

Multifactorial Analysis of the Dose Dependency of Desensitization of the 5-HT_{2C} Receptor in Oocytes of the African clawed frog, *Xenopus laevis*

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ABSTRACT

Previous research has shown that the 5-HT_{2c} G-protein coupled receptor undergoes agonist-induced desensitization. The purpose of this study was to determine the dose dependent nature of desensitization of 5-HT_{2c} receptors during and across applications of 5-HT. Using voltage clamp techniques, two 5-HT induced current responses, separated by a 4 minute inter-application interval, were measured and characterized by various metrics, including peak current amplitude, integration and reduction phase slope. It was hypothesized that initial inward chloride current responses, as measured by its peak amplitudes and integrations, would increase as the concentration of 5-HT is increased. Additionally, it was predicted that the current reduction rate of the response elicited by the initial application of 5-HT would increase with concentration, while the peak current amplitude and integration of peaks elicited by subsequent applications of 5-HT would decrease compared to peaks elicited by earlier applications. In accordance with previous work, our results showed that the initial amplitude and integration of chloride currents increase with increasing 5-HT concentration and that the amplitude and integration of the subsequent peaks at each 5-HT concentration are smaller than their respective initial peaks. Furthermore, it was found that the rate of current reduction observed in the second peaks at each 5-HT concentration decreases as 5-HT concentration increased. Taken together, our results that the initial agonist-induced 5-HT_{2C} response and subsequent desensitization scale with 5-HT concentration are likely mediated by two signal transduction pathways due to a common pathway intermediate, likely the release in internal calcium mediates both the opening of calcium-dependent chloride channels and the activation of PKC that eventually phosphorylates and closes chloride channels acting as a brake on the system.

INTRODUCTION

The 5-HT_{2C} receptor is a seven transmembrane spanning protein coupled to G proteins. The 5-HT_{2C} receptor is found in moderate density throughout the forebrain and the hindbrain and in high density in the choroid plexus (Van Oekelen, Luyten, & Leysen, 2003). The responsiveness of the 5-HT_{2C} receptor has been widely studied because there is considerable evidence for the receptor's role in regulating many physiological and behavioral processes such

as sleep, affective state, feeding behavior, and thermoregulation (Berg, Stout, Maayani, & Clarke, 2001; Stout, Clarke, & Berg, 2002). Research of the 5-HT_{2C} receptor is also warranted because of the receptor's potential to be a therapeutic target for various neuropsychiatric disorders. 5-HT_{2C} receptors are expressed along the mesocorticolimbic and nigrostriatal dopaminergic systems and their behavior has been shown to modulate the function of the dopamine system (Di Matteo, De Blasi, Di Giulio, & Esposito, 2001). Understanding the behavior of these receptors and how they may regulate dopaminergic systems would contribute to the development of new treatments for depression, schizophrenia, and drug addictions—neuropsychiatric disorders related to dysfunction of dopaminergic neurons (Gavarini et al., 2006).

Previous research has characterized the signal transduction pathway associated with the 5-HT_{2C} receptor. According to these studies, the 5-HT_{2C} receptor is coupled to G $\alpha_{q/11}$ proteins, which activate phospholipase C (PLC). Active PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ then diffuses into the cytoplasm and binds to Ins3P receptors on the endoplasmic reticulum (ER). Binding to Ins3P receptors on the ER opens internal calcium channels on the ER, increasing the internal calcium concentration. This increase in calcium concentration opens calcium-dependent chloride channels in the cell membrane and allows for an influx of chloride ions into the cell (Werry, Gregory, Sexton, & Christopoulos, 2005; Levitan & Kaczmarek, 2002). This influx of chloride ions can be measured as an increase in current using voltage clamp and can be used as a proxy for the activity of the 5-HT_{2C} receptor, as could measurements of levels of proteins involved in the signal transduction pathway.

It has been determined that repeated activation of seven transmembrane spanning proteins, and the 5-HT_{2C} receptor, specifically leads to desensitization of cellular responsiveness (Berg et al., 2001). Desensitization is the progressive decrease in response of a certain neurotransmitter during repeated exposures to the same transmitter (Stout et al., 2002). It has been shown that desensitization of the 5-HT_{2C} receptor is due to phosphorylation of the receptor by various G protein receptor kinases (GRKs). Phosphorylation of the receptor leads to the binding of arrestin, which uncouples the receptor from the G protein and causes the receptor to be removed from the cell membrane via endocytosis (Berg et al., 2001; Gavarini et al., 2006). Internalization of the receptors leads to a decrease in response to a transmitter because by decreasing the number of receptors to bind to ligand, fewer signal transduction pathways may be activated.

It has also been suggested that agonist-induced desensitization may be mediated by other kinases than GRKs. Studies have shown that phosphorylation of the 5-HT_{2C} receptor by protein kinase A (PKA), protein kinase C (PKC), or calcium/calmodulin-dependent kinase (CaMK) may result in desensitization. PKA is activated by cyclic AMP (cAMP), PKC is activated DAG and calcium ions, and CaMK is activated by calcium ions (Parker & Murray-Rust, 2004; Kawasaki et al., 1998; Braun & Schulman, 1995). Phosphorylation of the 5-HT_{2C} receptor uncouples the receptor from its G protein, thereby shutting down further activation of the PLC pathway (Van Oekelen et al., 2003). For each of the kinases mentioned above, their activation, and eventual effect of decoupling the 5-HT_{2C} receptor from its G protein, is caused by second messengers of an agonist-induced signal transduction pathway, which follows the model of agonist-induced desensitization. Chloride channels themselves can be phosphorylated by these kinases, but the effect that phosphorylation has on the channels is unclear. Li et al. (1989) studied the regulation

of chloride channels by PKC in airway epithelia and found that at high calcium concentrations, PKC inactivated the chloride channels but at low calcium concentrations, PKC activated the channels. Following the model of the PLC pathway outlined above, Li et al.'s (1989) findings would support the PKC-mediated mechanism of desensitization, as continued activation of the PLC pathway would cause the cytoplasm of the cell to acquire a high concentration of calcium that would activate PKC to phosphorylate chloride channels and inactivate them.

In a study of the desensitization of the 5-HT_{2C} receptor, Stout et al. (2002) used Chinese hamster ovary (CHO) cell lines to express 5-HT_{2C} receptors and pretreated the cell lines by incubating them in with a solution of 5-HT (10 nM, 30 nM, 100 nM, or 10 μM). The time of incubation was also varied from 0 to 60 minutes. The activity of the 5-HT_{2C} receptor was inferred by measuring the accumulation of IP protein as a percentage of the control; decreases in IP accumulation were indicative of desensitization by arguing that the activation of the 5-HT_{2C} receptor signal transduction pathway was decreasing. Stout et al. (2002) found that as the concentration of 5-HT used to incubate increased, the IP accumulation decreased compared to control and as the time of incubation increased, the IP accumulation decreased. Incubating with the 10 nM 5-HT did not yield desensitization even after incubation for up to one hour.

The results of studies by Gavarini et al. (2006) support Stout et al.'s (2002) findings. Gavarini et al. (2006) measured intracellular calcium responses by loading COS-7 cells with Fura-2/acetoxymethyl ester that fluorescently tags calcium ions. These cells were treated with 1 μM 5-HT twice sequentially with a washout period of 3 to 30 minutes. As the washout period increased, the calcium response (quantified as ratio of the second Fura-2 Ca²⁺ response amplitude divided by the first amplitude) increased, indicating that the amount of desensitization decreased. Gavarini et al. (2006) also measured chloride currents in *X. laevis* oocytes using two-

electrode voltage clamp with a holding potential of -60 mV. These oocytes were exposed to successive 1 μ M applications of 5-HT for 30 seconds each, separated by a 3 minute washout period, which yielded a decrease in the second peak chloride current amplitude, indicative of desensitization (Gavarini et al., 2006). These results support the notion that desensitization of the 5-HT_{2C} receptor increases with increasing concentrations of applied 5-HT and decreasing time intervals between applications of 5-HT. Both of these variations in ligand application increase the frequency of ligand bind to receptor, which likely facilitates the phosphorylation of the 5-HT_{2C} receptor, leading to more (earlier onset, more intense, more prolonged) desensitization.

In the current study, we will characterize the 5-HT_{2C} receptor by analyzing the dose-dependency of its desensitization. Using two-electrode voltage clamp, we will measure chloride currents in 5-HT_{2C} receptors expressed in immature oocytes from the African clawed frog, *Xenopus laevis*, through injection of mRNA from rat brain. Oocytes provide an attractive experimental system for the study of isolated neuronal proteins, such as G-protein coupled receptors, due the ease and specificity with which these proteins of interest are expressed. The oocytes themselves contain very few native receptors that cannot be removed by enzymatic treatments, but still contain the necessary molecular machinery for the faithful translation of injected RNA. Concentrations of perfused 5-HT were chosen to be 30 nM, 100 nM and 300 nM of 5-HT based on prior testing, which produced significant responses within range of our recording systems and current traces characteristic of desensitization upon repeated application of 5-HT. We predict that (1) the amplitude and integration of the inward chloride current trace produced by initial application of 5-HT will increase as the concentration of 5-HT increases, (2) the current reduction rate of the peak elicited by initial application of 5-HT would increase with increasing concentration, (3) the peak current amplitude and integration of peaks elicited by

subsequent applications of 5-HT would decrease compared to peaks elicited by earlier applications, and (4) the current reduction rate of the peaks elicited by subsequent applications of 5-HT would decrease as concentration increased (i.e. the rate of desensitization upon second 5-HT application would not decrease as much for higher 5-HT concentrations).

METHODS

Oocytes Preparation and 5-HT Solutions

In these experiments, 5-HT_{2c} receptors expressed in immature oocytes from the African clawed frog, *Xenopus laevis* via injection of mRNA from rat brain. Oocytes were removed from the *Xenopus laevis* frogs, treated with collagenase to remove the outer layer of follicle cells and shipped by Ecocyte Bioscience US LLC. The rat brain mRNA was purchased from Clontech or Zymagen and injected 2-4 days prior to recording to allow for full expression of rat brain receptors on the oocytes. The presence and properties of these receptors was assessed with electrophysiological techniques, specifically a two-microelectrode voltage clamping technique. Application of the varying 5-HT solutions and Modified Barth's Saline (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 10 mM HEPES, 0.91 mM Ca(NO₃)₂, 0.82 mM MgSO₄, pH 7.4) were carried out by a perfusion system developed in-house. MBS solutions of 30 nM, 100 nM and 300 nM of 5-HT were made.

Technique

Voltage clamping affords the investigator the ability to hold the membrane potential of a cell at a particular value and record the movement of ions initiated by agonist activation of receptors. In this experimental setup, the voltage electrode contained 3.0 M KCl and the current electrode contained 0.5 M KCl and the resistance of the voltage and current electrodes were between 1 and 4 M Ω and between 2 and 10 M Ω , respectively. The Warner Oocyte Clamp

amplifier (OC-725C) and a digital storage oscilloscope (Tektronix) were used in these experiments. Oocytes were voltage clamped at -50 mV or lower depending on the current induced in the cell at rest. A current of -0.25 μ A or less was tolerated. Current traces were recorded in LabChart7 (ADInstruments).

Experimental Design

A 20-second baseline current recording was taken before the first application of 5-HT. Oocytes were perfused three times with 5-HT of varying concentrations for 20-seconds separated by a four-minute IAI washout perfusion with MBS. Per oocyte, 5-HT concentration and IAI were kept constant. The 5-HT concentration (30 nM, 100 nM and 300 nM) was varied between oocytes. At least two oocytes were tested at each concentration.

Data Analysis

All data analyses were carried out in MATLAB, after raw data was imported into MATLAB from LabChart7. Peak amplitudes were calculated by taking the the absolute minimum of the response current trace and subtracting the baseline holding current observed prior to a agonist-elicited response. Peak current amplitudes elicited by first application of 5-HT were compared across concentrations. Additionally, the percent peak amplitude reduction was calculated for the current trace elicited by the second application of 5-HT by normalizing the second current amplitudes to their respective initial amplitudes. Normalizing the second amplitudes to their respective initial amplitudes allows for comparisons of percent peak reduction across concentrations. Lastly, we measured current integrations of each application of 5-HT, for each oocyte to obtain a more accurate calculation of total charge movement. Discrete numerical integration of the current trace over the 20 second application interval was performed using the trapezoid method. The integrations of the first 5-HT concentration application were

compared across different 5-HT concentrations. The percent integration reduction was calculated for the second application of each 5-HT concentration, by normalizing the integration of the current trace elicited by second application of 5-HT to the integration of the current trace of the first 5-HT application. When possible, average peak amplitude, percent peak amplitude reduction, integration and percent integration reduction for each 5-HT concentration were calculated depending on the acquisition of an adequate sample size. Figures and statistical results were generated using GraphPad Prism 6 software.

RESULTS

Inward chloride currents were taken to reflect the degree of receptor activity (Fig. 1), and this assumption was used to characterize the dose-dependent nature of desensitization exhibited by 5-HT_{2C} receptors during a single application of 5-HT and upon subsequent applications. Comparing the results of the first application of 5-HT revealed that increasing the concentration by one half log unit, from 30 nM to 100 nM, produced a response with approximately twice the peak amplitude and 1.5 times the total charge movement (Fig. 2A/B). Curiously, we observed a substantial reduction in the peak amplitude and total charge movement of the 300 nM condition compared to the 30 and 100 nM treatments (Fig. 2A/B). We suspect that this was due to receptor underexpression. Interestingly, the rate of desensitization during a single application appeared to be inversely proportional to the concentration, with the slope of current reduction decreasing as application concentration increased (Fig 2C

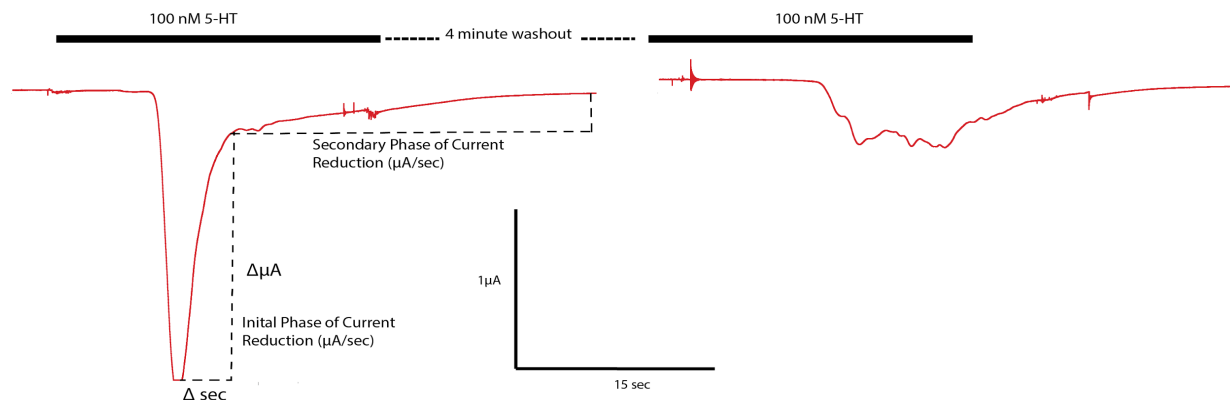


Figure 1. Sample inward chloride current traces produced by two 20 sec applications of 5-HT separated by a 4 minute IAI of 5-HT_{2c} receptors expressed in *Xenopus laevis* oocytes. Initial and secondary reduction phases are indicated by dashed lines; however, only the slope of the initial phase was calculated and considered.

To investigate the properties of desensitization of 5-HT_{2c} receptors over longer time intervals, the properties of the current responses elicited by a second application of 5-HT were studied and compared to the responses of the first application. Across treatments, desensitization appeared to increase with concentration. Specifically, a 61% reduction in peak current amplitude was observed in the second application of 100 nM 5-HT, compared to only a 53% in the 30 nM application (Fig. 3A). Similarly, a 38% reduction in the total charge movement was observed in the 100 nM application, while only 20% reduction occurred with second application of 30 nM 5-HT (Fig. 3B). When considering peak amplitude and total charge movement, the 300 nM condition exhibited unexpected behavior. In both cases, the second application elicited a response equal or larger in magnitude to the first treatment (Fig 3A/B). This effect was particularly pronounced for peak amplitude measurements where in some trials, we observed a second response over 4 times as large as the first. We suspect that these surprising results can again be attributed to receptor underexpression.

Lastly, we wondered how the rate of desensitization during an application, as measured by the slope of current reduction, would change with additional applications. Our results showed

that as concentration was increased, the rate of reduction in the slope across applications decreased (i.e. upon second application, the slope remains steeper and desensitization remains large for higher concentrations) (Fig. 3C). In particular, the mean percentage change in current reduction slopes was found to be significantly higher for the 300 nM condition than the 30 nM and 100 nM conditions found after performing Tukey's HSD post- hoc test ($p < 0.05$).

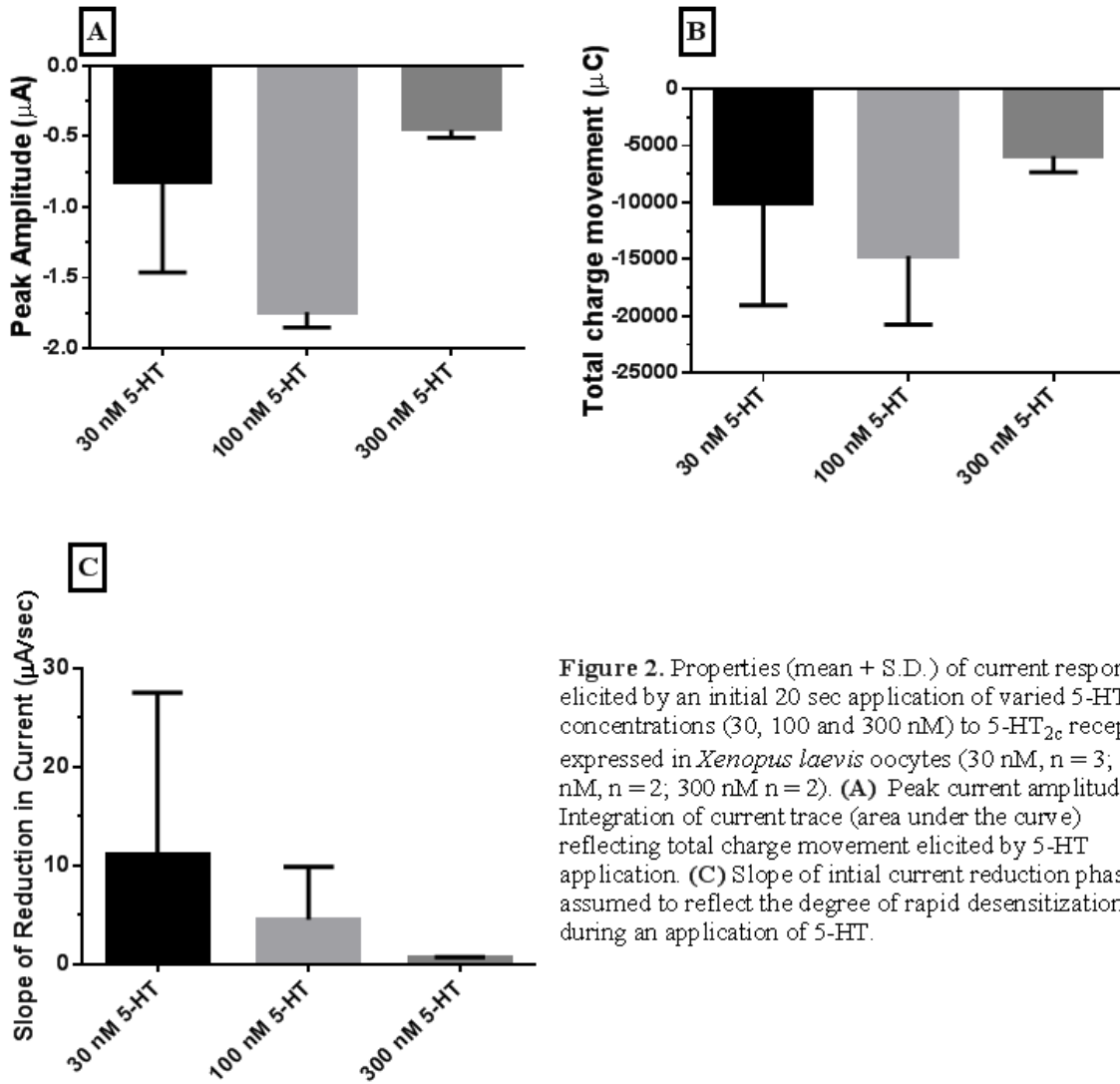


Figure 2. Properties (mean + S.D.) of current responses elicited by an initial 20 sec application of varied 5-HT concentrations (30, 100 and 300 nM) to 5-HT_{2c} receptors expressed in *Xenopus laevis* oocytes (30 nM, n = 3; 100 nM, n = 2; 300 nM n = 2). **(A)** Peak current amplitude. **(B)** Integration of current trace (area under the curve) reflecting total charge movement elicited by 5-HT application. **(C)** Slope of initial current reduction phase assumed to reflect the degree of rapid desensitization during an application of 5-HT.

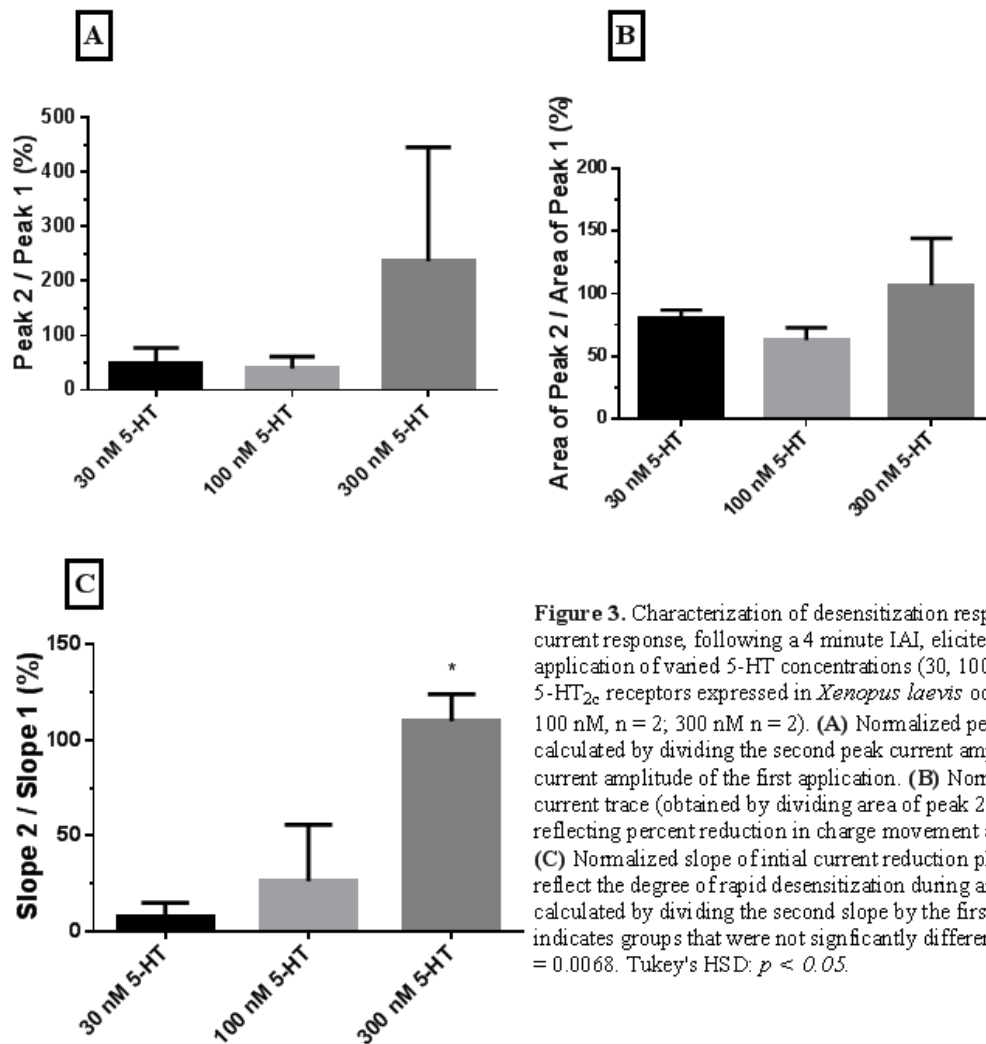


Figure 3. Characterization of desensitization response (mean + S.D.) of current response, following a 4 minute IAI, elicited by the second 20 sec application of varied 5-HT concentrations (30, 100 and 300 nM) to 5-HT_{2c} receptors expressed in *Xenopus laevis* oocytes (30 nM, n = 3; 100 nM, n = 2; 300 nM n = 2). (A) Normalized peak current amplitude calculated by dividing the second peak current amplitude by the peak current amplitude of the first application. (B) Normalized integration of current trace (obtained by dividing area of peak 2 by area of peak 1) reflecting percent reduction in charge movement across the membrane. (C) Normalized slope of initial current reduction phase assumed to reflect the degree of rapid desensitization during an application of 5-HT, calculated by dividing the second slope by the first. Line over bars indicates groups that were not significantly different. * $F(2, 4) = 22.34$, $p = 0.0068$. Tukey's HSD: $p < 0.05$.

DISCUSSION

General Conclusions

The results of the current study support hypotheses (1) that the initial amplitude and integration of the chloride currents increase with increasing 5-HT concentrations, (3) that the amplitude and integration of the second peaks at each 5-HT concentration would be smaller than their respective initial peaks, and (4) that the rate of current reduction of the second peaks at each 5-HT concentration would decrease as the 5-HT concentration increased. Our results did not

support hypothesis (2) that the current reduction rate of initial peaks would increase with increasing 5-HT concentration for we saw the exact opposite trend.

Our finding that the 100 nM 5-HT solution elicited an initial larger peak amplitude and peak integration than the 30 nM solution agrees with our conception that the PLC pathway mediates the opening of calcium-dependent chloride channels, thus applying more ligand to the system would activate more PLC and cause more chloride channels to open resulting in larger current deflections. Finding that the 300 nM 5-HT solution elicited a smaller initial response (in both peak amplitude and integration) was surprising, but may be explained by the fact that the batch of cells used in the 300 nM condition only had two days to express receptor proteins, while those used in the 30 nM and 100 nM conditions had four days. Having less time to express proteins, we assume that less receptor proteins were integrated into the membrane; thus, a lower cellular response was observed as there were simply less binding sites despite the higher concentration of ligand.

Our findings that both the peak current amplitude and the rate of charge flow across the membrane decreased in the second peak compared to the first peak were characteristic of a desensitizing response as reported by Gavarini et al. (2006) who observed a decrease in the second current peak elicited by a second 1 μ M application of 5-HT compared to the first current peak. More interesting were the results when we compared the second peaks across concentrations of 5-HT (the 100 nM to the 30 nM) and observed that as the concentration increased, the amplitude and the integration of the second peaks decreased as the concentration increased. These results support those of Stout et al. (2002) who saw that pretreatment of the CHO cell lines with higher concentrations of 5-HT, IP accumulation decreased, indicative of increased desensitization. Our results and Stout et al.'s (2002) findings suggest that shutdown of

the PLC pathway is involved in desensitization of the 5-HT_{2c} receptor. This suggests that either the GRK-arrestin internalization of receptors, phosphorylation of 5-HT_{2c} receptors, or phosphorylation of chloride channels may mediate desensitization of this receptor. Our proposed relationship between concentration and degree of desensitization is not conclusive however, as the highest 5-HT concentration elicited the highest secondary peak compared to its initial. These results are perplexing and may be explained by the limited sample size where biological variation and experimental error may have heavily skewed our results.

The rate of current reduction in the second peaks were expected to decrease when compared to the initial peaks as concentration increased. Our conception that increasing ligand concentration induces more cellular response as more membrane receptors are bound supports our findings that the higher concentrations of 5-HT continued to exhibit larger chloride currents upon subsequent applications of 5-HT, in other words, less effect of desensitization was observed as there was more ligand to bind to 5-HT_{2c} receptors, despite potentially uncoupling or phosphorylating a number of receptors after the initial 5-HT application. Interestingly, the oocytes exposed to the 300 nM 5-HT elicited the least reduction in desensitization as expected despite having the least amount of time to express 5-HT_{2c} receptors, assumedly.

In general, we have observed that the metrics employed in this study are related in specific ways, and these relationships are most likely supported by the underlying signal transduction pathways. In particular, the slope of current reduction appears to be highly related to the peak amplitude. For example, larger initial peak amplitudes often brought about increased reduction slopes, and as those peak amplitudes decreased with subsequent applications, so often did the reduction slopes (i.e. initial responses maintained sharper and larger peaks, whereas subsequent responses were smaller and flatter). Furthermore, we frequently observed two phases

of current reduction during the first application, with a rapid reduction occurring in the first phase, and more gradual reduction in the secondary phase. Interestingly, in most cases the initial rapid reduction phase was absent in subsequent applications, and only a gradual secondary phase was observed. We propose that a subpopulation of Ca^{2+} -dependent chloride channels, which initially provide the additional capacity for inward current, become phosphorylated by Ca^{2+} -activated PKC, and these phosphorylated chloride channels likely do not become dephosphorylated during the 4 minute IAI. Thus, we do not observe the increased peak amplitude rapid initial phase of current reduction in subsequent applications. It seems probable, however, that GRK-arrestin mechanism mediates the gradual, secondary phase of desensitization observed in all current responses.

Limitations

One potential confounding factor in this study was that sets of oocytes underwent different preparations. The oocytes used to test the 30 nM and 100 nM 5-HT concentrations had four days after being injected with mRNA to express receptor proteins; however, the oocytes in the 300 nM condition only had two days to express due to complications acquiring the oocytes.

Additionally, biological variations in individual oocytes, such as type and number of receptors, may account for differences in chloride current across oocytes trials independent of the perfused concentration of 5-HT. In addition to this, issues in the shipping and handling of the oocytes, damaged oocytes used during certain lab days. Damaged oocytes were difficult to impale with the microelectrodes and their ability to hold voltage across their membranes was compromised.

Suggestions For Future Research

Future research should be conducted to further characterize the dose-dependency of desensitization of the 5-HT receptor and the mechanism of desensitization. To elucidate which signal transduction pathways are implicated in desensitization of the 5-HT_{2C} receptor, oocytes could be pretreated with various kinase inhibitors (e.g. staurosporine, a PKC inhibitor) and then perfused under voltage clamp. If PKC was a second messenger involved in desensitizing the response of the 5-HT_{2C} receptor, then applying staurosporine would likely decrease desensitization of the chloride current. Various kinase inhibitors could be used to determine the different signal transduction pathways involved in desensitization of the 5-HT_{2C} receptor.

Additionally, to further characterize the behavior of the 5-HT_{2C} receptor, an investigation of the time dependency of desensitization in this receptor is warranted. Towards this end, applications of a constant 5-HT concentration could be maintained while varying the IAI. The procedure used by Gavarini et al. (2006) could be used as a model for future research on time dependency of desensitization; Gavarini et al. (2006) tested IAIs of 3, 6, 10, 15, and 30 minute washout intervals. Even with a 15 minute washout period, desensitization was observed, though very small. We would suggest, given our experimental design and set up in comparison with Garavini et al., comparing the percent current peak reduction across IAIs of 1, 3, 10, and 15 minutes.

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