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Role of protein kinase C in agonist-induced desensitization of 5-HT_{1A} receptor coupling to calcium channels in F11 cells

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Abstract

The 5-Hydroxytryptamine 1A receptor (5-HT_{1A}) is expressed both as a pre- and post-synaptic receptor in neurons. The presynaptic receptor preferentially desensitizes compared to post-synaptic receptors, suggesting different underlying mechanisms of agonist-induced desensitization. Using F11 cells as a model of post-synaptic neurons, the present study examined the role of protein kinase C (PKC) and protein kinase A (PKA) in desensitization of the 5-HT_{1A}-receptor by agonist. Desensitization in whole cell experiments was dependent on internal [Ca²⁺] and was blocked by chelation of intracellular Ca²⁺. Using the perforated patch technique, desensitization was reduced when Ba²⁺ was used as the conducting cation. Selective inhibitors of conventional PKC isoforms prevented 5-HT-induced desensitization, whereas an inhibitor of PKA did not. In cells in which 3 PKC/PKA sites located in the third intracellular loop (i3) of the 5-HT_{1A} receptor were mutated (i3, T229A-S253G-T343A), 5-HT-mediated desensitization was reduced (and abolished in the absence of intracellular Ca²⁺). In cells in which a fourth mutation was added (T149 in the second i2 loop), the cells responded similarly to the triple mutants suggesting that phosphorylation of T149 does not contribute greatly to the desensitization induced by 5-HT-mediated activation of PKC. Thus agonist-induced uncoupling of the 5-HT_{1A}-receptor is PKC-dependent, but requires a different set of phosphorylation sites than phorbol ester-mediated PKC activation, suggesting differential recruitment of PKC. Furthermore, these studies reveal that 5-HT_{1A}-receptor desensitization utilizes a different kinase in F11 cells and serotonergic neurons, which may in part account for their differential sensitivity *in vivo*.

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Keywords

Protein kinase C; Serotonin; Calcium current; 5-HT_{1A}-receptor; desensitization

1. Introduction

The 5-Hydroxytryptamine 1A (5-HT_{1A}) receptor functions as an inhibitory autoreceptor on serotonin neurons, and is expressed post-synaptically throughout the nervous system where its dysfunction is implicated in affective disorders such as anxiety and depression (Albert, 2012; Pineyro and Blier, 1999). Antidepressants may preferentially desensitize presynaptic 5-HT_{1A} autoreceptors while post-synaptic 5-HT_{1A} receptors are resistant. The mechanisms for desensitization of 5-HT_{1A} receptor coupling to ion channels remain unclear.

In non-neuronal cells, agonist-mediated uncoupling of 5-HT_{1A}-induced inhibition of cyclic adenosine monophosphate (cAMP) was shown to require Protein kinase C (PKC) activation (Harrington et al., 1994). PKC activity has been shown to phosphorylate the 5-HT_{1A} receptor (Raymond, 1991) and uncouple it from the activation of phospholipase C (PLC) and N-type Ca²⁺ channel inhibition (Liu and Albert, 1991; Swartz, 1993; Wu et al., 2002). Protein kinase A (PKA) also phosphorylates the 5-HT_{1A} receptor and potentiates its desensitization by PKC (Raymond and Olsen, 1994). Prolonged application of 5-HT has also been shown to phosphorylate the 5-HT_{1A} receptor, but in some preparations this appears to occur on sites different from those at which PKC acts (Nebigil et al., 1995; Raymond et al., 1999), raising the possibility that other kinases may be involved. Recently, it was shown that agonist-induced desensitization of 5-HT_{1A}-autoreceptor coupling to inhibition of N-type calcium channels in serotonergic neurons requires both increased [Ca²⁺]_i and PKA activation (Yao et al., 2010). To address desensitization of post-synaptic 5-HT_{1A} receptors, F11 cells were chosen as model of dorsal root ganglion (DRG) neurons (Boland et al., 1991) since they express 5-HT_{1A} receptors that are coupled to Ca²⁺ current inhibition (Cardenas et al., 1997). Unlike in raphe cells, we found that [Ca²⁺]_i and PKC but not PKA were required for 5-HT-mediated desensitization of the 5-HT_{1A} receptor in F11 cells.

In order to study the sites required for agonist-induced desensitization, consensus PKC/PKA phosphorylation sites on the second (i2) and third intracellular (i3) loops of the 5-HT_{1A} receptor were mutationally inactivated and the effect on 5-HT-induced desensitization of receptor coupling to Ca²⁺ channels was examined after transfection into F11 cells. A triple mutant lacking three consensus i3 PKC/PKA sites, or a quadruple mutant lacking those sites and the single PKC site (T149) in the i2 domain were compared to wild-type 5-HT_{1A} receptors. It was previously shown that all three sites on the i3-loop of the receptor were required for maximal (>70%) attenuation of phorbol ester-induced uncoupling to 5-HT_{1A} receptor-induced calcium mobilization (Lembo and Albert, 1995). These mutations did not affect phorbol ester-induced uncoupling of 5-HT_{1A} receptor signaling to N-type Ca²⁺ channel inhibition in F11 cells (Wu et al., 2002) however, mutation of a single site in the second intracellular loop (T149A) attenuated the inhibitory effect of phorbol esters on this response. In contrast we find that agonist-induced desensitization in F11 cells was dependent on sites in the i3 domain, but not altered by additional mutation of the i2 site, suggesting a different PKC specificity for phorbol ester- and agonist-induced desensitization.

2. MATERIALS AND METHODS

2.1 Tissue Culture and Cell Lines

All cells were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% carbon dioxide (Banerjee et al., 1993). The media was changed every day. The 1.9 kb BamHI/XbaI fragment of the wild-type or mutant rat 5-HT_{1A} receptor was subcloned into BamHI/XbaI-cut pcDNA3 (Invitrogen) to generate p3-DBX or mutants, for details see (Wu et al., 2002). The quadruple 5-HT_{1A} receptor mutant (qm) containing mutations at all putative PKC phosphorylation sites, was generated using the triple mutant (i3) plasmid as a template (Kushwaha and Albert, 2005). F11 cells were stably transfected as described previously (Wu et al., 2002) with wild-type receptor (clones designated wt-1 cells) or receptor mutants (i2-mutants: T149A receptor clone designated i2-1 cells, and i3-triple mutants: T229A/S253G/T343A of which one clonal line was called i3-8 cells). The 5-HT_{1A} receptor density of these lines (measured by binding of [³H]-8-hydroxy-2-(di-n-propylamino)tetratin (8-OH-DPAT), the selective 5-HT_{1A} agonist) with the exception of the qm1 line has been described previously (Wu et al., 2002). For the qm1 5-HT_{1A} receptor, the density measured 150 ± 11.9 fmol/mg protein. Values shown are the mean $\pm$ standard error of at least three independent experiments. The percent inhibition of forskolin-stimulated (10 μ M) cAMP accumulation by 8-OH-DPAT (1 μ M) of the qm1 5-HT_{1A}-expressing F11 cells was also measured and cAMP accumulation was inhibited by 55 ± 5 % as compared to 50 ± 7 % in wt-1 cells (Wu et al., 2002). For electrophysiological experiments, F11 cells were differentiated by serum removal from the media. All the groups of F11 clones, transfected or non-transfected, were thus treated the same way. After two days the cells stopped dividing and began extending processes, at which time they were gently re-plated onto glass cover slips. This had the effect of removing the long processes, which facilitated voltage clamp. The current recording was performed one day after re-plating and all of the effects of 5-HT were dose-related, had an ED₅₀ of approximately 10 nM and were competitively antagonized by the 5-HT_{1A}-selective antagonist *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridyl)cyclohexanecarboxamide (WAY100635, 100nM).

Before differentiation the stably transfected F11 cells were treated with 10 μ g/ml puromycin for 24 hours to select for the transfected clones. Periodic treatment with HAT medium (100 μ M hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine) prevented the growth of any cells that were reverting to the parental N18TG2 neuroblastoma phenotype (Greene et al., 1975). The method for the stable transfection of F11 cells has been described previously (Wu et al., 2002).

2.2 Whole-cell Recording

In this study, buffering [Ca^{2+}]_i close to 100 nM allowed desensitization by the agonist of the 5-HT_{1A} receptor expressed in neuronal F11 cells (Wu et al., 2002). Experiments designed to measure Ca^{2+} currents used as a pipette solution (in mM): for [Ca^{2+}]_i ~ 1nM: CsCl 110, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 40, ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) 10, MgATP 4, MgCl₂ 1, GTP 300 μ M, phosphocreatine 14 mM, pH 7.3 CsOH); for [Ca^{2+}]_i ~100nM the following changes were made: HEPES 20, EGTA 4, and CaCl₂ 2. The pH was adjusted to 7.3 using CsOH. Estimates of intracellular [Ca^{2+}] were obtained using the Maxchelator software (webmaxc, standard constants) to be found on the Internet at: <http://www.stanford.edu/~cpatton/maxc.html> (accessed 2/2013).

The extracellular solution was continually perfused at a rate of about 2ml/minute into a bath containing about 1 ml of recording solution. In order to eliminate the contribution of Na⁺

ions to the inward current, 0.1 μM TTX was added to all recording solutions. To establish seals for whole cell recording the following solution was used: this contained (in mM) NaCl 134, HEPES 20, Glucose 10, Sucrose 20, CaCl_2 2, KCl 2.5, and MgCl_2 2. The external recording solution, designed to isolate calcium channel currents contained (mM): TEACl 140, BaCl_2 30 or CaCl_2 30, HEPES 10, Sucrose 20, pH 7.3 with TEAOH. Recordings were carried out at room temperature 22–25°C. The osmolarity of the external solution was adjusted with sucrose such that the pipette solution was 20 mOsmols hypo-osmotic compared to the recording solution (300 mOsmol/liter). This usually prevented cell swelling. Drugs that dissolve in the extracellular solution were added to the perfusate.

2.3 Perforated Patch Solutions

Our successful attempts to obtain perforated patches in cultured F11 cells were carried out using the polyene antibiotic amphotericin B that produces a lower access resistance than nystatin (Rae et al., 1991). Large pipettes were used (approximately 2 M Ω). The pipette was filled with CsMeSO₃ 75mM, CsCl 55mM, MgCl_2 8mM and HEPES 10mM, pH 7.3. 5 μl of a stock of amphotericin B (6mg/100 μl in fresh dimethyl sulfoxide (DMSO) was added to 1.25ml of the pipette solution and the mixture was sonicated for 30 seconds. The pipette tip was filled with solution containing no amphotericin B. The same external solutions were used as in conventional whole cell recording. It was possible to tell that a perforated patch recording was obtained since if the cell recording went whole cell, the Ca^{2+} current ran down completely, within one minute, due to the lack of ATP in the pipette.

An Axopatch 200A patch clamp amplifier was used to voltage-clamp neurons with truncated dendrites and a cell soma with one dimension of at least 20 μm ; using the whole cell configuration. Electrodes, pulled from soda-lime glass capillary tubes, were regularly coated with Sylgard and ranged in resistance from 1.8–2.5 M Ω . The series resistance circuit of the amplifier was used to compensate 80% of the apparent series resistance. Clamp settling time was typically less than 300 μs . When measuring Ca^{2+} currents in TEA, the seal resistance is often greater than 5 G Ω . Subtraction of the leak and capacitance from the current records was done using the Axobasic software system. During the experiment, at regular periods, we obtained leak sweeps. Leak sweeps consisted of 16 averaged hyperpolarizing steps of 10mV. The leak sweep currents were scaled to the appropriate size and then subtracted from the individual current records. The voltage clamp data (measurement of Ca^{2+} current) was filtered at 2 KHz then digitized at 100 μs per point. Voltage protocols were generated and analyzed by an IBM PC clone using the Axobasic patch clamp software and the resultant data written to disk for analysis off line.

2.4 Statistics

The measurements of Ca^{2+} current are expressed as mean $\pm$ S.E.M., unless noted otherwise and in some cases the means were tested for equality using a student's t-test or paired t analysis. The peak calcium current, during a depolarizing step, was measured isochronally in the presence and absence of 5-HT. The data were displayed as peak current against time plots. Calcium current inhibition by 5-HT was expressed as the size of the inhibition by 5-HT, as a percentage of the baseline Ca^{2+} current, followed by the S.E.M. Desensitization was expressed as the reduction in the inhibition compared to the initial effect as a percent measurement. This approach normalizes the response so that it can be averaged over cells. To minimize any effect of Ca^{2+} current rundown, desensitization was measured after three minutes of the application of 5-HT and compared with the initial inhibition. This generally allowed for the full recovery of the baseline Ca^{2+} current after washing off the 5-HT compared to that measured before application of the 5-HT. Comparisons of multiple groups were done using an analysis of variance (ANOVA) followed by Student-Newman-Keul's (SNK) t-test.

3. RESULTS

3.1 Wild type 5-HT_{1A} receptors expressed in F11 cells desensitized after application of 10 μ M 5HT

As shown in Fig. 1A, when calcium (30 mM) was present in the extracellular solution, 5-HT (10 μ M) inhibited calcium current by 55–60% in the F11 cell line (wt-1), expressing wild type 5-HT_{1A} receptors (Wu et al., 2002). After a 3 minute incubation with 5-HT (10 μ M), 5-HT-induced inhibition of calcium current was significantly reduced (c–d), compared to the initial inhibition (a–b) ($P < 0.05$, $n = 6$) (Table 1). The results presented below suggest that this effect is due to receptor desensitization. The 5-HT-induced desensitization measured 34.7 ± 9.5 % (see methods). At submicromolar concentrations of 5-HT, this rapid desensitization was not observed. Since the desensitization was obtained with $[Ca^{2+}]_i$ set to ~ 100 nM in order to mimic the natural $[Ca^{2+}]_i$ we re-examined the effect using the perforated patch technique in which $[Ca^{2+}]_i$ can be considered to be maintained at close to intact cell levels.

Similar results were obtained when the perforated patch clamp technique was applied to the wt-1 cells (Fig. 1B). Using this technique, 5-HT (10 μ M) also induced an inhibition of calcium current when calcium (30 mM) was included in the extracellular solution. After 3 minutes of the application of 5-HT (10 μ M) the inhibition of calcium current by 5-HT was significantly reduced (Table 2, $P < 0.01$). The inhibitory effect of 5-HT was desensitized by 52.7 ± 11.1 % ($n = 7$) but this was not statistically different from the size of the effect measured using whole cell recording. The recordings did not usually last long enough to follow fully the recovery from desensitization but there was a clear trend towards a slow recovery of the original degree of inhibition by 5-HT.

3.2 Calcium and desensitization

To test whether 5-HT-induced desensitization involved channel inactivation Ba^{2+} (30 mM) replaced Ca^{2+} in the extracellular solution, since unlike Ca^{2+} , Ba^{2+} does not inactivate calcium channels. 5-HT was applied to wt-1 cells and the effect was measured as the inhibition peaked, and after 3 minutes of the application of 5-HT (10 μ M). The 5-HT-induced inhibition desensitized by 32.4 ± 5.9 % (Table 1), not significantly different than when Ca^{2+} was present extracellularly. Similar results were obtained in wt-1 cells when the perforated patch technique was used and barium (30 mM) was included in the extracellular solution. Compared to the initial peak of inhibition of calcium current, the inhibition after 3 minutes of the application of 5-HT (10 μ M) was reduced (Table 2) and the desensitization measured 31.6 ± 11.3 % ($n = 7$). These results are summarized in Table 2, which shows that 5-HT-induced desensitization was not reduced significantly when Ba^{2+} carried the current in place of Ca^{2+} in the wt-1 cells, indicating that the desensitization was not due to channel inactivation.

When Ca^{2+} was used as the charge carrier it could potentially induce Ca^{2+} -dependent channel inactivation whose relief on application of 5-HT, as a result of Ca^{2+} current inhibition, might resemble desensitization. This possibility was examined by using longer (100 ms) or shorter (10 ms) duration voltage pulses to elicit the Ca^{2+} current but the peak Ca^{2+} current was measured in the same way in either case. There was no significant difference between the data sets obtained using these protocols suggesting that relief of Ca^{2+} -induced channel inactivation is not a factor in our results and so the data were pooled. The finding that the data obtained using the non channel-inactivating cation Ba^{2+} as permeating divalent were essentially similar, providing that internal Ca^{2+} was buffered close to 100 nM, supported this conclusion.

3.3 10 mM 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) abolished the desensitization to 5-HT of wt-1 cells

In order to test directly whether intracellular Ca^{2+} was required in order to observe the desensitization to 5-HT, 10 mM BAPTA was used in the patch pipette and no Ca^{2+} was added to the pipette solution to buffer the intracellular Ca^{2+} concentration to very low levels <1 nM (not shown). In 5 cells 5-HT-induced desensitization measured only $2.2 \pm 13\%$ and was essentially abolished. This result was compared to a group of 6 cells where Ca^{2+} was buffered to ~ 100 nM, using EGTA (4 mM) and Ca^{2+} (2 mM), added to the pipette solution. In the control group the desensitization measured $34.7 \pm 9.5\%$ ($n=6$, $P < 0.05$ ANOVA and SNK t-test). Ca^{2+} was used as the charge carrier in these experiments. Thus 5-HT-induced desensitization in these cells is calcium-sensitive.

3.4 A Ca^{2+} -dependent PKC mediates the desensitization

Since in other studies of desensitization of the 5-HT_{1A} receptor there was a large reduction in homologous desensitization as a result of phosphorylation by the kinases such as PKA and PKC (Yao et al., 2010) we examined the effect of selective kinase inhibitors on the degree of desensitization to 5-HT in the wt-1 line (Fig. 2).

In a group of wt-1 cells, 200 nM PKA inhibitory peptide PKAI₅₋₂₄ peptide was included in the pipette solution and Ba^{2+} (30 mM) was used as the charge carrier. The desensitization measured $23.8 \pm 7.8\%$ ($n=6$, Fig. 2A) not significantly different from the control value of $32.4 \pm 5.9\%$ ($n=9$, ANOVA for multiple comparisons). The 200 nM concentration is selective for PKA and it was found to be effective at inhibiting PKA under similar conditions in studies of serotonergic neurons (Yao et al., 2010) and in other studies (Cheng et al., 1986).

Since the internal concentration of Ca^{2+} appears to be critical for the appearance of 5-HT-mediated desensitization in these cells we reasoned that a Ca^{2+} -dependent kinase such as PKC might be involved. In 6 cells the selective PKC inhibitor peptide PKCI₁₉₋₃₆ was included in the pipette solution at 20 μM (Diverse-Pierluissi and Dunlap, 1993). In this group of cells the desensitization measured $9.2 \pm 3\%$ (Fig. 2B) which was significantly smaller ($P < 0.05$) than the control group (ANOVA/SNK test).

In order to confirm a role for PKC in the desensitization to 5-HT in F11 wt-1 cells expressing the wild-type 5-HT_{1A} receptor, the effect of 5-HT was measured using the perforated patch technique and, as in the previous experiments, Ba^{2+} was used as the charge carrier. In the control group, the desensitization measured $31.6 \pm 11.3\%$ ($n=7$). In another 5 wt-1 cells the selective PKC inhibitor Go6976 was added to the bath at 200 nM (Fig. 3). 5-HT (10 μM) was then applied and the desensitization was significantly reduced or abolished, measuring $0.7 \pm 1.9\%$ ($P < 0.05$).

3.5 Removing three phosphorylation sites significantly decreased the extent of agonist-induced 5-HT_{1A} receptor desensitization

The i3-8 cell line expressed 5HT_{1A} receptors in which three phosphorylation sites on the i3 loop were mutated. In the i3-8 cells, the desensitization caused by 5-HT (10 μM) was still present although it was much reduced compared with 5-HT-induced desensitization in wt-1 cells expressing wild type 5-HT_{1A} receptors ($P < 0.05$) (Fig. 4). When Ca^{2+} was the external divalent cation, 5-HT inhibited the calcium current (Table 1). In i3-8 cells, after 3 minutes of the application of 5-HT (10 μM) the inhibition of calcium current by 5-HT was significantly reduced ($P < 0.05$), measuring only $14.6 \pm 3.7\%$, significantly less than in the wt-1 cells ($P < 0.05$). When the perforated patch clamp technique was employed (Fig. 4B), 5-HT (10 μM) also inhibited the calcium current when calcium (30 mM) was included in the extracellular

solution. After a 3-minute pretreatment of i3-8 cells with 5-HT (10 μ M), 5-HT-mediated inhibition of calcium current was significantly reduced ($P < 0.05$, Table 2). The desensitization measured $21.5 \pm 7.1\%$ (not different from the whole cell recordings). Thus in both whole cell and perforated patch experiments, the i3 mutant displayed a reduced desensitization of coupling to calcium channels compared to the wild-type 5-HT_{1A} receptor.

When the i3-8 line was examined using the whole cell recording configuration and Ba²⁺ was the charge carrying divalent cation (Fig. 4C), the effect of 5-HT was observed to desensitize by $19.0 \pm 2.2\%$ (Table 1, $P < 0.01$). By comparison, when the perforated patch recording configuration was used and Ba²⁺ was the charge-carrying divalent cation, 5-HT-induced desensitization was abolished, measuring $-2.1 \pm 4.7\%$ ($n=10$) (Table 2, Fig. 4D and Fig. 5 (2)). Thus homologous desensitization of the 5-HT_{1A} receptor, observed using Ba²⁺ as the divalent ion, is entirely dependent on the i3 PKC sites.

3.6 Expression of 5-HT_{1A} receptors with quadruple mutations

In order to address whether the residual desensitization of the i3-8, 5-HT_{1A} triple mutant, was due to T149 of the i2 loop, the additional T149A mutation was incorporated in the 5-HT_{1A} receptor yielding a quadruple mutant line qm (one clonal line was called qm-1). The quadruple mutant responded to 5-HT (10 μ M) with a similar desensitizing profile to the i3-8 cells when recorded using the whole cell configuration. In three qm-1 cells, recorded using Ca²⁺ (30 mM) as the charge carrying divalent, 5-HT-mediated desensitization measured 21% after three minutes of the application of 5-HT. Thus inactivation of the additional i2 loop PKC site did not confer any further resistance to agonist-induced desensitization.

4. DISCUSSION

4.1 Calcium-dependence of homologous 5-HT_{1A}-receptor desensitization

The desensitization observed in the present study was elicited by the agonist 5-HT, and is by definition homologous. 5-HT_{1A} receptors are present in DRG cells *in vivo* and so DRG-derived F11 cells transfected with 5-HT_{1A} receptors were used to model post-synaptic 5-HT_{1A} heteroreceptor regulation. The first finding of note is that the inhibitory effect of 5-HT on Ca²⁺ current undergoes desensitization only when intracellular Ca²⁺ was maintained at a physiologically relevant level of 100 nM (Zucker et al., 1991), as observed in acutely isolated dorsal raphe neurons (Yao et al., 2010). This desensitization is usually overlooked since most whole cell patch studies of Ca²⁺ currents are carried out with [Ca²⁺]_i buffered to very low levels in order to avoid Ca²⁺-dependent Ca²⁺ channel inactivation. In cells expressing wild type 5-HT_{1A} receptors (wt-1), the desensitization measured about 35% in whole cell patch, and about 53% using the perforated patch technique in Ca²⁺ containing medium. By chelating internal calcium using BAPTA, it was found that 5-HT-induced desensitization was calcium-dependent. Similar levels of desensitization were observed when Ba²⁺ was substituted as the charge-carrying divalent to eliminate Ca²⁺ influx, suggesting that it was internal [Ca²⁺] that is critical for desensitization.

A second key finding is the critical role of consensus sites for the phosphorylation of the 5-HT_{1A} receptor on the i3 loop at T229A-S253G-T343A in 5-HT-induced desensitization of the receptor. These sites have previously been shown to be involved in phorbol ester-induced uncoupling of the receptor to calcium mobilization in non-neuronal cells indicating their importance in G $\beta\gamma$ signaling (Lembo and Albert, 1995). Their removal resulted in a significant reduction in the degree of desensitization induced by 5-HT, but a complete block when using Ba²⁺ as the permeant ion. The residual desensitization (20%) seen with Ca²⁺ in the medium in cells expressing the 5-HT_{1A}-i3 triple mutant suggests that additional desensitization may occur downstream of the receptor. This residual desensitization was Ca²⁺-dependent since it was completely abolished when using Ba²⁺ as the permeant ion,

ruling out relief of Ca^{2+} -dependent Ca^{2+} -channel inactivation. Furthermore the same degree of desensitization of the effect of 5-HT on peak Ca^{2+} current was obtained when 10 ms depolarization was used to elicit Ca^{2+} influx as with 100 ms depolarization, suggesting a lack of Ca^{2+} -dependent Ca^{2+} -channel inactivation. On inspection of the perforated patch data, it is interesting to note that the sum of the desensitization measured in i3-8 cells in Ca^{2+} (presumed downstream component) plus that in wt-1 cells in Ba^{2+} (receptor-dependent component) measured 53.1% compared to 52.7 % for wt-1 cells bathed in Ca^{2+} (both components). This suggests that in addition to receptor phosphorylation, mechanisms downstream of the receptor may occur, such as Ca^{2+} -dependent uncoupling of the G-protein or PKC-mediated phosphorylation of the $\alpha 1\text{B}$ subunit of the N-type Ca^{2+} channel (Tosetti et al., 2003; Zamponi et al., 1997). Consistent with PKC-induced inactivation of calcium channels, Ewald (Ewald et al., 1988), showed that PKC down regulation reduced Ca^{2+} current inhibition by neuropeptide Y in cultured DRG cells.

4.2 Roles of PKC sites in homologous 5-HT_{1A}-receptor desensitization

Given the Ca^{2+} dependence of agonist-mediated uncoupling of the 5-HT_{1A} receptor, peptide inhibitors were used to test the specific role of PKA and PKC. In whole cell recordings, PKAI₅₋₂₄ peptide reduced the desensitization by about one-third but this effect did not reach statistical significance. PKCI peptide however, reduced the desensitization by two thirds and the effect was statistically significant (ANOVA SNK t-test, $P < 0.05$). To confirm the role of PKC, the selective membrane permeable PKC inhibitor drug Go6976 was shown to completely block homologous 5-HT_{1A}-receptor desensitization. The sensitivity of the desensitization to PKC inhibitors suggests that the Ca^{2+} sensitivity of the desensitization to 5-HT lies in the ability of 5-HT to activate PKC and not an indirect effect of Ca^{2+} on the Ca^{2+} channel. If the data obtained using Ba^{2+} in perforated patch recording preferentially detects the effect of PKC on the receptor alone, the block by Go6976 indicates that PKC mediates agonist-induced receptor phosphorylation at the i3 sites to mediate desensitization. The Ca^{2+} sensitivity of the desensitization to 5-HT probably lies in the ability of 5-HT to activate a Ca^{2+} -sensitive PKC isoform since the desensitization was sensitive to PKC inhibitors. Activation of PKC has been shown to phosphorylate the 5-HT_{1A} receptor (Raymond, 1991), and these results suggest that agonist-induced phosphorylation at the i3 sites of the 5-HT_{1A} receptor by Ca^{2+} -dependent PKC activation desensitizes receptor-induced Ca^{2+} current inhibition.

4.3 Mode of PKC activation and 5-HT_{1A}-receptor desensitization

An interesting finding from our studies is that the importance of PKC sites in receptor desensitization depends on the mode of PKC activation (directly by phorbol ester or indirectly via 5-HT/PLC). The potential site of phosphorylation T149 in the i2 loop of the receptor has been identified as a high affinity site at which PKC, activated by a phorbol ester, uncouples the 5-HT_{1A} receptor from the G-protein (Wu et al., 2002). However, we find that the absence of T149 does not further attenuate the desensitization induced by 5-HT-mediated activation of PKC. By contrast, the i3 sites of the 5-HT_{1A} receptor were dispensable for PMA-induced uncoupling, whereas these sites were important for agonist-induced desensitization. This suggests that different PKC sites of the 5-HT_{1A} receptor are targeted by PMA versus 5-HT, and that the two signaling pathways must elicit differences in PKC activation. 5-HT activates PKC via a short-lived generation of diacylglycerol (DAG), compared to the persistent phorbol ester - induced activation (Nishizuka, 1995). If activation of the 5-HT_{1A} receptor produces a localized increase in DAG near the i3 loop of the receptor, activating a PKC isoform locally to act on the i3 sites of the receptor; whereas low levels of phorbol ester may activate a range of isoforms more globally within the cell that result in a preferential action on T149 and perhaps other sites. Alternately, activation by agonist treatment of other kinases such as G-protein receptor kinase (GRK) may synergize

with PKC, to mediate additional phosphorylation and homologous desensitization, which would not occur with direct PKC activation.

4.4 Cell specificity of agonist-induced desensitization

An interesting observation was the difference in the requirement for PKA and PKC in models of pre- and post-synaptic 5-HT_{1A} receptors: in raphe neurons. PKA was implicated in Ca²⁺-dependent homologous desensitization of the 5-HT_{1A} receptor (Yao et al., 2010), in contrast to the predominant role of PKC in F11 cells. The relative importance of PKA vs. PKC could depend on the relative levels of kinase or adenylyl cyclase isoforms, particularly of Ca²⁺ sensitive forms of adenylyl cyclase. We have previously observed that 5-HT_{1A} receptors can trigger PLC activation, which occurs in raphe cells (Kushwaha and Albert, 2005), and this pathway could locally activate calcium-sensitive PKA isoforms to mediate autoreceptor phosphorylation. Consistent with this, 5-HT_{1A} agonists increased basal PKA activity in raphe cells (Yao et al., 2010). The combination of PKC and PKA activation could potentially augment 5-HT_{1A} autoreceptor desensitization (Raymond and Olsen, 1994), perhaps accounting for the increased desensitization of the 5-HT_{1A} autoreceptor compared to post-synaptic 5-HT_{1A} receptors following chronic antidepressant treatment *in vivo* that appears crucial for clinical improvement (Blier and de Montigny, 1987; 1990; Kennett et al., 1987). Thus, the mechanism of homologous desensitization may depend critically on the localization and expression of protein kinases and adenylyl cyclases in a given cell type. However, since 5-HT_{1A} receptor desensitization appears to depend critically on the type of coupling of the 5-HT_{1A} receptor to different second messengers in a specific cell type, it will be important to address whether differential desensitization occurs to multiple signaling pathways *in vivo*.

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Figure 1. Homologous desensitization of 5-HT_{1A}-mediated inhibition of calcium current in F11 cells

Prolonged application of 5-HT inhibited the Ca²⁺ current of cultured F11 cells stably transfected with the 5-HT_{1A} receptor and the inhibitory effect desensitized. Throughout this figure the charge carrying divalent was Ca²⁺ and the internal [Ca²⁺] was buffered close to 100 nM. A. Whole cell patch-clamp recording from an F11 cell expressing the wild type 5-HT_{1A} receptor. At points a, b, c and d the maximal inward current was measured and the corresponding time point is indicated by the same letter on the peak current against time plot below the current traces. This applies also to subsequent figures. B. Data obtained from a cell recorded using the perforated patch configuration. In this and all other figures the holding potential was -80 mV with a step to +10 or 0 mV (the peak of the I/V relationship) every 20 seconds. Leak currents have been subtracted.

Figure 2. PKC, but not PKA, mediates desensitization to 5-HT in F11 cells

A. In whole cell recordings, using Ba^{2+} as the external divalent, addition of the selective inhibitor of protein kinase A, PKA inhibitory peptide₅₋₂₄ (200nM) to the patch pipette, on average, did not significantly reduce the desensitization of the inhibition of Ca^{2+} current observed after 3 minutes of the application of 5-HT. B Under the same conditions however, addition of the peptide inhibitor of PKC₁₉₋₃₆ to the patch pipette (at 20 μM) blocked the desensitization completely.

Figure 3. Block of homologous desensitization using PKC inhibitor Go6976

The role of PKC was confirmed using Go6976, a selective inhibitor of conventional (Ca^{2+} -dependent) PKC isoforms. In F11 cells recorded from using the perforated patch configuration and which expressed the wt-1 5-HT_{1A} receptor, pretreatment with 200 nM Go6976 applied to the bath, prevented the desensitization to 10 μM 5-HT.

Figure 4. Reduced 5-HT-induced desensitization of a 5-HT_{1A} receptor lacking PKC sites in the third intracellular loop

With Ca²⁺ as the external divalent (A and B) and in the case of whole cell recordings (A) when internal Ca²⁺ was buffered at a physiologically relevant level, the i3-8 triple mutant receptor exhibited a smaller desensitization than the wild type cells (Fig 1). When Ba²⁺ was the external charge carrier (C and D), a smaller desensitization was also observed but on average there was no desensitization at all in the i3-8 expressing cells when the perforated patch technique was used.

A. In whole cell recordings F11 cells expressing 5-HT_{1A} receptors containing a triple mutation in the third intracellular loop of the receptor (i3-8 cells) also exhibited some desensitization on prolonged application of 5-HT but the size of the desensitization was significantly smaller than in the wild type cells. The charge carrying divalent ion was Ca²⁺.

B. A similar desensitization was observed when the perforated patch configuration was employed. C. In whole cell recordings, when the external charge carrying divalent was Ba²⁺ there was an observable desensitization after 3 minutes of application of 5-HT. When the cells were recorded from using the perforated patch configuration (D) the i3-8 line on average exhibited no desensitization of the inhibitory effect of 5-HT.

Figure 5. Whole Cell and Perforated Patch Data

The degree of desensitization was measured in wild-type or i3-8 lines using Ca^{2+} or Ba^{2+} as the charge carrying divalent in whole cell recordings on the left, or perforated patch recordings on the right. Each column labeled with a “*” was significantly different from the column indicated at the $P < 0.05$ level using ANOVA and SNK’s t-test. Using Ca^{2+} as the divalent the desensitization in wt-1 cells was greater than in the i3-8 line and when using Ba^{2+} the wt-1 desensitization was greater than that in the i3-8 line, measured using Ca^{2+} as the charge carrier. Referring to the data obtained with the perforated patch technique: the wt-1 (30 mM Ca^{2+}) desensitization was larger ($P < 0.05$) than the two i3-8 columns but not different from the wt-1 (Ba^{2+}) column. The i3-8 (external Ca^{2+} condition) was different from the external Ba^{2+} condition.

Whole Cell Data

Table 1

Data from whole cell recording experiments performed as described in Fig. 1 are summarized for F11 clones wt-1 and i3-8 transfected with 5-HT_{1A} receptor (wild-type or triple mutant). 5-HT (10 μM)-induced inhibition (% inhibition) of calcium currents using 30 mM Ca²⁺ or Ba²⁺ as charge carrier was calculated before and after 240 seconds of 5-HT treatment to induce desensitization, and the % desensitization is calculated from the ratio of these data. Data are shown as mean ± S.E.M. of the indicated number of experiments (N). For statistical significance see fig. 5.

	N	30 mM Ca ²⁺ [extracellular]			30 mM Ba ²⁺ [extracellular]		
		% Inhib. before desensitization	% Inhib. after desensitization	% desensitization	N	% Inhib. before desensitization	% Inhib. after desensitization
wt-1	6	54.3 ± 10.5	33.6 ± 6.6	34.7 ± 9.5	9	57.3 ± 6.5	40.1 ± 6.9
i3-8	16	60.5 ± 2.4	51.9 ± 3.1	14.6 ± 3.7	13	53.2 ± 4.4	44.2 ± 4.6

Perforated Patch Data

Table II

Data from perforated patch recording experiments performed as described in Fig. 1 are summarized for F11 clones wt-1 and i3-8 transfected with 5-HT_{1A} receptor (wild-type or triple mutant). Other details are the same as in Table 1. For statistical significance see fig. 5.

		30 mM Ca ²⁺ [extracellular]			30 mM Ba ²⁺ [extracellular]		
	N	% Inhib. before desensitization	% Inhib. after desensitization	% desensitization	N	% Inhib. before desensitization	% desensitization
wt-1	7	64.1 ± 7.1	32.5 ± 8.6	52.7 ± 11.1	7	44.4 ± 4.9	29.3 ± 5.9
i3-8	9	70.8 ± 7.1	54.8 ± 7	21.5 ± 7.1	10	58.9 ± 5.8	59.9 ± 5.8