Journal of Biomedicine and Biotechnology • 2004:1 (2004) 10–15 • PII. S1110724304302020 • http://jbb.hindawi.com

RESEARCH ARTICLE

Patch Clamp Study of Serotonin-Gated Currents via 5-HT Type 3 Receptors by Using a Novel Approach SHAM for Receptor Channel Scanning

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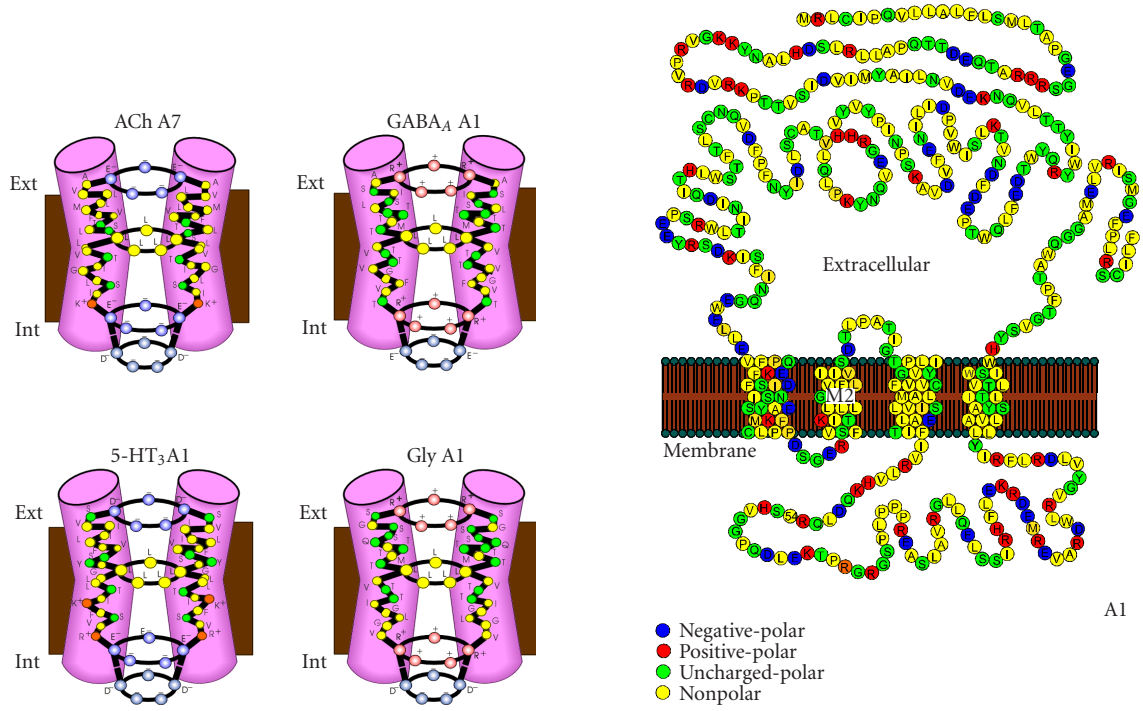
Received 22 February 2003; revised 26 April 2003; accepted 3 June 2003

We studied 5-hydroxy tryptamine type 3 (5-HT_3) receptors transfected in tsA-201 cell line to examine serotonin-induced whole cell currents. Using the site-directed mutagenesis technique, we individually mutated each residue in the membrane-spanning M2 segment to histidine. A high proportion of tsA-201 cells cotransfected with the cDNAs of 5-HT_3R and CD8 produced large amplitude responses (0.5–7.0 nA) to serotonin. The dose-response curve of wild-type (WT) receptor ranging from 0.5 to 500 μmole increases its K_d values, and V_{max} of 5-HT_3R falls at low external pH as if protonation of an acid group is enough to block the channel. Lysine at position 281, a basic residue, is more susceptible to acidification-induced blockade of the 5-HT_3R channel. Dose-response curves of K281S (replacing lysine at the 281 position with serine) at different pH are not significantly modulated, and histidine substitutions at the three consecutive positions 293, 294, and 296 eliminate the pH block of the channel.

INTRODUCTION

Serotonin (5-HT) is a neurotransmitter in the central nervous system (CNS) of vertebrates and invertebrates [1]. In vertebrates, 5-HT participates in the regulation of various physiological functions, including pain perception, blood pressure, sleep, homeothermia, and sexual activity. It is also believed that 5-HT may participate in the expression of symptoms of certain psychiatric disorders such as depression and anxiety [2]. In 1991, however, evidence was presented indicating that the 5-HT_3 receptor is a ligand-gated ion channel which, when activated, causes fast depolarizing responses in neurons [3]. Thus 5-HT_3, like acetylcholine, GABA, and glutamate, activates both G protein-coupled receptors and ligand-gated ion channels. The four hydrophobic putative transmembrane segments, found in other ligand-gated channel sequences and traditionally denoted as M1 through M4, are also present in the deduced amino acid sequence of the 5-HT_3 receptor [4]. The M2 segment shows particularly strong homology with other ligand-gated channels (Figure 1a). Similar to other (ligand-gated ion channels) LGIC receptors, more than one subunit have been identified. Two splice variants of A subunit [5] and B subunit have been cloned [6]. In this study we have endeavoured to identify the amino acids lining the A homomeric 5-HT_3 receptor channel. Using site-directed mutagenesis in combination with patch clamp technique, we have identified the amino acids that appear to line the channel and are critical for the interaction with the ions passing through the channel.

Karlin's group has developed an approach termed the substituted cystine accessibility method (SCAM) [7] which they have used to show that the M2 domain of the A subunit of the mouse AChR is in alpha-helical conformation interrupted by an extended structure in the centre. We propose to examine the structure of the M2 region of the A 1 subunit of 5-HT_3R (Figure 1b) using a slightly different yet potentially more powerful approach. We introduced histidine substitutions in the M2 region of the 5-HT_3R to create a proton binding site (as the imidazole side chain of histidine can reversibly coordinate with Zn, Ni, and proton) at predetermined positions within the M2 domain, an approach we just had to term the substituted histidine accessibility method (SHAM). Any proton blockade observed in the mutant channels will be due to binding at the introduced site. We created a series of single histidine substitutions along the M2 region of the 5-HT_3R (residues 258–277 or positions 1–20, Figure 2) for examining the proton binding site, and this periodicity provides the information for the secondary structure of the protein in the M2 domain.



(a) Rings of charged amino acids.

(b) A1 subunit of 5-HT$_3$ receptor.

FIGURE 1. (a) Four hydrophobic transmembrane segments are found in all members of Ligand-Gated Ion Channels (LGICs) sequence and traditionally denoted as M1–M4, modified from [4]; strong homology was found in the M2 region of all the four members, regarding the presence of charged amino acids at either end of the channel and at hydrophobic leucine ring in the centre of the channel. These charged rings are responsible for channel conductance and anion/cation selectivity. (b) Representation of A1 subunit of 5-HT type 3 receptor. Five of such subunits can form a functional homomeric serotonin type 3 receptor.

MATERIALS AND METHODS

5-HT$_3$R isolation and site-directed mutagenesis

We isolated a complementary DNA clone containing the coding sequence of one of these rapidly responding channels; a 5-HT$_3$R subtype has already been isolated by screening a neuroblastoma expression library for expression of serotonin-gated currents in a tsA-201 cell line.

Briefly, a pair of oligonucleotides derived from the published sequence of the original 5-HT$_3$R cDNA was used to amplify a 460-bp fragment from RNA isolated from the murine neuroblastoma line NIE-115 using a reverse transcription/PCR kit (RNA PCR kit, P/E Express). This fragment was used to screen a plasmid cDNA library made from NIE-115 mRNA. A full-length cDNA clone corresponding to the 5-HT$_3$RAs [3] was isolated and subcloned into pALTER (Promega, Madison, Wi).

K281S affects pH and channel conductance

Before generating SHAM mutants, we tested wild-type (WT) 5-HT$_3$R for any pH sensitivity, as we will be using protons for probing the introduced histidine sites in the M2 region. Wild-type 5-HT$_3$R appeared to be pH sensitive (see results), which could complicate our interpretation. For that reason we scanned the channel for the potential candidate or candidates responsible for this pH sensitivity. When we replaced lysine with serine (K281S), this mutant abolished the pH sensitivity observed in WT and there was no significant increase in the single channel conductance as recently documented [8]. This mutant does not show any alteration in the channel native properties. We used this mutant (K281S) as our control "WT" for generating SHAM mutants. Each of the 22 amino acid mutants generated is in fact a double mutant (ie, K281S/293H or K281S/294H, etc).

Site-directed mutagenesis

This process was performed by using the altered sites mutagenesis system from Promega. All mutants were sequenced to confirm the correct mutation have been inserted. The mutant 5-HT$_3$R cDNA was then subcloned into vector PCI (Promega) for transfection studies.

Receptor transfection

Transfection was carried out on cultures of tsA-201 cells. These cells which are derivatives of the widely used HEK-293 cell line were grown in Dulbecco's modified Eagles medium (D-MEM) containing 10% FBS and 100

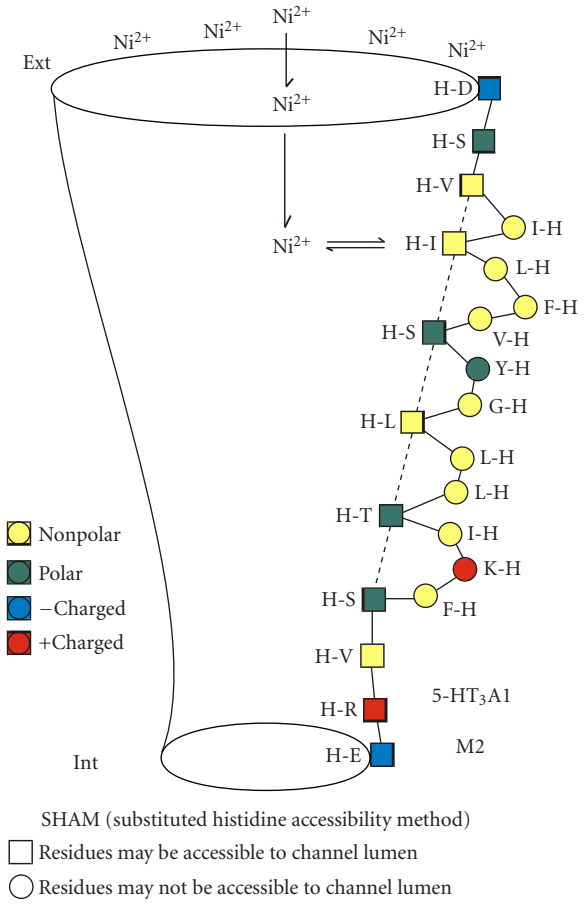


FIGURE 2. Using the site-directed mutagenesis techniques, we have individually mutated each amino acid residue in the membrane-spanning M2 segment to histidine and we call this method SHAM. Every single mutation is a double mutation of K281S. This cartoon (based on Karlins experiments on nicotinic acetylcholine receptor) exhibits hypothetical structure of the amino acid residues lining the A homomeric 5-HT_3R channel. In this diagram, Ni^{2+} (an ideal candidate) is shown to be entering into the channel to react with I293H. In our experiments, we were able to test the substituted histidine only with protons.

unit/mL penicillin and streptomycin (New Life Technologies, NY). Cultures were maintained in humidified atmosphere of 5% CO_2 at 37°C.

Binding studies

The tsA-201 cells were plated at a density of 5×10^6 cells/75 cm^2 and grown for 9 hours prior to transfection. Cells were transfected with 20 μg of 5-HT_3RAs cDNA using calcium phosphate coprecipitation (New Life Technologies), and then incubated for 36 to 72 hours prior to harvesting. Transfected cells were scraped from dishes, washed twice with Dulbecco's PBS (New Life Technologies), then resuspended in 1.0 mL PBS per 100 mL dish. Cells were then homogenized in PBS using a glass homogenizer then centrifuged at 35000 × g for 30 minutes in a Beckman JA20 rotor. Membranes were again washed and resuspended in PBS (1 mL per 100 mm dish). To determine K_d, 100 μL homogenate was incubated at 37°C for 1 hour with varying concentration of [^{3}H] granisetron (NEN, Mass). Specific binding of [^{3}H] granisetron was determined as the bound [^{3}H] granisetron could not be displaced by a saturating concentration of a competing ligand (100 μM of mCPBG or 10 μM MDL-72222 from RBI). K_d values were determined by fitting the binding data to the following equation using Sigma Plot 5.0 (SDR, Australia): $\phi = B_{max}(L)^n/[(L)^n + k_d]$, where ϕ is bound ligand, B_{max} is the maximum binding at equilibrium, L is the free ligand concentration, and n is the Hill coefficient. For k_i estimation, 100 μL of homogenate was incubated at 37°C for 1 hour with varying concentration of inhibitors and [^{3}H] granisetron (NEN, Mass). Binding was terminated by rapid filtration onto a GF/B filter. The IC_{50} value was calculated by fitting the data to the following equation using Sigma Plot 5.0 (SDR): $\phi = 1/(1 + [L/k_i])$, where ϕ is the ratio of [^{3}H] granisetron bound in the presence of inhibitor at concentration L as compared to the amount of [^{3}H] granisetron bound in the absence of inhibitor. See Table 1 for all the values.

Whole cell patch clamp experiments

The tsA cells were plated at a density of 0.25×10^6 cells/27 cm^2 dish. When confluent, cells were split into 100 mm culture dishes at a density of 30%–40% confluence and cotransfected 6–12 hours later with 20 μg cDNAs (10 μg of each cDNAs for 5-HT_3R and immunological marker CD4). Dyna beads attached with the cells expressing this marker and hence the cells with 5-HT_3R expression could be identified and grown 12 hours prior to transfection. Maximal transfection was observed 36 to 72 hours after transfection.

Transfected tsA-201 cells were transferred to a recording chamber and submerged in symmetrical extracellular recording buffer containing 140 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, pH 7.4, whereas the values of pipettes resistance were between 2–5 MΩ when filled with 140 mM NaF, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH 7.4. Cells were left immersed in the bath solution for 10–15 minutes to allow for cellular stabilization before electrophysiological experimentation was conducted. Cells were clamped in whole cell configuration at a holding potential of −70 mV. An *EPC 9 amplifier* (HEKA) was used for measuring the current elicited in response to the application of agonist (5-HT from RBI), and was best used in combination with the *PULSE software* (HEKA, Mass). Agonists and antagonists were dissolved in extracellular solution and delivered to cells using an in-house rapid perfusion system. For K_d values, current responses for each concentrations were normalized to the maximum response obtained from serotonin and fitted to the equation $\phi = 1/(1 + K_d/[C]^n)$, where ϕ is the normalized current at serotonin concentration [C], K_d is the concentration of serotonin needed to obtain half maximal activation, and n is the Hill coefficient. Cells were exposed to antagonist

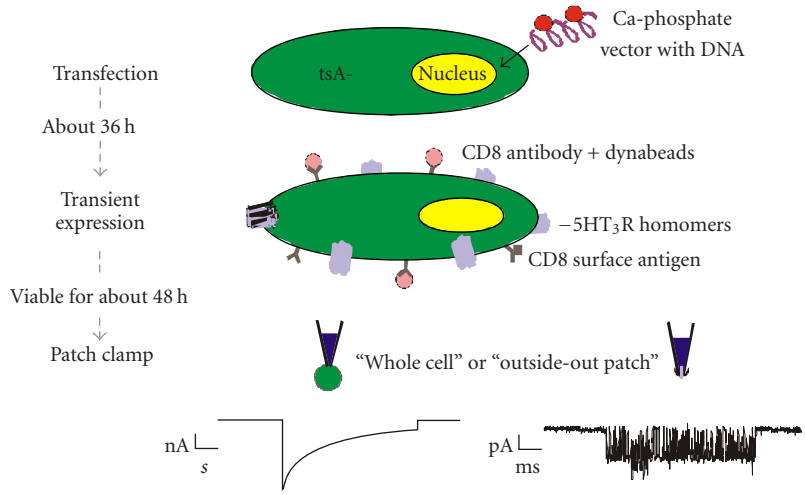


Figure 3. This figure exhibits the methodology used; tsA-201 cells were cotransfected by cDNAs of 5-HT$_3$R and CD8. Patch clamp technique (both whole cell and single channel configurations) was used to determine whether the expressed A1 receptors are responsible for serotonin-induced fast currents.

Table 1. This table outlines the electrophysiological and binding assay results for wild-type (WT) K281S and histidine-substituted resultant double mutants of the amino acid residues lining the 5-HT$_3$ receptor channel.

Receptor type/pH	I_{max}(nA) 500 μM	Time to peak (s)	E_{rev} (mV)	τ (s)	K_d (μM)	Hill const (n)	V_{max}(μM)	B_{max} (fmols)	K_d (nM)	ki (nM)
WT/7	7.65 ± 0.17	0.0581	−3.5	5.6	7.9	1.83	70.41	5.2	8.1	60
WT/5	3.51 ± 0.12	0.134	−4.12	5.9	12.07	2.08	30.28	1.1	7.2	91
WT/9	6.87 ± 1.10	0.586	−2.1	8.4	14.79	1.76	60.48	3.9	7.1	80
K281S/7	9.1 ± 1.06	0.093	+1.2	11.7	8.86	1.68	90.34	6.2	9.6	90
K281S/5	8.82 ± 1.12	0.254	−1.01	13.6	10.1	1.54	80.87	2.3	10.3	145
K281S/9	8.9 ± 0.98	0.638	−2.1	15.3	10.5	1.75	80.87	4.3	11.5	136
KS293H/7	5.46 ± 0.13	0.791	−3.6	14.6	10.4	1.81	50.42	5.2	9.8	111
KS293H/5	3.01 ± 0.11	0.852	+1.1	15.5	13.6	2.44	20.54	3.6	5.6	123
KS293H/9	4.79 ± 0.21	0.907	−3.1	16.9	14.6	1.98	40.33	8.9	6.8	89
KS294H/7	5.37 ± 0.16	0.689	−2.4	10.0	11.1	2.05	100.12	9.9	6.6	103
KS294H/5	3.264 ± 0.12	0.876	+2.1	11.6	16.8	2.24	60.88	7.3	4.2	76
KS294H/9	4.717 ± 0.21	0.885	−5.1	13.8	13.9	1.56	70.43	6.5	5.8	99
KS296H/7	4.46 ± 0.13	0.143	−2.6	11.8	10.6	1.86	90.67	9.4	7.8	89
KS296H/5	3.08 ± 0.03	0.564	+2.1	14.1	12.5	1.93	70.54	7.5	6.7	90
KS296H/9	3.74 ± 0.14	0.767	−3.6	16.3	16.4	2.21	80.67	8.9	7.3	115

(10 nM of d-tubocurarine) for 30 seconds before coapplying agonist to see the inhibitory responses, which were calculated as a ratio of the serotonin response. Dose response curve obtained by the coapplication of both agonist and antagonist was plotted using Sigma plot version 5 (SDR). Procedure outline from transfection to current generation due to cation passing through 5-HT$_3$R channel is shown in Figure 3 and resultant values in Table 1.

RESULTS AND DISCUSSION

Binding studies experiments were performed to test the receptor expression of 5-HT$_3$ in tsA-201 cell line, whereas patch clamp experiments mostly in whole cell configuration were done to establish the functional properties of WT K281S and double mutants and to identify which amino acids are essential for ion interactions.

A high proportion of tsA-201 cells cotransfected with the cDNAs of 5-HT$_3$R and CD8 produced large amplitude of current (0.5–7.0 nA) in response to serotonin in symmetrical solution (Figure 4a) with an E_{rev} close to zero (Table 1). Nontransfected cells never showed a response to 5-HT (n = 20). The dose-response curve of WT receptor ranges from 0.5 to 500 μmole. K_d values and I_{max} of A homomeric WT 5-HT$_3$R fall at low external pH (Figure 4b), as if *protonation of an acid group* is enough to block the channel. Lysine at position 281, a basic residue, is more

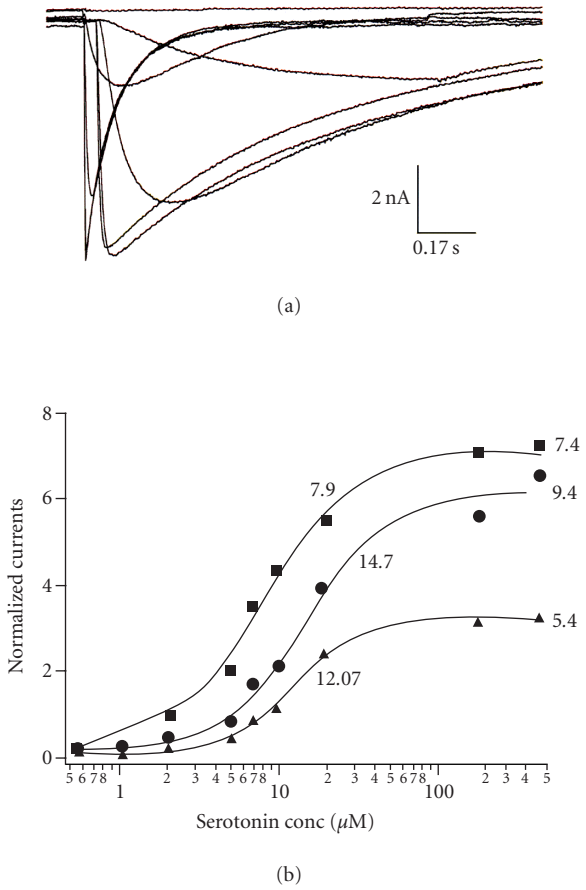


FIGURE 4. (a) WT 5HT$_3$ currents at −70 mV in symmetrical solution in response to different doses (0.5–500 μM) of serotonin at pH 7.4, amplitude of whole cell current increases with increasing serotonin concentration. (b) Dose response curve of I (nA) against log of serotonin concentration, indicating differences in K_d and I_{max} values for WT serotonin receptors at pH 5.4, 7.4, and 9.4.

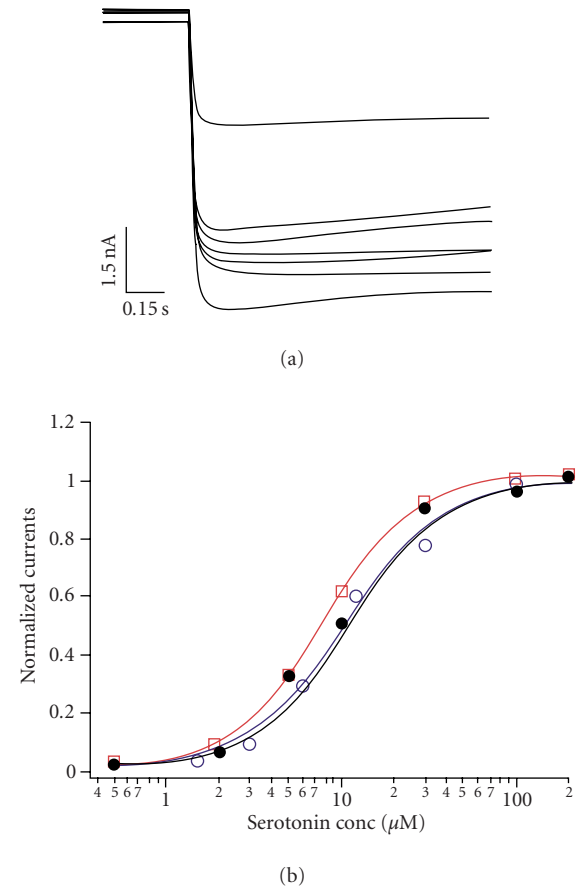
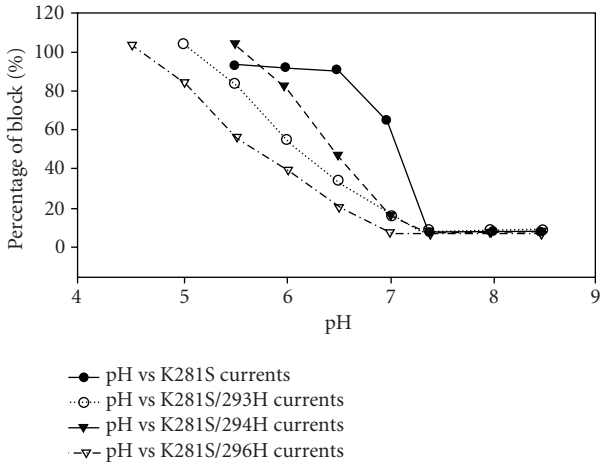


FIGURE 5. (a) A sample of current responses of mutant (K281S) 5-HT$_3$ receptor channels at different serotonin concentrations at pH 7. (b) Dose response curves of mutant 5-HT$_3$ receptor channel at different pH values. Similar K_d and V_{max} values for K281S at pH 5.4, 7.4, and 9.4 ($n = 4$). See Table 1 for comparison between WT and mutant receptor channel activities.

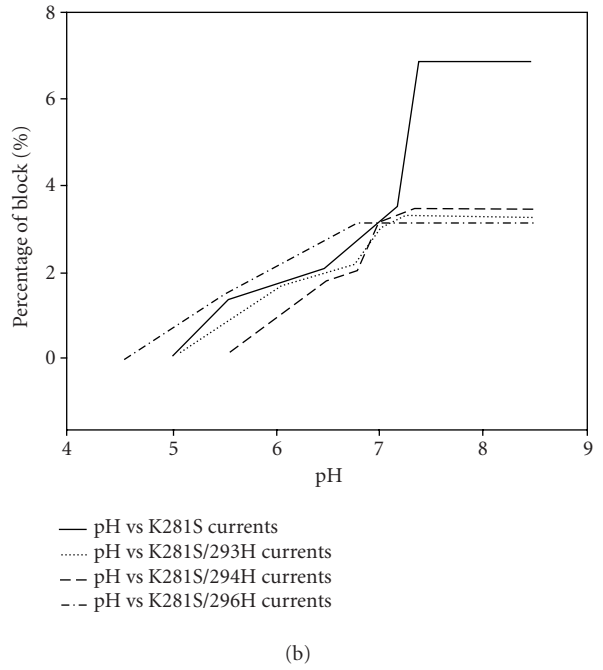
susceptible to acidification-induced blockade of the 5-HT$_3$R channel. Dose-response curves of K281S (replacing lysine at the 281 position with serine) at different pH are not significantly modulated (Figure 5b). Decay time constant is increased in mutant receptors as compared to WT (Figure 5a and Table 1).

Our preliminary studies show that receptors with histidine substitutions at one of three consecutive positions near the extracellular end of the M2 domain (positions 16, 17, and 19) are functional and eliminate the pH block (Figures 6a and 6b) of the channel. I_{max} and K_d values of serotonin currents for K281S at −70 mV at pH 5.4, 7.4, and 9.4 were very similar, and replacing histidine at positions I293H, I294H, and S296H (along with serine in place of lysine 281) in the channel lumen partially removes the pH block especially in case of S296H. Our data indicated that a properly placed histidine residue is an important structural element for functional expression as well as for pH regulation of 5-HT$_3$R. A brief electrophysiological and binding assay profile of A homomeric 5-HT$_3$R (both WT and SHAM mutants) is present in Table 1.

The present study investigated the amino acid critical for ion channel interaction of 5-HT$_3$R A subunit. 5-HT (type 3) is the only member of serotonin family which when activated opens the channel and responses within milliseconds. Recent studies indicated serotonin receptor type 3 involvements in both peripheral and central activities such as emesis, antiarrhythmic activity, pain, age-associated memory impairment, drug and alcohol abuse, migraine, psychosis, and fibromyalgia [9, 10, 11]. We are trying to identify the amino acid lining the channel and exploring the potential of one or more than one amino acid critical for the blockage of channel and hence the resultant pathology such as in the case of Startle disease which results due to a single amino acid mutation in the Glycine receptor channel [12]. Furthermore a complete scanning of 5-HT$_3$ receptor channel will also provide us



(a)



(b)

FIGURE 6. (a) Illustration of the percentage of the removal of current blockage at different pH values of the bath solution by single mutant K281S and double mutants K281S\293H, K281S\294H, and K281S\296H. (b) Variations in the current amplitude with changes in pH for the same mutant of 5-HT3 receptor channels are exhibited. See Table 1 for more details.

with a stronger tool for the specific antagonism of this receptor activity.

ACKNOWLEDGMENT

This work was supported by The Australian Research Council (ARC), Australia, The National Institutes of Health (NIH), USA, and the University of United Arab Emirates.

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