Electrophysiological consequences of ligand binding to the desensitized 5-HT_3 receptor in mammalian NG108-15 cells

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1. Using the whole-cell variation of the patch-clamp technique to record from mammalian NG108-15 cells, we have studied the ligand-gated ion channel current activated by a high concentration (100 μM) of local pressure-applied 5-hydroxytryptamine (5-HT). The response was induced at intervals of at least 90–120 s, which allowed the receptor to fully recover between activations.

2. The rapid inward current induced by pressure-applied 5-HT was reproducibly inhibited by the superfusion of low concentrations of 5-HT which evoked little or no detectable inward current alone (0·01–0·3 μM). This inhibitory effect was most likely to be due to a direct action on the 5-HT_3 receptor as it could be recorded using intracellular solutions with or without adenosine triphosphate (ATP) and guanosine triphosphate (GTP).

3. The maximum inhibitory effect of a given concentration of 5-HT was not dependent on its superfusion time but on the number of activations of the receptor by pressure-applied 5-HT. This activation dependence was clearly evident, since the first inward current in the presence of 0·1 μM 5-HT was often unaffected in amplitude.

4. The inhibitory effect of 5-HT was evident at holding potentials of +60 and −60 mV; with the calcium chelator BAPTA in the recording pipette and with the nominal removal of extracellular calcium and magnesium ions.

5. The inhibitory effect was concentration dependent, with 50% inhibition of the inward current amplitude occurring at ~50 nM 5-HT. The slope factor of the inhibition curve was 1·3. The effect was mimicked by two other 5-HT_3 receptor agonists, 2-methyl-5-HT and *m*-chlorophenylbiguanide (*m*CPBG) which gave 50% inhibition at ~600 nM and ~20 nM, respectively. These values are similar to the affinity values for these ligands determined in radioligand binding assays.

6. The 5-HT_3 receptor 'antagonists' (+)-tubocurarine and quipazine (both at 3 nM) reduced the inward current amplitude by ~50%. The rate of onset of the inhibitory effect of bath-applied 5-HT was slowed in the presence of (+)-tubocurarine but not in the presence of quipazine. This difference might be explained by the agonist properties seen only with quipazine.

7. The inhibition of the 5-HT_3 receptor mediated inward current by low concentrations of bath-applied 5-HT_3 receptor agonists is compatible with the cyclic model of receptor activation and desensitization. We conclude that we have been studying the high-affinity binding of agonists to the desensitized form of the 5-HT_3 receptor.

Biological adaptation to a continued stimulus is a common phenomenon. Various voltage-gated ion channels, including sodium, calcium and potassium channels, inactivate with prolonged membrane potential changes (Hille, 1992) and virtually all neurotransmitter-gated receptors desensitize to prolonged or repeated agonist application (Katz & Thesleff, 1957; Huganir & Greengard, 1990). Ligand-gated ion channels are a superfamily of heteromeric ion channels directly activated by the binding of neurotransmitter to sites on one or more of their subunits. It is perhaps not surprising that they desensitize since they are both receptors and ion channels. An alteration of the

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desensitization of these channels could have significant repercussions since they mediate fast chemical synaptic transmission in the peripheral and central nervous systems.

The 5-HT_3 receptor is an early evolutionary member of the ligand-gated ion channel superfamily comprising the nicotinic acetylcholine (nACh), γ-aminobutyric $acid_A$ ($GABA_A$) and glycine receptors (Ortells & Lunt, 1995). 5-HT_3 receptors are present on neurones in the autonomic (Wallis & North, 1978; Peters, Malone & Lambert, 1993), enteric (Derkach, Surprenant & North, 1989) and central nervous systems (Kilpatrick, Jones & Tyers, 1987; Yakel & Jackson, 1988; Ropert & Guy, 1991; Sugita, Shen & North, 1992). They are also endogenously expressed on certain neuroblastoma-derived cell lines: NG108-15 (Yakel & Jackson, 1988; Yakel, Shao & Jackson, 1991), N1E-115 (Neijt, Te Duits & Vijverberg, 1988; Neijt, Plomp & Vijverberg, 1989) & N18 (Yang, 1990). 5-HT_3 receptor-mediated responses are characterized by a rapid non-selective cation current that desensitizes rapidly in the continued presence of its natural agonist, 5-HT. The use of a variety of techniques has led to suggestions that there are subtypes of the 5-HT_3 receptor. Richardson & Engel (1986) first proposed subtypes based on apparent affinity differences of 5-HT_3 receptor antagonists in different tissues, and single channel conductances determined in different cells have been shown to vary between 0·3 and 15 pS (Peters, Malone & Lambert, 1992). Subsequently, short and long form variants of one of the 5-HT_3 receptor subunits have been cloned and their amino acid sequences show close homology with the other members of this ligand-gated ion channel family (Maricq, Peterson, Brake, Myers & Julius, 1991; Hope, Downie, Sutherland, Lambert, Peters & Burchell, 1993).

The 5-HT_3 receptor has recently been shown to exhibit a pentameric quaternary structure similar to the nACh receptor (Boess, Beroukhim & Martin, 1995). In common with the nACh receptor, there is general agreement that 5-HT_3 receptor activation results from the binding of at least two agonist molecules (Peters *et al.* 1992) and theoretical determination of the channel pore size gives a similar value for both receptors (Yang, 1990). In addition, both 5-HT_3 and nACh receptors are antagonized by (+)-tubocurarine, which shows a particularly high affinity for 5-HT_3 receptors in some species (Newberry, Cheshire & Gilbert, 1991). Site-directed mutagenesis of the nACh and 5-HT_3 receptors indicates that they may undergo similar conformational changes during desensitization (Yakel, Lagrutta, Adelman & North, 1993). Furthermore, the close similarity between the two receptors has allowed a functional chimaeric construct, with the receptor properties of the nACh receptor and the channel properties of the 5-HT_3 receptor, to be expressed in *Xenopus* oocytes (Eiselé, Bertrand, Galzi, Devillers-Thiéry, Changeux & Bertrand, 1993).

Desensitization of the 5-HT_3 receptor has been studied in detail in neuroblastoma-derived cells e.g. NG108-15 (Yakel & Jackson, 1988; Yakel *et al.* 1991) and N1E-115 (Neijt *et al.* 1988, 1989). Yakel *et al.* (1991) described the biphasic nature of desensitization of the 5-HT_3 receptor induced by high concentrations of 5-HT in NG108-15 cells. Neijt *et al.* (1988, 1989) also showed that low concentrations of 5-HT, below those necessary to activate the receptor, reduced the amplitude of the 5-HT_3 receptor-mediated inward current. In NG108-15 cells, we have observed a similar inhibitory effect of low concentrations of 5-HT. We have investigated this inhibitory effect in detail since there is evidence that the 5-HT_3 receptor in N1E-115 cells may differ from that in NG108-15 cells in terms of ligand binding (Boess, Sepúlveda, Lummis & Martin, 1992). In studying the time course of this phenomenon we have observed that it is concentration- and use-dependent. We have studied the actions of calcium ions and different 5-HT_3 receptor ligands, both agonists and antagonists, on this inhibitory effect in order to further our understanding of the properties of the 5-HT_3 receptor. We conclude that we have been studying the high-affinity binding of 5-HT to the desensitized 5-HT_3 receptor in these cells. A preliminary account of some of these findings has been published (Bartrup & Newberry, 1995).

METHODS

Cell culture

The NG108-15 cell line, a rat glioma × mouse neuroblastoma hybrid, was obtained from Dr Sarah Lummis (MRC Molecular Neurobiology Unit, Cambridge, UK) at 'passage' 30. Aliquots were stored in liquid nitrogen until needed. They were then thawed and cultured in Dulbecco's modification of Eagle's medium (DMEM) with 10% v/v fetal calf serum (FCS) and 1% w/v glutamine. The cells were incubated at 37 °C in 5% CO_2–95% air. They were diluted and plated out in fresh medium approximately twice a week for 7–8 weeks. At alternate dilutions, hypoxanthine (100 μM), aminopterin (0·4 μM) and thymidine (16 μM) (HAT) were added to the culture medium. In order to record from them, the cells were seeded at a low density onto 13 mm polylysine-coated circular coverslips (~1000 cells per coverslip) and used 'undifferentiated' 1–2 days later.

Electrophysiological recording

For whole-cell patch-clamp recordings, individual coverslips were transferred to an inverted stage microscope where they formed the base of a small bath (~0·2 ml) which was superfused with medium (2–3 ml min^{-1}) at 24–26 °C. The extracellular medium contained (mM): NaCl, 140; Hepes, 10; KCl, 5; dextrose, 5; $CaCl_2$, 2; $MgCl_2$, 1 (pH 7·4 with NaOH). Patch electrodes were fabricated from borosilicate glass capillaries (Clark Electromedical). These had an external tip diameter of ~2 μm and, when filled, had a resistance of 3–6 MΩ. They were filled with an intracellular solution of (mM): CsCl, 125; Hepes, 10; $MgCl_2$, 1; EGTA, 1; $CaCl_2$, 0·58 (pH 7·4 with NaOH) (junction potential, +3 mV). The free calcium ion concentration was calculated as 10 nM using the REACT programme (G. L. Smith, University of Glasgow, UK). In some experiments, EGTA and $CaCl_2$ were replaced with 10 mM BAPTA.

Some recordings were made with a more physiological intracellular solution of (mM): KCl, 125; Hepes, 10; $MgCl_2$, 1; Na_2ATP, 2; Na_2GTP, 1, to which a calcium ion chelator was not added (pH 7·4 with NaOH) (junction potential, +4 mV).

Recordings were made from cells using a List EPC-7 patch-clamp amplifier in voltage-clamp mode at a holding potential of −60 mV, allowing for the junction potential indicated above. The signal was filtered through a 3 kHz low pass filter and recorded on a Gould Windograf chart recorder. Cell membrane potential was measured in current-clamp mode in those cells from which recordings were made using the KCl-based pipette solution. The series resistance was determined by measuring the current required to hold the cell at −60 mV. Cell capacitance was determined by conventional means.

Drug application

5-HT$_3$ receptors were activated by local pressure application of 5-HT (100 μM, 10–15 p.s.i., 100–500 ms) from a pipette (tip diameter ~2 μm) situated ~20 μm from the cell soma at right angles to the direction of flow of the superfusate. This concentration of 5-HT was chosen since it would give a maximum response under our standard recording conditions (EC_{50}, 3 μM, Yakel *et al.* 1991). Before the experiment on a given cell, this pipette was sometimes moved to optimize the inward current amplitude. The interval between pressure applications of 5-HT was long enough to allow the receptors to recover from desensitization, normally 90–120 s. The short application time together with the high rate of superfusion and small bath volume ensured that excess 5-HT was rapidly removed. The 5-HT-containing pipette was normally left in place to avoid undesirable disturbance of the recording. However, the experiments to be described show the inhibitory effects of very low concentrations of 5-HT so we needed to ensure that there was no detectable leak of 5-HT from the 5-HT-containing pipette situated close to the recorded cell. If a leak was suspected, this pipette was moved well away from the cell and then repositioned close to it just before the pressure application of 5-HT. If there was a detectable reduction in the responses thereafter, we assumed that the pipette was leaking and it was replaced.

The amplitude of the pressure-applied 5-HT-induced inward current generally ran down throughout the recording. With most cells, the initial response was the largest and subsequent responses gradually reduced in amplitude until a steady response size was reached. This typically occurred within 10 min of starting the whole-cell recording. 5-HT was bath applied via the superfusion system after at least three or four consecutive inward currents of a similar amplitude had been recorded.

Low concentrations of 5-HT and other substances were bath-applied via the rapid superfusion system using a three-way tap connection. The superfused 5-HT was usually timed to reach the cell 30 s before the next pressure application of 5-HT. The delay time between turning the tap and a solution reaching the cell was ~8 s. We allow for this delay when indicating the time at which 5-HT reached the cell. This delay was determined by recording the time taken for 10 mM potassium chloride, applied via the superfusion system, to induce an inward current. The two methods of applying 5-HT will be referred to as 'pressure-applied' for activating the receptor with the high concentration of 5-HT (100 μM) and 'bath-applied' for the low superfused concentrations of 5-HT (0·01–0·3 μM).

Data analysis

The peak amplitude of the ionic current induced by the local application of 5-HT was the parameter measured throughout the experiments. Data are normally presented as median and range, except in graphs where points represent means ± standard error of the mean (S.E.M.). The data in graphs are expressed as a percentage of the response amplitude prior to applying the agonist via the bath. The concentration of substance reducing the amplitude of the fast inward current by 50% (the IC_{50}) was determined by constructing cumulative concentration–response curves for individual cells followed by interpolation of the data points. Least squares regression curve fitting to the pooled data was carried out using the RS1 programme (BBN Software Products, West Drayton, UK) and the equation $f(x) = 100/(1 + (x/IC_{50})^{**}n)$ where n is the slope factor.

In the experiments to be described, clear inhibitions of the pressure-applied 5-HT-induced inward current were seen; but we wished to determine whether the first response in the presence of 5-HT was significantly reduced. This was done by comparing the amplitude of the response induced in the presence of bath-applied 5-HT with that predicted, by extrapolation, from the responses just before the bath application. The amplitudes of three or four, usually four, responses before the drug was applied, were fitted by a straight line using the least squares regression programme (RS1). The first response in the presence of bath-applied 5-HT was only considered to be significantly different from the control responses if its amplitude lay outside the extrapolated 95% confidence limits of the fitted line.

Reagent sources

Reagents were obtained from the following sources: Analar grade salts and Hepes from BDH (Poole, UK); DMEM, FCS and HAT from Flow Laboratories (Thame, UK); 5-HT hydrochloride, CsCl, EGTA, BAPTA, adenosine triphosphate, disodium salt (Na_2ATP), guanosine triphosphate, disodium salt (Na_2GTP) and (+)-tubocurarine chloride from Sigma; 2-methyl-5-HT maleate and quipazine dimaleate from Research Biochemical Inc. (RBI, Semat, UK); *m*-chlorophenylbiguanide hydrochloride (*m*CPBG) from Cookson Chemicals (Bristol, UK).

RESULTS

Recordings were made from 262 NG108-15 cells. With the KCl-based pipette solution, these cells had a median resting membrane potential of −20 mV (range −3 to −40 mV) and an input resistance of 910 MΩ (300–7400 MΩ) ($n = 70$). With the CsCl-based pipette solution the input resistance was 1045 MΩ (123–4600 MΩ, $n = 192$). The median cell capacitance of all cells was 38 pF (10–100 pF). A brief (100–500 ms) pressure application of 5-HT onto a cell elicited a rapid inward current in 90% of cells studied. The response began ~30–40 ms after the start of the pressure pulse, peaked ~140–420 ms later and returned to baseline within 25 s (2–110 s, $n = 236$) (Fig. 1*A* and *B*). The recovery phase was often, although not always, biphasic (Fig. 1*B* and *C*, cf. Yakel *et al.* 1991). The median peak amplitude of these responses at a holding potential of −60 mV was 655 pA (35–3425 pA, $n = 236$). The reversal potential for this current was +7 mV (+3 to

+12 mV, $n = 6$) using a CsCl-based recording electrolyte. Rectification of the current was evident, but this was variable (Fig. 1*E* and 3*A*).

Desensitization of the 5-HT_3 receptor, indicated by a reduction in amplitude of the response to pressure-applied 5-HT, could be demonstrated in two ways. Firstly, if the receptor was activated by pressure-applied 5-HT at short intervals (Fig. 1*C*) and, secondly, if 5-HT was pressure-applied during a bath application of 5-HT at a concentration which evoked a clear inward current itself (Fig. 1*D*). In both cases, the responses to pressure-applied 5-HT were dramatically reduced. However, the fast inward current was also reduced by superfusing concentrations of 5-HT which evoked little or no detectable inward current. As shown in Fig. 2*A*, 0·1 μM 5-HT reduced the fast inward current by ~65% without producing a detectable inward current. When rinsing the bath with 5-HT-free medium, the response rapidly returned, usually to near-control levels. These effects were seen when recording with the KCl- or CsCl-based recording pipette solutions, the former containing ATP and GTP (see Methods, Figs 2*A* and *B*). Bath application of a slightly higher concentration of 5-HT (0·3 μM) induced a small inward current of 15 pA (5–150 pA) in twelve out of fifteen cells and reduced the response to pressure-applied 5-HT by 88 ± 2% (mean ± S.E.M., $n = 15$) (e.g. Fig. 2*B*).

We wished to determine whether the maximum inhibition by 0·1 μM 5-HT was dependent on the superfusion time or

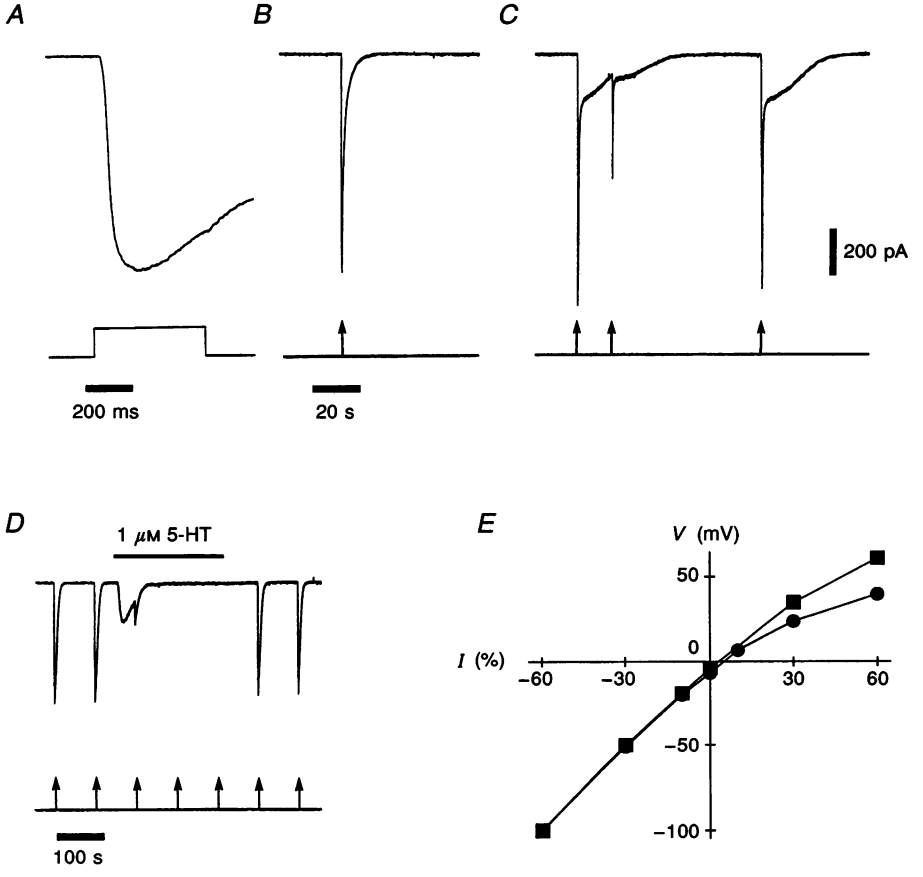

Figure 1. The electrophysiological response of NG108-15 cells to 5-HT

Pressure-applied 5-HT (100 μM) induced a rapid inward current at a holding potential of −60 mV. The same response is shown at two time bases in *A* and *B*. The time course of the recovery phase was variable, with the example in *C* being clearly biphasic. A reduction in the amplitude of the inward current, presumably due to desensitization, was apparent if 5-HT was reapplied during the recovery phase of the previous response to pressure application (*C*) or during a bath application of a concentration of 5-HT which induced an inward current itself (*D*). The voltage dependence of the 5-HT-induced current in two individual cells is shown in *E*. The reversal potential and variable rectification of the current can be seen (see also Fig. 3). The data presented in *E* are expressed as a percentage of the inward current recorded at −60 mV in each cell. In *A–D*, and also in following figures, the time of pressure application of 5-HT is indicated by the deflection and the arrows on the lower line. The current calibration of the upper trace applies to the whole figure. The time calibration in *B* is the same as for *C*. A CsCl-based pipette solution was used in all these experiments, except in *D*, in which a KCl-based electrolyte was used (see Methods).

the frequency of activation of the receptor. This was done by increasing the interval between the pressure applications of 5-HT from 120 to 240 or 480 s, allowing the superfusion time of 5-HT, before the receptor was activated, to be increased from 30 to 150 or 390 s, respectively. These changes did not alter the maximum inhibitory effect of bath-applied 5-HT. Rather, this effect appeared to depend on the number of times that the receptor was activated (Fig. 2*C*).

When activating the receptor at 120 s intervals, the inhibitory effect of the low concentration of 5-HT often did not begin until after the receptor had been activated. In the case of 0·1 μM 5-HT, which did not appear to induce an inward current, the first response in its presence was not significantly reduced in eleven out of twelve cells. However, this lack of effect on the first response occurred less often if the bath-applied 5-HT was seen to activate the receptor. With 0·3 μM 5-HT, which often induced a small inward

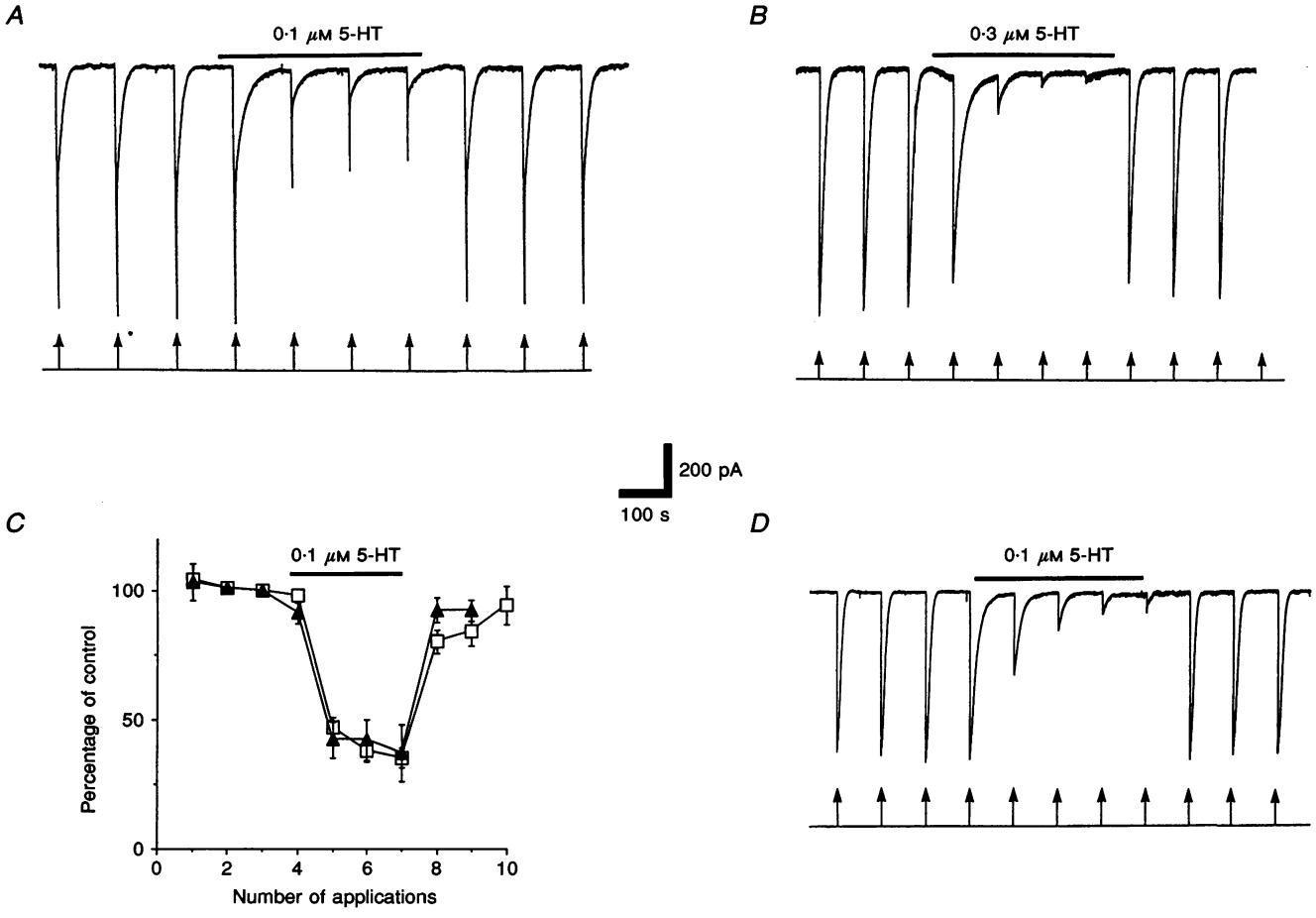

Figure 2. The inhibitory effect of low concentrations of bath-applied 5-HT on the 5-HT$_3$ receptor-mediated inward current

Bath application of low concentrations of 5-HT inhibited the response to pressure-applied 5-HT. This effect occurred when the bath-applied 5-HT (0·1 μM) evoked no detectable response (*A*) but was greater with 0·3 μM 5-HT (*B*), which induced a small inward current itself. In *A* it can be seen that the first response in the presence of bath-applied 5-HT was unaffected in amplitude but its duration was increased. The graph in *C* shows that the inhibition of the response by 0·1 μM 5-HT was unaffected by the interval between the pressure applications of 5-HT or the superfusion time. Increasing the interval between the responses from 120 (□) to 240 s (▲) allowed the 5-HT superfusion time, before the next inward current was evoked, to be increased from 30 (□) to 180 s (▲). Response amplitudes were normalized to the response prior to the bath application of 5-HT. Points are the means ± S.E.M. for 12 (□) and 5 (▲) experiments. The first response in the presence of the bath-applied 5-HT was not significantly affected in 11 out of 12 cells when the superfusion time was 30 s. This lack of effect became less obvious when the superfusion time was increased to 180 s (4 out of 5 cells) and 360 s (only 1 out of 4 cells, not shown). However, the inward current was immediately reduced if the 5-HT was bath applied during the recovery phase of the previous response to pressure-applied 5-HT (*D*). In *A*, a KCl-based pipette solution was used, whereas in *B* and *C*, a CsCl-based recording electrode solution was used. The holding potential was −60 mV in all cases. Calibration bars apply to all original traces.

current, the first response was unaffected in only seven out of fifteen cells. The clear dependence on activation was apparent if the 5-HT was superfused during the recovery phase of the response, when the amplitude of the next inward current was reduced immediately (Fig. 2*D*). Although, under certain conditions, the amplitude of the first inward current in the presence of the superfused 0·1 μM 5-HT was unaffected, its duration was often increased, in nine out of twelve cells by 38% (14–150%, $n = 9$). These effects may be explained by assuming that the low concentrations of 5-HT are preventing the recovery of the 5-HT_3 receptor following its activation. Since desensitization follows activation it may be that the desensitized form of the receptor is maintained in the presence of the low concentrations of 5-HT.

Desensitization of the 5-HT_3 receptor can be altered by changing the membrane potential or by divalent cations, particularly calcium ions (Yakel *et al.* 1993). We found that 0·1 μM 5-HT gave a similar activation-dependent percentage reduction in the amplitude of the inward current at holding potentials of +60 and −60 mV (Fig. 3*A* and *B*, $n = 4$). This indicated that the direction of flow of current through the channel did not affect the inhibitory effect of 5-HT. Furthermore, the holding potential of +60 mV would reduce the inward flow of calcium ions

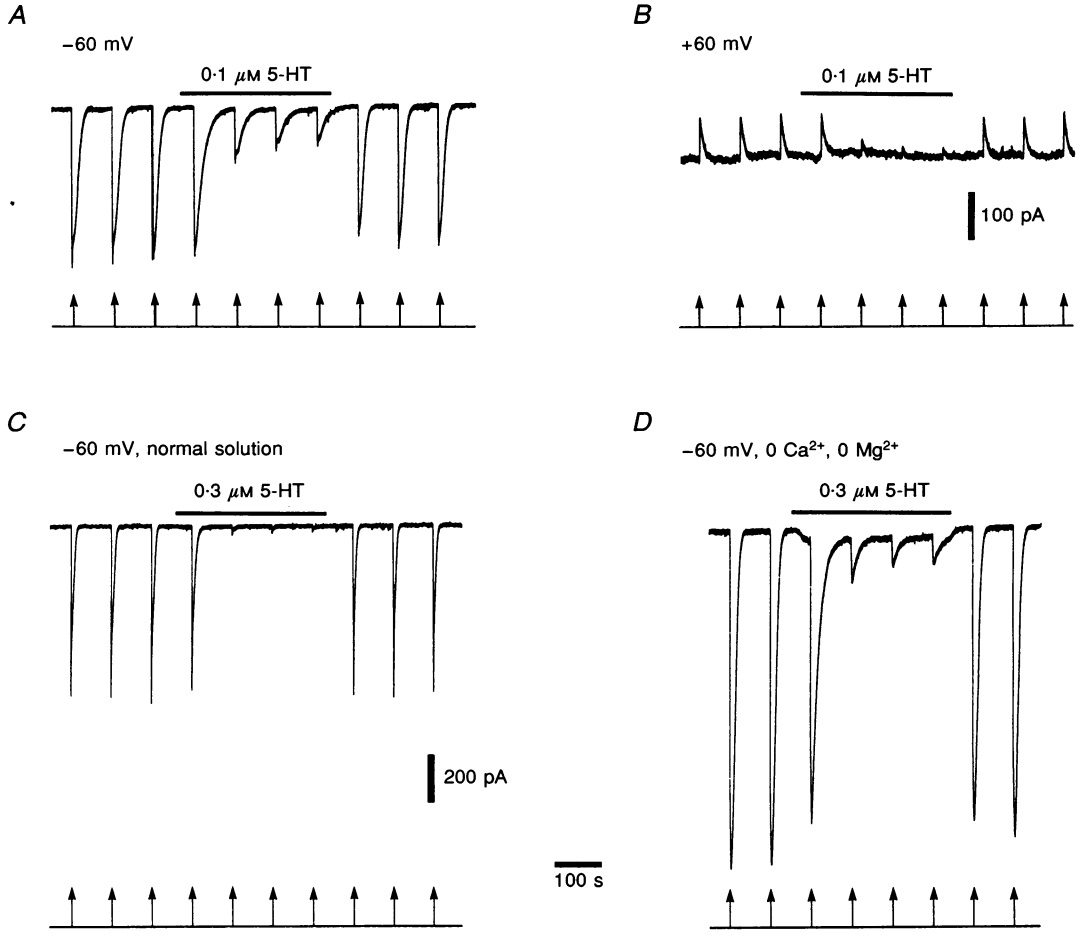

Figure 3. The inhibitory effect of 5-HT is largely unaffected by the membrane potential or the nominal removal of calcium ions

The inhibitory effect of 5-HT was apparent in the same cell at a holding potential of −60 mV (*A*) and +60 mV (*B*). The 5-HT-induced inward current in this cell shows marked rectification (see also Fig. 1*E*). In a second cell, held at −60 mV, nominal removal of the divalent cations from the superfusate is shown to markedly potentiate the pressure- and bath-applied 5-HT-induced inward currents, but the inhibitory effect of bath-applied 5-HT is still clearly present (*C* and *D*). It can be seen that lowering the divalent cation concentration did have a slight effect on the percentage inhibition of the inward current by 5-HT, but this was smaller than the effect of altering the concentration of bath-applied 5-HT by 3-fold (see Fig. 4*C*). The calcium chelator BAPTA (10 mM) was present in the recording electrode in both experiments. The time calibration bar applies to all traces. A CsCl-based pipette solution was used in all of these experiments.

known to permeate the channel (Peters *et al.* 1992). The presence of the calcium chelator BAPTA in the patch pipette in this and other experiments further reduced a possible effect of this ion on the internal side of the receptor ($n = 4$). In four experiments, we found that the nominal removal of calcium and magnesium ions from the superfusate increased the pressure-applied 5-HT response by 53% (0–89%), but that the inhibitory effect of 0·3 μM 5-HT was similar (Fig. 3*C* and *D*). These data indicate that the probable binding of 5-HT to the desensitized receptor was not dramatically affected by membrane potential or divalent cations.

If the inhibitory effect of 5-HT is due to it binding to the desensitized receptor, then other agonists of this receptor may have similar actions. The actions of two other well-characterized 5-HT$_3$ receptor agonists were studied: 2-methyl-5-HT and *m*-chlorophenylbiguanide (*m*CPBG) (e.g. Sepúlveda, Lummis & Martin, 1991). Like 5-HT, they reduced the pressure-applied 5-HT-induced inward current at concentrations that produced little or no detectable inward current alone (Fig. 4*A* and *B*). 2-Methyl-5-HT was less potent, and *m*CPBG more potent than 5-HT. The onset of action and washout of 2-methyl-5-HT were similar to those of 5-HT with the washout being more rapid, but the onset and washout of *m*CPBG were much slower. The effect

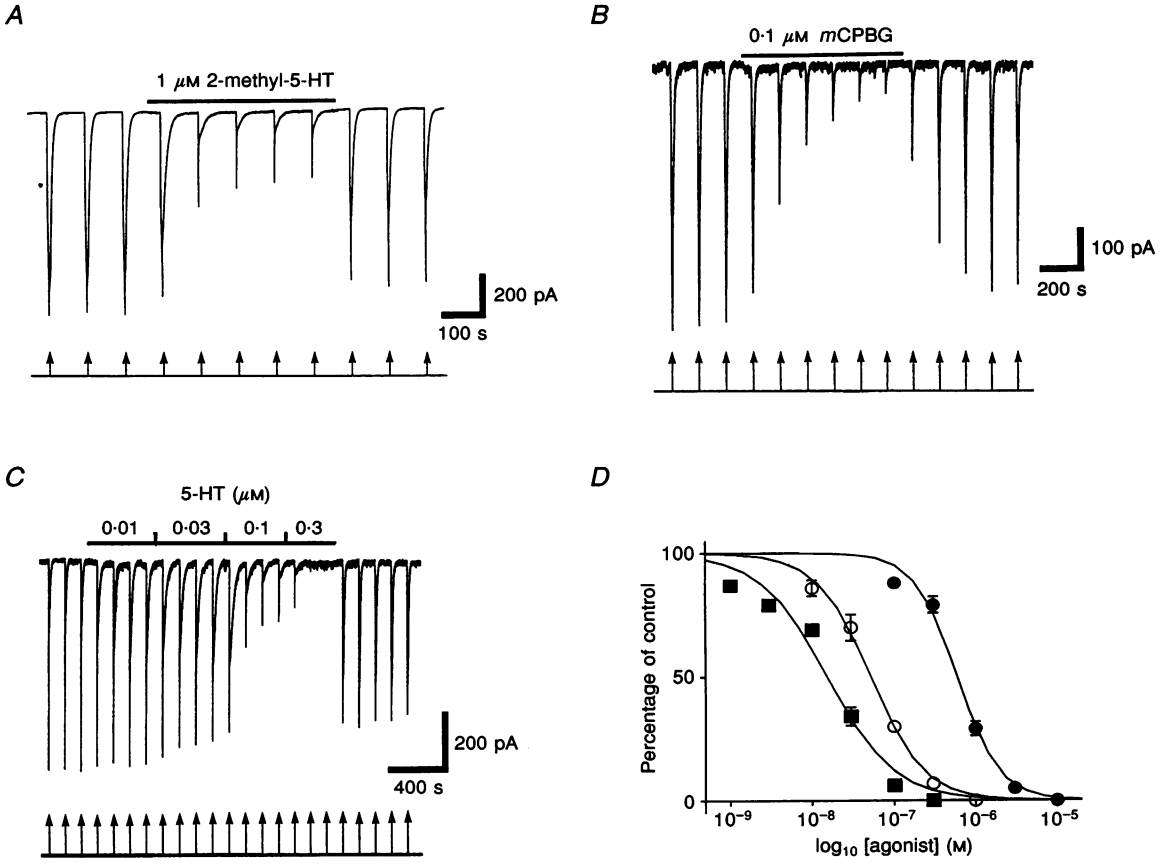

Figure 4. The 5-HT$_3$ receptor-mediated inward current is reduced by 5-HT$_3$ receptor agonists in a concentration-dependent manner

The effect of 2-methyl-5-HT (1 μM, *A*) was similar to that of 5-HT, but that of *m*-chlorophenylbiguanide (*m*CPBG, 0·1 μM, *B*) was much slower in onset and washout. In addition, the effects of *m*CPBG varied more between cells. An example of the concentration-dependent inhibition by bath-applied 5-HT is shown in *C*. Note that as in Fig. 2*C*, the inhibitory effect had largely peaked after four inward currents in the presence of each agonist concentration. Experiments such as this were used to construct concentration–inhibition curves for 2-methyl-5-HT (●), 5-HT (○) and *m*CPBG (■). The data were normalized to the response amplitude prior to applying the agonist via the bath. The data were fitted with an iterative curve fitting procedure (see Methods) giving IC_{50} values (slope factor) of 600 nM (1·7) for 2-methyl-5-HT; 50 nM (1·3) for 5-HT and 20 nM (1·1) for *m*CPBG. These values compare favourably with the IC_{50} values obtained by interpolating the original data points (see Results) and those in radioligand binding assays on these cells of 811, 170 and 14 nM, respectively (Boess *et al.* 1992). A CsCl-based pipette solution and a holding potential of −60 mV was used in all of these experiments.

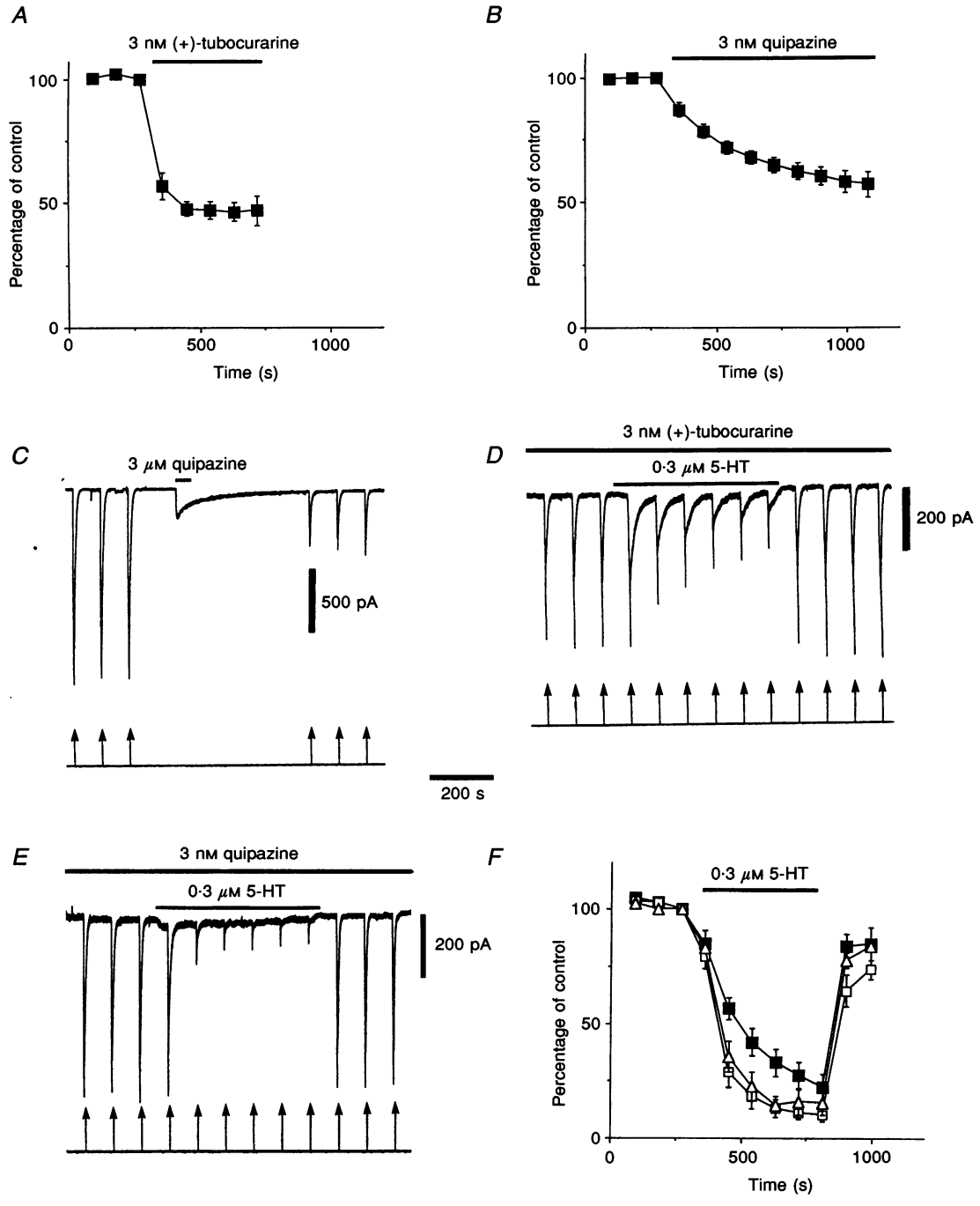

Figure 5. The effects of 5-HT_3 receptor antagonists

The 5-HT_3 receptor antagonists (+)-tubocurarine (*A*) and quipazine (*B*) produced similar reductions of the 5-HT_3 receptor-induced inward current: 48 ± 3% ($n = 8$) and 57 ± 5% ($n = 10$), respectively; however, the effect of tubocurarine was considerably faster in onset. At a higher concentration, quipazine induced a small desensitizing inward current and reduced the amplitude of the inward current induced by pressure-applied 5-HT (*C*). The inhibitory effect of bath-applied 5-HT was slowed in the presence of (+)-tubocurarine (*D*) but the time course was unaffected by quipazine (*E*). The pooled data of these effects are shown in *F*: tubocurarine (■), quipazine (□) and no antagonist (△). The pulse interval was 90 s, points are means ± S.E.M. of 8, 10 and 7 experiments, respectively. Time calibration bar refers to *C*, *D* and *E*. A CsCl-based pipette solution and a holding potential of −60 mV was used in all of these experiments.

of *m*CPBG was also more variable than that of the other agonists. Concentration–response curves were constructed for the three 5-HT$_3$ receptor agonists. This was done by sequentially increasing their concentration to reduce the influence of the slow time-dependent rundown of the response (Fig. 4*C*). The IC_{50} values for these agonists, interpolated from the original data (see Methods), were calculated to be: 2-methyl-5-HT, 600 nM (500–740 nM); 5-HT, 50 nM (30–64 nM) and *m*CPBG, 20 nM (14–28 nM) (median and range, $n = 6–7$). The pooled data were also fitted to logistic equations giving similar IC_{50} values and slope factors of 1·7, 1·3 and 1·0, respectively (Fig. 4*D*). It should be noted that the curve did not fit the data for *m*CPBG very well. It is possible that a two site fit would have been better, but there were insufficient data points to realistically test this. These data support the idea that 5-HT acts by binding to the 5-HT$_3$ receptor and prevents its recovery from desensitization.

Having demonstrated that other 5-HT$_3$ receptor agonists could mimic the inhibitory action of low concentrations of 5-HT, we wished to determine whether 5-HT$_3$ antagonists would influence this effect. We used 3 nM (+)-tubocurarine or quipazine which reduced the response to pressure-applied 5-HT by ~50%. The antagonist action of (+)-tubocurarine was much faster in onset than that of quipazine (Fig. 5*A* and *B*). The maximum inhibitory effect of 0·3 μM 5-HT was similar in the presence of these antagonists but the rate of onset of this effect was reduced by (+)-tubocurarine but not by quipazine (Fig. 5*D* and *E*). This effect is best seen in the overlaid cumulated graphs in Fig. 5*F*. The responses inhibited by 5-HT in the presence of (+)-tubocurarine were considerably longer in duration than those in quipazine (Fig. 5*D* and *E*). There was one other clear difference between these antagonists: at higher concentrations (30 nM to 3 μM), quipazine directly evoked an inward current (Fig. 5*C*). This action was not shared by (+)-tubocurarine.

DISCUSSION

Local pressure application of 5-HT onto an NG108-15 cell activates a rapid ionic current through 5-HT$_3$ receptors. The simultaneous bath application of low concentrations of 5-HT, even those which did not induce a detectable inward current, results in a marked reduction in the amplitude of the response induced by pressure-applied 5-HT. We believe that this inhibitory effect is consistent with previous models of receptor activation and desensitization (Katz & Thesleff; 1957; Rang & Ritter, 1970; Feltz & Trautmann, 1982), the continued presence of a low concentration of 5-HT preventing the normal recovery of the receptor from its desensitized form.

The inhibitory effect of low concentrations of superfused 5-HT was probably due to a direct action on the 5-HT$_3$ receptor. It could be recorded with the whole-cell patch-clamp technique using intracellular solutions with or without ATP and GTP, indicating that it was not mediated indirectly via GTP-binding protein-coupled 5-HT receptors. In addition it was also mimicked by two other agonist ligands of the 5-HT$_3$ receptor.

The inhibitory effect did not appear to be dependent on the direction of flow of current through the ligand-gated ion channel, or on membrane potential, since it could be recorded at holding potentials of +60 and −60 mV. Attempts to remove internal or external calcium ions had minimal effects on this type of inhibition. Internal calcium ions have been shown to modify the desensitization of another ligand-gated ion channel, the glutamate NMDA receptor (Vyklický, 1993) but our effect could be recorded with patch pipettes containing the calcium chelator BAPTA. External divalent cations have been shown to inhibit 5-HT$_3$ receptor-mediated responses (Peters, Hales & Lambert, 1988; Robertson & Bevan, 1991; Eiselé *et al.* 1993) and their removal did potentiate this 5-HT$_3$ receptor-mediated response; however, the inhibitory effect of bath-applied 5-HT was still present in their absence. This is consistent with the findings of Eiselé *et al.* (1993) who observed that it was the channel domain of the 5-HT$_3$ receptor which was modulated by calcium ions, rather than the agonist binding site. The most likely explanation for the inhibitory effect, therefore, is that the 5-HT binds to the external surface of the receptor to exert its effect. The low concentrations of 5-HT required to inhibit the amplitude of the inward current indicates that 5-HT was binding with a high affinity.

It is generally believed that receptors switch into a desensitized form following their activation. For the purposes of this discussion, the cyclic model of desensitization is shown (Katz & Thesleff, 1957) (Fig. 6). For simplicity, only one binding site for the agonist is indicated, although it is likely that two agonist molecules may bind and the model has been modified accordingly by others (e.g. Feltz & Trautmann, 1982). The present experiments do not allow us to determine whether the cyclic model or a complex co-operative model is most appropriate for this receptor (cf. Neijt *et al.* 1989).

As with other ligand-gated ion channels, relatively high concentrations of the ligand are necessary to activate the 5-HT$_3$ receptor, and this process is fast and transient because it involves a low-affinity binding site for 5-HT (AR) (see Fig. 6). The prolonged recovery time of the receptor indicates that 5-HT may subsequently bind to a high-affinity site on the desensitized receptor (AD), from which it slowly dissociates to allow full recovery. We will now discuss our findings in these terms. The inhibitory effect of bath-applied 5-HT was dependent on the activation of the receptor and the number of times it was activated, rather than the superfusion time of 5-HT or the

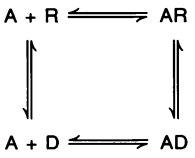

Figure 6. The cyclic model of desensitization (Katz & Thesleff, 1957)

The unoccupied activatable (R) and desensitized (D) forms of the receptor. In the absence of an agonist (A), the receptor is normally in the R form. An agonist molecule (A) normally binds to R, giving AR (and the response) with desensitization following. Under normal conditions, the recovery from AD, the agonist-bound desensitized form, is largely AD → D → R. After activation another agonist ligand can bind to D.

frequency of receptor activation. This may be explained if a certain number of activations of the population of receptors are needed to increase the probability that 5-HT will bind to their desensitized form. The activation dependence was particularly apparent if the superfused 5-HT did not evoke a detectable inward current and it reached the cell after the receptor had recovered. Under these conditions, the peak response of the next activation was usually unaffected with short superfusion times but it was reduced after prolonged superfusions. The latter may be explained if it is possible, albeit with a low probability, for 5-HT to bind to the desensitized form of the receptor. This could occur in one or both of two ways. Firstly, that the receptor is being activated (A + R → AR → AD) but at an undetectable level. Secondly, the desensitized form could occur without the receptor being activated (R → D). Evidence for this second possibility has been provided by single-channel recordings of another ligand-gated ion channel, the glutamate NMDA receptor (Lin & Stevens, 1994). Therefore, it seems likely that we are studying the high-affinity binding of 5-HT to the desensitized form of the 5-HT_3 receptor. Following activation, 5-HT is presumed to bind to the desensitized form of the receptor with a high affinity, from which 5-HT dissociates allowing recovery. Here, the 5-HT in the superfusate is presumably binding to the vacated high-affinity site (D) preventing subsequent recovery. This suggests that the receptor normally recovers via the route AD → D → R.

The IC_{50} values we obtained for the inhibitory effects of the three agonists in this functional assay probably reflect their affinity for the desensitized form of the receptor (D). Indeed, the IC_{50} values we determined for the three agonist ligands are reasonably close to their affinity values in radioligand binding assays (see legend to Fig. 4 and Boess *et al.* 1992). Interestingly, Sepúlveda *et al.* (1991) came to the conclusion that *m*CPBG has a high affinity for a desensitized form of the 5-HT_3 receptor in N1E-115 cells. On those cells Neijt *et al.* (1988) showed that low concentrations of 5-HT inhibited the activation of the 5-HT_3 receptor with a slope factor of 1·3 (IC_{50}, 130 nM). Here we find a similar slope factor for 5-HT. As previously shown, the onset of desensitization for each agonist was faster than the recovery. Earlier studies have shown that recovery from desensitization proceeds at a constant rate for different agonists (Rang & Ritter, 1970) and, more recently, that it may be better described by two rate constants (Feltz & Trautmann, 1982). In N1E-115 cells, Neijt *et al.* (1989) reported that the receptors recovered from desensitization with a single exponential function. Here we found that the recovery times from 5-HT and 2-methyl-5-HT were similar and short, but that the recovery from *m*CPBG was clearly longer. If, like 5-HT, these other ligands are preventing recovery from desensitization by binding to the vacated desensitized form (D) after 5-HT has dissociated, then the agonist-dependent recovery could be explained if *m*CPBG has a higher affinity for the desensitized receptor (AD) than the other ligands. The high affinity of *m*CPBG confers a slower dissociation from the desensitized form of the receptor (AD → D) and therefore the overall recovery (AD → D → R) is prolonged. The recovery from the unoccupied desensitized form to the unoccupied activatable form (D → R) should not depend on the ligand.

An increasing number of compounds have been shown to influence the desensitization of the 5-HT_3 receptor. These include potassium channel blockers such as tetraethylammonium (TEA; Kooyman, Zwart & Vijverberg, 1993), antidepressants (Fan, 1994) and 5-hydroxyindole (Kooyman, van Hooft & Vijverberg, 1993). We wished to see whether 5-HT_3 receptor antagonists could interact with this process. We chose (+)-tubocurarine and quipazine, which are generally accepted to be antagonists of 5-HT_3 receptor-induced responses. (+)-Tubocurarine acts as a competitive antagonist of peripheral 5-HT_3 receptors (Newberry *et al.* 1991; Newberry, Watkins, Sprosen, Blackburn, Grahame-Smith & Leslie, 1993) and in N1E-115 cells it acts in a voltage-independent manner (Peters, Malone & Lambert, 1990). Quipazine is an unusual antagonist of this receptor, since it displaces some radioligands of 5-HT_3 receptors with a slope factor of 1·6, like an agonist (Kilpatrick *et al.* 1987), but other radioligands with a slope factor close to unity (Barnes & Barnes, 1993). (+)-Tubocurarine appeared to slow the onset of the inhibitory action of 5-HT. A similar action of (+)-tubocurarine was observed on the desensitization of nicotinic receptors in chick muscle (Rang & Ritter, 1970). This action was not shared by quipazine. One possibility is that quipazine could have agonist-like properties and

'antagonize' by binding to the desensitized form of the receptor like the other agonists. We observed that it could activate the receptor at high concentrations so it might also bind to the desensitized form of the receptor at low concentrations. Indeed, there is other evidence that quipazine can act as an agonist at 5-HT_3 receptors. Quipazine promoted the uptake of [^{14}C]guanidinium into NG108-15 cells (Emerit, Riad, Fattaccini & Hamon, 1993) and produced a small inward current in *Xenopus* oocytes expressing the long form of the 5-HT_3 receptor subunit (Downie *et al.* 1995).

In conclusion, we believe we have been studying the consequences of the binding of 5-HT and other substances to a high-affinity binding site on the desensitized form of the 5-HT_3 receptor. 5-HT and other agonist ligands inhibit 5-HT_3 receptor-mediated ionic currents by preventing full recovery from desensitization which naturally follows activation. With low concentrations of 5-HT, this mainly occurs after the receptor has been activated. The functional model we use has similarities with the likely pulsatile nature of 5-HT_3 receptor-mediated neurotransmission, such as from a 5-HT-containing raphe neurone to a neurone in the amygdala (Sugita *et al.* 1992). A small rise in the extracellular level of 5-HT at this synapse, caused by the rapid firing of the raphe neurone and/or a serotonin reuptake inhibitor (SSRI), could inhibit transmission through it. However, this desensitization-induced inhibition would be most effective after the synapse had been used.

Barnes, J. M. & Barnes, N. M. (1993). Differential binding characteristics of agonists at 5-HT_3 receptor recognition sites in NG108-15 neuroblastoma-glioma cells labelled by [3H]-(*S*)-zacopride and [3H]granisetron. *Biochemical Pharmacology* **45**, 2155–2158.

Bartrup, J. T. & Newberry, N. R. (1995). Desensitization of 5-HT_3 receptors in the presence of low concentrations of 5-HT_3 receptor agonists. *British Journal of Pharmacology* **114**, 378*P*.

Boess, F. G., Beroukhim, R. & Martin, I. L. (1995). Ultrastructure of the 5-hydroxytryptamine$_3$ receptor. *Journal of Neurochemistry* **64**, 1401–1405.

Boess, F. G., Sepúlveda, M.-I., Lummis, S. C. R. & Martin, I. L. (1992). 5-HT_3 receptors in NG108-15 neuroblastoma × glioma cells: Effect of the novel agonist 1-(*m*-chlorophenyl)-biguanide. *Neuropharmacology* **31**, 561–564.

Derkach, V., Surprenant, A. & North, R. A. (1989). 5-HT_3 receptors are membrane ion channels. *Nature* **339**, 706–709.

Downie, D. L., Hope, A. G., Belelli, D., Lambert, J. J., Peters, J. A., Bentley, K. R., Steward, L. J., Chen, C.-Y. & Barnes, N. M. (1995). The interaction of trichloroethanol with murine recombinant 5-HT_3 receptors. *British Journal of Pharmacology* **114**, 1641–1651.

Eiselé, J.-L., Bertrand, S., Galzi, J.-L., Devillers-Thiéry, A., Changeux, J.-P. & Bertrand, D. (1993). Chimaeric nicotinic–serotonergic receptor combines distinct ligand binding and channel specificities. *Nature* **366**, 479–483.

Emerit, M. B., Riad, M., Fattaccini, C. M. & Hamon, M. (1993). Characteristics of [^{14}C]guanidinium accumulation in NG108-15 cells exposed to serotonin 5-HT_3 receptor ligands and substance P. *Journal of Neurochemistry* **60**, 2059–2067.

Fan, P. (1994). Effects of antidepressants on the inward current mediated by 5-HT_3 receptors in rat nodose ganglion neurones. *British Journal of Pharmacology* **112**, 741–744.

Feltz, A. & Trautmann, A. (1982). Desensitization at the frog neuromuscular junction: a biphasic process. *Journal of Physiology* **322**, 257–272.

Hille, B. (1992). *Ionic Channels of Excitable Membranes*. Sinauer Associates Inc., Sunderland, MA, USA.

Hope, A. G., Downie, D. L., Sutherland, L., Lambert, J. J., Peters, J. A. & Burchell, B. (1993). Cloning and functional expression of an apparent splice variant of the murine 5-HT_3 receptor A subunit. *European Journal of Pharmacology* **245**, 187–192.

Huganir, R. L. & Greengard, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron* **5**, 555–567.

Katz, B. & Thesleff, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *Journal of Physiology* **138**, 63–80.

Kilpatrick, G. J., Jones, B. J. & Tyers, M. B. (1987). Identification and distribution of 5-HT_3 receptors in rat brain using radioligand binding. *Nature* **330**, 746–748.

Kooyman, A. R., van Hooft, J. A. & Vijverberg, H. P. M. (1993). 5-Hydroxyindole slows desensitization of the 5-HT_3 receptor-mediated ion current in N1E-115 neuroblastoma cells. *British Journal of Pharmacology* **108**, 287–289.

Kooyman, A. R., Zwart, R. & Vijverberg, H. P. M. (1993). Tetraethylammonium ions block 5-HT_3 receptor-mediated ion current at the agonist recognition site and prevent desensitization in cultured mouse neuroblastoma cells. *European Journal of Pharmacology* **246**, 247–254.

Lin, F. & Stevens, C. F. (1994). Both open and closed NMDA receptor channels desensitize. *Journal of Neuroscience* **14**, 2153–2160.

Maricq, A. V., Peterson, A. S., Brake, A. J., Myers, R. M. & Julius, D. (1991). Primary structure and functional expression of the 5-HT_3 receptor, a serotonin-gated ion channel. *Science* **254**, 432–437.

Neijt, H. C., Plomp, J. J. & Vijverberg, H. P. M. (1989). Kinetics of the membrane current mediated by serotonin 5-HT_3 receptors in cultured mouse neuroblastoma cells. *Journal of Physiology* **411**, 257–269.

Neijt, H. C., Te Duits, I. J. & Vijverberg, H. P. M. (1988). Pharmacological characterization of serotonin 5-HT_3 receptor mediated electrical responses in cultured mouse neuroblastoma cells. *Neuropharmacology* **27**, 301–307.

Newberry, N. R., Cheshire, S. H. & Gilbert, M. J. (1991). Evidence that the 5-HT_3 receptors of the rat, mouse and guinea-pig superior cervical ganglion may be different. *British Journal of Pharmacology* **102**, 615–620.

Newberry, N. R., Watkins, C. J., Sprosen, T. S., Blackburn, T. P., Grahame-Smith, D. G. & Leslie, R. A. (1993). BRL 46470 potently antagonizes neural responses activated by 5-HT_3 receptors. *Neuropharmacology* **32**, 729–735.

Ortells, M. O. & Lunt, G. G. (1995). Evolutionary history of the ligand-gated ion-channel superfamily of receptors. *Trends in Neurosciences* **18**, 121–127.

PETERS, J. A., HALES, T. G. & LAMBERT, J. J. (1988). Divalent cations modulate 5-HT_3 receptor-induced currents in N1E-115 neuroblastoma cells. *European Journal of Pharmacology* **151**, 491–495.

PETERS, J. A., MALONE, H. M. & LAMBERT, J. J. (1990). Antagonism of 5-HT_3 receptor mediated currents in murine N1E-115 neuroblastoma cells by (+)-tubocurarine. *Neuroscience Letters* **110**, 107–112.

PETERS, J. A., MALONE, H. M. & LAMBERT, J. J. (1992). Recent advances in the electrophysiological characterization of 5-HT_3 receptors. *Trends in Pharmacological Science* **13**, 391–397.

PETERS, J. A., MALONE, H. M. & LAMBERT, J. J. (1993). An electrophysiological investigation of the properties of 5-HT_3 receptors of rabbit nodose ganglion neurones in culture. *British Journal of Pharmacology* **110**, 665–676.

RANG, H. P. & RITTER, J. M. (1970). On the mechanism of desensitization at cholinergic receptors. *Molecular Pharmacology* **6**, 357–382.

RICHARDSON, B. P. & ENGEL, G. (1986). The pharmacology and function of 5-HT_3 receptors. *Trends in Neurosciences* **9**, 424–428.

ROBERTSON, B. & BEVAN, S. (1991). Properties of 5-hydroxytryptamine$_3$ receptor-gated currents in adult rat dorsal root ganglion neurones. *British Journal of Pharmacology* **102**, 272–276.

ROPERT, N. & GUY, N. (1991). Serotonin facilitates GABAergic transmission in the CA1 region of rat hippocampus *in vitro*. *Journal of Physiology* **441**, 121–136.

SEPÚLVEDA, M.-I., LUMMIS, S. C. R. & MARTIN, I. L. (1991). The agonist properties of *m*-chlorophenylbiguanide and 2-methyl-5-hydroxytryptamine on 5-HT_3 receptors in N1E-115 neuroblastoma cells. *British Journal of Pharmacology* **104**, 536–540.

SUGITA, S., SHEN, K.-Z. & NORTH, R. A. (1992). 5-Hydroxytryptamine is a fast excitatory transmitter at 5-HT_3 receptors in rat amygdala. *Neuron* **8**, 199–203.

VYKLICKÝ, L. (1993). Calcium-mediated modulation of NMDA responses in cultured rat hippocampal neurones. *Journal of Physiology* **470**, 575–600.

WALLIS, D. I. & NORTH, R. A. (1978). The action of 5 hydroxytryptamine on single neurones of the rabbit superior cervical ganglion. *Neuropharmacology* **17**, 1023–1028.

YAKEL, J. L. & JACKSON, M. B. (1988). 5-HT_3 receptors mediate rapid responses in cultured hippocampus and a clonal cell line. *Neuron* **1**, 615–621.

YAKEL, J. L., LAGRUTTA, A., ADELMAN, J. P. & NORTH, R. A. (1993). Single amino acid substitution affects desensitization of the 5-hydroxytryptamine type 3 receptor expressed in *Xenopus* oocytes. *Proceedings of the National Academy of Sciences of the USA* **90**, 5030–5033.

YAKEL, J. L., SHAO, X. M. & JACKSON, M. B. (1991). Activation and desensitization of the 5-HT_3 receptor in a rat glioma × mouse neuroblastoma hybrid cell. *Journal of Physiology* **435**, 293–308.

YANG, J. (1990). Ion permeation through 5-hydroxytryptamine-gated channels in neuroblastoma N18 cells. *Journal of General Physiology* **96**, 1177–1198.

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