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Benzamide derivatives provide evidence for the involvement of a 5-HT_4 receptor type in the mechanism of action of serotonin in frog adrenocortical cells

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We have previously shown that serotonin (5-HT) is a potent stimulator of corticosterone and aldosterone secretion by frog adrenocortical cells and we have demonstrated that the action of 5-HT is not mediated by the classical 5-HT receptor subtypes i.e. 5-HT_1, 5-HT_2 and 5-HT_3. Recently, a non-classical 5-HT receptor (termed 5-HT_4) has been characterized using 4-amino-5-chloro-2-methoxy-benzamide derivatives as serotonergic agonists. In the present report, we have investigated the possible involvement of the 5-HT_4 receptor subtype in the mechanism of action of 5-HT on steroid secretion. Increasing concentrations of benzamide derivatives (zacopride, cisapride and BRL 24924) gave rise to a dose-related stimulation of corticosteroid production, zacopride being the most potent compound of this series to enhance steroidogenesis. Prolonged administration (230 min) of zacopride induced a rapid increase in corticosterone and aldosterone output followed by a gradual decline of corticosteroid secretion. During prolonged exposure of adrenal tissue to zacopride (10^{-5} M), the corticotropic activity of 5-HT (10^{-6} M) was totally abolished. The stimulatory effects of 5-HT and zacopride were abolished by the non-selective 5-HT_3 antagonist ICS 205 930. In contrast methysergide, a 5-HT_1 receptor antagonist, and MDL 72222, a selective 5-HT_3 antagonist did not block zacopride-induced corticosteroid secretion. Both 5-HT and zacopride induced a dose-related increase in cAMP production by frog adrenal slices. Taken together, these results indicate that the stimulatory effect of 5-HT on frog adrenocortical tissue is mediated by activation of a 5-HT_4 receptor subtype positively coupled to adenylate cyclase.

INTRODUCTION

It is now well established that the physiological effects of serotonin (5-HT) in the central nervous system (CNS) or in peripheral tissues are mediated through different types of receptors. Until recently, it was generally accepted that 3 classes of specific 5-HT binding sites exist in brain membranes based on interactions with various agonists and antagonists[6]. The 5-HT_1 class is subdivided into 4 receptor subtypes called 5-$HT_{1A,1B,1C}$ and $_{1D}$ which are all characterized by a nanomolar affinity for 5-HT and selected agonists, but a micromolar affinity for most antagonists[19]. The 5-HT_2 class has in contrast a nanomolar affinity for selective antagonists and a micromolar affinity for 5-HT and related agonists[3]. The 5-HT_{1A} and 5-HT_{1B} receptor subtypes have been reported to be respectively positively and negatively coupled to adenylate cyclase[5,16,34,35]. Conversely, activation of 5-HT_{1C} and 5-HT_2 receptors causes stimulation of phosphatidylinositol turnover[17,18]. In addition to 5-HT_1 and 5-HT_2 receptors, a 6th category of 5-HT receptors has been identified. These receptors, which mediate many of the excitatory actions of serotonin in the peripheral nervous system[11,30], have been called the 5-HT_3 subtype. Very recently, Bockaert et al.'s group[4,13,14], reported the existence, in the central nervous system, of a 5-HT receptor having pharmacological properties different from those of 5-HT_1, 5-HT_2 and 5-HT_3 receptors. This newly discovered receptor subtype which is positively coupled to adenylate cyclase has been called 5-HT_4[4,13–15].

In amphibians the presence of 5-HT in adrenal chromaffin cells has been demonstrated by immunohistochemical and biochemical techniques[10]. We have recently shown that 5-HT stimulates corticosteroid secretion by frog adrenocortical tissue in vitro[11]. Preliminary pharmacological characterization revealed that the action of 5-HT in the frog adrenal gland is not mediated by the classical receptor subtypes i.e. 5-HT_1, 5-HT_2 and 5-HT_3[20].

In the present study, we provide evidence for the

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involvement of 5-HT_4 receptors positively coupled to adenylate cyclase, in the stimulatory effect of 5-HT on steroid secretion.

MATERIALS AND METHODS

Secretagogues and reagents

5-Hydroxytryptamine (5-HT, serotonin), HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) and EDTA were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). 8-Hydroxy-2-(di-*N*-propylamino)-tetraline (8-OH-DPAT) was obtained from Research Biochemical Inc. (Wayland, MA, U.S.A.). ICS 205 930, 2-methyl serotonin and methysergide were generously provided by Sandoz (Basel, Switzerland), cisapride was given by Janssen Research Fondation (Beerse, Belgium), BRL 24924 was obtained through the courtesy of Beecham Pharmaceuticals (Harlow, U.K.), zacopride was kindly provided by Robins Company Research Labs. (Richmond, VA, U.S.A.) and MDL 72222 was a generous gift of Merrell Dow Research Center (Strasbourg, France). [1,2,6,7-^{3}H]corticosterone and [1,2,6,7-^{3}H]aldosterone were purchased from Amersham Int. (U.K.)

Perifusion system technique

For each experiment, 8 interrenal (adrenal) glands from male frogs (*Rana ridibunda* Pallas) were dissected free of kidney tissue. The glands were sliced and preincubated in Ringer's buffer. The interrenal slices were then transferred into a siliconized glass column between several beds of Bio-Gel P2 (Bio-Rad Labs.; Richmond, CA, U.S.A.). Each chamber had a packed bed volume of 0.3 ml and a head volume of 0.5 ml. The tissues were continuously perifused with gassed Ringer solution at a constant flow rate (200 μl/min) and temperature (24 °C). The glands were allowed to stabilize for 1 h, and then the experimental manipulation commenced. Test substances were dissolved in Ringer's solution and infused into the column at the same flow rate as Ringer alone by means of a multichannel peristaltic pump (Desaga, Heidelberg, F.R.G.). Fractions of the perifusate were set apart every 5 min, and the tubes were immediately frozen until corticosteroid assays.

Static incubations of adrenal tissue

For each experiment, two interrenal glands were dissected, sliced and preincubated in 1 ml gassed Ringer's solution. Interrenal fragments were rinsed and then incubated in 200 μl of Ringer's buffer in the absence or presence of graded concentrations of 5-HT or zacopride (from 10^{-7} to 10^{-4} M) for 2 min at room temperature. The reaction was stopped by transferring adrenal fragments in 150 μl of ice-cold 5% perchloric acid. The tissues were homogenized and centrifuged (10,000 *g*; 2 min; 4 °C). The supernatant was separated and the pellet was frozen until DNA quantification. An aliquot of the supernatant (120 μl) was transferred into a 5 ml tube and neutralized with 120 μl of 1 M $KHCO_3$. After centrifugation (10,000 *g*; 2 min; 4 °C), 200 μl of the supernatant was diluted in 600 μl acetate buffer (0.05 M) and stored at −20 °C before submission to cAMP radioimmunoassay.

Radioimmunoassays of corticosteroids

The concentrations of corticosterone and aldosterone in each fraction were measured by use of sensitive and specific radioimmunoassays[22,24]. The assays were sensitive enough to detect 20 pg of corticosterone and 5 pg of aldosterone. The specificity of the antibodies has been evaluated by determining their cross-reactivities with 34 different steroids and related compounds. The assay was directly performed on the effluent perifusate without any extraction or purification procedure[22]. None of the secretagogues showed any interference in the assays.

Radioimmunoassay of cAMP

The concentration of cAMP in the tissue extracts was determined using a commercial kit without extraction or acetylation. (code RPA 509; Amersham, U.K.). The sensitivity of the assay is such that 13 fmol/tube cAMP can be detected.

DNA quantification

DNA assay was carried out according to Labarca and Paigen[21] method slightly modified. The tissue pellets and calf DNA standards were treated with EDTA (10 mM, pH 12.3). Twenty μl of all samples and of DNA standard (10 μg/ml) were diluted in 2 ml Hoescht solution (0.1 μg/ml) containing 100 mM Tris base, 10 mM EDTA and 1 M NaCl. Fluorescence was then measured for each sample using TKO 100 Dedicated mini Fluorometer (Hoefer Scientific Instruments, San Francisco) with emission wavelength set at 458 nm.

Calculations

Each perifusion pattern was calculated as the mean profile of corticosteroid secretion (± S.E.M.) established over at least three independent experiments. The levels of corticosterone and aldosterone released were expressed as percentages of the basal values, calculated as the mean of eight samples (40 min) just preceding administration of the first secretagogue. Statistical significance of differences between values was calculated using Student's *t*-test.

RESULTS

Effect of benzamide derivatives on corticosteroid secretion

The effect of graded concentrations of zacopride (from 10^{-7} to 10^{-5} M) on corticosterone and aldosterone production is shown in Fig. 1. Infusion of 20-min pulses of zacopride induced a dose-related increase of corticosterone (a) and aldosterone (b) secretion. The lag period of the responses was 10–15 min and the maximum effect on steroid output was achieved 25 min after the beginning of the administration of the benzamide derivative. Administration of a concentration of 10^{-5} M of zacopride induced a 134% and a 154% increase in corticosterone and aldosterone, respectively.

Series of experiments similar to those presented in Fig. 1 were conducted with different serotonergic agonists. Increasing concentrations of various benzamide derivatives (zacopride, cisapride and BRL 24924, from 10^{-8} to 10^{-4} M) gave rise to a dose-related stimulation of corticosterone (Fig. 2a) and aldosterone (Fig. 2b) production. In contrast, 8-OH-DPAT (5-HT_1 agonist) and 2-methyl-5-HT (5-HT_3 agonist) were totally devoid of effect on steroid secretion. Similar results were obtained with corticosterone secretion (data not shown).

Adrenocortical response to 5-HT during prolonged infusion of zacopride

Fig. 3 shows the effect of 5-HT alone or during prolonged infusion of zacopride on corticosterone (a) and aldosterone (b) secretion. As shown before[11], a 20-min pulse of serotonin (10^{-6} M) induced a marked stimulation of corticosteroid release. Administration of zacopride (10^{-5} M) during 230 min induced a rapid increase in corticosterone and aldosterone release which peaked 50 min after the onset of zacopride infusion. Then steroid

secretion declined gradually. During prolonged exposure of adrenal slices to zacopride, the stimulatory effect of 5-HT on corticosteroid secretion was totally abolished.

Effect of serotonergic antagonists on 5-HT- or zacopride-induced stimulation of corticosteroid secretion

The effects of 5-HT (10^{-6} M) on corticosterone and aldosterone secretion in basal conditions and during prolonged administration of the 5-HT_3 antagonist ICS 205 930 are illustrated in Fig. 4. At a concentration of 10^{-5} M, ICS 205 930 caused a slight stimulation of corticosterone (Fig. 4a) and aldosterone (Fig. 4b) release but did not affect the response of adrenal tissue to serotonin. At a concentration of 5×10^{-4} M, ICS 205 930 caused a marked stimulation of corticosterone (Fig. 4c) and aldosterone (Fig. 4d) release followed by a rapid return to the basal level. During infusion of 5×10^{-4} M ICS 205 930, the stimulatory effect of 5-HT was totally abolished.

The effects of zacopride in the presence of methysergide (a non selective 5-HT_1 and 5-HT_2 antagonist), MDL 72222 and ICS 205 930 (two 5-HT_3 antagonists) on corticosteroid secretion are compared in Fig. 5. Prolonged infusion of methysergide (Fig. 5a,b) or MDL

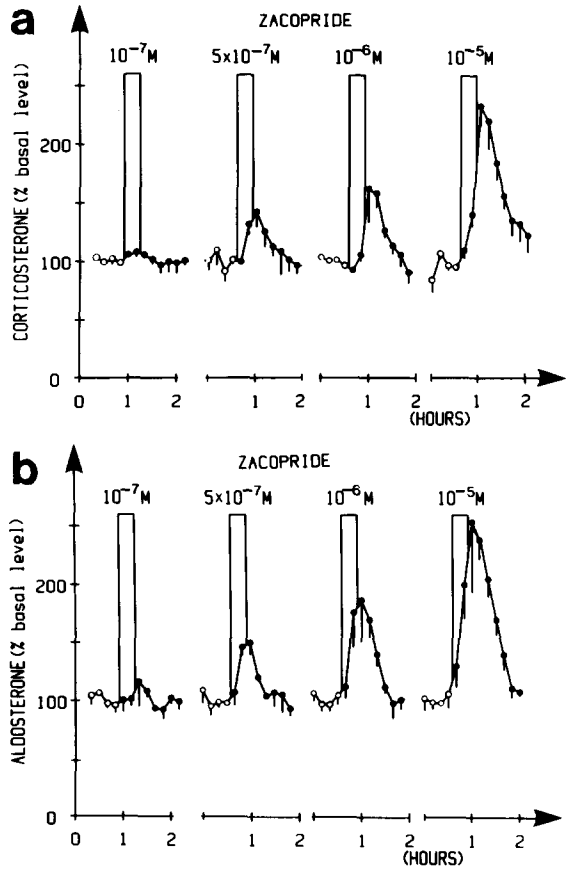

Fig. 1. Effect of increasing concentrations of zacopride (from 10^{-8} to 10^{-4} M) on corticosterone (a) and aldosterone (b) secretion by perifused frog interrenal fragments. After a 120-min equilibration period, zacopride was administered for 20-min. The data represent the means (± S.E.M.) of three independent perifusion experiments. Each point is the mean corticosteroid production of two consecutive fractions collected during 5 min. The spontaneous level of corticosterone and aldosterone release (100% basal level) was calculated as the mean of 8 consecutive fractions (40 min; O———O) just preceding the infusion of each dose of zacopride. The mean secretion rates of corticosterone and aldosterone in these experiments were 117 ± 20 and 101 ± 21 fmol/interrenal gland per min, respectively.

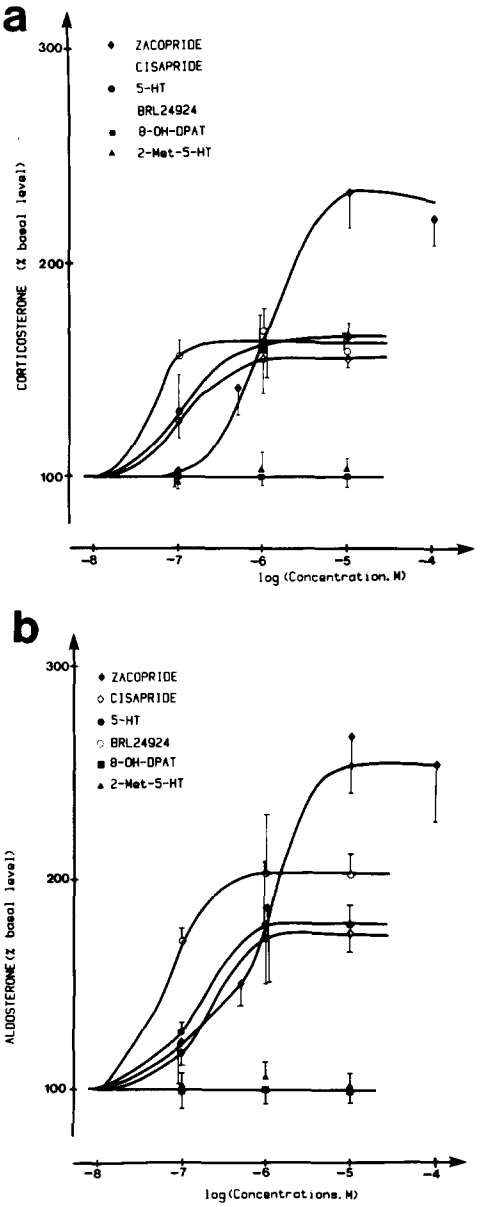

Fig. 2. Semi-logarithmic plot comparing the effect of 5-HT, different benzamide derivatives, 8-OH-DPAT (a 5-HT_1 agonist) and 2-methyl serotonin (a 5-HT_3 agonist) on corticosterone (a) and aldosterone (b) production by frog interrenal slices. All experimental values were calculated from data similar to those presented in Fig. 1. The mean corticosteroid concentration in 2 consecutive fractions collected just after the pulses of 5-HT or benzamide derivatives (peak height) were compared to the mean corticosteroid levels observed just prior to the infusion of each dose of secretagogues.

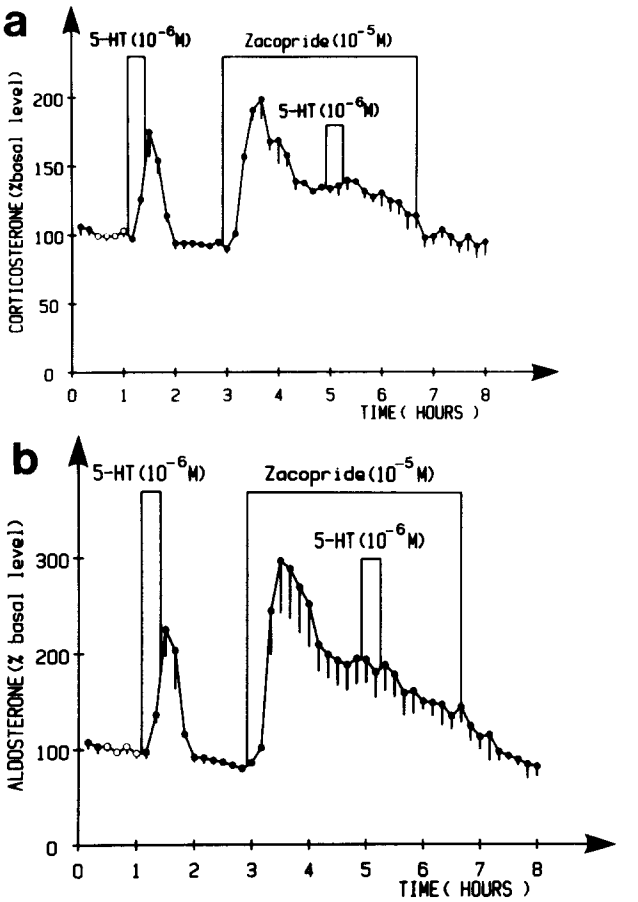

Fig. 3. Effects of 5-HT alone and during prolonged infusion of zacopride on corticosterone (a) and aldosterone (b) secretion. After a 120-min equilibration period, a first pulse of 5-HT (10^{-6} M) was administered for 20 min and the interrenal tissue was allowed to stabilize for another 100-min period. Then, zacopride (10^{-5} M) was infused during 230 min. During infusion of zacopride a second pulse of 5-HT (10^{-6} M) was added for 20 min. The mean secretion rates of corticosterone and aldosterone in basal conditions (100% basal level) were 446 ± 104 and 181 ± 55 fmol/interrenal gland per min. See legend to Fig. 1 for other designations.

72222 (Fig. 5c,d) did not affect the response of adrenal gland to zacopride. In contrast, ICS 205 930 totally blocked the response of the interrenal tissue to zacopride (Fig. 5e,f).

Effect of serotonin and zacopride on cAMP production

Incubation of interrenal slices with 5-HT (Fig. 6a) or

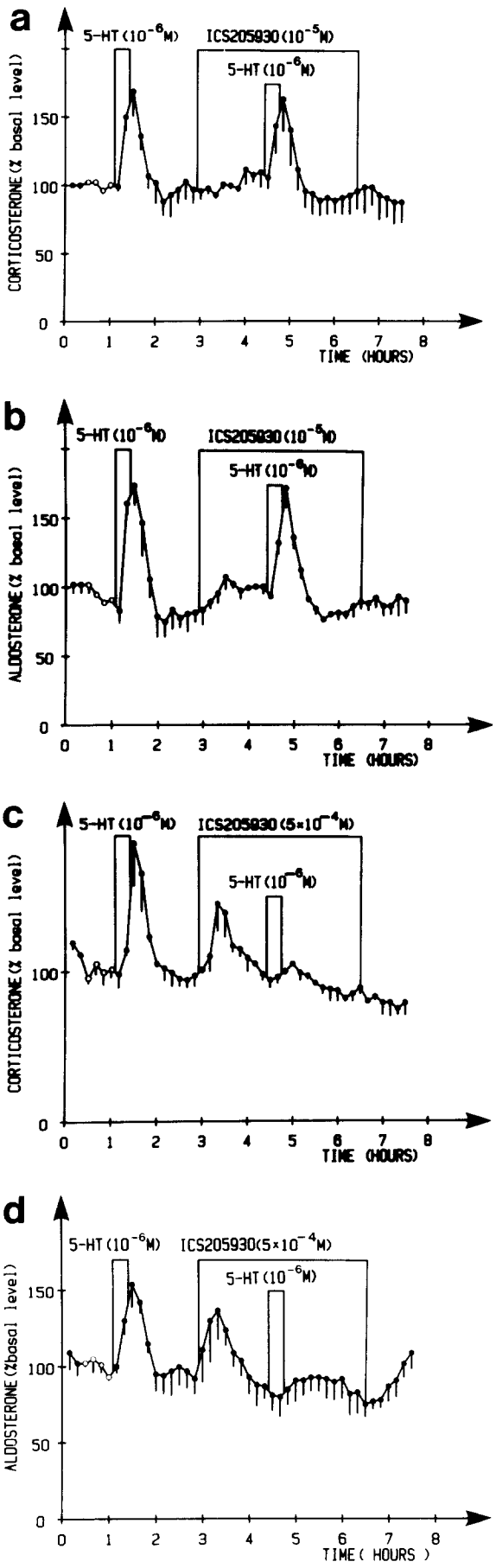

Fig. 4. Effect of 5-HT alone or during prolonged infusion of ICS 205 930 on corticosterone (a,c) and aldosterone (b,d) secretion. After a 120-min equilibration period, a first pulse of 5-HT (10^{-6} M) was administered for 20 min and the interrenal tissue was allowed to stabilize for another 100-min period. Then, ICS 205 930 (10^{-5} M, a and b or 5×10^{-4} M, c and d) was infused during 220 min. During infusion of ICS 205 930, a second pulse of 5-HT (10^{-6} M) was added for 20 min. The mean secretion rates of corticosterone and aldosterone in basal conditions were 105 ± 5 (a), 315 ± 27 (c) and 113 ± 10 (b), 159 ± 28 (d) fmol/interrenal gland per min, respectively. See legend to Fig. 1 for other designations.

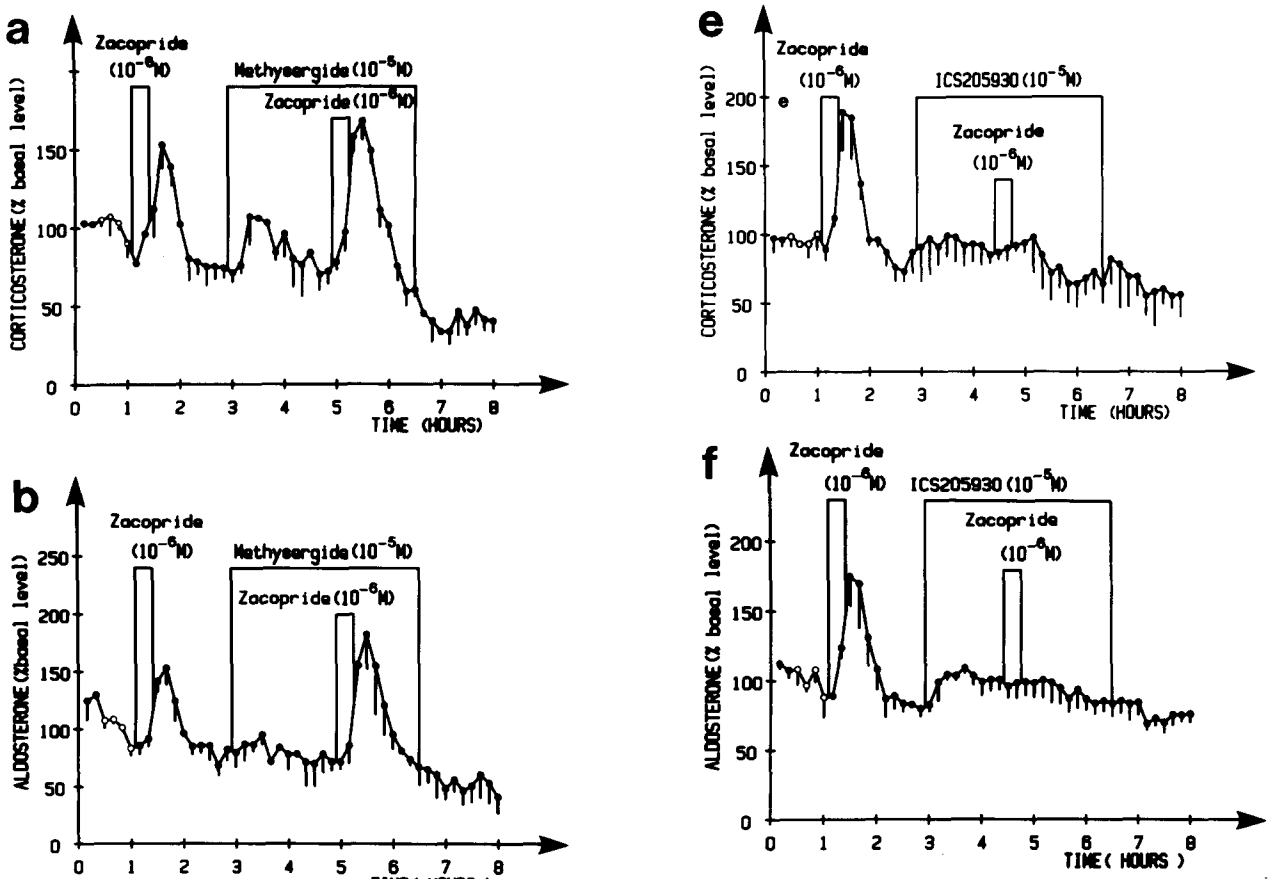

Fig. 5. Effect of zacopride alone or during prolonged infusion of methysergide (a,b), MDL 72222 (c,d) or ICS 205 930 (e and f) on corticosterone and aldosterone secretion. After a 120-min equilibration period, a first pulse of zacopride (10^{-6} M) was administered during 20 min and the interrenal tissue was allowed to stabilize for another 90-min period. Then, the different 5-HT antagonists were infused during 220 min (10^{-5} M each). During infusion of the antagonists, a second pulse of zacopride (10^{-6} M) was added for 20 min. In these experiments, the mean secretion rates of corticosterone and aldosterone under basal conditions were 89 ± 6 and 182 ± 22 fmol/interrenal gland per min. See legend to Fig. 1 for other designations.

zacopride (Fig. 6b) induced a dose-related increase of the content of cyclic AMP in the tissue. At the highest concentrations used, both 5-HT and zacopride induced a two-fold increase of cAMP levels.

DISCUSSION

5-HT has been reported to stimulate aldosterone secretion both in vivo[26] and in vitro[1,27,28]. We have previously shown the presence of 5-HT in secretory vesicles of chromaffin cells in the frog adrenal gland[10], and we have observed that 5-HT stimulates corticosterone and aldosterone production in a dose-dependent manner[11]. More recently, using pharmacological approaches, we demonstrated that the effect of 5-HT on corticosteroid secretion is not mediated through the classical serotonin receptor subtypes, i.e. 5-HT_1 and

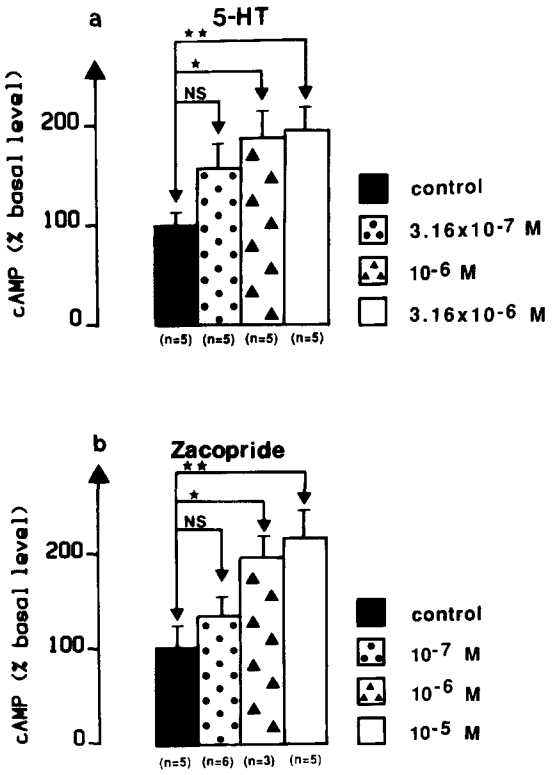

Fig. 6. Effect of graded concentrations of 5-HT (a) and zacopride (b) from 10^{-7} to 10^{-5} M on cyclic AMP production by frog interrenal slices. The tissue cAMP content was measured after a 2-min incubation with the drug and calculated as a percentage of the control level. The number of experiments is indicated under each column. Asterisks denote groups which differ significantly from the corresponding control group as determined by Student's *t*-test. $^{*}P < 0.02$; $^{**}P < 0.01$. Basal cyclic AMP content was 602.5 ± 86 (a) and 440 ± 100 (b) fmol cAMP/100 ng DNA.

5-HT_2[20]. In particular, we showed that the 5-HT_1 and 5-HT_2 antagonists, metergoline and ketanserin, do not affect 5-HT-evoked corticosteroid secretion[20] and that the 5-HT_1 agonist 8-OH-DPAT is totally devoid of activity on our model.

Based on studies performed either in vivo[2] or in vitro[27,32,36] it has been proposed that, in mammals, 5-HT stimulates corticosteroid production through activation of a 5-HT_2 receptor subtype. Surprisingly, however, these authors observed that in rat zona glomerulosa cells, 5-HT increases cAMP formation[27,36] while it is clearly established that stimulation of 5-HT_2 receptors is coupled to an increase of the turn-over of membrane phosphoinositides[7,8]. Alternatively, Lorens et al.[25] have proposed that 5-HT_{1A} receptors could play a role in the regulation of corticosterone in the rat. Our data[20] also indicated that 5-HT-evoked corticosteroid secretion is not mediated through the 5-HT_3 receptor subtype inasmuch as: (a) 2-methyl-5-HT, a potent 5-HT_3 agonist, could not mimic the stimulatory action of 5-HT in our preparation and (b) the 5-HT_3 receptor antagonist MDL 72222 did not affect the response of adrenocortical cells to 5-HT[20]. The present data, showing that 5-HT stimulates cAMP production in a dose-dependent manner, also argues against the involvement of 5-HT_3 receptors, since the activation of this receptpr subtype is known to activate cationic channels[3,12]. These data confirm the existence in the adrenal gland of a 5-HT receptor subtype having unique pharmacological properties clearly different from those of 5-HT_1, 5-HT_2 and 5-HT_3 receptors.

Previous studies performed on mouse embryo colliculus neurons[15] and guinea pig hippocampal membranes[4] showed that 5-HT-stimulated adenylate cyclase activity could be blocked by a recognized 5-HT_3 receptor antagonist, ICS 205 930. However, these authors observed that much higher concentrations of ICS 205 930 were required to inhibit the effect of 5-HT in their preparation than those generally needed to block 5-HT_3 receptors[13]. The group of Bockaert et al. also showed that the stimulatory effect of 5-HT on cAMP production could be mimicked by 4-amino-5-chloro-2-methoxy-substituted benzamide derivatives[15]. The non-additivity of 5-HT and the substituted benzamide derivatives led these authors to propose that all these compounds act on a new receptor subtype termed 5-HT_4[15].

Several lines of evidence suggest that the effect of 5-HT on frog adrenocortical cells is mediated through 5-HT_4-like receptors. (i) All the benzamide derivatives tested were full or partial agonists of this receptor, including those which have been described as 5-HT_3 antagonists. The order of potency of these compounds was zacopride > BRL 24924 > cisapride. (ii) The stimulatory effects of 5-HT and zacopride on corticosteroid production were not additive. (iii) Only ICS 205 930 antagonized the stimulatory effect of zacopride while the selective 5-HT_3 receptor antagonist MDL 72222 did not affect the stimulatory action of the benzamide derivative. Consistent with the high potency of 5-HT, as compared to zacopride, high concentrations of ICS 205 930 were necessary to block the stimulatory effect of 5-HT on frog adrenal gland. Similar results have recently been reported in the central nervous system by Dumuis et al.[13]. At these high concentrations, ICS 205 930 had by itself a stimulatory effect which could be ascribed either to an agonistic activity or to some non-specific effect of this drug, in particular on potassium or sodium channels. The observation that both 5-HT and zacopride induced stimulation of cAMP production provides additional support for the involvement of a 5-HT_4 receptor subtype in our model. These results are in agreement with the findings of Bockaert et al. who showed that 5-HT_4 receptors are positively coupled to the adenylate cyclase system in colliculi neurons[15] and hippocampal membranes[4].

In other peripheral organs including the ileum[9] and the gut[33], the action of 5-HT is not mediated by the classical 5-HT_1, 5-HT_2 or 5-HT_3 receptors and it is conceivable that some of the effects of 5-HT on these tissues could be mediated by 5-HT_4 receptors.

In conclusion, the results presented herein provide evidence for the involvement of the recently characterized 5-HT_4 receptors outside the central nervous system. Preliminary studies conducted with human adrenocortical cells indicate that, in mammals, serotonin also stimulates corticosteroid secretion through activation of a 5-HT_4-like receptor[23].

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