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Characterization of Homologous 5-Hydroxytryptamine$_4$ Receptor Desensitization in Colliculi Neurons

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SUMMARY

Exposure of mouse colliculi neurons to selective 5-hydroxytryptamine (5-HT)$_4$ agonists was accompanied by a rapid desensitization of the receptor-stimulated adenylyl cyclase response. Half-maximal desensitization occurred after 2 min. Only exposure of neurons to selective 5-HT$_4$ agonists led to a potent desensitization of the 5-HT$_4$-mediated response. Neurons exposed to other agents, like isoproterenol, vasoactive intestinal peptide, or forskolin, that increase cAMP levels did not undergo any desensitization of 5-HT$_4$ receptors. Activation of protein kinase A with either 8-bromo-cAMP or dibutyryl-cAMP or application of inhibitors of protein kinase A-dependent phosphorylation did not change the rate of 5-HT$_4$-induced desensitization. No shift to lower potency of 5-HT$_4$ agonists in the concentration-response curve was observed. These results suggest that 5-HT$_4$ receptor agonists induced homologous but not cAMP-mediated heterologous desensitization. A good correlation was found between the affinities of nine 5-HT$_4$ agonists and their abilities to desensitize the adenylyl cyclase response. This may indicate that homologous desensitization is a function of the mean occupancy time of the receptors by agonists. When permeabilized neurons were loaded with heparin, an inhibitor of the β-adrenergic receptor kinase (βARK), 5-HT$_4$ receptor desensitization was reduced by 30–40%. Interestingly, Zn^{2+}, an other inhibitor of βARK, totally prevented 5-HT$_4$-induced desensitization. Pretreatment of neurons with concanavalin A, reported to inhibit sequestration of β-adrenergic receptors from the cell surface, reduced the desensitization process by 70%. These data suggest that both sequestration and phosphorylation by βARK, or another specific agonist-dependent receptor kinase, are involved in homologous desensitization of 5-HT$_4$ receptors coupled to adenylyl cyclase.

Over the past 3 years, many investigations have reported the existence of a new 5-HT receptor, termed 5-HT$_4$, that has a wide tissue distribution and is involved in several functional responses (1). Even though it clearly belongs to the G protein-coupled receptor family, its pharmacological profile does not fit into the 5-HT$_1$, 5-HT$_2$, and 5-HT$_3$ classification as defined by Bradley *et al.* (2). Recent studies have shown that the 5-HT$_4$ receptor is not limited to the brain (3–10) but is also present in guinea pig ileum (11–15), ascending colon (16), rat esophagus (17, 18), human and porcine heart (19–22), and frog adrenocortical cells (23).

The 5-HT$_4$ receptor has been characterized by a positive coupling to adenylyl cyclase recently shown in three models, brain (4, 8), heart (20), and esophagus (24). In brain, activation of 5-HT$_4$ receptors led to K^+ channel blockade via a cAMP-dependent mechanism (25). In human myocytes, we have reported that the increase in the L-type Ca^{2+} channel current produced by 5-HT is mediated by an elevation of intracellular cAMP due to 5-HT$_4$ receptor activation (22). More recently, Ford *et al.* (24) observed cAMP accumulation in rat esophagus after 5-HT$_4$ receptor stimulation.

We have previously reported that activation of 5-HT$_4$ receptors in colliculi neurons was followed by a steady desensitized state of the receptors (6, 7). Desensitization has been used to discriminate between 5-HT$_4$ and 5-HT$_3$ receptors in guinea pig ileum. Indeed, 5-methoxytryptamine was able to completely desensitize 5-HT$_4$ receptor responses without affecting 5-HT$_3$-mediated responses. Reciprocally, 2-methyl-5-HT desensitized 5-HT$_3$ responses without affecting the 5-HT$_4$ responses (26, 27).

However, the mechanism involved in the process of desensitization of 5-HT$_4$ receptors remains to be characterized. The best known model for desensitization is the βAR-adenylyl cyclase system, in which two major mechanisms have been implicated in the process. Homologous agonist-specific desen-

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); G protein, GTP-binding protein; PKA, protein kinase A (cAMP-dependent protein kinase); βAR, β-adrenergic receptor; βARK, β-adrenergic receptor kinase; VIP, vasoactive intestinal peptide; FK, forskolin; 8-Br-cAMP, 8-bromo-cAMP; Bt$_2$-cAMP, dibutyryl-cAMP; G$_s$ coupling protein, stimulatory guanine nucleotide-binding regulatory protein of adenylyl cyclase; Con A, concanavalin A; PKI, protein kinase inhibitor (peptide corresponding to the inhibitory region of the heat-stable inhibitor of protein kinase A); IBMX, 3-isobutyl-1-methylxanthine; ISO, isoproterenol; H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N,N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

sitization involves phosphorylation of the receptor by a cAMP-independent βARK (28, 29). Heterologous desensitization, which could involve stimulation of other types of receptor, implicates phosphorylation of βAR by PKA (30–32) or protein kinase C (33). In both types of desensitization, phosphorylation of the βAR is followed by sequestration and down-regulation of the receptor. A decrease in mRNA coding for the βAR has also been reported during desensitization (34, 35). Sequestration of the receptors away from the cell surface can also occur independently of other desensitizing steps and does not require the presence of the G_s coupling protein (36–39).

In the present report, we provide results (based on a rapid loss of signal-transduction capacity of the receptor) that clearly demonstrate that rapid desensitization of 5-HT$_4$ receptors occurs exclusively through cAMP-independent and agonist-selective desensitization. The desensitization process can be blocked by Zn^{2+} or heparin, both reported to be inhibitors of βARK (28, 39), and by Con A, which may prevent receptor sequestration at the cell surface (38).

Experimental Procedures

Materials. The following drugs were purchased from Sigma Chemical Co. (St. Louis, MO): serotonin creatin sulfate, (−)-ISO, heparin, PKI, Con A, IBMX, Bt_2-cAMP, 8-Br-cAMP, and H7. VIP was from Peninsula Laboratories (San Carlos, CA). [^{3}H]Adenine (24 Ci/mmol) was from Amersham (UK). Trans-Port, a transient cell permeabilization kit, was from GIBCO BRL Life Technologies (European Division, Cergy Pontoise, France).

The following drugs were generously donated: BIMU 1 [(*endo*-*N*-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-ethyl-2-oxo-1*H*-benzimidazol-1-carboxamide hydrochloride] and BIMU 8 [*endo*-*N*-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-isopropyl-2-oxo-1*H*-benzimidazol-1-carboxamide hydrochloride] were from Boehringer Ingelheim (Milan, Italy), cisapride (*cis*-4-amino-5-chloro-*N*-[1-[3-(4-fluorophenoxy)propyl]-3-methoxy-4-piperidinyl]-2-methoxybenzamide) was from Janssen Pharmaceutica (Beerse, Belgium), renzapride ([(±)-*endo*]-2-methoxy-4-amino-5-chloro-*N*-(1-azabicyclo[3.3.1]non-4-yl)benzamide monohydrochloride) was from Beecham Pharmaceuticals (Harlow, UK), (*RS*)-zacopride [(*RS*)-4-amino-*N*-(1-azabicyclo[2.2.2]oct-3-yl)-5-chloromethyoxybenzamide HCl], (*R*)-zacopride, and (*S*)-zacopride were from Delalande Laboratories (Rueil-Malmaison, France), and metoclopramide was from Delagrange Laboratories (Paris, France).

Cell cultures. Cells from colliculi neurons from 14–15-day-old Swiss mouse embryos were dissociated and plated in serum-free medium, in 12-well Costar tissue culture dishes that had been previously coated with poly-L-ornithine. Cultures were maintained for 6 days at 37° in a humidified atmosphere in 6% CO_2/94% air. Under these culture conditions, already described in detail by Weiss *et al.* (40) and Dumuis *et al.* (4), >95% of the cells are represented by neurons.

cAMP formation. Intracellular cAMP levels were determined by measuring the conversion of the [^{3}H]adenine nucleotide precursor [^{3}H]ATP to [^{3}H]cAMP, as described previously (4). On the sixth day of culture and before each experiment, neurons were incubated at 37° for 2 hr with culture medium containing 2 μCi/ml [^{3}H]adenine (24 Ci/mmol) (Amersham, UK). After 2 hr, the cultures were washed and incubated with 0.75 mM IBMX, 0.1 μM FK, and test agents (agonists or antagonists prepared in culture medium), in a volume of 1 ml, for 5 min at 37°. The reaction was stopped by aspiration of the medium and addition of 1 ml of ice-cold 5% trichloroacetic acid. Cells were loosened with the aid of a rubber scraper and 100 μl of 5 mM ATP/5 mM cAMP were added to the mixture. Cellular protein was centrifuged at 500 × *g* and the supernatant was eluted through sequential chromatography on Dowex and alumina columns, which separated [^{3}H]ATP from [^{3}H]cAMP. We have previously shown that, in neuronal cultures, 0.1 μM FK does not modify basal cAMP concentrations but increases neurotransmitter efficacy in cAMP production; potency remains unaffected (40).

Homogenate preparation. Colliculi neurons were grown in Falcon Petri dishes for 6–8 days, as described above. After removal of the culture medium, cells were washed four times at room temperature with an isotonic solution (50 mM Tris maleate, 2 mM EGTA, 10% (w/v) sucrose, pH 7.4). Cells (20×10^6) were scraped and harvested in 0.5 ml of the same medium. The suspension was homogenized at 4° in a Dounce homogenizer (10 strokes).

Adenylyl cyclase assay. Adenylyl cyclase was initiated by addition of the homogenate (5–15 μg of protein) in an incubation mixture of 100 μl (50 mM Tris maleate, pH 7.4, 2 mM $MgSO_4$, 0.2 mM ATP, 10 μM GTP, 1 mM cAMP, 5 mM creatine phosphate, 200 μg/ml creatine kinase, 0.5 mM IBMX, 2 μCi of [α-^{32}P]ATP, 2×10^{-3} μCi of [^{3}H]cAMP). Incubation was carried out at 30° for 5 min and the reaction was stopped by addition of 900 μl of 5.5 mM Tris·HCl, pH 7.6, 0.4 mM ATP, 0.6 mM cAMP, 10 mM $CaCl_2$, 0.1 N HCl. The [^{32}P]cAMP formed was isolated according to the method of Salomon *et al.* (41).

Desensitization of the 5-HT$_4$ receptor-adenylyl cyclase system in colliculi neurons. Whatever the duration of the desensitization period, (0–24 hr), neurons were incubated in culture medium containing 2 μCi/ml [^{3}H]adenine for 2 hr before the cAMP accumulation period, which immediately followed the desensitization period. The cAMP accumulation was started by addition of culture medium containing IBMX (0.75 mM) plus FK (0.1 μM) and cAMP-stimulating agents. The accumulation was measured for 5 min as described above (cAMP accumulation period). During the desensitization period, performed in the absence of IBMX and FK, no significant increase was observed in the presence of 5-HT.

Permeabilization of colliculi neurons. Neurons grown for 6 days first were labeled with [^{3}H]adenine as described above and then were made permeable with a commercial kit (Trans-Port transient cell permeabilization kit; GIBCO BRL), following the indicated protocol for attached cells. Neurons (10^6 cells/well) in 12-well Costar tissue culture dishes were rinsed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and were incubated at 37° for 20 min with the permeabilization solution, containing 360 μl of intracellular buffer (an isotonic high-potassium HEPES buffer that mimics intracellular ion concentration), 10 μl of transport reagent, 5 mM glucose, and 2 mM ATP. PKI or heparin was added immediately after the transport reagent. After 20 min at 37°, the permeabilization reaction was stopped by addition of 20 μl of stop solution to each well. The solution was removed and rinsed once with phosphate-buffered saline. The permeabilized cells were incubated for 15–30 min in serum-free medium containing 2 μCi/ml [^{3}H]adenine and, after this period, labeled cells were incubated with or without 5-HT$_4$ agonists or other drugs for indicated periods of time (desensitization period). All the following experiments were carried out as described for intact cells. As observed by microscope, neurons retained their normal morphological appearance and >95% of the cells were trypan blue positive.

Data analysis. In all figures, the mean values of at least three experiments performed in duplicate have been displayed. Standard errors are represented by vertical bars. EC_{50} refers to the agonist concentrations yielding 50% of the maximal activation, determined directly on each concentration-response curve. pEC_{50} is the negative logarithm of EC_{50}. *V* is the adenylate cyclase activity minus basal activity. The maximal efficacy (E_{max}) of the agonists is the maximal stimulating effect on cAMP formation as a percentage of the maximal stimulatory effect of 5-HT.

Results

Time course of 5-HT$_4$ receptor desensitization and resensitization in colliculi neurons. We observed that cAMP accumulation, subsequently obtained during a 5-min

incubation period with a saturating dose of 5-HT (10 μM), IBMX (0.75 mM), and FK (0.1 μM), was reduced when colliculi neurons were preincubated with 5-HT (10 μM) or BIMU 8 (10 μM) (a potent 5-HT$_4$ agonist) (7) for the indicated periods of time (desensitization period) (Fig. 1A). After a 5-min drug exposure period, the maximal stimulation of adenylyl cyclase

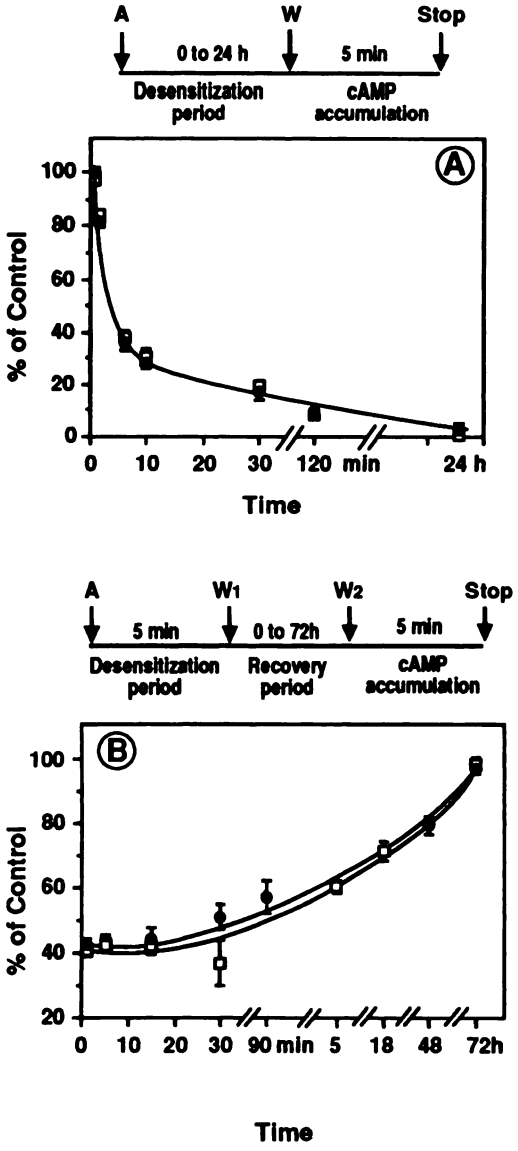

Fig. 1. Time course of desensitization and resensitization of 5-HT$_4$ receptors in colliculi neurons. A, Neurons were preincubated with 5-HT (10 μM) (□) or BIMU 8 (10 μM) (●) for the indicated time (0–24-hr desensitization periods) (*A* to *W*). After three washes with culture medium (*W*), cAMP accumulation was started by addition of culture medium containing IBMX (0.75 mM) plus FK (0.1 μM) plus 5-HT (10 μM) (□) or BIMU 8 (10 μM) (●) and was measured for 5 min (cAMP accumulation period). B, Neurons were preincubated for 5 min (desensitization periods) (*A* to W_1) with 5-HT (10 μM) (□) or BIMU 8 (●) (10 μM) and then washed three times with culture medium (W_1). cAMP accumulation was started with 5-HT at W_2, after a recovery period in fresh medium (0 to 72 hr) (W_1 to W_2). Results are expressed as a percentage of residual stimulation relative to the maximal stimulation by each agonist, 5-HT (10 μM) (□) or BIMU 8 (10 μM) (●), taken as 100%. The basal conversion of [^{3}H]ATP to [^{3}H]cAMP was 0.8 ± 0.3%. 5-HT (10 μM)- and BIMU 8 (10 μM)-stimulated conversion was 2.4 ± 0.35 and 2.7 ± 0.4%, respectively. The results are the mean ± standard error of three separate experiments performed in duplicate.

by 5-HT was equal to only 40% of the control value. A drug exposure period of 2 hr was required to totally abolish the 5-HT-evoked response. As shown in Fig. 1A, desensitization occurred with a half-time of 2 min and clearly proceeded in two steps, a rapid step (0 to 10 min) and a slower one (10 min to 2 hr). After a 5-min exposure to 5-HT (10 μM) or BIMU 8 (10 μM), a 72-hr recovery period in the presence of fresh medium was required to fully recover the maximal 5-HT-induced adenylyl cyclase stimulation (Fig. 1B). After a 30-min exposure to 5-HT$_4$ agonists, 5-HT-induced adenylyl cyclase stimulation was only partially recovered after 72 hr. After this period of time, 80% of the enzymatic activity was recovered when desensitization was induced by 5-HT and only 50% when it was induced by BIMU 8 (data not shown).

Effect of 5-HT pretreatment on the EC_{50} and E_{max} of the 5-HT dose-concentration curves. When colliculi neurons were preincubated with 5-HT (10 μM) for different periods of time (2, 5, or 10 min) and assayed for agonist-induced cAMP accumulation with increasing concentrations of the same drug (10^{-8} to 10^{-4} M), we detected no shift in the EC_{50} for 5-HT, as shown by the Eadie-Hofstee representation (Fig. 2B). The EC_{50} for 5-HT was 78, 68, 64, and 66 nM, respectively, after 0-, 2-, 5-, and 10-min desensitization periods. Only the maximum responses (E_{max}) were reduced by 38, 56, and 68% after the same drug exposures (Fig. 2A).

Relationship between the potency of 5-HT$_4$ agonists and their ability to desensitize 5-HT$_4$ receptors. We tested the ability of nine 5-HT$_4$ agonists to desensitize the 5-HT$_4$ receptor. These agonists belong to either the indol (5-HT), the benzamide (cisapride, renzapride, zacopride, or metoclopramide), or the benzimidazolone groups (BIMU 8 or BIMU 1). Colliculi neurons were preincubated with 5-HT$_4$ agonists (10 μM), at concentrations that have previously been shown to induce maximal cAMP accumulation for all compounds (4, 6, 7). As shown in Fig. 3A, 5-min applications of potent full agonists, such as 5-HT, BIMU 8, or cisapride, reduced the maximal efficacy of 5-HT by 63, 64, and 50%, respectively. In contrast, the same applications of weak agonists, such as (*R*)-zacopride and metoclopramide, reduced the maximal efficacy of 5-HT by only 18 and 3%, respectively. Preincubation of neurons with the weak partial agonist metoclopramide, at a concentration of up to 1 mM, did not increase the desensitization obtained with 5-HT (data not shown). We compared the percentage desensitization obtained with nine 5-HT$_4$ agonists with their respective affinity in stimulating cAMP formation (pEC_{50} values reported in Fig. 3B). As shown in Fig. 3D, a significant correlation was found between the pEC_{50} and the percentage desensitization (5-min preincubation) ($r = 0.95$) but not between the maximum efficacy (E_{max}) (values reported in Fig. 3C) and the percentage desensitization (5-min preincubation) ($r = 0.5$) (Fig. 3E).

Relationship between the ability of a compound to stimulate intracellular cAMP production and to desensitize 5-HT$_4$ receptors. βAR and VIP receptors positively coupled to adenylyl cyclase are expressed by colliculi neurons. We compared the ability of a series of drugs (added alone or in combination) to increase cAMP levels over a 5-min incubation period, i.e., 5-HT (10 μM), BIMU 8 (10 μM) (both in the absence of FK), ISO (10 μM), VIP (1 μM) (both in the presence of 0.1 μM FK), high concentrations of FK (10 μM), and high concentrations of FK (10 μM) plus VIP (1 μM) (Fig. 4A).

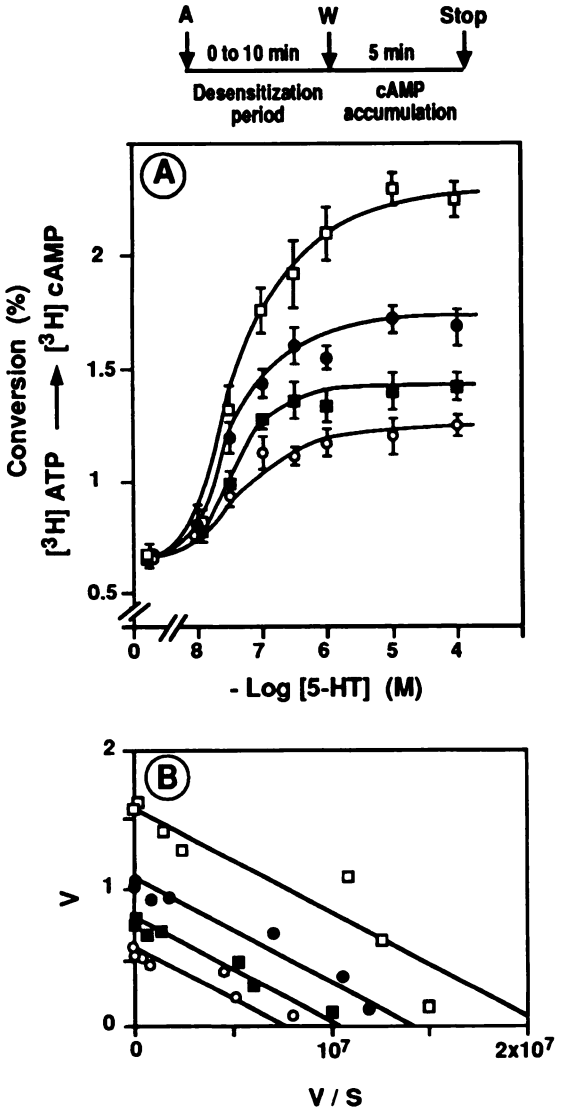

Fig. 2. Effect of 5-HT treatment on the 5-HT concentration-response curves. A, Neuronal cells were incubated in the absence (□) or presence of 5-HT (10 μM) for 2 (●), 5 (■), or 10 min (○). The desensitization (2-, 5-, and 10-min) periods (*A* to *W*) were terminated by three washes (*W*), and cAMP accumulation was started by addition of culture medium containing IBMX (0.75 mM) plus FK (0.1 μM) and increasing concentrations of 5-HT (10^{-8} top 10^{-4} M) for 5 min, as described in Experimental Procedures. Results are expressed as the percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP (mean ± standard error) from three separate experiments, each performed in duplicate. B, Eadie-Hofstee transformation of the dose-response curves of 5-HT (illustrated in A). *V* (on the *y*-axis) represents the net activity (minus basal activity) and *S* (in *V*/*S* on the *x*-axis) the 5-HT concentration.

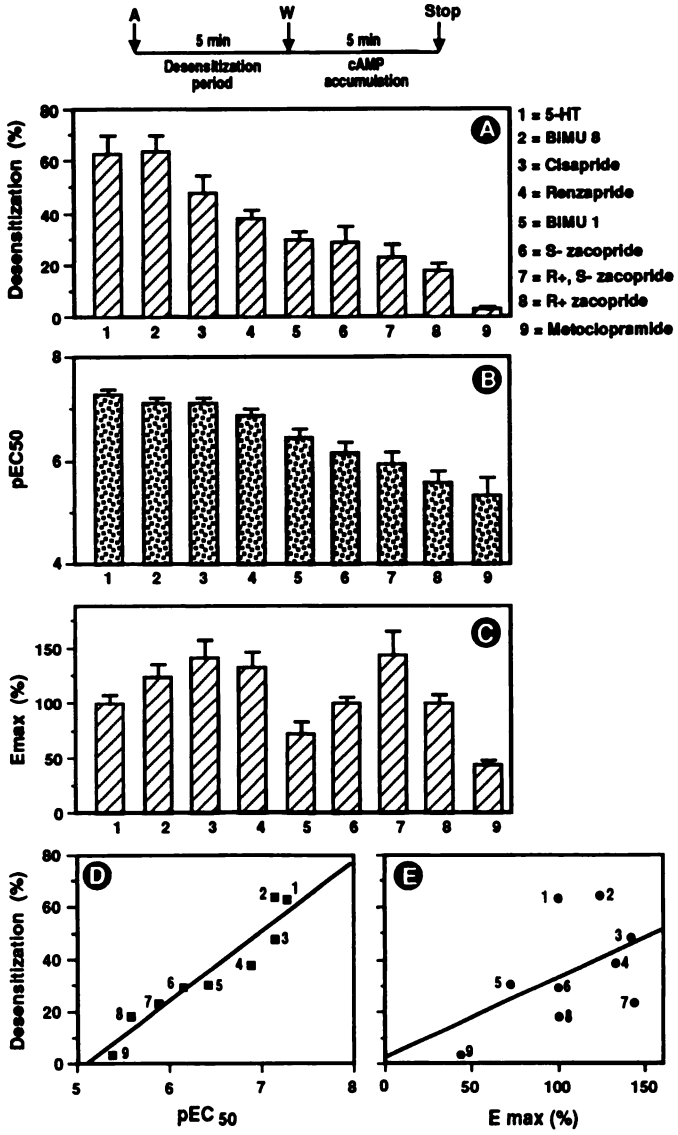

Fig. 3. Desensitization of the 5-HT$_4$ receptor induced by different 5-HT agonists. A, Neurons were incubated with 10 μM concentrations of one of the indicated 5-HT$_4$ agonists for a 5-min desensitization period (*A* to *W*) before measurement of cAMP accumulation. Neurons were washed three times with culture medium (*W*) and cAMP accumulation in the presence of 5-HT (10 μM) was started as described for Fig. 1. Desensitization was measured as percentage loss of stimulation by 5-HT (10 μM) total loss of stimulation taken as 100%. Basal conversion of [^{3}H]-ATP to [^{3}H]cAMP was 0.7 ± 0.2% and 5-HT (10 μM)-stimulated conversion was 2.3 ± 0.4%. The results are the mean ± standard error of four to six separate experiments. B, Representation of the pEC_{50} values, taken from Dumuis *et al.* (4, 6, 7) and Bockaert *et al.* (1). Each compound is numbered as in A. C, Representation of the E_{max} values, taken from Dumuis *et al.* (4, 6, 7) and Bockaert *et al.* (1). Each compound is numbered as in A. D, Correlation between the potencies (pEC_{50}) of 5-HT$_4$ agonists and their abilities to desensitize the 5-HT$_4$ receptor-mediated adenylyl cyclase response in colliculi neurons. The pEC_{50} values of nine 5-HT$_4$ agonists (on the *x*-axis) for stimulating cAMP production are correlated with the percentage desensitization mediated by the corresponding 5-HT$_4$ agonists (on the *y*-axis). The correlation coefficient (*r*) is 0.95 after a desensitization period of 5 min. E, Correlation between the maximum efficacies (E_{max}) of the same nine agonists as in A and their abilities to desensitize the 5-HT$_4$ receptor. The maximum efficacy (E_{max} on the *x*-axis) of the agonists is the maximal stimulatory effect on adenylyl cyclase activity as a percentage of the maximal stimulatory effect of 5-HT (taken as 100%). E_{max} values are correlated with the percentage desensitization mediated by the corresponding 5-HT$_4$ agonist (on the *y*-axis). The correlation coefficient (*r*) is 0.5 after a desensitization period of 5 min.

When the cells were stimulated with 5-HT$_4$ agonists alone, the cAMP accumulation was relatively low (1% conversion), whereas it varied from 4 to 8% when cells were stimulated with other drugs; 8% conversion represented the highest intracellular cAMP level that could be obtained in colliculi neurons with FK plus VIP (Fig. 4A, *bar 6*). When the same drugs were applied for 5 min before cAMP accumulation, only 5-HT$_4$ agonists induced (as already shown) a 60% reduction in subsequent 5-HT responses (Fig. 4B, *bars 1* and *2*), whereas the reduction was only 5–9% with other drugs (Fig. 4B, *bars 3–6*).

The cAMP accumulation induced by VIP (10 μM) during a

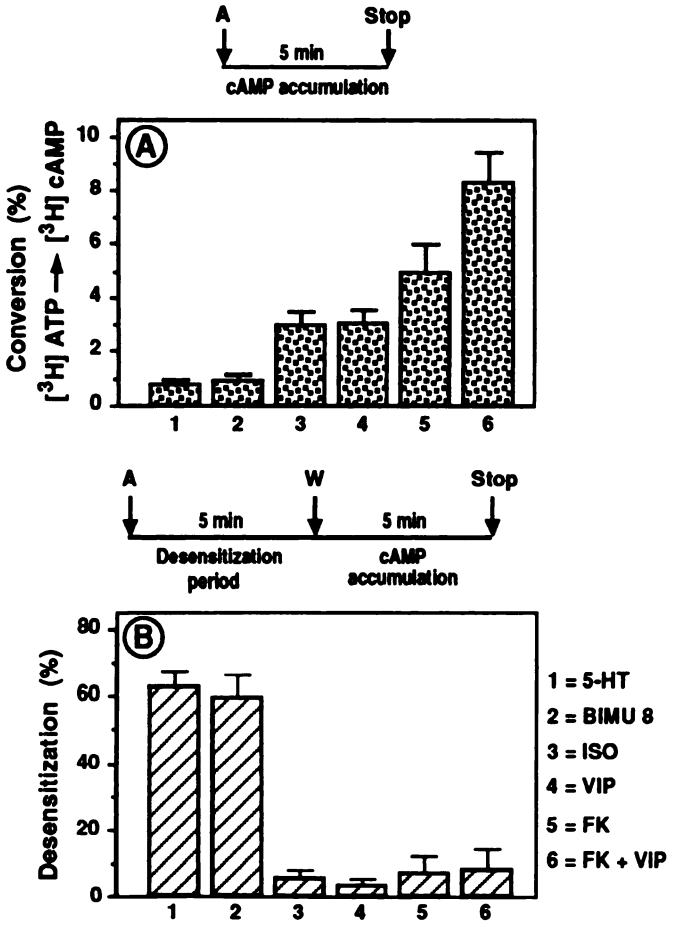

Fig. 4. Absence of correlation between the capacity of drugs to mediate adenylyl cyclase stimulation and to desensitize 5-HT_4 receptors in colliculi neurons. A, cAMP accumulation during 5 min with the following agents: 5-HT (10 μM) (*bar 1*), BIMU 8 (10 μM) (*bar 2*) (both in the absence of FK), ISO (10 μM) (*bar 3*), VIP (1 μM), (*bar 4*) (both in the presence of 0.1 μM FK), FK (10 μM) (*bar 5*), or FK (10 μM) plus VIP (1 μM) (*bar 6*). cAMP accumulation was measured as described in the legend to Fig. 1. Results are expressed as the percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP. Data are the mean ± standard error of four separate experiments, each performed in duplicate. B, Neurons were pretreated for 5 min (*A* to *W*) with the same cAMP-stimulating agents as indicated in A. Cells were washed (*W*) and as described for Fig. 1, cAMP accumulation in the presence of 5-HT (10 μM) was measured. Desensitization was quantified as the percentage loss of cAMP formation obtained with 10 μM 5-HT.

5-min incubation period in the presence of IBMX (0.75 mM) and FK (0.1 μM) was identical whether or not colliculi neurons were preincubated with 5-HT (10 μM) for 30 min (269 ± 24% and 254 ± 31% stimulation over basal after preincubation or not with 5-HT, respectively). Similar results were obtained with ISO.

These results clearly show that rapid desensitization of 5-HT_4 receptors was not dependent on intracellular cAMP levels. These results were confirmed by the following experiments. Treatment of the cells with the permeant cAMP analogues 8-Br-cAMP or Bt_2-cAMP at 1 mM, two compounds that directly activate PKA (implicated in the heterologous desensitization process) (30), had no effect on the 5-HT-induced cAMP accumulation. Moreover, desensitization of the 5-HT_4 receptor was not affected by phorbol-12,13-dibutyrate, a direct activator of PKC (data not shown).

To ascertain whether desensitization of the 5-HT_4 receptor can be followed by measuring adenylyl cyclase activity using homogenates of colliculi neurons, we first treated intact cells with either 5-HT (10 μM) or ISO (10 μM) for 10 min and then assayed for 5-HT-stimulated adenylyl cyclase activity (10 μM) using homogenates prepared as described in Experimental Procedures. Under these conditions, homogenates prepared from 5-HT-treated cells exhibited a loss of 66 ± 7% of the 5-HT-stimulated adenylyl cyclase activity, whereas homogenates prepared from cells treated with ISO (10 μM) retained their maximal 5-HT-stimulated adenylyl cyclase activity (Fig. 5). These results indicate that rapid homologous desensitization of 5-HT_4 receptors was not due to an indirect effect of cAMP accumulation when the experiments were carried out using intact cells.

Effects of protein kinase inhibitors. In order to further explore the possible implication of PKA in the desensitization of the 5-HT_4 receptor, H7 (1–100 μM), an inhibitor of PKA and protein kinase C, was added 15 min before and during the 5-min pretreatment with 5-HT_4 agonists. As reported in Table 1, H7 did not prevent any desensitization of the 5-HT response. We confirmed this result using PKI, a selective PKA inhibitor. Because PKI is unable to enter intact cells, neurons were permeabilized as described in Experimental Procedures. They were loaded with 1–10 μM PKI and then exposed to 5-HT (10 μM) before cAMP accumulation measurement. As shown in Table 1, PKI did not affect desensitization induced by a 5-min exposure to 5-HT (10 μM). We have previously shown (25) in these neurons that H7 and PKI inhibit the cAMP-dependent inhibition of K^+ channels by 5-HT_4 receptors; these results suggested that PKA-dependent phosphorylation is not likely to be involved in the 5-HT_4 receptor desensitization process.

When permeabilized neurons were loaded with heparin (1–10 μM), an inhibitor of βARK (39), and then exposed to 5-HT (10 μM) for 5 min, we obtained a reduction of 30–40% in the desensitization rate (Table 1).

Zn^{2+} has been reported to be an *in vitro* inhibitor of βARK (28). As shown in Fig. 6A, low concentrations of Zn^{2+} (25 μM to 1 mM) markedly reduced the 5-HT-mediated desensitization. This effect was dose dependent, and a 100% blockade of desensitization could be obtained with 1 mM Zn^{2+}.

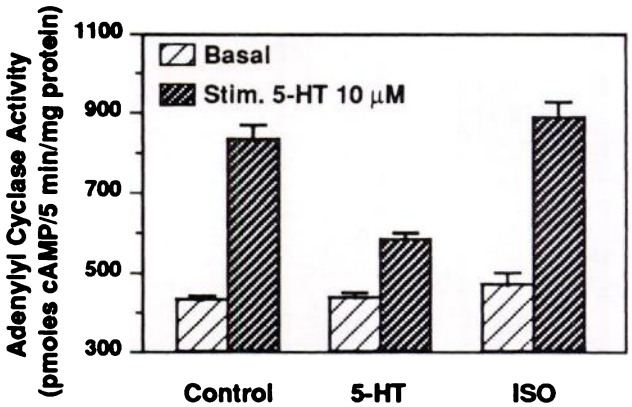

Fig. 5. Effect of 5-HT and ISO pretreatment on 5-HT-stimulated adenylyl cyclase activity measured in colliculi neurons. Cells were incubated for 10 min in the absence (control) or in the presence of either 10 μM 5-HT or 10 μM ISO in culture medium; cells were then washed four times in 50 mM Tris maleate, 2 mM EGTA, 10% (w/v) sucrose, pH 7.4, and lysed in the same buffer. Homogenates were prepared and adenylyl cyclase activity was measured after 10 μM 5-HT stimulation for 5 min, as described in Experimental Procedures. Data are the mean ± standard error of three experiments, each performed in triplicate.

TABLE 1

Effect of inhibitors on agonist-mediated desensitization of adenylyl cyclase activity in intact and permeabilized neurons

Intact or permeabilized neurons were pretreated before desensitization, as follows: control without inhibitor, H7 (10 μM) for 15 min, PKI (10 μM) for 15 min, heparin (10 μM) for 15 min, Zn^{2+} (400 μM) for 15 min, Con A (100 μg/ml) for 4 hr, Con A (100 μg/ml) for 4 hr plus heparin (10 μM) for 15 min, or Con A (100 μg/ml) for 4 hr plus Zn^{2+} (400 μM) for 15 min. After the indicated time of pretreatment with one or two agents, neurons were incubated with 5-HT (10 μM) for a desensitization period of 5 min. They were then washed three times and assayed for 5-HT (10 μM)-stimulated cAMP formation as described in the legend to Fig. 1. Data are presented as percentage desensitization, where 100% represents the maximum desensitization obtained with 5-HT (10 μM) during a 5-min desensitization period. In intact neurons the basal percentage of conversion of [^{3}H]ATP to [^{3}H]cAMP was 1.1 ± 0.3%. 5-HT (10 μM)-stimulated conversion was 3.6 ± 0.4 and 2.02 ± 0.3% before and after desensitization, respectively. In permeabilized neurons the basal percentage of conversion was 0.8 ± 0.1%. 5-HT (10 μM)-stimulated conversion was 2.43 ± 0.3 and 1.7 ± 0.2% before and after desensitization. Data are the mean ± standard error of at least three experiments, each performed in duplicate.

Inhibitors	Desensitization	
	Intact neurons	Permeabilized neurons
	%	
None	100 ± 2	100 ± 5
H7, 10 μM	98 ± 3	
PKI, 10 μM		100 ± 4
Heparin, 10 μM		61 ± 15
Zn^{2+}, 400 μM	12 ± 7	
Con A, 100 μg/ml	30 ± 15	49 ± 28
Con A, 100 μg/ml, + heparin, 10 μM		12 ± 11
Con A, 100 μg/ml, + Zn^{2+}, 400 μM	3 ± 2	

In order to verify that Zn^{2+} did not inhibit the coupling between 5-HT$_4$ receptors and adenylyl cyclase, we studied the 5-HT-stimulated cAMP formation in the absence and presence of 400 μM Zn^{2+}. As shown in Fig. 6B, Zn^{2+} did not decrease the 5-HT-stimulated cAMP formation but, as expected, the maximum efficacy of 5-HT was found to be higher in the presence of Zn^{2+}. This was probably the consequence of inhibition of 5-HT-mediated desensitization by Zn^{2+}, rather than the consequence of stimulation of adenylyl cyclase, because increasing concentrations of Zn^{2+} (25 μM to 1 mM) had no effect on basal and FK-induced cAMP formation (data not shown).

Effect of Con A on desensitization of 5-HT$_4$ receptors in intact and permeabilized cells. To study the relative contribution of receptor sequestration in the desensitization process, neurons were pretreated with Con A, a lectin reported to inhibit βAR desensitization by preventing receptor sequestration (42). After a preincubation period of 4 hr at 37° in medium containing 100 μg/ml Con A, cells were washed before exposure to 10 μM 5-HT for 5 min and then 5-HT-induced cAMP formation was measured. As shown in Table 1, pretreatment with Con A inhibited rapid desensitization induced by 10 μM 5-HT by 70%. A similar reduction in desensitization was obtained in permeabilized cells pretreated with Con A. When neurons pretreated with Con A were permeabilized and then preincubated with 10 μM heparin, desensitization induced by 5-HT was totally abolished; similar results were obtained when neurons pretreated with Con A were preincubated with Zn^{2+} (400 μM) (Table 1).

Discussion

Agonist-induced desensitization of 5-HT$_4$ receptors coupled to adenylyl cyclase in colliculi neurons occurs via mechanisms that are not dependent on intracellular cAMP. Only exposure of neurons to selective 5-HT$_4$ agonists led to a potent desensitization. Neurons exposed to other agents, like ISO, VIP, or FK, that increase cAMP levels did not undergo any desensitization of 5-HT$_4$ receptors. Symmetrically, incubation of neurons with 5-HT did not desensitize ISO or VIP responses. We have clearly shown that there is no correlation between the percentage of conversion of ATP to intracellular cAMP and the percentage desensitization (Fig. 4). The absence of 5-HT$_4$ receptor desensitization after treatment with either ISO or VIP could be due to a different cellular localization of the adenylyl cyclase-coupled receptors. This hypothesis can be ruled out by the experiments performed either with FK (a direct activator of adenylyl cyclase) or with VIP plus FK (Fig. 4). This latter combination produced the highest level of cAMP (8% conversion of ATP to cAMP) that can be reached in colliculi neurons. Whatever the neurotransmitter tested, its effect on cAMP production in neurons in primary culture was not additive with that obtained using VIP plus FK (data not shown) (43). Moreover, when neurons were incubated with high concentrations of 8-Br-cAMP or Bt$_2$-cAMP (1–3 mM), compounds that are

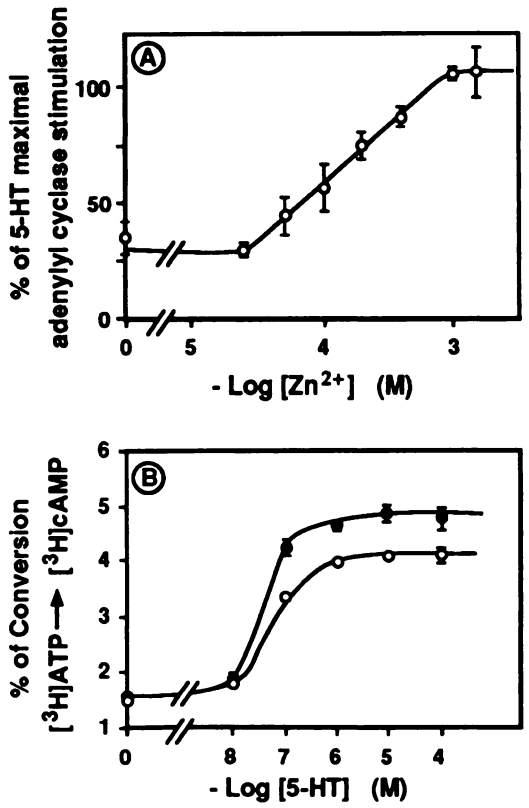

Fig. 6. Effect of Zn^{2+} on 5-HT$_4$-induced cAMP accumulation and desensitization. A, Neurons were pretreated with various concentrations of Zn^{2+} (0–1 mM) for 15 min. 5-HT (10 μM) was then added for a 5-min desensitization period. The cells were then washed three times and cAMP accumulation was started by addition of 10 μM 5-HT and was measured as described in the legend to Fig. 1. Results are expressed as a percentage of residual activity relative to the maximal stimulatory effect of 10 μM 5-HT, taken as 100%. The basal conversion of [^{3}H]ATP to [^{3}H]cAMP was 1.54 ± 0.05%. The 5-HT (10 μM)-stimulated conversion was 4.06 ± 0.02 and 2.48 ± 0.09% before and after desensitization, respectively. Data are the mean of three experiments, each performed in duplicate. B, Effect of Zn^{2+} on the stimulation of cAMP formation in colliculi neurons by 5-HT. Cells were incubated with increasing concentrations of 5-HT and FK (0.1 μM) in the absence (○) or presence (●) of Zn^{2+} (400 μM). Conversion of [^{3}H]ATP to [^{3}H]cAMP was determined after 5 min at 37°, as described in Experimental Procedures.

direct activators of PKA, desensitization of the 5-HT_4 receptor-adenylyl cyclase system was not observed.

Zhou and Fishman (44), in a study of human β_1-AR desensitization, suggested that agonist-occupied receptors may be better substrates for PKA than unoccupied receptors. However, when neurons were loaded with PKI or H7 (both PKA inhibitors) and then exposed to 5-HT_4 agonists, the agonist-induced desensitization remained unchanged. The fact that we observed no shift in the EC_{50} of adenylyl cyclase activation by 5-HT, but only a massive reduction in maximum responses, suggests that PKA does not mediate the desensitization of the 5-HT_4 receptor-mediated response. This suggestion is based on the reports by Clark *et al.* (32, 45), Kandel *et al.* (46), Zhou and Fishman (44), and Lohse *et al.* (39), who showed that the shift in the EC_{50} of adenylyl cyclase activation by βAR agonists could be attributed to phosphorylation of βAR by PKA. The shift in EC_{50} for agonists after desensitization of βAR was related to the fact that PKA plays a predominant role in βAR desensitization when the receptors are exposed to low agonist concentrations (39). However, Lohse *et al.* (39) and Lefkowitz *et al.* (31) reported that even at high agonist concentrations both kinases (PKA and βARK) are involved in the desensitization process for β_2AR (60% is attributed to βARK and 40% to PKA) (46).

The absence of PKA expression in colliculi neurons after 6 days in culture can also be ruled out. Our laboratory has recently shown that in colliculi neurons (6 days *in vitro*) (25) some outward K^+ currents (30–40%) can be inhibited by 5-HT_4 receptors. This 5-HT_4-mediated response was blocked by PKI and H7 and was mimicked by cAMP. The fact that 5-HT_4 receptor desensitization is not PKA mediated is consistent with the suggestion made by Roth *et al.* (47) and Dohlman *et al.* (48), who reported that PKA-dependent process may be too slow to mediate rapid desensitization required during neuronal signaling when high concentrations of a neurotransmitter are released at a synapse but may be more adequate in peripheral tissues.

Our results show that desensitization of 5-HT_4 receptors occurs exclusively after exposure to potent 5-HT_4 agonists. This process is termed homologous desensitization. In the βAR system, the desensitization process involves phosphorylation of the agonist-occupied form of the receptor by a kinase independent of intracellular cAMP, termed βARK (39). In the present study, we found a good correlation between the potencies of 5-HT_4 agonists and the percentage of agonist-induced receptor desensitization. The correlation coefficient was $r = 0.95$ after 5-min exposure to the indicated 5-HT_4 agonists (Fig. 3). Few studies have reported a relationship between the efficacy and potency of a drug and its ability to induce desensitization. Su *et al.* (49) reported that in 132N1 astrocytoma cells a partial agonist of the β_2ARK, such as zinterol or soterenol, produced a partial desensitization, compared with that obtained with ISO. Zinterol is more potent but less efficacious (partial agonist) than ISO. Similarly, Pittman *et al.* (50) showed that the rate of βAR loss is a function of intrinsic activities of the agonists used to induce desensitization. However, in these βAR systems homologous and heterologous desensitization and down-regulation occur. In contrast, we found that, in the 5-HT_4 receptor adenylyl cyclase system, the desensitization power of a drug is a function of its potency rather than its efficacy. This is particularly clear with zacopride enantiomers, which are less potent than 5-HT but have the same efficacy (1) and produce 50% of the 5-HT-induced desensitization. It is possible that heterologous desensitization, which is dependent on cAMP, would be a function of drug efficacies. In contrast, homologous desensitization, which is due to the phosphorylation of occupied receptors by an agonist-specific kinase, is expected to be related to the mean occupancy time of the receptor by the agonist; the higher the mean occupancy time, the greater could be the phosphorylation and thus the homologous desensitization. At equilibrium, the mean occupancy time is a direct function of $1/k - 1$, a value that generally increases when the potency of a drug increases. The apparent discrepancy between the results obtained by Pittman *et al.* (50) and those obtained here could be simply due to the different steps in desensitization studied in these two models.

Several inhibitors of βARK, such as heparin and Zn^{2+}, have been described previously (39, 28). Inhibition by Zn^{2+} has also been observed in studies of rhodopsin phosphorylation by rhodopsin kinase (51). Both compounds caused a decrease in 5-HT_4 receptor desensitization. Heparin was able to partially inhibit desensitization induced by 5-HT when neurons were permeabilized and loaded with the inhibitor (about 40%), whereas Zn^{2+} markedly inhibited the 5-HT-induced desensitization (about 100% inhibition) in intact neurons. This is the first demonstration showing that Zn^{2+} can be a useful tool to inhibit homologous desensitization in intact cells.

Zn^{2+} can either inhibit a receptor-specific protein kinase as demonstrated *in vitro* by Benovic *et al.* (28), or like other divalent cations (Mg^{2+}, Mn^{2+}) activate a phosphoprotein phosphatase (52). In both situations, either inhibition of a kinase or stimulation of a phosphatase will lead to a decrease in receptor phosphorylation and consequently to a decrease in 5-HT_4 receptor desensitization.

Whether the well studied βARK is involved in the homologous desensitization of other adenylyl cyclase-coupled receptors is unknown. Strasser *et al.* (53) reported that the same kinase may act on multiple adenylyl cyclase-coupled receptors. Despite the fact that 5-HT_4 receptor kinase has not been identified, our results suggest that homologous desensitization of the 5-HT_4 receptor involves a pattern of events similar to that described for homologous desensitization of βAR.

A fast desensitization response to 5-HT is sensitive to pretreatment with Con A, which is known to prevent sequestration of βAR (38). Desensitization induced by a 5-min exposure to 5-HT was reduced by about 70% after pretreatment of the neurons with Con A. These results suggest that sequestration is a rapid and important step in homologous desensitization of the 5-HT_4 receptor. In βAR-induced desensitization, the role of sequestration is controversial. Some studies suggest that sequestration accounts for little or no desensitization and consider that it is a relatively slow process to play a role (47, 44); others have claimed that the role of sequestration accounts for the entire desensitization process (54). It is fair to say that we did not control whether the mechanism of Con A-induced reduction of 5-HT_4 receptor desensitization was also a sequestration process. In addition to reducing the desensitization of G protein-coupled receptors, pretreatment with Con A also reduces desensitization of ionic channel receptors such as (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors (55, 56). These receptors mediate very fast responses (milliseconds); here again, the mechanisms of action of Con A are

unknown. It is clear that, in experiments using Zn^{2+} and Con A, we cannot exclude effects unrelated to the known actions on receptor-specific kinases and sequestration, respectively.

In conclusion, we have shown that 5-HT$_4$ receptor-mediated cAMP accumulation rapidly desensitizes. This desensitization is not triggered by cAMP but appears to be strictly of the homologous type. Homologous desensitization of the 5-HT$_4$-mediated response is rapid, as expected for a process occurring at synaptic levels. The model described here is also interesting for further investigations, because it constitutes a system in which homologous desensitization is not complicated by heterologous desensitization.

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