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Acute Ca^{2+} -Dependent Desensitization of 5-HT_{1A} Receptors is Mediated by Activation of Pka in Rat Serotonergic Neurons

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Abstract

This report investigates acute changes in the sensitivity of 5-HT_{1A} receptors in dorsal raphe (dr) neurons in response to elevated serotonin. DR neurons were isolated from adult rats and measurements of inhibition of Ca^{2+} current by 5-HT were obtained using the whole cell patch clamp technique. During a 10-minute application of 5-HT (with normal $[\text{Ca}^{2+}]_i \sim 100 \text{ nM}$) a desensitization occurred. The response to 20 nM 5-HT decreased by 66% relative to control and remained depressed for about 30 min. When the internal $[\text{Ca}^{2+}]$ was buffered to $<1 \text{ nM}$ only a weak transient desensitization occurred that was surmountable with higher [5-HT]. Adenylyl cyclase activation with forskolin mimicked the desensitization and selective inhibition of PKA, but not PKC, partially antagonized the desensitization induced by 5-HT. To measure the activity of PKA and phosphatase enzymes, dr slices were incubated with the selective agonist DP-5-CT ($1 \mu\text{M}$) for 10 minutes and the phosphorylation of the PKA substrate Kemptide was followed using ATP- γP^{32} . DP-5-CT inhibited the cAMP stimulated maximal activity of PKA but raised basal PKA activity, thus increasing the percentage of PKA in the active state (activity ratio), an effect that was prevented by the selective 5-HT_{1A} antagonist WAY100635. DP-5-CT also caused a significant inhibition of phosphatase activity. These data support a model in the dr where 5-HT_{1A}-receptor stimulation of PKA promotes phosphorylation of a target and phosphatase inhibition leading to heterologous desensitization. The effect would be expected to have physiological consequences for 5-HT-mediated IPSPs and the Ca^{2+} component of the action potentials of dr neurons.

Keywords

Calcium Current; desensitization; 5-Hydroxytryptamine; PKA; Phosphatase; Serotonin

The desensitization of the 5-HT_{1A} receptor (5-HT_{1A}R) has been a topic of intense study because of its clinical implications. The 5-HT_{1A}R is a somatodendritic serotonin receptor of serotonergic cells in the dorsal raphe nucleus (DRN). Neurons in the various raphe nuclei project widely to brain areas including the forebrain, hypothalamus and hippocampus

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(Jacobs and Azmitia, 1992), thus changes in the excitability of raphe neurons affects the serotonin level in those brain areas and may have an impact on psychological states (Delgado et al., 1994). Activation of the 5-HT_{1A}R on DRNs leads to inhibition of cell firing but the 5-HT_{1A}R becomes desensitized in the persistent presence of 5-HT, which leads to the recovery of excitability (Blier et al., 1990). Activation of 5-HT_{1A} receptors on dr neurons has been shown to open GIRK K⁺ channels and to inhibit Ca²⁺ current (Aghajanian and Lakoski, 1984, Penington and Kelly, 1990, Penington et al., 1993a).

Another consequence of the activation of 5-HT_{1A} receptors in some preparations is reported to be the inhibition of forskolin-stimulated adenylate cyclase resulting in an inhibition of cyclic AMP levels (De Vivo and Maayani, 1986). Recently however the stimulation of basal cAMP levels and PKA activity has been observed after activation of the 5-HT_{1A} signaling pathway even in the dr. (Andrade, 1993, Clarke et al., 1996, Wischmeyer and Karschin, 1996, Johnson et al., 1997, Tang and Hurley, 1998, Liu et al., 1999). Consequently, it becomes important to know whether 5-HT_{1A} receptor stimulation in dr neurons results in inhibition or stimulation of cAMP levels and PKA activity in the dr.

The majority of previous work in the DRN has examined desensitization of the 5-HT_{1A}-R after chronic application of 5-HT reuptake inhibitors (Blier and De Montigny, 1983) with the exception of some studies by Riad et al who found that the receptor internalizes within 15 minutes after continued agonist stimulation (Riad et al., 2001). In the present study, we examined the immediate adaptive response of isolated dorsal raphe serotonergic neurons. Since the activation of the 5-HT_{1A}R can be measured by voltage-dependent Ca²⁺ channel inhibition, we monitored 5-HT_{1A}R-mediated inhibition of Ca²⁺ current and report that the 5-HT_{1A}R desensitizes in 10 minutes after 5-HT is applied to the neurons, in a Ca²⁺-dependent manner. Since stimulation of PKA appears to contribute to the mechanism of this desensitization, the role of PKA and cAMP in 5-HT_{1A}R desensitization was investigated using electrophysiological observations and a specific assay of PKA-activity. To examine the role of phosphatases in regulating the response to 5-HT, an electrophysiological study, using a selective inhibitor of protein phosphatases was coupled with a biochemical technique to measure the activity of PP1 and PP2A, in slices of the dr. These data provide clarification of the acute actions of 5-HT on the activity of PKA and 5-HT_{1A}-receptor desensitization in dr neurons.

Experimental procedures

Animals were anesthetized with Halothane and then decapitated with a small animal guillotine in accordance with our local Animal Care and Use Committee regulations. Three coronal slices (500 µm) through the brain stem at the level of the dorsal raphe nucleus were prepared from young adult rats 200-250g, using a “vibroslice” in a manner that has previously been described (Penington et al., 1991). A piece of gray matter 2×2 mm was cut from immediately below the cerebral aqueduct containing the dorsal raphe nucleus. The pieces of tissue were then incubated in a PIPES buffer solution containing 0.2mg/ml trypsin (Sigma Type XI) under pure oxygen for 120 minutes. The pieces of tissue were then triturated in Dulbecco's modified Eagles's medium.

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, we added 0.1 µM TTX to all recording solutions. The following solutions were routinely used to establish seals for whole cell recording; this contained (in mM) NaCl 135, HEPES 20, Glucose 10, Sucrose 20, CaCl₂ 2, KCl 2.5, and MgCl₂ 2. The external recording solution, designed to isolate calcium channel currents (carried by Ba²⁺),

contained: TEACl 160, BaCl₂ 5 (or CaCl₂ 5), HEPES 10, and Sucrose 20 :pH 7.3 with TEOH.

Pipette Solutions: For Ca²⁺ current: ~1nM free [Ca²⁺]_i in mM: CsCl 130, HEPES 10, EGTA 10, MgCl₂ 1, CaCl₂ 0, Mg-ATP 4, GTP 0.3 and phosphocreatine 14. For [Ca²⁺]_i ~100 nM the following changes were made: HEPES 20, EGTA 4, and CaCl₂ 2. The pH was adjusted to 7.3 using CsOH. Estimates of intracellular [Ca²⁺] were obtained using the Maxchelator software (webmaxc, standard constants) to be found on the Internet at: <http://www.stanford.edu/~cpatton/maxc.html>. All drugs were obtained from Sigma Chem. Co. or Calbiochem USA. Forskolin was dissolved in DMSO and diluted to a final DMSO concentration of 0.01% which had no effect by itself.

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 will range in resistance from 1.5-2.0 MΩ. 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 Ca²⁺ currents in TEA; the seal resistance was often greater than 5 GΩ. Subtraction of the leak and capacitance from the current records was done using the Axobasic software system. During the experiment, at regular periods, we obtain leak sweeps. Leak sweeps consist 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 Ca²⁺ current) was filtered at 2 KHz then digitized at 100 μs per point. Voltage protocols will be generated and analyzed by an IBM PC Pentium clone using the Axobasic 1 patch clamp software and the resultant data written to disk for analysis off line. 5-HT was applied using a gravity fed system connected via a manifold to a small bore glass capillary tube other drugs were applied through bath perfusion.

After achieving whole-cell patch, the dr neurons were held at -60 mV and Ca²⁺ current was elicited by a depolarizing step to +10 mV, a voltage that yield the largest current. When the Ca²⁺ current became stable, 5-HT was applied briefly to obtain the baseline inhibition of Ca²⁺ current. 5-HT then was applied (in the desensitizing concentration) for 10 minutes to induce desensitization. 5-HT was then washed off and the acute response of dr neurons was subsequently measured (with the challenging concentration) at fixed time points.

When measuring the effects of desensitization on the response to low challenging concentrations of 5-HT, (1 nM, 10 nM, 20 nM and 100 nM), the desensitization was induced by treatment with a desensitizing concentration of 100 nM 5-HT. For all other experiments, 10 μM 5-HT was applied to induce desensitization unless otherwise specified. The term "desensitizing conditions" means [Ca²⁺]_i was buffered to 100 nM and Ca²⁺ was used as the charge carrier.

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 mean size of the inhibition by 5-HT as a percentage of the baseline Ca²⁺ current followed by the S.E.M. This approach normalizes the response so that it can be averaged over cells. Using % inhibition as a measure of the effect of 5-HT is justified for several reasons. The first is that the channel population in the dr, inhibited by 5-HT, is homogenous with respect to this response (Penington et al., 1991) even after various Ca²⁺ channel blockers. Second the absolute reduction in the effect of 5-HT after desensitization recovered completely in cells that did not exhibit current run down. Data that includes control periods in the same cell as the

experimental period were compared by paired t-test but if the data was from different cell groups an un-paired t-test was applied. Multiple groups were compared using ANOVA (SPSS software Chicago IL, release 11.5.0) followed by either the Scheffe test of significance or a t-test for repeated measurements.

Protein kinase a assay

Dorsal raphe punches from individual midbrain coronal slices were briefly sonicated at low power, on ice, in 300 μ L of homogenization buffer, according to the method of Roberson and Sweatt (Roberson and Sweatt, 1996) see supplemental methods. [γ - 32 P]ATP, was used in the reaction to phosphorylate Leu-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) a PKA substrate, with or without 10 μ M cyclic AMP, a saturating concentration. 32 P incorporation into Kemptide was then quantitated by liquid scintillation counting. Assays of the activity of PKA were carried out as follows. First, the ability of cAMP to fully stimulate the basal activity of PKA was assessed and the ratio of basal (without cAMP) to total PKA activity was measured (the activity ratio) being an assessment of the percentage of PKA in the tissue that is in the active state (Corbin et al., 1973). Following this, the catalytic subunit of PKA (cPKA) was added to assay tubes, without dr tissue, to act as a positive control. In certain assay tubes the selective inhibitor of PKA (PKA inhibitory peptide, PKAI) was added at 0.2 or 2 μ M to confirm that the phosphorylation of kemptide (a specific substrate of PKA) was due to the activity of PKA.

Phosphatase assay

Six 500 micron thick coronal slices of brain containing the dr nucleus were obtained from two 170-200 g rats. The region of the dr nucleus was cut out. Three of these slices were kept in 250 μ L of aCSF for 20 minutes at 30°C with the 5-HT_{1A} agonist DP-5-CT at 1 μ M, and three were incubated in the same volume without the agonist. Cellular reactions were stopped by freezing the tissue in liquid nitrogen and the tissue was then stored at -70°C until it was assayed. A Protein Serine/Threonine Phosphatase (PSP) Assay System (#P0780S, New England BioLabs Inc.) was used to measure the phosphatase activity in dr tissue (see supplementary methods). The substrate, 32 P labeled Myelin Basic Protein, was prepared by phosphorylation on multiple serine and threonine residues with protein kinase (PKA) in the presence of γ - 32 P labeled ATP (2000 Ci/mmol). The phosphatase activity in dr extracts of rat brain was then determined by measuring the release of radioactive inorganic phosphate from the labeled protein.

Results

Desensitization to 5-HT was dependent on the internal free calcium concentration

In this study, acutely isolated DRN neurons were voltage clamped at -60 mV and Ca²⁺ current was elicited with a 20 ms depolarizing step to +10 mV. 5-HT_{1A}R activation inhibits this elicited Ca²⁺ current; however, in the continuous presence of 5-HT (10 μ M), the depressed Ca²⁺ current showed a gradual increase in size when the intracellular Ca²⁺ was buffered at 100 nM. Under conditions similar to physiological [Ca²⁺]_i, with 100 nM free Ca²⁺ in the pipette solution and Ca²⁺ used as charge carrier, the response to brief challenging pulses (30 s) of 5-HT were measured before and after a desensitizing concentration of 5-HT (10 μ M or 100 nM) was applied continuously for 10 minutes. Fig 1 (a) illustrates Ca²⁺ current traces extracted at different time points. The response to the agonist, decreased from 59.4 $\pm$ 3.0 % inhibition at the beginning of the application to 45.6 $\pm$ 3.5 % at the end of the application. (p<0.001, n=7, paired t-test, Fig 1b). Desensitization was expressed as the reduction in inhibition of Ca²⁺ current as a percentage of the initial inhibition. The average desensitization to 5-HT measured 23.7 $\pm$ 3.2%. After the desensitization was induced, the I/V curve of Ca²⁺ current did not shift significantly in the

presence or absence of 5-HT (not shown), ruling out the possibility that the effects seen were due to a drift in the voltage-dependence of the Ca^{2+} current.

The apparent desensitization was dependent on $[\text{Ca}^{2+}]_i$. When the pipette Ca^{2+} was buffered to <1 nM, the inhibition of Ca^{2+} current at the beginning and end of the 10-minute application of the agonist measured $62.1 \pm 2.6\%$ and $58.5 \pm 3.0\%$ ($n=6$) respectively. There was no significant difference between the inhibitions (Fig 1b). The effect of a higher concentration of free Ca^{2+} in the pipette solution was also examined (Fig 1c). With 300 nM free Ca^{2+} in the pipette, the desensitization measured $30.1 \pm 4.7\%$ ($n=6$), which was not significantly different from that induced with 100 nM free Ca^{2+} in the pipette. There was no significant difference in the initial acute ability of 5-HT to inhibit the Ca^{2+} current before desensitization in the three conditions.

The Ca^{2+} required for the induction of desensitization could come from the release of internal Ca^{2+} stores. In order to reduce the influence of any release of internal Ca^{2+} from stores, BAPTA a fast Ca^{2+} chelator, was used in place of EGTA with the $[\text{Ca}^{2+}]_i$ maintained at ~ 100 nM, and Ba^{2+} was the charge carrying divalent (Table 1 group 3). Under these conditions, the desensitization measured $19.7 \pm 4.1\%$ ($n=5$), not different from the standard desensitizing conditions.

Potentially, if the $[\text{Ca}^{2+}]_i$ was high enough it could exert a tonic Ca^{2+} -dependent inactivation of Ca^{2+} channels (Eckert and Chad, 1984). During a prolonged desensitizing application of the agonist, Ca^{2+} influx is reduced. The inhibition of Ca^{2+} influx, in turn, could result in a lowering of internal Ca^{2+} concentration and cause the relief of any potential tonic Ca^{2+} -dependent inactivation of Ca^{2+} channels. Such an effect might lead to a slow increase in the Ca^{2+} current, which would mimic response desensitization. This possibility was considered unlikely, since on average the baseline Ca^{2+} current did not increase after the relief of Ca^{2+} current inhibition; however this possibility was investigated further below.

To determine whether the response required Ca^{2+} influx from the channels, Ba^{2+} was used as a substitute for Ca^{2+} as the charge carrier, since Ca^{2+} channel activity shows less inactivation by the Ba^{2+} ion. With 100nM free pipette Ca^{2+} , 5-HT inhibited Ba^{2+} current by $55.7 \pm 2.3\%$ at the beginning and $44.8 \pm 3.1\%$ at the end of a 10-min application of 5-HT ($p<0.05$, paired t-test, $n=5$), the desensitization measured $19.6 \pm 4.1\%$, not significantly different from that induced with Ca^{2+} influx. We next completely eliminated Ca^{2+} influx by not depolarizing the membrane, thus preventing Ca^{2+} current during the 10-min application of 5-HT (Table 1, Group 2). The Inhibitory effect of 5-HT decreased to the same level as when eliciting Ca^{2+} current; 5-HT inhibited Ca^{2+} current by $58.9 \pm 1.4\%$ at the beginning and $48.2 \pm 1.3\%$ at the end of the 10 min application of 5-HT, and the desensitization measured $18 \pm 2.2\%$, not different from that induced with Ca^{2+} influx. In contrast, in the absence of a 10 min 5-HT application, not eliciting Ca^{2+} current for 10 min did not affect 5-HT_{1A}R-mediated Ca^{2+} current inhibition. Brief pulses of 5-HT inhibited Ca^{2+} current by $66.9 \pm 1.8\%$ and $67.3 \pm 1.7\%$ ($p>0.05$, paired t-test, $n=6$) respectively before and after a 10 min period. The absence of Ca^{2+} influx (either by not eliciting the current or by applying 0 mM Ca^{2+} /5 mM Mg^{2+} with the 5-HT) did not induce desensitization by itself, reveal an outward current, increase in leak current, or alter the response of the neurons to 5-HT after Ca^{2+} influx was restored (supplemental figures 1 and 2). Thus, 5-HT_{1A}R activity was required for the desensitization, whereas Ca^{2+} influx through the channels was not.

Prolonged application of 100 nM, but not 20 nM 5-HT induced desensitization

In the presence of 100 nM $[\text{Ca}^{2+}]_i$ in the patch pipette, a brief challenging concentration of 100 nM 5-HT was used to measure desensitization after a prolonged (desensitizing) application of lower concentrations, 20 nM and 100 nM 5-HT. When 5-HT was applied at

20 nM for 10 minutes it produced an inhibition of $30.5 \pm 3.2\%$ at the beginning, and $30.2 \pm 3.2\%$ at the end of the application ($n=6$), but failed to induce any desensitization. A desensitizing concentration of 5-HT at 100 nM, applied for 10 minutes, was as effective as 10 or 100 μM 5-HT in eliciting the desensitization to a challenge with 100 nM 5-HT (Figs. 2a & 3a). The desensitization to 100 nM averaged $25.6 \pm 1.8\%$, not different from that obtained when using 10 μM 5-HT. The desensitization induced by 100 nM 5-HT recovered with a similar time course to its onset (recovery $t_{1/2} \sim 2.5$ min, Fig 2b).

Desensitization greatly affected the response to low concentrations of 5-HT

When the desensitization was induced by treatment with 100 nM 5-HT, the response to a challenging concentration of 20 nM 5-HT following desensitization was dramatically reduced by $66.4 \pm 5\%$ ($n=6$, Fig. 2a). This effect was long lasting (recovery $t_{1/2} > 16$ minutes, Fig. 2b).

Dose response curves were generated for the 5-HT-mediated inhibition of Ca^{2+} current before and after the induction of the desensitization. Fig 3a shows that the response desensitized in an apparently non-surmountable fashion, with the curve shifted to the right and the EC_{50} increased from 24 nM to 44 nM. The maximal response of dr neurons to 5-HT was reduced after the desensitization, even at high concentrations of 5-HT.

When the same protocol was repeated using 0 nM Ca^{2+} in the pipette solution and the desensitizing concentration of 5-HT was 100 nM applied for ten minutes, there was a significant desensitization only when the challenging concentration was 20 nM (not shown). The desensitization of dr neurons challenged with 20 nM 5-HT measured $32.4 \pm 6.3\%$ ($n=5$, $p < 0.01$, paired t-test) but in comparison to the same experiment carried out in the presence of 100 nM internal Ca^{2+} the effect recovered quickly. Under these conditions, dose response curves were generated, before and after the induction of desensitization. After the induction of desensitization, although the dose-response curve shifted to the right and the EC_{50} increased from 21 nM to 32 nM, the maximum inhibition by 5-HT could be achieved after a challenge with 100 nM or 10 μM 5-HT. This result suggested that there was a Ca^{2+} -independent component of the desensitization that can be overcome by a high dose of the agonist.

Desensitization was induced by 5-HT_{1A} receptor activation

Although the somatodendritic serotonin receptor of dr neurons is predominantly the 5-HT_{1A}R, other subtypes of serotonin receptors are present in the cell membrane. To confirm that it was 5-HT_{1A}R activation that caused desensitization, a specific 5-HT_{1A}R agonist, 8-OH-DPAT (100 nM) was used to induce desensitization. The desensitization seen with 5-HT was also observed with 8-OH-DPAT. In 5 cells, the average desensitization induced with 100 nM 8-OH-DPAT applied for 10 minutes was $19.0 \pm 3.6\%$. Recovery was complete after seven minutes of wash. 8-OH-DPAT has been shown to bind to 5-HT₇ receptors. To rule out the possibility that the 5-HT₇ receptor played a role in desensitization, SB 269970 (300 nM), a 5-HT₇ selective antagonist (Thomas et al., 2000) was co-applied with 5-HT (100 nM) for 10 minutes when the desensitization was induced. In the presence of the 5-HT₇ antagonist, the desensitization induced by treatment with 5-HT (100 nM) measured $20.4 \pm 6.6\%$ ($n=5$), which was not significantly different from that in the absence of the antagonist ($p > 0.1$).

In three recordings, held long enough to observe whether the desensitization seen to 5-HT also affected the response to baclofen (a GABA_B receptor agonist), it was found that the response to 5-HT desensitized by 21.3% and that to baclofen desensitized by 56.9%. Without the 10 minute application of 5-HT there was no noticeable difference between the

first and second application of baclofen (see supplemental Fig 3). This result suggests that the desensitization in response to 5-HT is heterologous since it decreases the response to more than one receptor.

Inhibition of PKA reduced the desensitization induced by application of the agonist

The human 5-HT_{1A}R has been shown to be a substrate for PKA (Raymond and Olsen, 1994), so we tested whether PKA played a role in the desensitization. PKA inhibitory peptide PKAI₅₋₂₄ (Cheng et al., 1986) partially antagonized the desensitization to 10 μ M 5-HT. With 200 nM PKAI in the pipette solution, the desensitization measured 13.3 ± 3.0 %, which was significantly smaller than control ($p < 0.05$, $n = 7$, Fig. 4a). In addition, the desensitization to a 10-minute application of 100 nM 5-HT followed by challenge with the 20 nM concentration of 5-HT was antagonized by a similar proportion (without PKAI peptide 66% desensitization, with PKAI, 49.6 ± 5.1 %, $n = 6$, $p < 0.01$). The recovery from desensitization was significantly faster with PKAI in the pipette since the desensitization had recovered fully by 7 minutes of wash off of the agonist. It is likely that the PKAI inhibitor peptide works via the cAMP/PKA cascade since another way of activating this cascade using forskolin had the same effect as 5-HT (see below).

The activation of conventional protein kinase C is dependent on Ca^{2+} (Nishizuka, 1995). In the dr however, the conventional PKCs did not play a role in the desensitization to 5-HT. With the selective PKC inhibitory peptide PKCI₁₉₋₃₆ in the pipette solution (200 μ M) the desensitization measured 22.4 ± 2.1 % ($n = 7$), not significantly different from the control condition (Fig 4a).

Activation of Adenylyl cyclase mimicked and occluded 5-HT-induced desensitization

Since blocking the activity of PKA antagonized the desensitization, the effect of activation of the kinase was tested on 5-HT_{1A}R-mediated inhibition of Ca^{2+} current. Previous attempts to activate adenylyl cyclase (AC) in isolated dr neurons with various cAMP analogs were carried out using a pipette solution with internal Ca^{2+} buffered close to or < 1 nM. These compounds were without any effect on Ca^{2+} current or its acute modulation by 5-HT (Penington et al., 1991, 1993b). Similarly Forskolin (40 μ M), an activator of AC, did not alter the effect of 5-HT under these conditions (Fig. 4b). However, when internal Ca^{2+} was buffered close to 100 nM, and 40 μ M forskolin was applied to the bath, there was a pronounced reduction in the ability of brief pulses of 5-HT (applied with forskolin) to inhibit Ca^{2+} current (Fig. 4b). Also forskolin mimicked the effect of the 10-minute desensitizing application of 5-HT. After 5 minutes of washing the desensitization measured -33.9 ± 9.8 % ($p < 0.05$ paired t-test, $n = 7$), and there was no appreciable recovery. When forskolin was applied before, and throughout the application of 5-HT for 10 minutes (standard desensitization protocol), the desensitization elicited with 5-HT plus forskolin was not significantly different from the effect of adding forskolin alone or 5-HT for 10 minutes alone ($n = 7$). Thus forskolin occluded the desensitizing effect of 5-HT.

Inhibition of PP1/2A removed the Ca^{2+} dependency of the agonist-induced desensitization

Since activation of PKA led to 5-HT_{1A}R desensitization, we tested whether inhibition of phosphatases had the same effect. Phosphatase1/2A was inhibited with 10 nM microcystin, a highly potent and selective inhibitor of PP1/2A (Herzig and Neumann, 2000) which allowed desensitization to occur with the intracellular concentration of Ca^{2+} buffered to nominally 0 nM; a condition that without microcystin would produce weak, transient and surmountable desensitization. When microcystin was included in the pipette at 10 nM, the response to 5-HT (10 μ M) desensitized during a 10-minute application. The desensitization measured 26.4 ± 7.1 % ($n = 6$, $p < 0.05$, paired t-test), which was not different from cells

perfused with 100 nM Ca^{2+} . Unexpectedly, the response to 5-HT recovered normally within 11 minutes (Fig. 5).

Microcystin in the pipette solution had no effect on the desensitization when the pipette Ca^{2+} was buffered to 100 nM. The desensitization measured $29.3 \pm 7.1\%$ ($n=6$, $p<0.01$, paired t-test), which was not different from control. Furthermore, the response to the agonist recovered fully within 11 minutes. Thus the desensitization to 5-HT may be limited by phosphatase activity when cell Ca^{2+} is low which raises the question does phosphatase activity similarly limit the effect of forskolin?

Microcystin did not reveal Forskolin-mediated desensitization in low Ca^{2+} conditions

It was possible that under low pipette Ca^{2+} conditions, the activity of phosphatases could be too prominent for the forskolin-activated kinase to overcome. To test this possibility, the effect of forskolin was tested using low pipette Ca^{2+} (a condition under which it was formally ineffective) but now 10 nM microcystin was included in the pipette solution. Addition of microcystin did not reveal the Ca^{2+} -dependent desensitizing effect of forskolin and there was no significant difference in the ability of brief pulses of 5-HT to inhibit the Ca^{2+} current with or without forskolin ($p>0.05$, $n=5$, not shown). The above results suggest that 5-HT_{1A}R activation could lead to inhibition of PP1/2A and stimulation of PKA; we next assayed PKA and phosphatase activity in dr tissue with or without incubation with the 5-HT_{1A}R agonist, DP-5-CT.

Incubation of dr tissue with a 5-HT_{1A}R agonist increased the activity ratio of PKA

The 5-HT_{1A}R is known in some preparations to inhibit adenylyl cyclase activation, thus presumably decreasing the activity of PKA. The following experiment analyzed biochemically, in dorsal raphe tissue, whether 5-HT_{1A}R activation leads to an increase or decrease in PKA activity.

The addition of cAMP to raphe tissue increased the phosphorylation of an exogenously added PKA substrate kemptide from 71.5 ± 16.4 pM P_i /min-mg to 535.1 ± 121 P_i /min-mg ($n=12$). PKA was responsible for this increased phosphorylation since the specific peptide PKA inhibitor (PKAI, 0.2 μM) inhibited phosphorylation by $88.8 \pm 4.4\%$ ($n=5$) Fig. 6a). Phosphorylation remaining in the presence of PKAI likely reflects phosphorylation of Kemptide that is due to kinase(s) other than PKA. Changes in PKA activity was defined as an activity ratio which is an assessment of the percentage of PKA in the tissue that is in the active state (see methods).

A role for 5HT_{1A} receptors in the regulation of PKA activity was tested using the 5-HT_{1A} selective agonist dipropyl-5-carboxamidotryptamine (DP-5-CT). Dorsal raphe tissue punches were incubated for 10 minutes at 30°C in the presence or absence of DP-5-CT and the tissue was then flash frozen. For the assay the tissue was rapidly thawed and sonicated followed by the assay of PKA activity. DP-5-CT significantly inhibited PKA activity $22.25 \pm 7\%$ ($p<0.004$, $n=12$, Fig. 6a) in the presence of cAMP. DP-5-CT did not significantly alter basal PKA activity. The PKA activity ratio however was significantly increased ($80.9 \pm 33\%$, from 0.143 ± 0.014 , control to 0.256 ± 0.049 , DP-5-CT; $p = 0.026$, Fig. 6b). This increase of PKA activity ratio was completely prevented by the selective 5-HT_{1A} receptor antagonist WAY100635 (1 μM). These data suggest that 5-HT_{1A}-receptor activation in the dr increases PKA activity.

The 5-HT_{1A} antagonist also prevented the effect of DP-5-CT on total PKA activity in cAMP in that the activity was inhibited by only $12.47 \pm 5.4\%$ which was not different from control. With an antagonist present the basal activity, instead of being increased was

decreased by $4.0 \pm 18\%$ N.S. The activity ratio in control measured 0.189 ± 0.03 and in DP-5-CT it measured 0.216 ± 0.04 not different from control (Fig. 6b).

Phosphatase activity in dr slices is inhibited by 5-HT_{1A}-selective agonists

Phosphatase activity in dr extracts was assayed by measuring the amount of ^{32}P liberated from ^{32}P -labelled myelin basic protein (^{32}P -MyBP). The non-selective kinase inhibitor K252a ($0.5\ \mu\text{M}$) was used to inhibit kinase activity during preparation of dr extracts. DP-5-CT was added to determine whether 5-HT_{1A} receptors regulated phosphatase activity in dr tissue. DP-5-CT significantly inhibited phosphatase activity in dr extracts, (Control, $48.64 \pm 0.97\ \text{pM Pi release / min-}\mu\text{g}$; DP-5-CT, $41.18 \pm 1.171\ \text{pM Pi release / min-}\mu\text{g}$, $n=3$ for both groups, $p=0.038$, Fig. 7a). Microcystin LR, a selective protein phosphatase 1 (PP1) and 2A (PP2A) inhibitor, reduced the release of ^{32}P by 75% suggesting that PP1 or PP2A activity was being regulated by 5-HT_{1A} receptor activation (Control, $253.3 \pm 21.3\ \text{pM Pi, release / min}$, $62.96 \pm 8.3\ \text{pM/min}$ ($n=4$) Fig. 7b).

Discussion

This report describes a Ca^{2+} -dependent desensitization of the response to 5-HT_{1A} receptor stimulation that occurs when the concentration of Ca^{2+} in the pipette solution was within the physiological range ($\sim 100\ \text{nM}$). After the desensitization was induced, the response to $20\ \text{nM}$ 5-HT was reduced by 66% and the maximum inhibition could not be achieved even with higher concentrations of 5-HT.

When $[\text{Ca}^{2+}]_i$ was buffered $<1\ \text{nM}$, application of $100\ \text{nM}$ 5-HT for 10 minutes resulted in a weak, transient and surmountable desensitization to challenge with $20\ \text{nM}$ 5-HT, but this was not evident when the cell was challenged with a $[\text{5-HT}] \geq 100\ \text{nM}$. The desensitization to a 5-HT agonist was heterologous since the effect of baclofen was also reduced (suppl. Fig 3); this is discussed further below in relation to the mechanism of the effect. Desensitization and percent baseline inhibition of Ca^{2+} current by 5-HT were unrelated to the degree of Ca^{2+} current rundown (suppl. Fig 5).

Ours results differed from those in cultured embryonic chick dorsal root ganglion (DRG) cells (Diverse-Pierluissi et al., 1996, Tosetti et al., 2003). In chick cells the desensitization was rapid and complete within 30-40 seconds, required Ca^{2+} influx through Ca^{2+} channels and was prevented when Ba^{2+} influx was measured in place of Ca^{2+} . In dr neurons, a fast component was not observed; the desensitization was not dependent on Ca^{2+} influx through the channels but on the basal level of intracellular Ca^{2+} . When Ba^{2+} was used as the charge carrier in place of Ca^{2+} it was capable of supporting the desensitization.

It is proposed that desensitization occurred in two steps. The first, a non Ca^{2+} -dependent conformational change in the 5-HT_{1A}R, as a result of a prolonged exposure to a high concentration of the agonist. In support of this, when internal Ca^{2+} was buffered to $<1\ \text{nM}$, treating dr cells with high concentrations of 5-HT transiently decreased the cell sensitivity to $20\ \text{nM}$ 5-HT but not $10\ \mu\text{M}$ 5-HT. The next step required internal Ca^{2+} and appeared to involve a phosphorylation event mediated by PKA, leading to a more long-term desensitization (Raymond and Olsen, 1994). The apparent decrease in 5-HT_{1A}R signaling strength could be due to i), a decrease in the number of 5-HT_{1A} receptors on the cell surface, ii), a decrease in the affinity between the 5-HT_{1A}R and its agonist or iii), a reduction in the coupling between the 5-HT_{1A}R and the G-protein. In supplemental figure 1, when the G-protein coupled to the 5-HT_{1A} receptor, was activated with GTP- γ -S, the inhibition remained stable and did not desensitize over 10 minutes, with or without 5-HT. The desensitization induced by 5-HT could be greatly reduced with external Cd^{2+} which likely forms “metal bridges” simulating the external disulphide bridges in the receptor that prevent

desensitization (Dohlman et al., 1990, Rosati and Traversa, 1999, Elliott et al., 2004). Together these results suggest that the 5-HT_{1A} receptor itself shows an acute desensitization to the natural agonist. This suggests the following simplest scenario:

Where A= agonist, R= receptor in high affinity (G-protein bound) state, AR' = desensitized receptors having a conformation that is incapable of efficient coupling to the G-protein and P = phosphate.

Desensitization might occur through a well-established pathway of GPCR desensitization involving the binding of arrestin to the phosphorylated receptors, which leads to receptor internalization (Krupnick and Benovic, 1998). Moreover, it has been shown that in dr neurons, treated with a high concentration of a 5-HT_{1A} agonist, the 5-HT_{1A} receptor internalized when observed at a 15 minute time point (Riad et al., 2001) and this effect was blocked by the 5-HT_{1A} selective antagonist WAY100635. In the present study the longer lasting desensitization that we observed with 100 nM internal Ca²⁺ may result in receptor internalization.

The kinase that phosphorylates the 5-HT_{1A} receptor is likely to be PKA since PKAi₅₋₂₄ partially antagonized the desensitization, and the adenylyl cyclase activator, forskolin, mimicked it. In contrast, conventional inhibitors of PKC were ineffective even though they blocked the effect of the phorbol ester PMA in dr neurons (Chen and Penington, 1996). Some of our conclusions, based on kinase inhibitors, are supported by biochemical evidence and the inhibitors of PKA, PKC and phosphatases are considered to be highly selective (Cheng et al., 1986, House and Kemp, 1987, Herzig and Neumann, 2000).

Inhibition of basal cAMP levels by 5-HT_{1A} receptors in the dr features in one theory of the action of antidepressant drugs (Honore, 2007). This suggestion was used to explain an antidepressant-like phenotype in knockout mice lacking a cAMP-sensitive background leakage K⁺ channel called TREK1 normally found in the dr (Heurteaux et al., 2006) and which is closed by increasing levels of cAMP. However, the usual negative coupling between the 5-HT_{1A} receptor and cAMP accumulation may not occur in dr neurons (Clarke et al., 1996, Johnson et al., 1997). Liu et al. (Liu et al., 1999) reported that in rat pituitary GH4C1 cells, depleted of G_{ai1} and transfected with 5-HT_{1A} receptors, activation of the receptor could lead to activation of AC II or AC IV via the release of G_{βγ} subunits (Andrade, 1993, Tang and Hurley, 1998). In the present work, physiological and biochemical evidence for PKA stimulation by 5-HT in dr neurons was obtained, although maximally stimulated PKA was inhibited by 5-HT (see below).

Without Ca²⁺ in the pipette solution, the desensitization to prolonged application of 5-HT was weak, transient and surmountable when the cell was challenged with 100nM 5-HT, but after inhibition of PP1/2A with microcystin the application of 5-HT resulted in desensitization to the same extent as when [Ca²⁺]_i was ~100 nM. Thus in the relative absence of [Ca²⁺]_i the 5-HT-induced desensitization appears to be limited by phosphatase activity. Our results reveal that 5-HT may both stimulate the kinase (not Ca²⁺-dependent) and inhibit the phosphatase in a Ca²⁺-dependent manner. A mechanism for the second possibility has been described in which the activity of PP1 can also be modulated by PKA (Walaas et al., 1983, Winder and Sweatt, 2001). Thus 5-HT-stimulated PKA activity could contribute to desensitization of the 5-HT_{1A} receptor by inhibiting phosphatase activity.

Activation of adenylyl cyclase by forskolin reduced the effect of 5-HT, but the effect of forskolin was Ca²⁺-dependent. Further, addition of microcystin to the pipette did not allow forskolin to decrease the effects of 5-HT in low Ca²⁺ pipette conditions (as it did with 5–

HT) so the action of forskolin was not the result of indirect phosphatase inhibition. The agonist-induced desensitization was capable of inducing the first step of desensitization in the absence of Ca^{2+} , and the full long-term effect was then revealed by phosphatase inhibition. However, in the absence of internal Ca^{2+} , forskolin could not induce the first stage, and phosphatase inhibition was ineffective. Thus, different adenylyl cyclase isoforms are potentially involved in the effects of forskolin and 5-HT.

The inhibition by an agonist of maximal cAMP-induced PKA activity is not a “physiological” measure, and the stimulation by an agonist of basal PKA activity is perhaps more meaningful. It was expected that stimulation of the basal level of PKA activity would occur after treatment with DP-5-CT with no change in the total activity (activity ratio increases). In the present study, there was a significant increase in the AR of PKA by 80 % caused by the 5-HT_{1A} agonist; however this was brought about by a significant decrease in the total activity and an average stimulation in basal levels of PKA activity with DP-5-CT. These effects were mediated by stimulation of the 5-HT_{1A} receptor since it was completely antagonized by incubation with WAY100635.

The decrease in the “total” PKA activity after treatment with the 5-HT agonist suggests that a saturating concentration of cAMP was not capable of producing as many free cPKA subunits. However, the increase in AR with the 5-HT agonist suggests that the percentage of total PKA in the slice that was active under basal conditions was greater than control after treatment with the 5-HT agonist. Since there appears also to be a stimulation of basal PKA activity there could be a differential effect of 5-HT receptor stimulation on the two main types of PKA (I and II isoforms).

It should be noted that the biochemical measurement of stimulation of PKA activity and inhibition of phosphatase activity observed in this study was a population response from the whole dr nucleus which contains several different types of cells. However a similar effect was also seen when recording from individual serotonergic cells isolated from the nucleus suggesting that the population response is a signal from the serotonergic neurons in the dr nucleus. Application of serotonin also desensitized the response to baclofen; this means that the desensitization was heterologous. This type of desensitization, rather than one specific for the 5-HT_{1A} receptor, would be expected given the mechanism of the desensitization, since activation of a freely diffusible messenger such as cAMP would be expected to influence the response to several transmitters and not just the 5-HT_{1A}R. It would be interesting to determine whether the application of baclofen would also desensitize the response to 5-HT although whether every G-protein coupled transmitter would activate this mechanism would presumably depend on the Ca^{2+} -sensitivity of the individual transduction mechanism.

The effect that we have described should have physiological consequences. A long burst of spikes in the dr could expose the raphe cells to at least 100 nM 5-HT, perhaps remaining in the synaptic space long enough to desensitize the 5-HT receptors. The response of the neurons to small amounts of 5-HT, producing IPSPS (Aghajanian and Lakoski, 1984, Williams et al., 1988) and inhibiting Ca^{2+} influx during the action potential (Penington et al., 1992), would be depressed for some time. This effect may be surmountable only if $[\text{Ca}^{2+}]_i$ is low enough, and a large amount of 5-HT was released, so that strong stimulation would continue to produce profound inhibition of the cell bodies and prevent action potential generation. The relationship between the acute effects that we have described and the effect of chronically flooding raphe neurons with 5-HT, such as occurs after prolonged application of re-uptake blockers, is a subject worthy of further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

5-HT	5-hydroxytryptamine
5-HT_{1A}R	5-hydroxytryptamine receptor
8-OH-DPAT	(±)-8-Hydroxy-2-(di-n-propylamino)tetralin hydrobromide
BAPTA	Ethylenedioxybis(o-phenylenenitrilo)tetraacetic acid
cAMP	cyclic adenosine monophosphate
DMSO	Dimethyl sulfoxide
DP-5-CT	dipropyl-5-carboxamidotryptamine
dr	dorsal raphe
EGTA	Ethylene-bis(oxyethylenenitrilo)tetraacetic acid
G protein	guanyl nucleotide-binding proteins
GPCR	G-protein coupled receptor
GTP	Guanosine 5'-triphosphate
GTP-γ-S	Guanosine-5'-[γ-thio]triphosphate
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic acid
IPSP	inhibitory post synaptic potential
PMA	phorbol-12-myristate 13-acetate
PKA	Protein kinase A
PKC	Protein kinase C
PP1/2A	protein phosphatase 1/2A
PP2B	protein phosphatase 2B
S.E.M.	standard error of the mean

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Figure 1.

The response of dr neurons to 5-HT (10 μM) desensitized during a ten-minute application of the agonist when the pipette solution contained nominally 100 or 300 nM free Ca^{2+} , and Ca^{2+} was used as the charge carrier. (a) Shows traces of Ca^{2+} current at different time points showing the inhibition of Ca^{2+} current and its desensitization at different pipette concentrations of Ca^{2+} . The small letters in italics show the time in (b) that corresponds with the traces in (a). (b) Is a current against time plot for two cells containing either nominally 0 or 100 nM Ca^{2+} with the current size normalized. Only in the cell containing 100 nM Ca^{2+} did the response to 5-HT decrease during the application. After 12 minutes of washing away the agonist, serotonin was able to inhibit the Ca^{2+} current to its original level (not shown). (c) Shows that the degree of desensitization increased with intracellular $[\text{Ca}^{2+}]$ but appeared to reach a maximum at 300nM. Despite the changes in pipette $[\text{Ca}^{2+}]$ the degree of initial Ca^{2+} current inhibition did not change significantly. The holding potential was -60 mV and the test potential was $+10$ mV unless otherwise noted.

Figure 2.

Desensitization induced by 100 nM 5-HT greatly affects the response to 20 nM 5-HT. **(a).** A response to 20 nM 5-HT, obtained after a 10 minute application of 100 nM 5-HT, was reduced to a greater extent than that seen when the cell was briefly tested with 100 nM 5-HT. **(b).** The graph shown by the diamond shaped symbols illustrates the rate of recovery from desensitization induced by 100nM 5-HT, applied for 10 min, and challenged briefly with 100 nM 5-HT. Desensitization recovered with a similar time course to its onset (recovery $t_{1/2} \sim 2.5$ min). The graph shown by circles, illustrates the rate of recovery of desensitization elicited with 100 nM 5-HT, for 10 min, and challenged with 20 nM 5-HT. The reduction in the effect of the 20 nM concentration of 5-HT was long lasting (recovery $t_{1/2} > 16$ minutes). The graph shown by triangles is the rate of recovery of desensitization elicited by 10 μ M 5-HT and challenged briefly with 10 μ M 5-HT. The rate of recovery was slower when desensitization was induced with 10 μ M 5-HT. The lines between the points were fit by eye and each point is an average of 3-6 observations in as many cells. Pipette Ca^{2+} was buffered to ~ 100 nM and the charge-carrying ion was Ca^{2+} .

Figure 3.

Dose response curves for the inhibition of Ca^{2+} current by 5-HT in dr neurons. **(a)** The filled circles show the control dose response curve which had an EC_{50} of 24 nM. The open circles show the dose response curve measured after 10 minutes in 5-HT after desensitization had occurred. The response was reduced in an apparently non-competitive fashion with a decrease in the maximal response and a shift to the right of the dose response curve. The EC_{50} was increased to 44 nM. **(b)** Subtracting the control from the desensitized curves yields the dose response curve for the desensitization. This clearly indicates that when induced by 100 nM 5-HT the desensitization was more substantial when the cell was challenged with lower concentrations of agonist. The concentration of 5-HT which was

inhibited by 50% by application of 100nM 5-HT was 29.7nM. Sigmoidal curves in figure 3 were fit to the equation: $f = (a-d) / (1-x/h) + d$ using Sigma Plot 8 where a is the minimum response, d is the maximum, x is the x-axis concentration and h is the EC₅₀. Note that the slope was constrained to a value of 1. The pipette and bath conditions were the same as in figure 2.

Figure 4.

(a) Reduction in the response of dr neurons to 5-HT after a 10-min application of 5-HT ($10\mu\text{M}$) where the pipette contained different kinase inhibitors. The selective PKA inhibitor PKAi₅₋₂₄ partially blocked the desensitization. PKCi₁₉₋₃₆ was without effect.

(b) When the pipette $[\text{Ca}^{2+}]$ was buffered at $\sim 1\text{ nM}$ forskolin was without effect on the response to 5-HT. The average inhibition induced by 5-HT in the presence of forskolin remained constant over time. The bar graph on the right illustrates a similar experiment to that on the left except the pipette $[\text{Ca}^{2+}]$ was buffered close to 100 nM . Treatment with forskolin ($40\mu\text{M}$), a potent adenylyl cyclase activator, alone mimicked the desensitization and the response did not readily recover. Ca^{2+} was the charge-carrying ion.

Figure 5.

When 10 nM of the selective PP1/2A phosphatase inhibitor microcystin was included in the pipette the response to 5-HT desensitized when pipette Ca^{2+} was buffered low ($\sim 1\text{nM}$). Without the microcystin the effect of 5-HT did not desensitize. This concentration of microcystin had no observable effect on the response to 5-HT or its desensitization when pipette $[\text{Ca}^{2+}]$ was buffered close to 100 nM.

Figure 6.

(a) The effect of DP-5-CT on PKA activity. The second column shows the action of the 5-HT_{1A} agonist on the Total cAMP stimulated activity of PKA. There was a small statistically significant inhibition. In the same experiments, when the action of DP-5-CT was examined on the basal level of PKA activity (without the addition of cAMP) there was on average a stimulation of PKA activity which did not reach statistical significance. The selective inhibitor of PKA (PKA inhibitory peptide, PKAI₅₋₂₄) was added at 0.2 or 2 μ M to confirm that the phosphorylation of kemptide (a specific substrate of PKA) was due to the activity of PKA. The specific phosphorylation of Kemptide by dr tissue, measured in the presence of cAMP, was inhibited by 88.8 ± 4.4 % (n=5) by addition of PKAI peptide. (b) shows the activity ratio of the effect of DP-5-CT on the activity of PKA which was a significant stimulation. This effect was completely antagonized by the 5-HT_{1A} selective antagonist WAY100635 (1 μ M).

Figure 7.

The modulation of phosphatase activity by Dipropyl-5-carboxamidotryptamine (DP-5-CT). An inhibition of phosphatase activity was observed in the presence of DP-5-CT. The graph (a) shows the inhibition caused by the agonist. (b) Microcystin LR is a selective inhibitor of PP1 and PP2A. When microcystin (20 nM) was added to the assay tubes containing dr tissue, approximately a 75% inhibition of phosphatase activity was observed.

Table 1

Showing experimental protocol

Group	[Ca ²⁺] _i	Charge carrier	Agonist appln.	Current elicited	Significance
1	100nM	Ca ²⁺	Yes	Yes	10 min ** +2.3 min * +7 min *
2	100nM	Ca ²⁺	Yes	No	10 min ** +2.3 min *
3	100nM BAPTA	Ba ²⁺	Yes	Yes	10 min * +2.3 min *
4	1nM	Ca ²⁺	Yes	Yes	N.S.
5	100nM	Ca ²⁺	No	No	N.S.

Significance was assigned by paired t-test compared to the effect of 5-HT (10 μM) for that group at 0 min, each cell was used as its own control

* = <0.05,

** = <0.01.

N.S. means no desensitization.