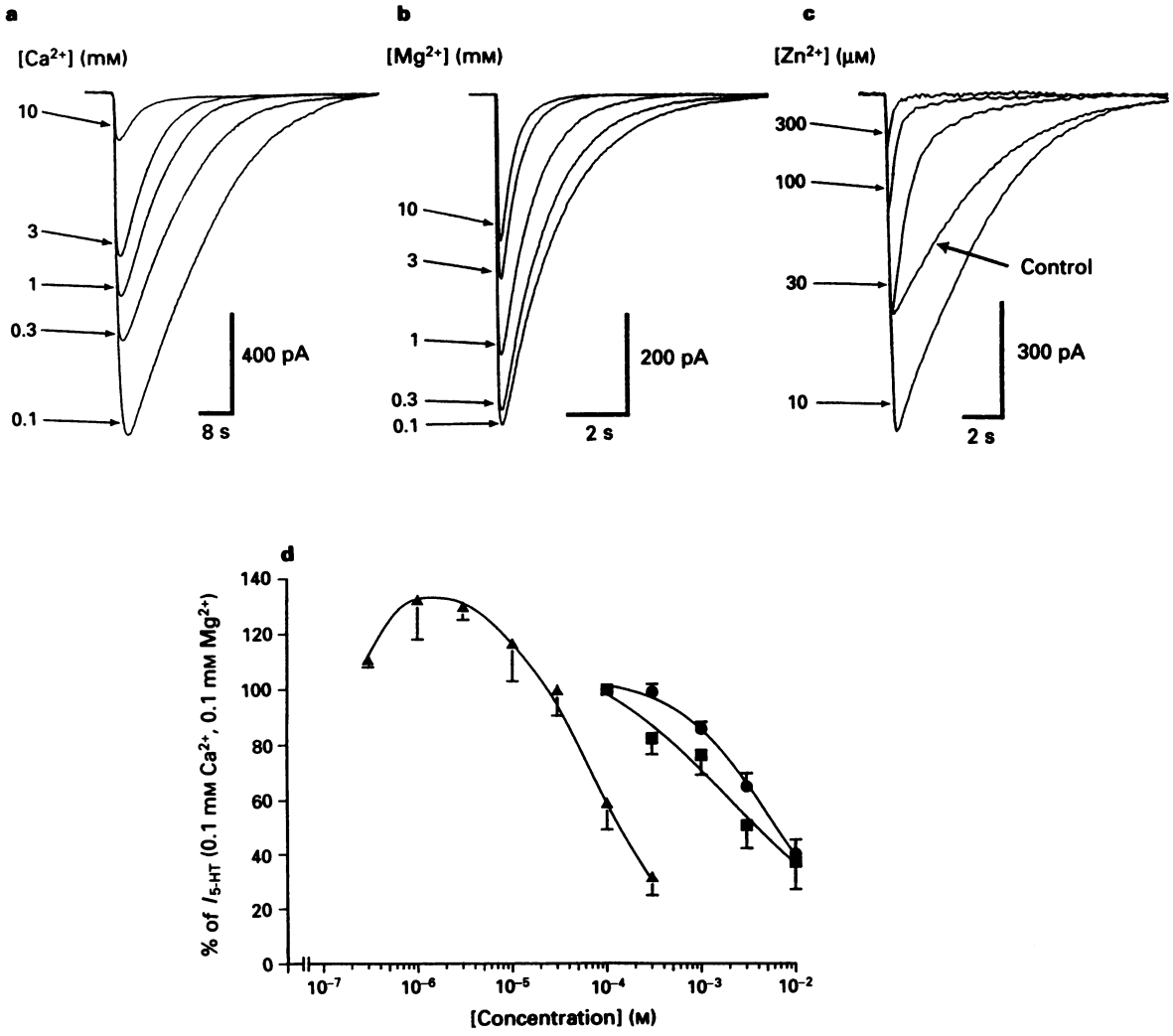

Figure 4 Divalent cations modulate the 5-HT$_3$ R-A-mediated current-response in HEK 293 cells. Superimposed responses to ionophoretically applied 5-HT recorded from 3 different cells illustrating the influence of extracellular Ca^{2+} (a), Mg^{2+} (b) and Zn^{2+} (c) upon current amplitude and duration. (a) Concentration-dependent reduction in current amplitude and duration by Ca^{2+} (0.1–10.0 mM). The concentration of Mg^{2+} was held constant at 0.1 mM. (b) Mg^{2+} exerts a depressant effect that is qualitatively similar to that observed with Ca^{2+}. The extracellular concentration of Ca^{2+} was maintained at 0.1 mM. (c) A low concentration (10 μM) of Zn^{2+} augments 5-HT-evoked currents, whereas higher concentrations (30–300 μM) depress both the amplitude and duration of the response. The effects of Zn^{2+} were examined in the presence of Ca^{2+} and Mg^{2+}, each at a concentration of 0.1 mM. All currents were recorded at a holding potential of -60 mV and each trace represents the computer generated average of 4 responses. (d) Graphical depiction of the concentration-dependent effects of Ca^{2+} (■), Mg^{2+} (●) and Zn^{2+} (▲) upon the amplitude of the response to 5-HT. Responses are expressed as a percentage of the control response amplitude observed in the presence of 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+}. For Ca^{2+} and Mg^{2+}, each point represents the mean of 4 observations performed on separate cells. Data points for Zn^{2+} were obtained from 4 to 7 cells. Error bars indicate s.e.mean.

solution. With $[Mg^{2+}]_o$ fixed at 0.1 mM, increasing $[Ca^{2+}]_o$ from 0.1 mM to 1 mM and 10 mM reduced the amplitude of the 5-HT-induced response to 76.4 ± 7% of control ($n = 4$) and 32.6 ± 11.8% of control ($n = 4$) respectively (Figure 4). Similarly, a blocking action of Mg^{2+} was observed with $[Ca^{2+}]_o$ held constant at 0.1 mM, the response to 5-HT being reduced to 86 ± 3% of control, ($n = 4$) and 40 ± 5% of control ($n = 5$) by 1 and 10 mM Mg^{2+} respectively (Figure 4). The inhibitory effect of Mg^{2+} and Ca^{2+} was voltage-independent and occurred in the absence of a discernible shift in $E_{5\text{-HT}}$ (Figure 5).

In a previous study (Lovinger, 1991), the divalent cation zinc was shown to be a potent antagonist of the 5-HT$_3$ receptor mediated current recorded from NCB-20 cells. However, in this investigation, Zn^{2+} (0.3–300 μM) exerted a biphasic effect upon the amplitude of 5-HT-evoked currents recorded from HEK 293 cells, with low concentrations potentiating, and higher concentrations depressing, the response (Figure 4). For example, in the presence of 3 μM Zn^{2+}, the current amplitude was enhanced to 135 ± 5% of control ($n = 4$), whereas with 300 μM Zn^{2+}, the response was reduced to 29 ± 9% of control ($n = 3$; Figure 4).

Fluctuation analysis of the 5-HT-evoked response

The conductance of the ion channel integral to the 5-HT$_3$ receptor varies markedly between preparations (Peters *et al.*, 1992). In undifferentiated cells of the neuroblastoma cell lines N1E-115 and N18, extremely small single channel conductances of less than 1 pS have been reported. In such cases, single channel currents cannot be directly discerned by the patch clamp technique, but some of their properties may be determined indirectly by fluctuation analysis of 5-HT-induced whole-cell noise (Lambert *et al.*, 1989; Yang, 1990). In the present study, relatively slowly rising and desensitizing inward current responses, suited to fluctuation analysis, were elicited by a low concentration of 5-HT (1 μM) applied by microperfusion. Figure 6 illustrates low gain d.c.- and high

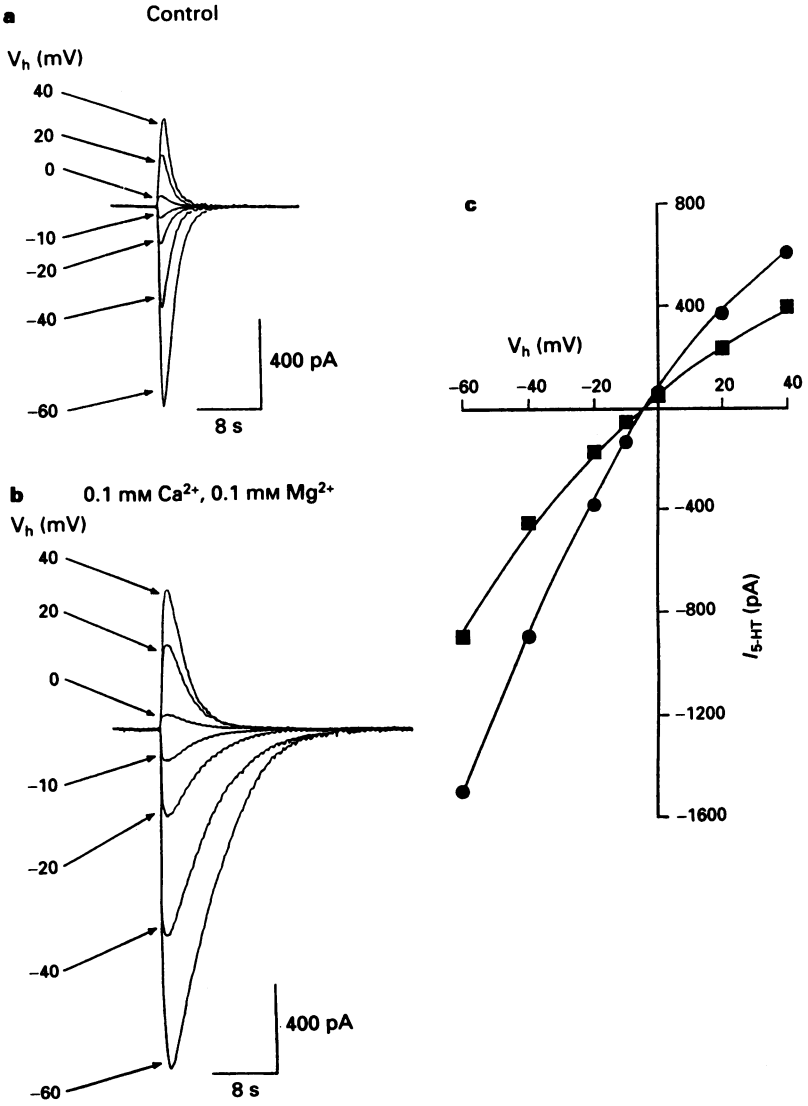

Figure 5 The effect of calcium and magnesium on the current-voltage relationship for the 5-HT$_3$ R-A-mediated electrical response in HEK 293 cells. (a) Current responses to ionophoretically applied 5-HT recorded at holding potentials (V_h) ranging between −60 and +40 mV. Currents were recorded in the standard extracellular saline (E1) containing 1 mM Ca^{2+} and 2 mM Mg^{2+}. (b) Current responses recorded from the same cell as in (a) but in the presence of a solution containing 0.1 mM Ca^{2+} and 0.1 mM Mg^{2+}. Note that the 5-HT-induced currents at all holding potentials are enhanced in amplitude and duration in the divalent cation deficient solution. In both (a) and (b), each current is the average of four responses to 5-HT and leakage currents have been subtracted. (c) Graphical representation of the data shown in (a) and (b) in the form of a current-voltage relationship. Note that in both the control (■) and divalent cation-deficient solutions (●) inward rectification is apparent. The reversal potential of the response to 5-HT is unaffected by the reduction in the extracellular concentration of divalent cations.

gain a.c.-coupled records associated with such a response. The inward current was accompanied by a small increase of current noise that is apparent in the high gain a.c.-coupled record (Figure 6). Fluctuation analysis of this response yielded a linear relationship between the noise variance (σ^2) and mean amplitude (I_m) of the inward current response to 5-HT. By fitting equation (3) to this data, the current i flowing through a single 5-HT activated ion channel was estimated to be 16.9 fA for the exemplar cell. From the holding potential (V_h) at which the recording was made (i.e. −60 mV) and the reversal potential ($E_{5\text{-HT}}$) of the response to 5-HT determined under the appropriate ionic conditions (ie. approximately -2 mV), the single channel conductance can be estimated from the relationship:

$$i = \gamma(V_h - E_{5\text{-HT}}) \quad (4)$$

For the example illustrated in Figure 6, the single channel conductance approximated to 290 fS. The mean single channel conductance derived by fitting either equations (2) or (3) to the data obtained from two and four additional cells respectively was estimated to be 420 ± 74 fS ($n = 7$).

Discussion

The cDNAs encoding the murine 5-HT$_3$ R-A and an alternatively spliced form of the subunit (5-HT$_3$ R-A$_s$) produce functional, presumably homomeric, receptor channel complexes when expressed in *Xenopus laevis* oocytes (Maricq *et al.*, 1991; Eiselé *et al.*, 1993; Hope *et al.*, 1993; Yakel *et al.*, 1993; Downie *et al.*, 1994; Uetz *et al.*, 1994). 5-HT-induced currents recorded from such oocytes exhibit many of the pharmacological and biophysical properties attributed to the 5-HT$_3$ receptor native to the murine N1E-115 and NCB-20 cell lines from which these cDNAs were first isolated. Similarly, the results reported here demonstrate that the 5-HT$_3$ R-A cDNA, when stably transfected into HEK 293 cells, directs the formation of functional 5-HT-activated ion channels.

A number of studies (see Peters *et al.*, 1992) have established that the pharmacological properties of 5-HT$_3$ receptors vary markedly across species. Consistent with previous studies utilizing *Xenopus* oocytes (Maricq *et al.*, 1991; Hope *et al.*, 1993; Downie *et al.*, 1994; Uetz *et al.*, 1994), the murine 5-HT$_3$ R-A expressed in HEK 293 cells was blocked by the selective 5-HT$_3$ receptor antagonist, ondansetron and the non-selective antagonists, metoclopramide and (+)-tubocurarine, at concentrations similar to those reported for murine neuronal cell lines (Lambert *et al.*, 1989; Peters *et al.*, 1990), mouse hippocampal (Yakel & Jackson 1988) and mouse nodose ganglion neurones (Malone *et al.*, 1991; Peters *et al.*, 1992). In particular, (+)-tubocurarine exhibits the characteristically high affinity established for the mouse 5-HT$_3$ receptor. The mechanism of action of (+)-tubocurarine is not known, but in murine N1E-115 neuroblastoma cells the antagonism is both use- and voltage-independent, making the associated ion channel an unlikely locus of action (Peters *et al.*, 1990). Consistent with these findings, functional experiments performed on a chimaeric construct of the N-terminal region of the chick nicotinic α_7 subunit and the C-terminal and the putative transmembrane domains of the 5-HT$_3$ R-A, reveals a receptor that is activated by acetylcholine which demonstrates the relatively low affinity for (+)-tubocurarine characteristic of the α_7 nicotinic subunit (Eiselé *et al.*, 1993). The isolation and sequencing of homologues of the 5-HT$_3$ R-A subunit from species in which the native receptor is known to exhibit divergent pharmacological properties may be instructive in defining ligand binding sites. In this respect, the recently identified rat 5-HT$_3$ R-A$_s$, which differs from the murine 5-HT$_3$ R-A$_s$ at least 100 fold in its sensitivity to (+)-tubocurarine, is of interest (Johnson & Heinemann, 1992; Isenberg *et al.*, 1993). The rat

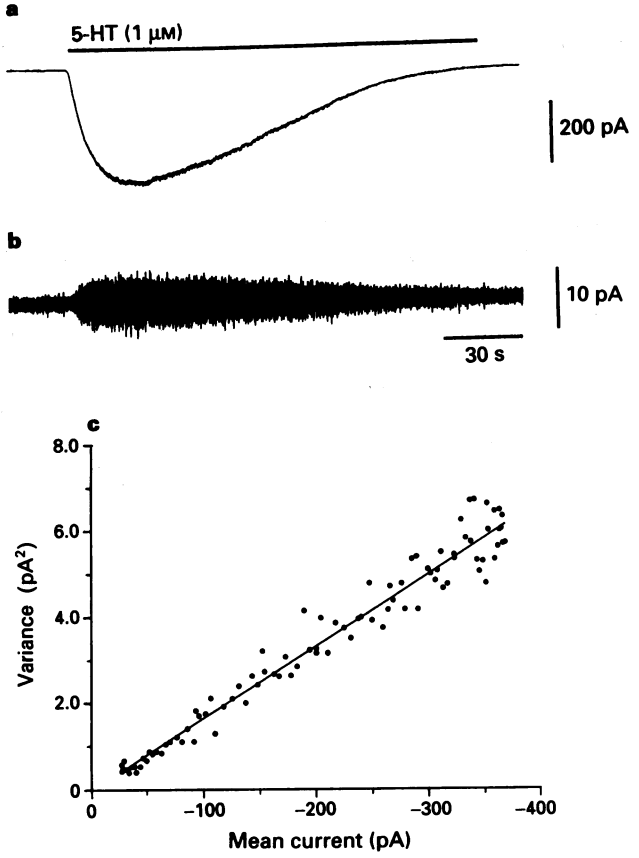

Figure 6 Fluctuation analysis of 5-HT-evoked whole cell currents mediated by the 5-HT$_3$ R-A expressed in HEK 293 cells. (a) Low gain d.c.-coupled record (500 Hz low-pass filtering) of an inward current response elicited by 1 μM 5-HT applied by microperfusion for the period indicated by the solid bar above the trace. (b) High-gain a.c.-coupled record (1 Hz–500 Hz band-pass) of the response illustrated in (a). Note that the changes in the amplitude of the current fluctuation (noise) parallel the development and desensitization of the response depicted in (a). (c). A plot illustrating the relationship between the variance of the a.c.-coupled current and the mean (d.c.) current elicited by 5-HT. Current variance in the absence of 5-HT has been subtracted. The slope of the line, fitted to the data points by least-squares linear regression analysis, indicates the elementary current flowing through a single 5-HT$_3$ R-A complex to be approximately 17 fA. From the holding potential of −60 mV and an $E_{5\text{-HT}}$ of −2 mV, the single channel conductance in this example is estimated to be 290 fS.

subunit exhibits only sixteen differences in primary amino acid sequence within the N-terminal domain (Isenberg *et al.*, 1993). Intriguingly, three of those are in a region corresponding to the C loop of nicotinic α subunits, a domain known to be involved in the binding of (+)-tubocurarine to nicotinic receptors (see Lambert *et al.*, 1995).

The reversal potential (~0 mV) of the 5-HT-induced current recorded from *Xenopus* oocytes expressing the murine 5-HT$_3$ R-A subunit is shifted to more negative potentials by the total replacement of extracellular Na^+ by the poorly permeant cation Tris, suggesting the homo-oligomeric complex to retain selectivity toward monovalent cations (Maricq *et al.*, 1991). The results described here confirm this suggestion, since Na^+ and Cs^+ were found to be approximately equipermeant and the partial replacement of either extracellular Na^+ by NMDG or intracellular Cs^+ by TEA produced large negative and positive shifts in $E_{5\text{-HT}}$ respectively. However, the magnitude of the shift in $E_{5\text{-HT}}$ occurring in response to these ionic substitutions was consistently less than that predicted from the Goldman-Hodgkin-Katz voltage equation (Hille, 1992). As chloride ions appear to be impermeant, and noting that the reduction in the extracellular concentrations

of Ca^{2+} and Mg^{2+} had a negligible effect upon $E_{5\text{-}HT}$, the latter may be expressed as:

$$E_{5\text{-}HT} = \frac{RT}{zF} ln \frac{P_{Na}/P_{Cs}[Na]_0}{P_{Na}/P_{Cs}[Na]_i + [Cs]_i} \quad (5)$$

where R, T, z and F have their usual meaning. From the above, and the value of $E_{5\text{-}HT}$ (−2 mV) determined with solutions E2 and I1, the permeability of Na relative to Cs (i.e. P_{Na}/P_{Cs}) is calculated to be 0.94. However, this ratio fails to predict accurately the observed shifts in $E_{5\text{-}HT}$. For example, reducing $[Na]_o$ to 75 mM and 20 mM would be expected to shift $E_{5\text{-}HT}$ to −18.7 and −52.0 mV respectively, rather than the observed values of −14.5 and −42.9 mV. Similarly, partial replacement of intracellular Cs^+ by TEA resulted in an $E_{5\text{-}HT}$ of +25.9 mV, a value considerably less than that predicted (i.e. +40.3 mV). The most likely explanation of these discrepancies is that, in common with 5-HT$_3$ receptors native to rabbit nodose ganglion neurones (Malone, 1992; Malone *et al.*, 1994) and N1E-115 neuroblastoma cells (Kooyman *et al.*, 1993), the 5-HT$_3$ R-A possesses a finite permeabilty to both NMDG and TEA. Indeed, if equation (5) above is extended to include terms for either P_{NMDG}/P_{Cs} or P_{TEA}/P_{Cs}, and the permeability ratios of 0.047 and 0.131, respectively, determined for nodose ganglion neurones (Malone, 1992; Malone *et al.*, 1994) are employed as estimates of their ability to permeate the 5-HT$_3$ R-A, then the predicted and measured reversal potentials agree in all cases to within 3 mV.

5-HT$_3$ receptors endogenous to murine neuronal cell lines and rabbit nodose ganglion neurones are blocked by the divalent cations calcium and magnesium in a concentration-dependent, voltage-independent manner (Peters *et al.*, 1988, 1993; Yang, 1990; Lovinger, 1991). Such observations are consistent with the results of the present study. Furthermore, inspection of the 5-HT$_3$ current-voltage relationship (Figures 2 and 3) reveals the response to exhibit the inward rectification that is characteristic of murine 5-HT$_3$ receptors (Lambert *et al.*, 1989; Yang 1990; Lovinger, 1991; Yang *et al.*, 1992). The mouse or rat homologues of the 5-HT$_3$ R-A, when expressed in *Xenopus* oocytes, are also blocked by magnesium and calcium, but the antagonism is reported to be voltage-dependent (Maricq *et al.*, 1991; Johnson & Heinemann 1992; Eiselé *et al.*, 1993). This difference is not readily explained by the expression system employed, as in one study performed with *Xenopus* oocytes (Yakel *et al.*, 1993), modest inward rectification of the 5-HT-evoked current in the presence of Mg^{2+} and Ca^{2+} was observed. However, in that study, Ca^{2+} was reported to enhance 5-HT$_3$ receptor desensitization in a voltage-dependent manner and an intracellular site of action was implicated (Yakel *et al.*, 1993). At present, there is no obvious explanation for the divergent observations regarding Ca^{2+}- and Mg^{2+}-induced blockade of recombinant 5-HT$_3$ receptors. Further studies designed to clarify the site(s) of action of these divalent cations are required, particularly in view of a recent report which demonstrated blockade of 5-HT$_3$ receptor mediated currents in rat dentate gyrus basket cells by Ca^{2+} and Mg^{2+} to be voltage-dependent (Kawa, 1994). In this respect, the results of experiments performed upon chimaeric α_7 nicotinic-5-HT$_3$ receptors, which suggest the ion channel as a locus of action, are of interest (Eiselé *et al.*, 1993).

The divalent cation zinc is known to interact with a variety of ligand-gated ion channels (Harrison & Gibbons, 1994; Smart *et al.*, 1994). Micromolar concentrations of Zn^{2+} cause inhibition of NMDA (Mayer *et al.*, 1989; Rassendren *et al.*, 1990) and $GABA_A$ receptor-evoked currents (Draguhn *et al.*, 1990; Smart *et al.*, 1994), but enhancement of ATP, AMPA and kainate-evoked currents (Rassendren *et al.*, 1990; Li *et al.*, 1993). At somewhat higher concentrations, Zn^{2+} antagonizes AMPA and kainate-evoked currents (Mayer *et al.*, 1989; Rassendren *et al.*, 1990). Flux assays suggest that Zn^{2+} also interacts with 5-HT$_3$ receptors, since the receptor-mediated influx of [^{14}C]-guanidinium in NG 108-15 cells is inhibited by Zn^{2+} (Emerit *et al.*, 1993). Under voltage-clamp conditions, Zn^{2+} produces a voltage-dependent inhibition of the 5-HT$_3$ receptor-mediated currents recorded from murine NCB-20 cells (Lovinger, 1991), with an IC_{50} of approximately 20 μM. The 5-HT$_3$ R-A subunit investigated here was isolated from this cell line (Maricq *et al.*, 1991) and it is of interest that low concentrations of Zn^{2+} (0.3–10 μM) enhanced the receptor mediated current with blockade being evident only with higher concentrations (30–300 μM) of Zn^{2+}. In preliminary experiments, a similar biphasic effect of Zn^{2+} was observed with *Xenopus* oocytes expressing the 5-HT$_3$ R-A subunit (D. Belelli, C.H. Gill, J.J. Lambert & J.A. Peters, unpublished observations). By analogy to the influence of subunit composition upon the sensitivity of $GABA_A$ receptors to blockade by Zn^{2+} (Smart *et al.*, 1994), it is conceivable that additional subunits are present within the 5-HT$_3$ receptor expressed by NCB-20 cells. The only additional subunit thus far identified in these cell lines is an alternatively spliced form of the 5-HT$_3$ R-A, termed the 5-HT$_3$ R-A$_s$ (Hope *et al.*, 1993; Hussy & Jones 1993). Whether the 5-HT$_3$ R-A$_s$ expressed alone, or in combination with the 5-HT$_3$ R-A, exhibits a similar biphasic response to Zn^{2+} remains to be determined. However, it is known that alternatively spliced forms of the NMDAR-1 subunit are differentially modulated by this divalent cation (Hollman *et al.*, 1993; Zheng *et al.*, 1994). We are at present investigating the mechanism of Zn^{2+} action on the 5-HT$_3$ R-A in more detail. Furthermore, in view of the species-dependent pharmacological properties of this receptor, studies upon the 5-HT$_3$ receptors of rabbit, mouse and guinea-pig nodose ganglion neurones would be of interest (Peters *et al.*, 1992).

Fluctuation analysis of whole-cell currents mediated by the 5-HT$_3$ R-A suggests the homo-oligomeric complex to possess a single channel conductance of approximately 0.42 pS. It is unlikely that this extremely low value is a result of the exclusion of frequency components that would contribute significantly to the signal variance. Fluctuation analysis conducted over an extended frequency range (i.e. 0.5 Hz to 1 kHz) upon suitably slowly changing signals (see below) yielded estimates of single channel conductance similar to those obtained with the routinely employed bandwidth of 1 Hz–500 Hz. Nonetheless, several limitations to the analysis remain. The estimates of channel conductance were made by stationary analysis applied to a changing current signal in response to microperfused 5-HT (1 μM). For this approach to be valid, the current should not change appreciably within the period of a single variance calculation. Fluctuation analysis was routinely restricted to frequencies $\geq$1 Hz in an effort to avoid possible *overestimation* of the variance from trends that could be introduced by the lengthy data blocks that would be necessary to incorporate lower frequency components. In interpreting the results, we have assumed that neither desensitization nor agonist concentration are likely to affect single channel conductance. The conductance estimate obtained from fluctuation analysis is in fact a weighted mean that is influenced by the relative frequency of all conducting states. Although for simplicity, we have interpreted our data in terms of a 5-HT activated channel of unitary conductance, this need not necessarily be the case. Indeed, a comparison of the conductance of 5-HT$_3$ receptors of rat superior cervical ganglion neurones determined by direct observation of single channel currents and fluctuation analysis reveals a large discrepancy that probably reflects the existence of multiple conductance states (Yang *et al.*, 1992). Similarly, recordings made on membrane patches excised from rodent hippocampal neurones suggest the existence of two populations of channel: one with a conductance of approximately 10 pS, and a second which cannot be directly resolved, but which produces an inward current that is blocked by 5-HT$_3$ receptor antagonists (Jones & Surprenant, 1994). Multiple conductances have also been reported for 5-HT$_3$ receptors expressed by neurones of the guinea-pig submucous plexus (Derkach *et*

al., 1989) and mouse superior cervical ganglion (Hussey & Jones, 1993).

The conductance of the 5-HT$_3$ R-A described here is similar to that estimated for 5-HT$_3$ receptors native to NCB-20, N1E-115 and N18 cells (Lambert *et al.*, 1989; Yang, 1990; van Hooft *et al.*, 1994). It remains unclear as to why the single channel conductance in these systems is so low. The minimal diameter of the ion channel associated with the 5-HT$_3$ receptor, as assessed by studies employing permeant cations of differing sizes (Yang, 1990; Malone *et al.*, 1994), is similar to that established for nicotinic acetylcholine receptors which typically demonstrate conductances of 40–50 pS (Karlin, 1993). Previous suggestions that the 5-HT$_3$ ion channel may be longer, or less polarizable, than that of nicotinic receptors (Yakel *et al.*, 1990) are not supported by the structural information that has been derived from cloned 5-HT$_3$ receptor subunits. Indeed, over the M2 region that is proposed to line the ion channel, subunits of 5-HT$_3$ and nicotinic receptors exhibit a high degree of sequence homology (Maricq *et al.*, 1991). In particular, acidic amino acid residues which are thought to produce rings of negative charge with extracellular, intermediate and cytoplasmic locations, and which are known to influence the conductance of the nicotinic receptor complex, are common to both receptor types (Maricq *et al.*, 1991; Karin, 1993). However, the presence of a basic lysine residue located within the putative M2 domain of the 5-HT$_3$ R-A represents one obvious structural feature that is not shared with nicotinic receptor subunits. Mutation of this residue to glycine (the homologous amino acid found in the chick α_7 nicotinic subunit) dramatically slows the kinetics of desensitization of the 5-HT$_3$ R-A, expressed in *Xenopus* oocytes (A. Hope, D. Belelli, J.J. Lambert & J.A. Peters, unpublished observations). The effect of this mutation upon single channel conductance is currently under evaluation. A potential influence of a post translational modification upon the channel conductance is suggested by a preliminary report demonstrating the conductance of the 5-HT$_3$ receptor endogenous to N1E-115 neuroblastoma cells to be modified by an unidentified intracellular factor(s) (van Hooft & Vijverberg, 1994).

The small conductance associated with the 5-HT$_3$ receptor expressed by murine neuronal cell lines is probably not unique, because there are indications of a similar conductance state in neurones of the rat superior cervical (Yang *et al.*, 1992) and dorsal root (Robertson & Bevan, 1991) ganglia. Furthermore, 5-HT$_3$ receptor single channel mainstate conductances of 12 pS and 10 pS have recently been determined for mouse superior cervical ganglion neurones and hippocampal neurones, respectively, suggesting heterogeneity within this species (Hussy & Jones, 1993; Jones & Surprenant, 1994). The future isolation of cDNAs encoding 5-HT$_3$ receptor channels with divergent conductances should clarify the molecular determinants of ion transport through this channel.

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