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ACUTE Ca^{2+}-DEPENDENT DESENSITIZATION OF 5-HT_{1A} RECEPTORS IS MEDIATED BY ACTIVATION OF PROTEIN KINASE A (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-min application of 5-HT (with normal $[Ca^{2+}]_i \sim 100$ 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 $[Ca^{2+}]$ was buffered to <1 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 protein kinase A (PKA), but not protein kinase C (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 dipropyl-5-carboxamidotryptamine (DP-5-CT, 1 μM) for 10 min and the phosphorylation of the PKA substrate Kemptide was followed using ATP-γ^{32}P. 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 inhibitory post synaptic potentials and the Ca^{2+} component of the action potentials of dr neurons. Published by Elsevier Ltd on behalf of IBRO.

Key words: 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 (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 dorsal raphe (dr) neurons has been shown to open GIRK K^+ channels and to inhibit Ca^{2+} 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 protein kinase A (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 min 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 Ca^{2+} channel inhibition, we monitored 5-HT_{1A}R-mediated inhibition of Ca^{2+} current and report that the 5-HT_{1A}R desensitizes in 10 minutes after 5-HT is applied to the neurons, in a Ca^{2+}-dependent manner.

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Abbreviations: AC, adenylyl cyclase; AR, agonist ratio; BAPTA, ethylenedioxybis(o-phenylenenitrilo) tetraacetic acid; cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; DP-5-CT, dipropyl-5-carboxamidotryptamine; dr, dorsal raphe; DRN, dorsal raphe nucleus; 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; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate 13-acetate; PP1/2A, protein phosphatase 1/2A; PP2B, protein phosphatase 2B; SEM, standard error of the mean; 5-HT_{1A}R, 5-hydroxytryptamine receptor; 8-OH-DPAT, (±)-8-hydroxy-2-(di-n-propylamino) tetralin hydrobromide.

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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 PP1and 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

Sprague-Dawley rats (Charles River Labs, Wilmington, MA, USA) 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 DRN were prepared from young adult rats 200–250 g, using a "vibroslice" in a manner that has previously been described (Penington et al., 1991). A piece of gray matter 2×2 mm^2 was cut from immediately below the cerebral aqueduct containing the DRN. The pieces of tissue were then incubated in a PIPES buffer solution containing 0.2 mg/mL trypsin (Sigma Type XI) under pure oxygen for 120 min. 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 2 ml/min 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, $CaCl_2$ 2, KCl 2.5, and $MgCl_2$ 2. The external recording solution, designed to isolate calcium channel currents (carried by Ba^{2+}), contained: TEACl 160, $BaCl_2$ 5 (or $CaCl_2$ 5), HEPES 10, and Sucrose 20: pH 7.3 with TEAOH.

Pipette Solutions: For Ca^{2+} current: ~1 nM free $[Ca^{2+}]_i$ in mM: CsCl 130, HEPES 10, EGTA 10, $MgCl_2$ 1, $CaCl_2$ 0, Mg-ATP 4, GTP 0.3 and phosphocreatine 14. For $[Ca^{2+}]_i$~100 nM the following changes were made: HEPES 20, EGTA 4, and $CaCl_2$ 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. All drugs were obtained from Sigma Chem. Co. (St. Louis, MO, USA) or Calbiochem, (La Jolla, CA, USA). Forskolin was dissolved in dimethyl sulfoxide (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 to 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 Ca^{2+} 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 10 mV. 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 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 manifest 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 Ca^{2+} current was elicited by a depolarizing step to +10 mV, a voltage that yield the largest current. When the Ca^{2+} current became stable, 5-HT was applied briefly to obtain the baseline inhibition of Ca^{2+} current. 5-HT then was applied (in the desensitizing concentration) for 10 min 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 $[Ca^{2+}]_i$ was buffered to 100 nM and Ca^{2+} 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 Ca^{2+} current followed by the SEM. 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 Ca^{2+} 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 were from different cell groups an un-paired *t*-test was applied. Multiple groups were compared using ANOVA (SPSS software Chicago, IL, USA, 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 μl of homogenization buffer, according to the method of Roberson and Sweatt (Roberson and Sweatt, 1996) see supplemental methods. ATP-γ^{32}P, was used in the reaction to phosphorylate Leu–Arg–Arg–Ala–Ser–Leu–Gly (Kemptide) a PKA substrate, with or without 10 μM 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 μM to confirm that the phosphorylation of kemptide (a specific substrate of PKA) was due to the activity of PKA.

Phosphatase assay

Six 500-μm 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 μL of aCSF for 20 min at 30 °C with the 5-HT_{1A} agonist DP-5-CT at 1 μM, 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