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Zn^{2+} enhancement of the recombinant 5-HT_3 receptor is modulated by divalent cations

Peter C. Hubbard, Sarah C.R. Lummis *

Division of Neurobiology, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK

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Abstract

The modulation by Zn^{2+} of recombinant murine 5-hydroxytryptamine$_{3A}$ (5-HT_{3A}) receptor responses and its modification by Ca^{2+} or Mg^{2+} were studied using whole-cell voltage clamp and radioligand binding techniques. In the absence of other added divalent cations Zn^{2+} enhanced the response to 5-HT by increasing maximum peak current (I_{max}) to a maximum of 122.5%, decreasing the rate of desensitization (maximum $t_{1/2} = 210\%$), and decreasing the EC_{50} by approximately two fold. In the presence of Ca^{2+} or Mg^{2+}, the effects of Zn^{2+} on I_{max} and $t_{1/2}$ were still manifest, although higher Zn^{2+} concentrations were required; however, the effect on EC_{50} was abolished. Zn^{2+} also enhanced [^{3}H]agonist but not [^{3}H]antagonist binding. We propose there is more than one Zn^{2+} binding site on the 5-HT_3 receptor molecule, and that one or more of these sites may also bind Ca^{2+} and Mg^{2+}.

Keywords: 5-HT_3 receptor; Zn^{2+}; Cation, Divalent; Voltage-clamp, Whole-cell; Radioligand binding; Neuromodulation

1. Introduction

The transition metal ion, Zn^{2+}, which is present in the nervous system at concentrations up to 200–300 μM (Frederickson et al., 1983; Palm et al., 1983), is known to modulate the function of a number of neurotransmitter-gated ion channels. It has been shown to be inhibitory at NMDA and $GABA_A$ receptors (e.g. Westbrook and Mayer, 1987; Christine and Choi, 1990; Smart and Constanti, 1990), but can enhance the response of some glycine, glutamate and P2X receptors (Rassendren et al., 1990; Li et al., 1993; Bloomenthal et al., 1994). The 5-hydroxytryptamine$_3$ (5-HT_3) receptor belongs to the family of neurotransmitter-gated channels that includes the nicotinic acetylcholine, glycine and $GABA_A$ receptors (Maricq et al., 1991). 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 with distinctive biophysical properties as either homo-oligomeric 5-HT_{3A} or hetero-oligomeric 5-HT_{3A} and 5-HT_{3B} receptor subunit complexes (Davies et al., 1999). The 5-HT_{3B} receptor subunit is expressed in many different tissues (Dubin et al., 1999), suggesting that many native 5-HT_3 receptors may exist as heteromers; however, there is evidence that in some systems at least (e.g. mouse neuroblastoma N1E-115 cells and DRG neurones) 5-HT_3 receptors can exist as homo-oligomers of 5-HT_{3A} receptor subunits. Interestingly, there is evidence for both enhancement and inhibition of 5-HT_3 receptor responses by Zn^{2+}: Lovinger (1991), Emerit et al. (1993) and Uki and Narahashi (1996) proposed that Zn^{2+} was inhibitory, while Gill et al. (1995) reported a biphasic effect, enhancement followed by inhibition at higher concentrations. The recent discovery of the 5-HT_{3B} receptor subunit (Davies et al., 1999), which may be a constituent of native 5-HT_3 receptors, may explain at least some of these discrepancies. The aim of the current study was

* Corresponding author. Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, UK. Tel.: +44-1223-766047; fax: +44-1223-766002.

E-mail address: s.lummis@mole.bio.cam.ac.uk (S.C.R. Lummis).

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therefore to examine the effects of Zn^{2+} at homomeric 5-HT_{3A} receptors stably expressed in human embryonic kidney (HEK) cells, and, as Zn^{2+} is one of a number of divalent cations that modify 5-HT_3 receptor function, to examine the modification of these effects by Ca^{2+} and Mg^{2+}.

2. Materials and methods

2.1. Cell culture

HEK 293 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM)/F12 medium supplemented with 10% fetal calf serum. They were stably transfected with DNA coding for the 5-$HT_{3A(b)}$ receptor subunit cloned from NIE-115 murine neuroblastoma cells in the eukaryotic expression vector pRc/CMV (InVitrogen, Abingdon, UK) as previously described (Hope et al., 1993; Hargreaves et al., 1996). For electrophysiological recording, cells were plated at low density onto 35 mm culture dishes (Falcon, Becton Dickinson, UK, Oxford, UK) and used for experiments within 18–36 h. For radioligand binding, the cells were grown in 90 mm dishes until confluent (3–5 days). They were then removed from plates after washing with phosphate buffered saline by gentle scraping into HEPES buffer (10 mM, pH 7.5) containing the following protease inhibitors: 1 mM EDTA, 50 μg/ml soybean trypsin inhibitor, 50 μg/ml bacitracin and 0.1 mM phenylmethylsulphonyl fluoride. Following homogenisation, they were frozen at −20°C. On the day of assay, they were thawed, washed twice and then resuspended in HEPES buffer.

2.2. Electrophysiological recording

Currents were recorded from isolated single cells in the whole-cell attached configuration as previously described (Hargreaves et al., 1996). Briefly, cells were patched with borosilicate glass micropipettes (Clark Electromedical Instruments, Reading, UK) of resistance 2–4 MΩ, back-filled with intracellular solution containing 140 mM CsCl, 1.0 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM EGTA (10 nM free Ca^{2+}), 10 mM HEPES, pH 7.2 at 24°C. Currents were recorded using an EPC-9 amplifier (HEKA Electronik, Darmstadt, Germany) connected to a PC (Macintosh Power PC 7100/66) running ''Pulse'' software (version 7.85). Cells were routinely superfused with extracellular solution containing 140 mM NaCl, 2.8 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, pH 7.2 at 24°C (2 ml/min). Divalent cation-free solution, used for application of agonists, was the same as extracellular solution without added $MgCl_2$ or $CaCl_2$, or with either 1 mM $MgCl_2$ or 1 mM $CaCl_2$ as described; $ZnCl_2$ at 1, 10 or 100 μM was applied in these solutions. Agonists were applied via a U-tube with complete solution exchange around the cell being less than 100 ms (Sepúlveda et al., 1991). An interval of at least 3 min was given between applications to allow complete washout of 5-HT and recovery from desensitization of the receptors. All currents were recorded whilst the cells were voltage-clamped at −60 mV except where otherwise stated. All experiments were performed at room temperature (20–24°C).

2.3. Analysis of data

All data were normalised to the response to a maximal concentration of 5-HT (30 μM). These were applied at frequent intervals (10–20 min) throughout the experiment. Concentration–response data were fitted (by non-linear least squares regression) to the logistic equation:

$$\text{Normalised response} = I_{max}[\text{5-HT}]^{n_H} / \left(EC_{50}^{n_H} + [\text{5-HT}]^{n_H}\right)$$

using the graphing package ''KaleidaGraph™'' version 3.0 (Synergy Software, Reading, PA, USA) to give estimations of EC_{50}, I_{max}, and Hill coefficient (n_H). The half-time of desensitization, $t_{1/2}$, represents the time taken for the current to decay to half of its peak value in the continued presence of 5-HT (30 μM). Frequently, application of the higher concentrations of Zn^{2+} (10 and 100 μM) ruptured the seal between micropipette and cell membrane. Consequently, only data where the $t_{1/2}$ was measured at all concentrations of Zn^{2+} in the same cell were used for subsequent analysis. All data are shown as arithmetic mean ± S.E.M.

2.4. Radioligand binding

These were performed as previously described (Lummis et al., 1990, 1993) with minor modifications. Briefly cell membranes were incubated at 0°C in HEPES buffer (10 mM, pH 7.5) in a final volume of 1 ml for 1 h for binding of [^{3}H]granisetron, a 5-HT_3 receptor antagonist or 2 h for binding of [^{3}H]-meta-chlorophenylbiguanide ([^{3}H]mCPBG), a 5-HT_3 receptor agonist. Non-specific binding was defined using 100 nM GR67330 (1,2,3,9-te-tra-hydro-[(5-methyl-1*H*-imidazol-4-yl)methyl]-9-methyl-4*H*-carazol-4-one). Incubations were terminated by rapid dilution with ice-cold buffer followed by vacuum filtration onto prewetted GF/B filters with 2 × 2 ml washes using a Brandel cell Harvester. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard. Experimental data were analysed using ''PRISM'' (GraphPad™, San Diego, CA, USA).

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). [^{3}H]Granisetron (81

Ci/mmol) and [^{3}H]m-chlorophenylbiguanide (mCPBG; 26 Ci/mmol) were from DuPont (Stevenage, UK). All other reagents were obtained from Sigma.

3. Results

3.1. Effects of divalent cations

The 5-HT$_3$ receptor expressed in HEK 293 cells responded to application of 5-HT in a concentration dependent manner, with an inward current of between 200 pA and 2 nA. The effect of Zn^{2+} on 5-HT-induced responses was differentially modulated in the presence of Ca^{2+} or Mg^{2}; example traces are shown in Fig. 1.

Application of 5-HT in the presence of Zn^{2+} (1–100 μM) increased the maximal peak current (I_{max}) in a concentration dependent manner (Figs. 2 and 3A). 1 and 10 μM Zn^{2+} increased the apparent affinity of the 5-HT$_3$ receptor, as measured by EC_{50} (Fig. 3B), and also increased $t_{1/2}$ (Fig. 3C), while 100 μM Zn^{2+} had no significant effect. Zn^{2+} (1–100 μM) had no effect on the Hill coefficient of the 5-HT dose–response curves (Fig. 3D).

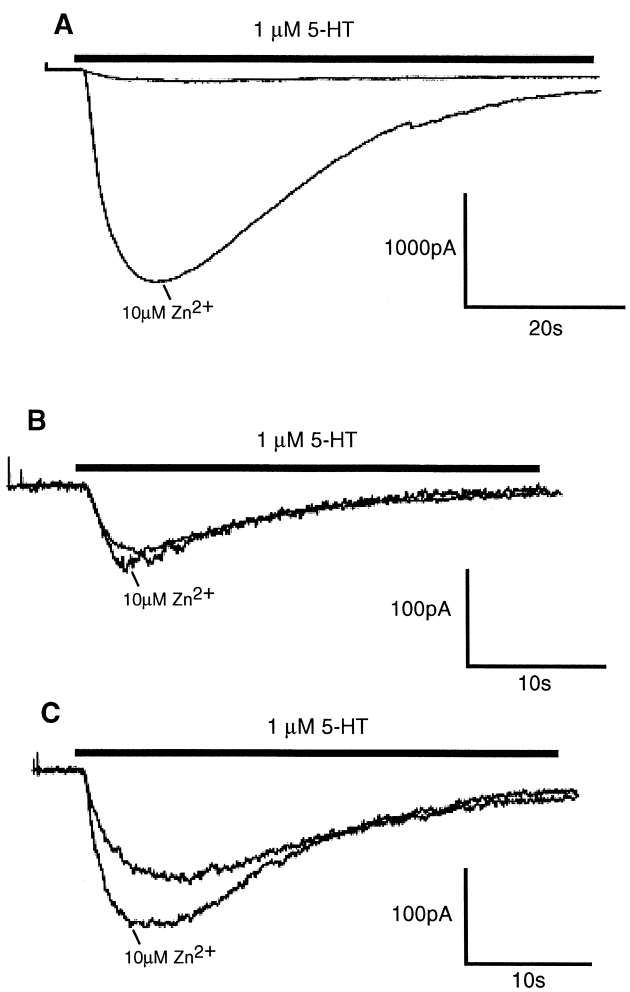

Fig. 1. 5-HT$_3$ receptor mediated responses are differentially modulated by Zn^{2+} in the presence of Ca^{2+} or Mg^{2+}. Representative responses to 1 μM 5-HT in the presence of 0 and 10 μM Zn^{2+} in divalent cation-free saline (A), plus 1 mM Ca^{2+} (B) or 1 mM Mg^{2+} (C) are shown.

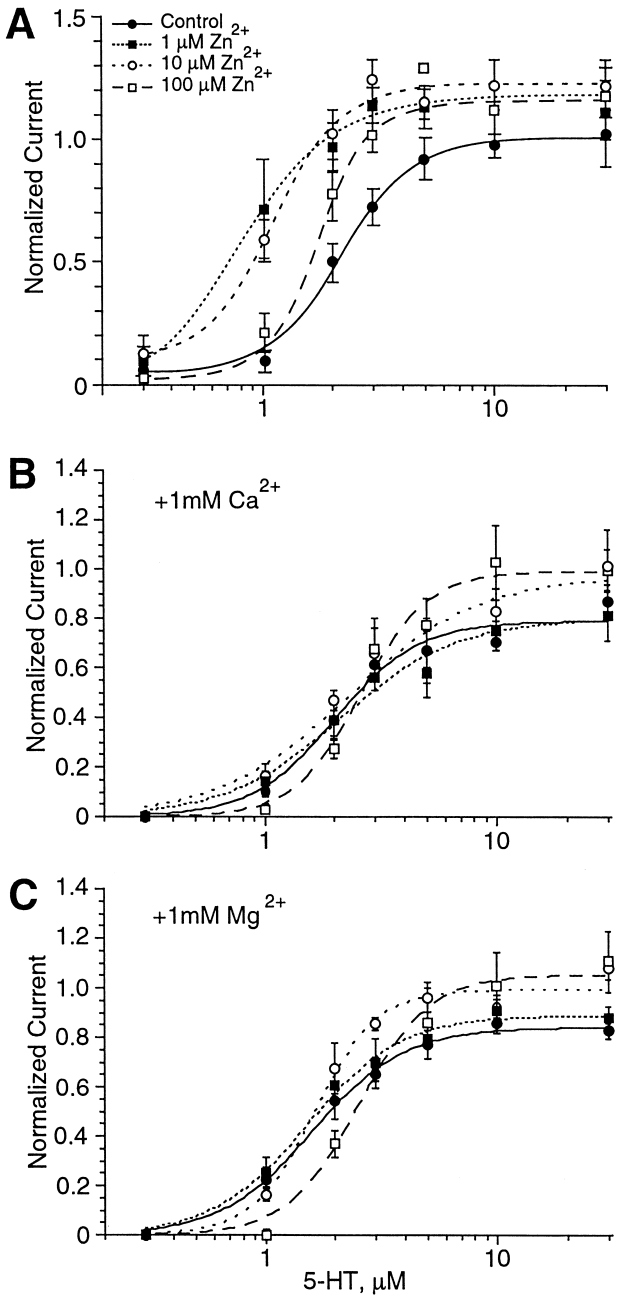

Fig. 2. Dose–response curves reveal differential modulation by Zn^{2+} in the presence of Ca^{2+} or Mg^{2+}. Concentration dependence of the 5-HT-evoked current in the presence of 0 (□), 1 (▲), 10 (■) and 100 (△) μM Zn^{2+} in divalent cation-free saline (A), plus 1 mM Ca^{2+} (B) or 1 mM Mg^{2+} (C) are shown. Data = mean ± S.E.M., $n = 4$ or 5.

Application of 5-HT in the presence of 1 mM Ca^{2+} or 1 mM Mg^{2+} reduced I_{max} and $t_{1/2}$, while the EC_{50} remained unaffected; the Hill co-efficient (n_H) was also unchanged (Fig. 3).

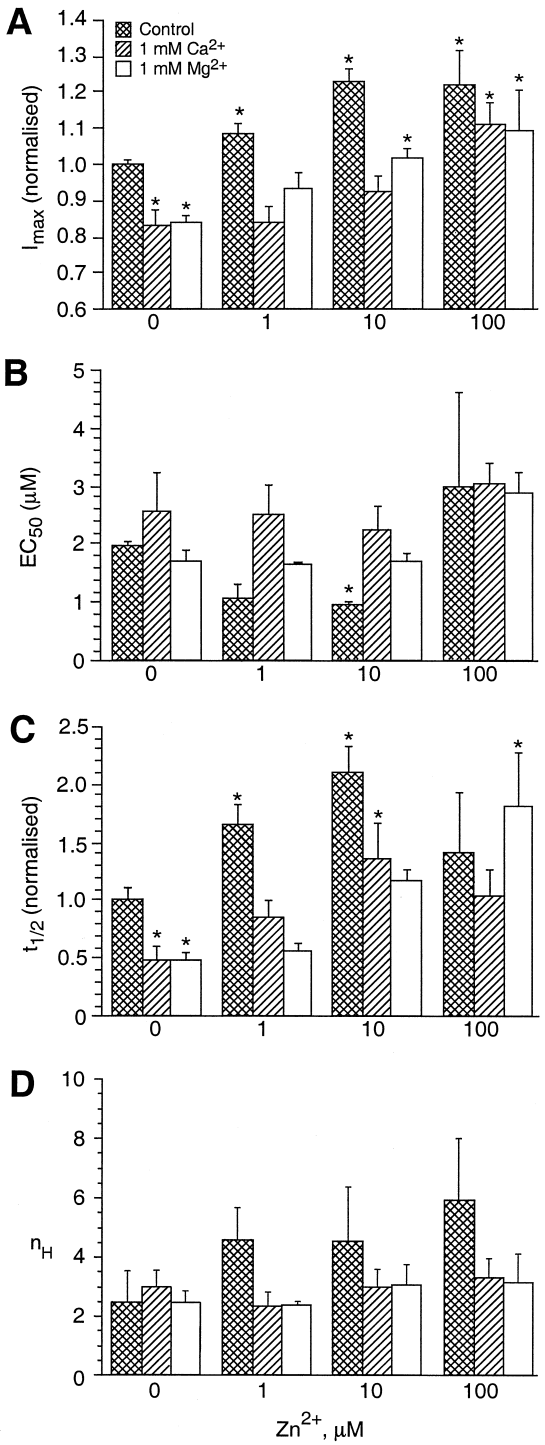

Fig. 3. The effect of Zn^{2+} on functional parameters in 5-HT_3 receptors and their modulation by Ca^{2+} and Mg^{2+}. Data obtained in divalent cation-free solution (filled bars), in the presence of 1 mM Ca^{2+} (hatched bars) or in the presence of 1 mM Mg^{2+} (open bars). Data have been normalised to maximal current evoked by 30 μM 5-HT in divalent cation-free solution. (A) Peak current (I_{max}); (B) EC_{50}; (C) half-time of desensitization ($t_{1/2}$); (D) Hill coefficient (n_H). Data = mean ± S.E.M., $n = 4$ or 5. * Significantly different $P < 0.05$, compared to control using repeated measures ANOVA followed by Dunnett's test for multiple comparisons.

3.2. Effects of Zn^{2+} in the presence of Ca^{2+} or Mg^{2+}

The enhancement by Zn^{2+} was modified in the presence of Ca^{2+} and Mg^{2+} (Fig. 1). To further explore the effects of these ions, functional parameters were investigated. Both Ca^{2+} or Mg^{2+} reduced I_{max} by approximately 20% in the absence of Zn^{2+}, but an increase caused by Zn^{2+} was still manifest in the presence of either ion (Fig. 3A), although a higher concentration of Zn^{2+} was required. The same pattern was reflected in the effect of Zn^{2+} on the desensitization rate (Fig. 3B): both Ca^{2+} and Mg^{2+} significantly reduced $t_{1/2}$, but the effect of Zn^{2+} to increase $t_{1/2}$ was still present, although again there was an apparent rightwards shift of the concentration–response curve. In contrast, the decrease in EC_{50} by 1 and 10 μM Zn^{2+} was abolished in the presence of 1 mM Ca^{2+} or 1 mM Mg^{2+} (Fig. 3C). Overall, the effects of Mg^{2+} tended to be similar to, but less than those of Ca^{2+}, as has been previously reported (e.g. Peters et al., 1988) suggesting that Mg^{2+} acts at the same site(s) as Ca^{2+}, but with a lower efficacy.

3.3. Effects of Zn^{2+} on radioligand binding

Examination of the effect of Zn^{2+} (1–100 μM) on the radioligand binding of a 5-HT_3 receptor antagonist or agonist revealed no effect on [^{3}H]granisetron binding, but an increase in [^{3}H]mCBPG binding at both 1 and 10 μM ($13 \pm 4\%$ and $28 \pm 7\%$, respectively, $n = 3$). Saturation plots of [^{3}H]mCBPG binding showed that there was no change in K_d in the presence of Zn^{2+} but an increase in the maximum number of [^{3}H]mCBPG binding sites was observed at both 1 and 10 μM Zn^{2+} (Table 1). No change in either parameter was observed at 100 μM Zn^{2+}.

3.4. The permeability of the 5-HT_3 receptor ion channel to Zn^{2+}

To assess the permeability of the ion-channel of the 5-HT_3 receptor to Zn^{2+}, the response of a supramaximal concentration of 5-HT (30 μM) was recorded in the presence of 140 mM Na^+ (and no other metal ions) and subsequently in 70 mM Zn^{2+} (and no other metal ions). When Zn^{2+} was the only cation available to carry the current, the inward current was either abolished or much reduced, being less than 5% of that when Na^+ was present (Fig. 4). This suggests that the 5-HT_3 receptor ion channel is probably impermeable to Zn^{2+}.

Table 1
[^{3}H]mCBPG binding parameters in the presence of Zn^{2+} ($n = 3$–6)

	Control	1 μM Zn^{2+}	10 μM Zn^{2+}	100 μM Zn^{2+}
K_d (nM)	1.00 ± 0.26	1.22 ± 0.17	1.17 ± 0.33	0.91 ± 0.13
B_{max} (pmol/mg protein)	2.46 ± 0.17	3.00 ± 0.32[a]	3.15 ± 0.24[a]	2.51 ± 0.22

[a] Significantly different to control, paired *t*-test.

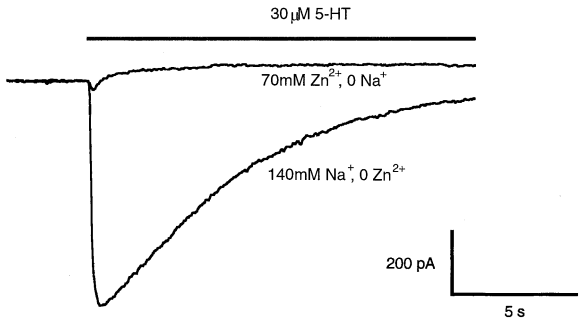

Fig. 4. Permeability of recombinant murine 5-HT_3 receptors expressed stably in HEK 293 cells to Zn^{2+}. The figure shows two consecutive traces from the same cell superimposed, one after exposure to 30 μM 5-HT in extracellular solution containing 140 mM Na^+ as the only metallic species, and the second after exposure to 30 μM 5-HT in extracellular solution containing 70 mM Zn^{2+} as the only metallic species (osmolality was maintained by substituting mannitol for Na^+). The peak current in Zn^{2+} only solution is only 4.0% of that obtained in Na^+ only solution. The long-term change in leak current seen in the second trace is a non-specific effect due the change in $[Ca^{2+}]_o$; untransfected HEK 293 cells showed a similar effect (data not shown). The data are from one cell but are representative of three different cells.

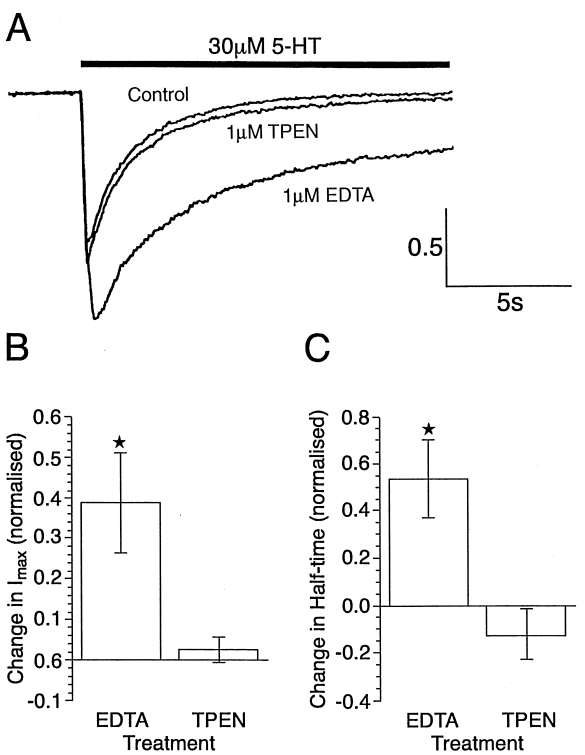

Fig. 5. Effect of Ca^{2+}/Mg^{2+}- and Zn^{2+}-free extracellular solutions on response of 5-HT_3 receptors. (A) Three consecutive superimposed traces recorded from the same cell after exposure to 30 μM 5-HT in extracellular solution containing no added Ca^{2+}, Mg^{2+} or Zn^{2+} (control), 30 μM 5-HT in Ca^{2+}- and Mg^{2+}-free extracellular solution containing 100 μM EDTA and Zn^{2+}-free extracellular solution containing 1 μM TPEN. Panels B and C show the pooled, normalised data of I_{max} and $t_{1/2}$ respectively of six similarly treated cells. $^*P < 0.05$, compared to control using repeated measures ANOVA followed by Dunnett's test for multiple comparisons.

3.5. There is no ''high affinity'' binding site for Zn^{2+}

Although all solutions were made up using analytical grade reagents and deionised water, there nevertheless exists the possibility of contamination by low (nanomolar) concentrations of Zn^{2+} and/or other heavy metal ions, and such low concentrations of Zn^{2+} have been shown to have effects on the NMDA receptor (Paoletti et al., 1997). To assess whether low concentrations of Zn^{2+} had any effects on the 5-HT_3 receptor, a maximal concentration (30 μM) of 5-HT was applied in the presence and absence of 1 μM TPEN (*N,N,N′N′*-tetrakis-(2-pyridylmethyl)-ethylenediamine, a chelator of heavy metal ions) or 100 μM EDTA (ethylenediamine tetra-acetic acid; a chelator of divalent cations) in nominal Ca^{2+}/Mg^{2+} and Zn^{2+}-free extracellular solutions. TPEN had no effect on either peak current or rate of desensitisation (Fig. 5) suggesting there is no high affinity binding site for Zn^{2+} on the 5-HT_3 receptor. By contrast, co-application of 100 μM of EDTA caused a marked increase in peak current and $t_{1/2}$, suggesting that sufficient concentrations of divalent cations lingered in the bath to affect both peak current and the rate of desensitization. As TPEN, which binds to a wide range of metal ions (although not to Ca^{2+} and Mg^{2+}) with much higher affinity than EDTA (Arslan et al., 1985), had no

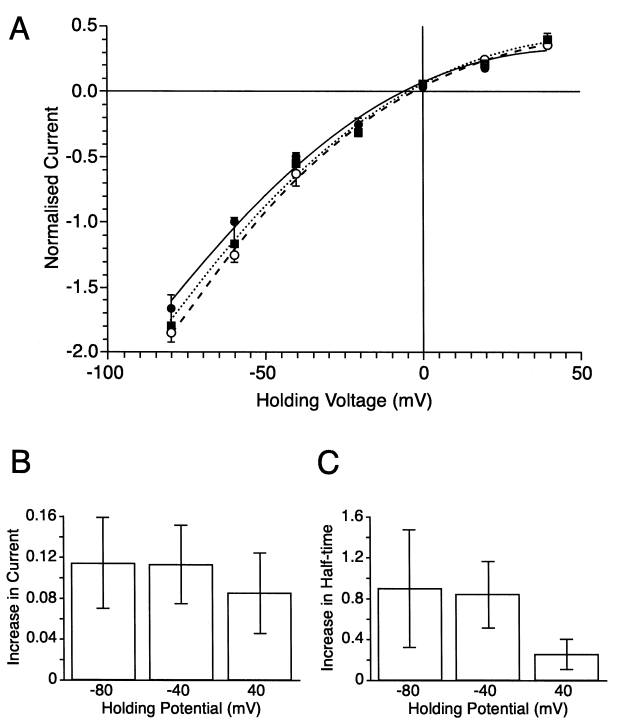

Fig. 6. Voltage dependence of the effects of Zn^{2+}. (A) Current/voltage plot of pooled, normalised data ($n = 3–7$ for each point). Reversal potentials are −3.7 mV (control), −4.4 mV (1 μM Zn^{2+}), and −3.0 mV (10 μM Zn^{2+}). (B) Increase of I_{max} caused by 1 μM Zn^{2+} at three different holding potentials (−80, −40 and +40 mV). (C) Increase of $t_{1/2}$ caused by 1 μM Zn^{2+} at three different holding potentials (−80, −40 and +40 mV).

effect, there is a high probability that these 'contaminant' divalents are Ca^{2+} and Mg^{2+}.

3.6. Voltage dependence of Zn^{2+} effects

To assess the voltage dependence of Zn^{2+}, its affects on I_{max} and $t_{1/2}$ were investigated at different membrane potentials (Fig. 6). The reversal potential and rectification properties of the 5-HT_3 receptor were also unaffected by 1 and 10 μM Zn^{2+} (Fig. 6A), and 1 μM Zn^{2+} had similar effects on I_{max} and $t_{1/2}$ at both positive and negative holding potentials (Fig. 6B and C).

4. Discussion

4.1. Effect of Zn^{2+} on 5-HT_3 receptor function

The action of Zn^{2+} (1–10 μM) on recombinant 5-HT_3 receptors in the absence of other divalent cations is to potentiate 5-HT induced responses; the potentiation is caused by an increase in maximum current, a decrease in the rate of receptor desensitization and a decrease in the apparent receptor affinity (EC_{50}). However, at a higher concentration (100 μM) only one of these effects was manifest, that of enhancing I_{max}. These data support and extend results obtained in a previous study on recombinant 5-HT_{3A} receptors (Gill et al., 1995), which showed enhancement of current amplitude (I_{max}) at low Zn^{2+} concentrations, although these authors also report inhibition at high Zn^{2+} concentrations. Interestingly, three studies, Lovinger (1991), Emerit et al. (1993) and Uki and Narahashi (1996), which used native 5-HT_3 receptors, reported only inhibition of the 5-HT_3 receptor-mediated response by Zn^{2+}. The recent identification of the novel 5-HT_{3B} receptor subunit (Davies et al., 1999) provides a possible explanation for these observations: hetero-oligomeric receptors have different characteristics to homo-oligomeric (5-HT_{3A} subunit only) receptors (Davies et al., 1999); and, although it has not yet been examined, it is a reasonable explanation that the ability of Zn^{2+} to enhance 5-HT_3 receptor function may be a property restricted to homomeric 5-HT_{3A} receptors.

Other neurotransmitter-gated channels which have enhanced function in the presence of low concentrations of Zn^{2+} are glycine (Bloomenthal et al., 1994; Lynch et al., 1998), α-amino-3-hydroxy-5-methylisovazole-4-propionic acid (AMPA) (Rassendren et al., 1990) and P2X (Li et al., 1993; Li et al., 1996) receptors. Although these receptors are all structurally distinct, the modulation of their responses by Zn^{2+} is broadly similar, and resembles the modulation observed in the present study, with low (0.1–100 μM) Zn^{2+} concentrations enhancing responses, while higher (0.1–10 mM) concentrations have no effect or cause inhibition. Some of these authors suggest that these proteins possess multiple Zn^{2+} binding sites, with either potentiatory or inhibitory properties, and for the glycine receptor at least, there is evidence to support this proposal from kinetic studies (Bloomenthal et al., 1994; Lynch et al., 1998). The latter authors have also examined the potentiation effects of Zn^{2+} on this receptor in more detail, and they conclude that when Zn^{2+} binds it acts allosterically on the agonist transduction pathway at a site remote from the agonist binding site (Lynch et al., 1998). Our radiolabeled agonist binding data, which shows an apparent increase in the number of binding sites with no change in binding affinity, would also be consistent with Zn^{2+} causing a structural change in the protein away from the binding site. This change apparently reveals more binding sites; there are potentially five binding sites on the molecule (one/subunit), but only two or three need be occupied to open the channel ($n_H = 2.4$), therefore, it is possible that some are unavailable to ligands. A structural change could expose these previously silent sites. The lack of effect observed for [^{3}H]antagonist binding may be because the change caused by Zn^{2+} is insufficient without the conformational change to the protein caused by agonist binding. Interestingly, a previous study examining the effect of Zn^{2+} on the binding of [^{3}H]GR65630 (3-(5-methyl-1*H*-imidazol-4-yl)-1-(1-methyl-1*H*-indol-3-yl)-1-propanone), another 5-HT_3 receptor antagonist, reported an increase in K_d but no change in B_{max} (Nishio et al., 1994). It is difficult to reconcile these results with our data, but the differences may have arisen because their experiments were performed on solubilized native (rat brain) 5-HT_3 receptors.

Specific amino acid residues that appear to form part of a Zn^{2+} binding site have been identified in several receptors; for example, a histidine residue (292 or the equivalent 267) that forms part of the pore lining region in the β1 and β3 subunits of $GABA_A$ receptors (Wooltorton et al., 1997; Horenstein and Akabas, 1998). Interestingly, however, a histidine residue located in a different region, the N-terminal domain, appears to be part of the Zn^{2+} binding site in GABA-activated ρ1 receptor subunit homomers (Wang et al., 1995), suggesting that Zn^{2+} binding sites have evolved at distinct locations even on closely related proteins. It also appears that even small changes in the amino acid sequence of the protein can lead to large changes in Zn^{2+} modulatory effects: in a recent report by Palma et al. (1998), Zn^{2+} inhibits acetylcholine-induced responses in α7 nACh receptors in a competitive, voltage-independent manner with an IC_{50} of approximately 10 μM. However, in the same receptor with a single amino acid change in the channel domain (L247T), low concentrations of Zn^{2+} (nM) now potentiate acetylcholine-induced effects, while high concentrations (mM) inhibit in a voltage-dependent manner. In addition, Zn^{2+} at concentrations as low as 1 pM acts as an agonist. Thus, it appears that the Zn^{2+} binding site is at or near the agonist binding site, one or both of which are presumably structurally modified by the

L247T mutation; alternatively more and/or perhaps different Zn^{2+} binding sites are revealed by the changed structure. It is therefore not surprising that Zn^{2+} may have quite different modulatory effects at recombinant (5-HT_{3A} receptor subunits only) and native (probably 5-HT_{3A} plus 5-HT_{3B}) receptor subunits.

4.2. Ca^{2+} and Mg^{2+} attenuate the effect of Zn^{2+}

The inhibitory effects of Ca^{2+} and Mg^{2+} on the 5-HT_3 receptor are well documented (Peters et al., 1988, 1993; Yang, 1990; Gill et al., 1995; Brown et al., 1998). We report similar effects in this study, with Ca^{2+} and Mg^{2+} causing a decrease in I_{max} and an increase in the rate of recovery from desensitization, both effects opposite to those observed with Zn^{2+}. The effects of Zn^{2+}, however, were still manifested in the presence of either ion, although there was an apparent shift to the right of the Zn^{2+} concentration–effect curve. This suggests possible competition of Zn^{2+}, Ca^{2+} and Mg^{2+} for a common binding site. Brown et al. (1998), who observed a depression of channel conductance in the presence of divalent ions, propose that there is a binding site for Ca^{2+} and Mg^{2+} within the channel. As they suggest that divalents bind to this site with higher affinity than monovalent ions, and thereby retard their flux, it is unlikely that this is the site through which Zn^{2+} mediates its enhancing properties; in addition the effects of Zn^{2+} at low concentrations are voltage-independent. However, it is possible that this channel site could be an alternative Zn^{2+} binding site, binding to which then results in inhibition in the same way as Ca^{2+} and Mg^{2}. Some support for this hypothesis comes from the studies of Lovinger (1991) on native 5-HT_3 receptors, where the inhibition by Zn^{2+} is voltage-dependent.

Ca^{2+} and Mg^{2+} also attenuate the effect of Zn^{2+} to slow the rate of recovery from desensitization, the former ions again causing an apparent rightward shift of the Zn^{2+} concentration–effect curve. How binding of divalent ions modulates receptor desensitization is not yet clear; the rate of recovery from desensitization of the 5-HT_3 receptor is a complex phenomenon and probably involves the interconversion of many states (Van Hooft and Vijverberg, 1996). Several modulators of 5-HT_3 receptors, including 5-hydroxyindole (Kooyman et al., 1993), ethanol (Zhou et al., 1998) and intracellular Ca^{2+} (Jones and Yakel, 1998) act by changing the rate of recovery from desensitization but their actual mechanisms and/or binding sites are poorly understood. Nevertheless, the rate of desensitization may be an important parameter in the regulation of 5-HT_3-mediated responses in vivo.

4.3. Permeability of the 5-HT_3 receptor to Zn^{2+} ions

The 5-HT_3 receptor is a non-selective cation channel (Jackson and Yakel, 1995) and, in vivo, the bulk of the 5-HT_3 receptor-mediated current is likely to be carried by Na^{+} ions. Nevertheless, it also has some permeability to divalent cations (Hargreaves et al., 1994) and recent reports suggest structural variants which may be highly permeable to Ca^{2+} (Rondé and Nichols, 1998; Van Hooft et al., 1998). In view of this, we tested the permeability of the 5-HT_3 receptor to Zn^{2+} ions by massively increasing the external concentration of Zn^{2+} to 70 mM, and removing all other metallic cations from the solution. Despite problems caused by Zn^{2+} ions breaking the seal between cell membrane and micropipette, our data suggest that the amplitude of current carried by Zn^{2+} in the absence of other metal ions was less than 5% of the magnitude of the current carried by Na^{+} (Fig. 4). Thus, Zn^{2+} entry into the cell via the 5-HT_3 receptor is likely to be negligible.

4.4. Conclusions

We conclude that Zn^{2+} at low concentrations (1–100 μM) can modify the responses of 5-HT_3 receptors, in both the presence and absence of physiological concentrations of Ca^{2+} and Mg^{2+}. These concentrations of Zn^{2+} are probably sufficient to modify 5-HT_3 receptor responses in vivo: estimates vary between the background concentration of 0.1–0.2 μM within the cerebro spinal fluid (CSF) (Palm et al., 1983) to possibly over 200 μM within the synapse (Frederickson et al., 1983), and Zn^{2+} has been shown to be present in nerve terminals and can be released upon stimulation (Assaf and Chung, 1984; Howell et al., 1984). The bimodal nature of Zn^{2+} effects suggests that there is more than one Zn^{2+} binding site, and the fact that Zn^{2+} effects can be modulated by Ca^{2+} and Mg^{2+} suggests one or more of the Zn^{2+} binding sites may be common to all three cations. As there is little or no Zn^{2+} entry through the 5-HT_3 receptor channel, the Zn^{2+} binding sites are unlikely to be intracellular. Combining our data with previous work, we propose that the site that modulates enhancement resides in the N-terminal extracellular domain, while the site that results in inhibition may be located in or close to the channel. The recent identification of Zn^{2+} binding sites on GABA (Wang et al., 1995; Wooltorton et al., 1997; Horenstein and Akabas, 1998) and NMDA receptors (Choi and Lipton, 1999) suggests that, in future, it should be possible to clarify the position of these sites in more detail.

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