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The role of the agonist binding site in Ca^{2+} inhibition of the recombinant 5-HT_{3A} receptor

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Abstract

The mechanism and site of action of Ca^{2+} at the recombinant murine 5-hydroxytryptamine $(5\text{-HT})_{3A}$ receptor were investigated using whole-cell voltage clamp, radioligand binding and single-cell Ca^{2+} imaging. Inhibition of the 5-HT (3 μM)-induced response by 10 mM Ca^{2+} reached a plateau at 68.5% inhibition, with half-maximal effect at 2.6 mM. This was due to an increase in EC_{50} from 2.35 to 3.87 μM and a 30% reduction in I_{max}. Ca^{2+} also resulted in the inhibition of binding of both 5-HT_3 receptor agonist [^{3}H]*m*-chlorophenylbiguanide and antagonist [^{3}H]granisetron due to an increase in K_d, with no change in B_{max}. An increase in EC_{50} from 2.6 (1 mM Ca^{2+}) to 4.7 μM (10 mM Ca^{2+}), with no change in maximal $[Ca^{2+}]_i$, was observed from Ca^{2+} imaging studies. Largely similar effects were observed with Mg^{2+}. The combined data suggest that Ca^{2+} acting at a site that directly or indirectly influences the agonist binding site plays a significant role in its inhibitory effect at the 5-HT_3 receptor. © 2001 Published by Elsevier Science B.V.

Keywords: 5-HT_3 receptor; Divalent cation modulation; Cation permeability; Ion channel, neurotransmitter gated; Ca^{2+}

1. Introduction

5-hydroxytryptamine $(5\text{-HT})_3$ receptors are members of the family of cys-loop neurotransmitter receptors, which includes nicotinic acetylcholine, γ-aminobutyric acid $(GABA)_A$ and glycine receptors. In common with other members of this family, the 5-HT_3 receptor is a pentameric assembly of subunits (Boess et al., 1995). Two 5-HT_3 receptor subunits, 5-HT_{3A} (Maricq et al., 1991) and 5-HT_{3B} (Davies et al., 1999), have been identified so far, and heterologously expressed receptors function as either homo-oligomeric 5-HT_{3A} or hetero-oligomeric 5-HT_{3A} and 5-HT_{3B} subunit complexes. Examination of the properties of the latter has substantially clarified a controversial area of 5-HT_3 receptor research: whether these proteins have significant Ca^{2+} permeability. It has become clear that hetero-oligomeric receptors have much lower permeabilities to divalent cations (Davies et al., 1999) and, combined with studies of very highly Ca^{2+} permeable 5-HT_3 receptors, which may incorporate certain nicotinic acetylcholine receptor subunits (Rondé and Nichols, 1998; Van Hooft et al., 1998), 5-HT_3 receptors can exhibit a wide range of Ca^{2+} permeability.

Ca^{2+} is also one of many modulators which affect the activity of the cys-loop family of neurotransmitter receptors by binding to distinct sites on the molecule (Galzi and Changeux, 1995; MacDonald and Olsen, 1994). This probably has a physiological role: Ca^{2+} is present in presynaptic vesicles and can be released with neurotransmitter upon stimulation, and the concentrations of Ca^{2+}_{ext} needed for the inhibition of agonist-evoked responses are within the physiological range. Ca^{2+} modulates various properties of ligand-gated ion channels and some effects, such as those on kinetics, may be due to the activation of Ca^{2+}-dependent intracellular enzymes such as protein kinases or phosphatases (see Jones and Westbrook, 1996; Yakel, 1997 for reviews). The lack of effect of inhibitors of these enzymes on 5-HT_3 receptor responses, however, suggests that the major effect of Ca^{2+} in this protein is via other mechanisms (Boddeke et al.,1996). There is evidence from the nicotinic acetylcholine receptor that some effects of Ca^{2+},

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such as modulation of activation, are the result of Ca^{2+} binding directly to the receptor, and a nicotinic acetylcholine receptor Ca^{2+} binding site has recently been localised to a region on the N-terminal domain of the nicotinic acetylcholine receptor α7 subunit (Galzi et al., 1996). This appears to be close to, and may partially overlap, the agonist binding site. The present study, which uses a combination of techniques to investigate the mechanism of action of Ca^{2+} at the 5-HT_3 receptor, also suggests that there is an interaction between a Ca^{2+} binding site on this protein and the agonist binding site. We propose that this interaction may be a significant factor in the inhibition of the response in the presence of Ca^{2+}.

2. Materials and methods

2.1. Cell culture

Tissue culture procedures and transfections were as described previously with minor modifications (Hargreaves et al., 1994). Briefly, human embryonic kidney (HEK) 293 cells were grown in 90-mm dishes in 50% Dulbecco's modified Eagles medium/Ham's F12 media at 7% CO_2. When the cells reached 50–80% confluency, they were transfected with the eukaryotic expression vector pRc/CMV (Invitrogen) containing the complete coding sequence for the 5-$HT_{3A(b)}$ receptor subunit cloned from N1E-115 neuroblastoma cells, using the Ca^{2+} phosphate precipitation method (Hargreaves et al., 1994). Stable cell lines were selected using geneticin (1 mg/ml) and are referred to as HEK/5-HT_{3A} cells.

2.2. Electrical recordings

Whole-cell currents were recorded using a Biologic Patch/Cell-clamp amplifier. Patch pipettes (3–5 MΩ) were made from thin-walled borosilicate glass capillary tubing (Clark Electromedical GC150TF-10). Unless otherwise specified, the pipette filling solution had the following composition (mM): KCl 140, $MgCl_2$ 2.0, $CaCl_2$ 0.1, EGTA 1.1 (free Ca^{2+} concentration 10^{-8} M), HEPES 10, pH 7.2. Cells were viewed through a Nikon TMS inverted microscope by phase contrast optics and continuously perfused (3–5 ml/min) with a solution of the following composition (mM): NaCl 140, KCl 2.8, $MgCl_2$ 0.5, $CaCl_2$ 0.5, D-glucose 10, HEPES 10, pH 7.2. Both intracellular and extracellular solutions had osmolarities of approximately 300 mosM. A salt bridge containing 3 M KCl was used throughout. Where appropriate, differences in junction potentials arising from solution changes were calculated (Barry, 1994) and corrections applied. Agonists were applied through a U-tube, which allowed a change of solution within 100 ms (Sepúlveda et al., 1991) and 5 min were allowed between applications to ensure recovery from desensitization. Cells were routinely clamped at −60 mV. pCLAMP (Burlingame, CA) software was used for acquisition and analysis of macroscopic currents. Current–voltage data were generated by voltage ramps. Leak current measured with voltage ramps applied just before addition of agonist was subtracted in each case. Curves resulting from concentration–response experiments were analysed using nonlinear regression curve fitting facilities using the SigmaPlot Scientific Graph System (Jandel Scientific), which uses the Marquardt–Levenberg algorithm.

2.3. Radioligand binding

This was performed as previously described with minor modifications (Lummis et al., 1990, 1993). Briefly, HEK/5-HT_{3A} cells were washed twice with phosphate-buffered saline and then scraped into HEPES buffer (10 mM, pH 7.5) containing 1 mM EDTA, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml bacitracin and 0.1 mM phenylmethylsulphonylflouride. The suspension was centrifuged (10,000 *g*, 5 min, 0 °C) and the membranes were then washed twice in HEPES buffer and used immediately for binding experiments. These were performed in a final volume of 500 μl of HEPES buffer (10 mM, pH 7.4) containing, unless otherwise stated, either 1 nM [^{3}H]*m*-chlorophenylbiguanide (mCPBG), a 5-HT_3 receptor agonist, or 0.1 nM [^{3}H]granisetron, a 5-HT_3 receptor antagonist. Nonspecific binding was defined using 1 μM D-tubocurarine. In saturation experiments, the concentrations of [^{3}H]granisetron or [^{3}H]mCPBG varied between 0.006 and 2 nM and 0.1 and 20 nM, respectively. Incubations were for 2 h at 0 °C and were terminated by rapid filtration through Whatman GF/B filters using a Brandel receptor binding harvester, followed by two washes with 4 ml of ice-cold HEPES buffer (5 mM, pH 7.4). Protein concentrations were determined using bovine serum albumin as the standard. Data were analysed using Prism software (Graphpad, San Diego, CA).

2.4. Ca^{2+} imaging

This was as described previously with minor modifications (Hargreaves et al., 1994). Briefly, HEK/5-HT_{3A} cells grown on coverslips were washed once with HEPES-buffered medium (HBM: 115 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 25 mM HEPES, 15 mM glucose, pH 7.4) and then incubated for 30 min at room temperature in HBM containing 2 μM fura-2/acetoxymetyl ester and 1 mg/ml bovine serum albumin. After washing (2 × 1 ml HBM) and at least 30 min further incubation, the cells were washed (2 × 1 ml) in Na^+-free HBM where Na^+ was replaced by *N*-methyl-D-glucamine (NMDG), and coverslips were placed in a perfusion chamber on the stage of a Nikon Diaphot inverted microscope. The equipment used was similar to that described previously (Hargreaves et al., 1994). In brief, cells on a round

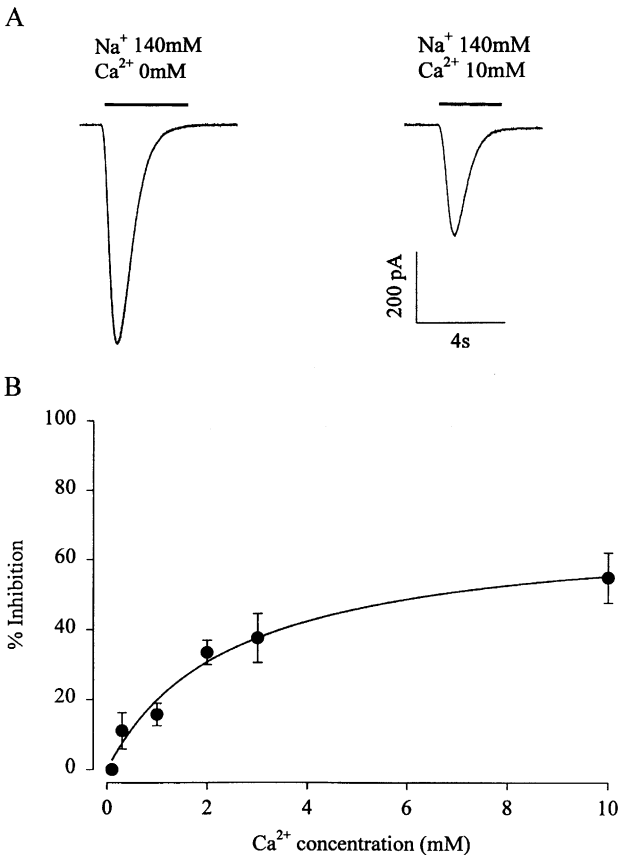

Fig. 1. Responses to 3 μM 5-HT in saline ± 10 mM Ca^{2+} obtained in the same cell at −60 mV (A). Response at the same agonist concentration with Ca^{2+} concentration ranging from 0.1 to 10 mM were used to construct the graph shown in (B). Data = means ± S.E.M. from 3–6 cells.

glass coverslip were mounted in a chamber on the stage of an inverted epifluorescence microscope. Excitation light from a 100-W xenon lamp passed through a rapidly rotatable housing holding narrow band interference filters (340 and 380 nm). Emitted light was captured by an ISIS-M CCD camera (Photonic Science, Robertsbridge, UK) after passage through a dichroic mirror (400 nm) and high-pass barrier filter (480 nm). Agonists were introduced directly into the chamber (exchange time < 5 s) in Na^+-free HBM until maximal responses were reached or for 30 s (whichever was the longer). Image pairs (340/380 nm) were collected at 5-s intervals using Metafluor software (Universal Imaging, PA, USA), and fluorescence ratios were formed by dividing pairs of images. These were converted to $[Ca^{2+}]_i$ by reference to a look-up table created using standard solution (Molecular Probes). The traces shown were produced by calculating the average $[Ca^{2+}]$ within the perimeter of individual cells. Concentration–response curves and parameters were obtained using Prism software (GraphPad, San Diego, CA).

2.5. Drugs and reagents

All cell culture reagents were obtained from Gibco BRL (Paisley, UK), except fetal calf serum, which was from Sigma (Poole, UK). 5-HT hydrochloride was from Research Biochemicals (St. Albans, UK) and mCPBG from Tocris Chemicals (Bristol, UK). Fura-2/acetoxymetyl ester was from Molecular Probes, (Eugene, OR, USA). Radiolabelled compounds were from DuPont/New England Nuclear. All other reagents were of the highest available quality.

3. Results

3.1. Effect of divalent cations on 5-HT_3 receptor activation

Whole-cell currents, measured in cells clamped at −60 mV, were evoked by 5-HT in a saline solution containing only monovalent cations. To study the effect of Ca^{2+} and Mg^{2+} on the agonist-stimulated responses, $CaCl_2$ or $MgCl_2$ were added to the saline at a final concentration of 10 mM. Fig. 1A illustrates typical responses to 3 μM 5-HT in the presence or absence of Ca^{2+} obtained in the

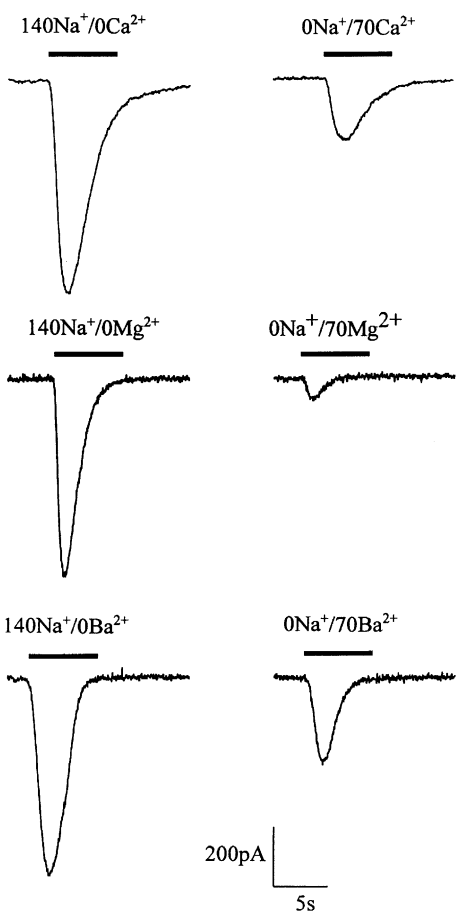

Fig. 2. Typical responses of 5-HT_3 receptors to 1 μM mCPBG. Left: responses elicited by mCPBG (shown by bar) in saline devoid of divalent cations of the following composition (in mM): NaCl 140, D-glucose 10, HEPES 10, pH 7.2. Right: response to the same concentration of agonist in a solution containing (mM) XCl_2 70, mannitol 70, D-glucose 10, HEPES 10, pH 7.2, where X is Ca^{2+}, Mg^{2+} or Ba^{2+} as indicated.

same cell. An agonist concentration close to its EC_{50} was used to avoid rapid desensitisation, which occurs at higher agonist concentrations, and also served to minimise the increase in desensitisation in the presence of divalents, which we observed when using maximal (e.g. 10 μM) 5-HT concentrations. In the presence of 10 mM Ca^{2+}, the peak amplitude of the response to 3 μM 5-HT was reduced to 45 ± 7% ($n = 4$) of the control response in divalent-free solution. The dependence of the inhibition of 5-HT-evoked responses on Ca^{2+} concentration is shown in Fig. 1B. Inhibition of the 5-HT (3 μM) response by Ca^{2+} could be described by a rectangular hyperbola that reached a plateau at 68.5 ± 6.6% inhibition. The Ca^{2+} concentration giving half-maximal effect was 2.6 ± 0.6 mM ($n = 6$).

3.2. Divalent cations permeate the 5-HT_3 ion channel

When HEK/5-HT_{3A} cells in a $CaCl_2$, $MgCl_2$ or $BaCl_2$ saline with no monovalent cation present (solution composition in mM: XCl_2 70, mannitol 70, D-glucose 10, HEPES 10, pH 7.2; X = Ca^{2+}, Mg^{2+} or Ba^{2+}) were challenged with agonist, an inward current was elicited with similar time course as that obtained in Na^+-containing saline but of reduced maximum amplitude. An example of these currents are illustrated in Fig. 2, which shows responses from cells stimulated with 1 μM *m*-chlorophenylbiguanide (mCPBG), a selective 5-HT_3 receptor agonist. Traces shown are from cells in NaCl-rich solution in the absence of divalent cations and then in XCl_2-rich solution in the absence of monovalent cations. The currents carried by divalent cations was a fraction of that carried by Na^+ using the same agonist concentration in the same cell: 0.24 + 0.03 ($n = 7$), 0.24 + 0.02 ($n = 5$) and 0.12 + 0.05 ($n = 4$) for Ca^{2+}, Ba^{2+} or Mg^{2+}, respectively.

3.3. Interaction of cations at the receptor pore

To evaluate the voltage-dependence of the divalent cation block of the activation of the 5-HT_3 receptor, we activated the receptor with 5-HT (2 μM) and applied voltage ramps from −80 to 60 mV. Fig. 3A shows the typical transient inward current evoked by activation of the 5-HT_3 receptor, together with the effect of simultaneously applying voltage ramps. In Fig. 3B, the current–voltage relation derived from the ramp labelled * in Fig. 3A is shown. Currents elicited by ramps in the absence of agonist have been subtracted. In Fig. 3C, the percent inhibition as a function of voltage is shown. The slope of the linear regression to the voltage-dependence of inhibition was −0.06 ± 0.02% per mV ($n = 4$), which is not significantly different from 0 ($P < 0.05$).

To obtain further confirmation for the lack of voltage-dependence of the Ca^{2+} inhibition of the 5-HT_3-mediated current, K^+ in the patch pipette was replaced by NMDG, an impermeant cation. Under these conditions, the effect of Ca^{2+} was voltage-independent, as shown in Fig. 4A and

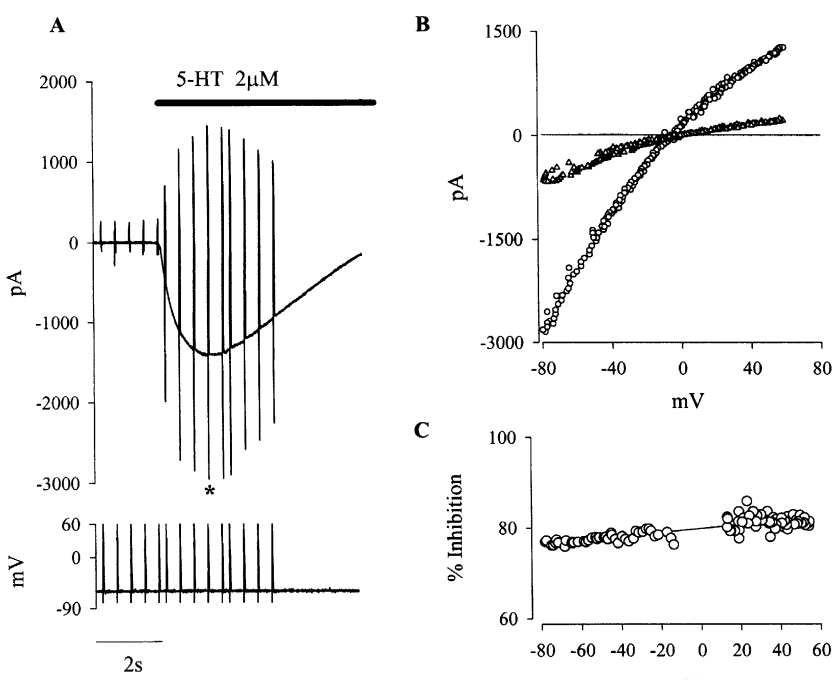

Fig. 3. Current/voltage relationships in the presence and absence of 10 mM Ca^{2+}. While applying 2 μM 5-HT (V_h −60 mV), voltage ramps were delivered. These lasted for 50 ms and started at −80 mV, carried the membrane potential to +60 mV and then repolarised back to −60 mV. (A) shows an example of this protocol. The ramp marked with an asterisk is expanded and the resulting current vs. voltage plot is presented in (B). Currents elicited by the voltage protocol in the absence of agonist have been subtracted. This plot also includes (triangles) the same protocol in the same cell in the presence of 10 mM Ca^{2+}. In (C), the inhibition of currents in the −80–60 mV range in 10 mM Ca^{2+} is plotted.

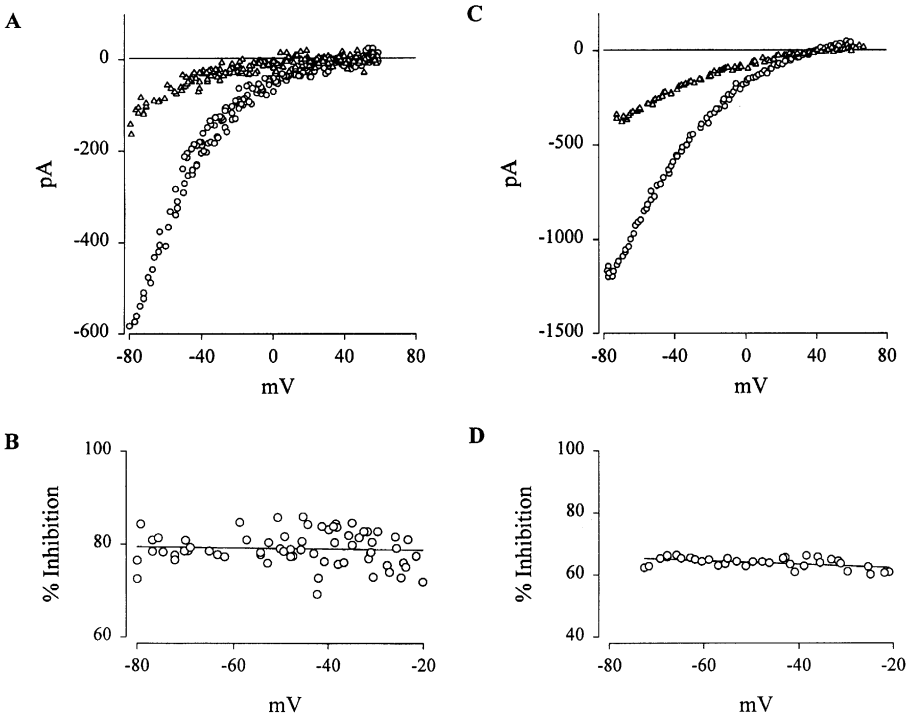

Fig. 4. Current/voltage relationship in the presence and absence of 10 mM Ca^{2+} using an impermeant intracellular cation. Experimental procedure was in all respects similar to that described in Fig. 3A except that in the pipette solution, all K^+ has been replaced by equimolar NMDG. (A) shows the current/voltage relationship in the presence and absence of 10 mM Ca^{2+} for an expanded voltage ramp chosen as in Fig. 3B while applying 2 μM 5-HT. In (B), inhibition of the currents by Ca^{2+} in the −80 to −20 mV range is plotted. (C) and (D) show an experiment in all respects identical to that in (A) and (B), but using 10 mM Mg^{2+} instead of Ca^{2+}.

B. The experimental procedure was similar to that used in Fig. 3, except that the pipette solution was NMDG-rich. The slope for the relationship between inhibition and voltage was $-0.05 \pm 0.03\%$ per mV ($n = 4$), which was not significantly different from 0 ($P < 0.05$) or from that measured in experiments with K^+-rich pipettes. Inhibition by Ca^{2+} was $72.8 \pm 4.2\%$ ($n = 6$) when NMDG was used as the main intracellular cation and was not significantly different from that measured with intracellular K^+ ($70.5 \pm 3.8\%$, $n = 6$). Fig. 4C and D shows a similar experiment but with addition of 10 mM Mg^{2+} instead of 10 mM Ca^{2+}. Mg^{2+} also caused an inhibition of 5-HT-induced current to a lesser extent than Ca^{2+} but similarly voltage-independent. The slope of the line describing the inhibition vs. voltage relation was not significantly different from zero (data not shown).

3.4. Effect of Ca^{2+} and Mg^{2+} on the affinity of 5-HT

To test for a possible modulation of the agonist-binding site, the dependence of the activation of the receptor upon agonist concentration in the presence or absence of divalent cations was studied. Fig. 5 shows normalised peak amplitude currents evoked by 5-HT in a divalent cation-free solution and the responses to the same agonist concentrations in the presence of 10 mM Ca^{2+} or Mg^{2+}. The

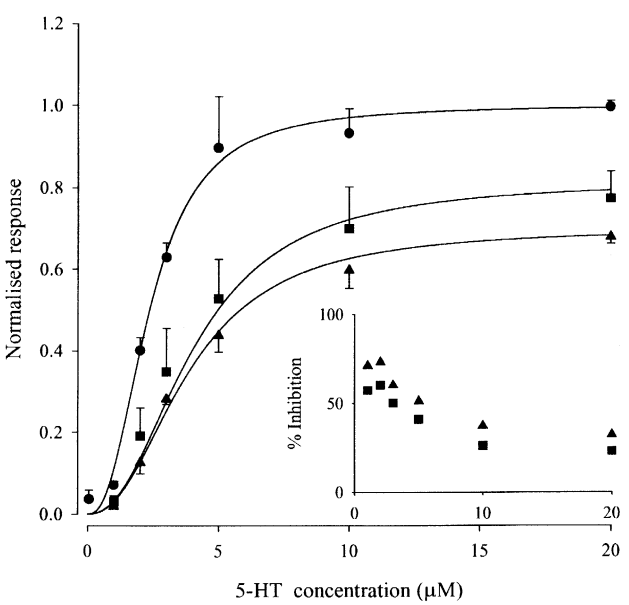

Fig. 5. Concentration–response curves of 5-HT-induced currents in the absence (circles) or presence of 10 mM Ca^{2+} (triangles) or 10 mM Mg^{2+} (squares). The peak currents measured at −60 mV holding potential are normalised to the maximum response elicited by saturating concentrations of the agonist in a divalent cation-free solution. Each point is the means ± S.E.M. of at least four separate measurements. The lines drawn are the best fit to the Hill equation. The inset shows the same data plotted as percent inhibition of 5-HT-induced current.

Table 1
Effect of 10 mM Ca^{2+} and Mg^{2+} on parameters obtained from whole-cell voltage clamp measurements

	Control	Ca^{2+}	Mg^{2+}
I_{max}	1.01 ± 0.01	0.70 ± 0.03[a]	0.81 ± 0.07[a]
EC_{50}	2.35 ± 0.26	3.87 ± 0.24[a]	4.04 ± 1.17
n_H	2.4 ± 0.3	2.3 ± 0.4	2.3 ± 0.5

Parameters were obtained from fits to Hill equation of the data in Fig. 5. Data = means ± S.E.M. from four independent concentration–response curves.
[a]Values that are significantly different from control, $P < 0.05$.

concentration–response relationship was well described by the Hill equation with EC_{50} of 2.35 ± 0.26 μM and n_H of 2.4 ± 0.3 in the absence of divalent cations. Ca^{2+} caused an increase in EC_{50} to 3.87 ± 0.24 μM ($n = 4$) but did not affect the Hill slope. Both Ca^{2+} and Mg^{2+} reduced I_{max} (Table 1). Analysis of the data as percent inhibition produced by the divalent cations revealed a decrease in inhibition as agonist concentration increased. This is shown in the inset to Fig. 5.

3.5. Effect of Ca^{2+} and Mg^{2+} on ligand binding to the 5-HT_3 receptor

Addition of 10 mM Ca^{2+} or Mg^{2+} resulted in inhibition of binding of both a radiolabelled agonist and antagonist to 5-HT_3 receptors. Initial experiments revealed inhibition of [^{3}H]granisetron binding of 54.3 ± 3.8% and 58.1 ± 3.2% by 10 mM Ca^{2+} and Mg^{2+}, respectively ($n = 3$). Saturation data showed that the inhibition was due to an apparent decrease in the affinity (K_d) of the binding site, while the maximum number of binding sites (B_{max}) was unchanged (Table 2). Quantitatively similar data were obtained using the radiolabelled agonist [^{3}H]mCPBG: K_d values almost doubled in the presence of 10 mM Ca^{2+} or Mg^{2+}, with no significant changes in B_{max}.

3.6. Effect of Ca^{2+} on $[Ca^{2+}]_i$ following activation of 5-HT_3 receptors

To examine the direct effect of Ca^{2+} on the movement of Ca^{2+} through the 5-HT-activated receptor, we used single-cell Ca^{2+} imaging in the presence of 1 and 10 mM Ca^{2+}. As has been previously described, Na^+ was completely replaced by the impermeant NMDG in these experiments and Mg^{2+} was 0.5 mM. Fig. 6A shows typical responses from a single cell to 4 μM 5-HT, where the agonist-induced increase in $[Ca^{2+}]_i$ is less in the presence of 10 mM Ca^{2+} than in 1 mM Ca^{2+}. Concentration–response curves reveal this is due to an effect on EC_{50} (Fig. 6B), which changes from 2.6 ± 0.3 μM in 1 mM Ca^{2+} to

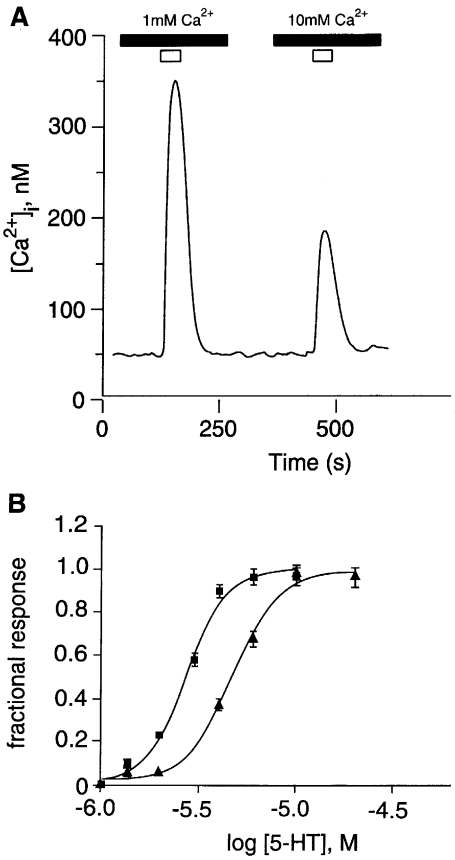

Fig. 6. Changes in $[Ca^{2+}]_i$ in response to 5-HT in the presence of 1 or 10 mM Ca^{2+}. Typical response from a single cell to 4 μM 5-HT (open bars) in the presence of 1 or 10 mM Ca^{2+} are shown in (A). Cells were routinely perfused in Na^+-free HBM (NMDG containing) with 2 mM Ca^{2+}, which was exchanged for Na^+-free HBM containing 1 or 10 mM before, during and after addition of 5-HT as shown (filled bars). Concentration response curves are shown in (B). Data are means ± S.E.M. from three separate experiments, each from 5–8 individual cells.

Table 2
Radioligand binding parameters in the presence and absence of 10 mM Ca^{2+} or 10 mM Mg^{2+}

	[^{3}H]granisetron			[^{3}H]mCPBG		
	K_d (nM)	B_{max} (pmol/mg protein)	n	K_d (nM)	B_{max} (pmol/mg protein)	n
Control	0.18 ± 0.04	2.4 ± 0.4	4	1.5 ± 0.4	2.4 ± 0.9	5
Ca^{2+}	0.43 ± 0.03[a]	2.6 ± 0.9	4	3.6 ± 0.6[a]	2.7 ± 0.8	5
Mg^{2+}	0.35 ± 0.09[a]	2.5 ± 1.0	4	2.5 ± 0.4[a]	2.7 ± 0.8	5

Data = means ± S.E.M.
[a]Values that are significantly different to control, $P < 0.05$.

4.7 ± 0.3 μM in 10 mM Ca^{2+} ($n = 3$). There was no significant difference in the maximal $[Ca^{2+}]_i$ levels reached under the two conditions, values in 10 mM Ca^{2+} were $99 \pm 18\%$ ($n = 3$) of those in 1 mM Ca^{2+}.

4. Discussion

The present study confirms and extends previous reports that show that recombinant 5-HT_{3A} receptors expressed in HEK cells are Ca^{2+} permeant and are also inhibited by Ca^{2+}. However, in contrast to previous work, our data from a variety of techniques suggests that the ability of Ca^{2+} to permeate the 5-HT_3 receptor channel may not be the sole or even the major mechanism by which it causes inhibition. Our results show that direct modulation of the agonist binding site may play a significant role in the inhibitory process.

4.1. Ca^{2+} permeation through 5-HT_3 receptors

In HEK cells stably expressing recombinant 5-HT_{3A} receptors, Ca^{2+} and the divalent cations Ba^{2+} and Mg^{2+} were permeant ions. We measured sizeable currents evoked by 5-HT or mCPBG under conditions where these are the only available extracellular cations, although the fraction of the current carried by Mg^{2+} was less than for Ca^{2+} and Ba^{2+}. This supports previous work (e.g. Brown et al., 1998; Yang, 1990), which show that 5-HT_3 receptors are permeable to divalent cations, with Ca^{2+} being more permeant than Mg^{2+}. The recent discovery of a second class of 5-HT_3 receptor subunit, 5-HT_{3B} (Davies et al., 1999), which, when expressed with the 5-HT_{3A} subunit, renders the receptor much less permeable to divalent cations, suggests that the controversy in the literature about the apparent lack of Ca^{2+} permeability of some native 5-HT_3 receptors can now be explained. For example, in NG108-15 cells and hippocampal basket cells addition of Ca^{2+} did not shift the reversal potential of agonist-evoked currents (Yakel et al., 1990; Kawa, 1994). Conversely in neuroblastoma N18 cells and dissociated rat superior cervical ganglion cells, similar studies revealed a significant contribution from divalent cations to receptor-mediated currents (Yang, 1990), and Ca^{2+} imaging techniques have shown large, transient increases in internal free Ca^{2+} concentration evoked by 5-HT_3 receptor agonists in synaptosomes from rat corpus striatum and N1E-115 neuroblastoma cells (Nichols and Mollard, 1996; Hargreaves et al., 1994). Thus, native 5-HT_3 receptors which are Ca^{2+}-impermeant may contain 5-HT_{3B} subunits, while others may be homomeric and be constituted solely of 5-HT_{3A} receptor subunits. It is also possible that the latter may possess other subunits: recent work suggests that nicotinic acetylcholine receptor α4 subunits can coassemble with 5-HT_{3A} receptor subunits and render the resulting receptor significantly more Ca^{2+} permeable (Van Hooft et al., 1998). Ca^{2+} does appear to permeate recombinant homomeric 5-HT_{3A} receptors when they are expressed in HEK cells (present study; Hargreaves et al., 1994; Brown et al., 1998), but not in oocytes (Gilon and Yakel, 1995; Glitsch et al., 1996). The apparent lack of permeability in the latter experiments, however, may be due to higher concentrations of permeant monovalent ions in these experiments perhaps indicating an interaction between monovalent and divalent ions in channel permeation.

4.1.1. Site of inhibition of the 5-HT_{3A} receptor by Ca^{2+}: the ion channel

The ability of divalent cations to inhibit 5-HT_3 agonist-elicited currents is widely accepted (e.g. Peters et al., 1988; Yang, 1990; Lovinger, 1991; Gill et al., 1995; Brown et al., 1998), although most of these reports do not provide evidence for a particular mechanism of action. One possibility is an interaction within the pore, for which the best evidence comes from the demonstration, by fluctuation analysis, of a decrease in single channel conductance upon Ca^{2+} addition (Brown et al., 1998). This would be consistent with masking of surface charges at the mouth of the channel or an interaction within the channel pore where the divalent cation would bind more tightly; the relatively slow dissociation from this site would result in a decrease in monovalent ion flux. An interaction with a site in the pore region, however, might be expected to be voltage-dependent, and we and others found that the blocking effect of Ca^{2+} and Mg^{2+} was not voltage-dependent in either recombinant or native 5-HT_3 receptors (present study; Brown et al., 1998; Gill et al., 1995; Lovinger, 1991; Peters et al., 1988; Yang, 1990), although there are some reports of voltage-dependent Ca^{2+} inhibition for recombinant 5-HT_{3A} receptors expressed in *Xenopus* oocytes (Maricq et al., 1991; Eiselè et al., 1993), and native receptors present in basket cells (Kawa, 1994). It is possible that small changes in voltage-dependence might be masked by the large inwardly directed driving force for Ca^{2+} present, but this explanation seems unlikely in view of the similar lack of voltage-dependence for Mg^{2+}, which is generally present at comparable extra- and intracellular concentrations. In Fig. 4, for example, the effects of Ca^{2+} and Mg^{2+} are qualitatively the same with concentration gradients of 10^6 and 5, respectively. There was also no voltage-dependence for the inhibition with NMDG as the main intracellular cation. If the Ca^{2+} or Mg^{2+} effect were exerted within the pore, it might be enhanced if the more physiological monovalent ions are replaced with the impermeant NMDG as the main intracellular cation. Such effects have been observed for pore inhibitors of K^+ channels, and may be due to a sweeping away effect of K^+ (MacKinnon and Miller, 1988).

Nevertheless, these data do not exclude the possibility that Ca^{2+} can bind to a site in or close to the pore. Indeed, in the presence of Na^+, which we used in our patch clamp

experiments, we would expect that if Ca^{2+} were to transiently bind to a site in the pore region, it would result in a decrease in monovalent ion flux, as proposed by Brown et al. (1998). As our Ca^{2+} flux experiments were performed in the absence of Na^{+}, we would not expect a similar result here. In support of this hypothesis is the block by Ca^{2+} of the chimeric α7-5-HT_3 receptor, which was constructed from subunits containing the N-terminal domain of the α7 nicotinic acetylcholine receptor subunit and the transmembrane domain of the 5-HT_{3A} receptor subunit (Eiselè et al., 1993).

4.1.2. Site of inhibition of the 5-HT_{3A} receptor by Ca^{2+}: the ligand-binding site

Thus, while the ion channel is a plausible location for a divalent cation binding site that causes inhibition, our data suggest that there is a significant contribution from Ca^{2+} acting at a site remote from the pore region and are consistent with this site being closely associated with the agonist binding site. We observed an apparent change in ligand binding affinity from both the patch clamp and Ca^{2+} imaging data, which show an approximate two-fold increase in their EC_{50} for 5-HT in the presence of 10 mM Ca^{2+}. This is not a large change, but the relatively high Hill coefficient of the response means that the effect on the observed current can be significant. For example, at 3 μM 5-HT, the concentration we have used in Fig. 2, the observed current, I, calculated from the Hill equation $I = I_{max}.[5\text{-HT}]^{n_H}/(EC_{50}^{n_H} + [5\text{-HT}]^{n_H})$, and using the data from Table 1, will be 64% of I_{max}. In 10 mM Ca^{2+}, I will be 35% I_{max}. Thus, the observed current will be decreased by 55%, a value not dissimilar to that observed in Fig. 1B.

Parameters calculated from ligand binding data, which show that the maximum number of binding sites is unchanged in 10 mM Ca^{2+} and Mg^{2+} but the affinities for both agonist and antagonist binding are decreased, are also consistent with a direct modification of the ligand binding site and, indeed, suggest competitive inhibition. Competitive inhibition of the related nicotinic acetylcholine receptor agonist binding site by monovalent cations has been previously reported (Gibson et al., 1977; Akk and Auerbach, 1996), and as 5-HT has an amino moiety that is cationic at physiological pH it is quite possible that here, there is also a competition between cations and the neurotransmitter, although further experiments are required to prove this. Further support for our hypothesis comes from experiments with monovalent tetraethylammonium ions, which have been shown to interact with the agonist binding site of the 5-HT_3 receptor (Hoyer and Neijt, 1988). Indeed, further exploration of the mechanism of action of these ions revealed that tetraethylammonium is both permeable through the receptor operated channel and acts externally, competing with 5-HT for the agonist binding site (Kooyman et al., 1993). Thus, tetraethylammonium and Ca^{2+} may act similarly on this receptor.

5. Conclusions

Thus, in conclusion, we have shown that Ca^{2+} and other divalent cations can both permeate 5-HT-gated channels and inhibit 5-HT_3 receptor function. Our data do not exclude the possibility that this inhibitory effect may be caused by an interaction of divalent ions within the pore, which would be noncompetitive, but they strongly suggest a significant contribution is via a site associated with the agonist binding site, which appears competitive. In the nicotinic acetylcholine receptor, a Ca^{2+} binding site has been identified in the N-terminal domain of the α7 subunit (Galzi et al., 1996). Binding of Ca^{2+} to this sequence results in enhancement of the acetylcholine-induced response and interestingly Ca^{2+} effects in this domain appear to be dominant over effects in the pore: insertion of the relevant sequence into the 5-HT_{3A} receptor results in the subsequent enhancement of agonist-induced responses in the presence of Ca^{2+}. Thus, examination of the homologous region of the 5-HT_3 receptor would be a logical place to start the search for an N-terminal 5-HT_3 receptor inhibitory Ca^{2+} binding site.

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