Cholecystokinin release mediated by 5-HT₃ receptors in rat cerebral cortex and nucleus accumbens

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1 The effects of 5-hydroxytryptamine (5-HT) on the release of cholecystokinin-like immunoreactivity (CCK-LI) were examined in synaptosomes prepared from rat cerebral cortex and nucleus accumbens and depolarized by superfusion with 15 mM KCl.

2 In both areas 5-HT, tested between 0.1 and 100 nM, increased the calcium-dependent, depolarization-evoked CCK-LI release in a concentration-related manner. The concentration-response curves did not differ significantly between the two brain areas (EC₅₀: 0.4 ± 0.045 nM and 0.48 ± 0.053 nM, respectively, in cortical and n. accumbens synaptosomes; maximal effect: about 60% at 10 nM 5-HT).

3 The 5-HT₁/5-HT₂ receptor antagonist methiothepin (300 nM) did not affect the CCK-LI release elicited by 10 nM 5-HT. However, the effects of 10 nM 5-HT were antagonized in a concentration-dependent manner by the 5-HT₃ receptor antagonists (3 α -tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205-930; 0.1–100 nM; IC₅₀: 3.56 ± 0.42 nM in the cortex and 3.90 ± 0.50 nM in the n. accumbens) and ondasetron (IC₅₀: 8.15 ± 0.73 nM in the cerebral cortex). 5-HT (10 nM) was also strongly antagonized by 100 nM 1 α H, 3 α 5 α H-tropan-3-yl-3,5-dichlorobenzoate (MDL 72222) another blocker of the 5-HT₃ receptor. Moreover, the 5-HT₃ receptor agonist 1-phenylbiguanide (tested in the cerebral cortex between 0.1 and 100 nM) enhanced CCK-LI release in a manner almost identical to that of 5-HT (EC₅₀ = 0.64 ± 0.071 nM).

4 It is concluded that 5-HT can act as a potent releaser of CCK-LI in rat cerebrocortex and nucleus accumbens through the activation of receptors of the 5-HT₃ type situated on the CCK-releasing terminals. This interaction may provide a rationale for the clinical development of both 5-HT₃ and CCK receptor antagonists as novel anxiolytic drugs.

Keywords: 5-Hydroxytryptamine; cholecystokinin release; 5-HT₃ presynaptic receptors; cerebrocortex; nucleus accumbens; 1-phenylbiguanide; ICS 205-930; MDL 72222; ondasetron; anxiety

Introduction

5-Hydroxytryptamine (5-HT) has long been implicated in the control of anxiety (Iversen, 1984; Chopin & Briley, 1987). In general, it seems that stimulation of the 5-HT system results in an anxiogenic effect, while a reduction in inappropriately elevated 5-HT neuronal activity leads to anxiolysis. Accordingly anxiolytic activity has recently been reported for 5-HT₃ receptor antagonists (Jones *et al.*, 1988; Lecrubier *et al.*, 1990).

Administration of cholecystokinin (CCK), a major neuropeptide in the central nervous system (Vanderhaeghen *et al.*, 1975; Dockray, 1976) has been found to induce anxiety both in the experimental animal and in man and CCK receptor antagonists have been shown to display anxiolytic activity (see review by Ravard & Dourish, 1990, and references therein).

This similarity between 5-HT and CCK prompted us to investigate whether the two systems could interact in the brain. The question was approached by examining the action of 5-HT on the release of cholecystokinin-like immunoreactivity (CCK-LI) from cerebral tissue. It is known that CCK is widely distributed throughout the brain (Beinfeld *et al.*, 1981; Emson & Marley, 1983), the highest amounts being found in the cerebral cortex and in limbic areas possibly involved in the expression of anxiety.

The peptide is concentrated in nerve endings from which it can be released upon depolarization (Pinget *et al.*, 1979). Thus we have used synaptosomal preparations obtained from rat cerebral cortex or nucleus accumbens which were exposed to 5-HT during depolarization. The data show that the release of CCK-LI, determined by radioimmunoassay, was potentiated by nanomolar concentrations of 5-HT via the activation of receptors of the 5-HT₃ type.

Methods

Preparation of synaptosomes

Adult male Wistar rats (200–250 g) were used. Crude synaptosomes were prepared from the cortex or from the nucleus accumbens as previously described (Raiteri *et al.*, 1984). Briefly, the tissue was homogenized in 40 vol of 0.32 M sucrose, buffered at pH 7.4 with phosphate. The homogenate was centrifuged (5 min, 1000 g at 0–4°C) to remove nuclei and debris and synaptosomes were isolated from the supernatant by centrifugation at 12,000 g for 20 min. The synaptosomal pellet was then resuspended in a physiological medium having the following composition (mM): NaCl 125, KCl 3, MgSO₄ 1.2, CaCl₂ 1.2, NaH₂PO₄ 1.0, NaHCO₃ 22 and glucose 10 (aeration with 95% O₂ and 5% CO₂ 37°C), pH 7.2–7.4.

Protein was measured according to Bradford (1976), with bovine serum albumin used as a standard.

Release experiments

Identical aliquots of the synaptosomal suspension (ranging between 0.8–1 mg protein in the different experiments) were layered at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri *et al.*, 1974). Superfusion was then started with standard medium supplemented with 0.1% gelatine and aerated with 95% O₂ and 5% CO₂, at a rate of 0.6 ml min⁻¹. After 36 min, to equilibrate the system, fractions were collected according to the following scheme: two 3-min samples (basal release) before and after one 6-min sample (evoked release). A 90 s period of depolarization was applied at the end of the first fraction collected. Depolarization of synaptosomes was performed with 15, 25, or 35 mM KCl, substituting an equimolar concentration of NaCl in the superfusion

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medium. 5-HT or 1-phenylbiguanide was added to the superfusion medium concomitantly with the depolarizing stimulus. Methiothepin, ICS 205-930, ondansetron or MDL 72222 was added 8 min before 5-HT. The Ca²⁺-free medium, when used, was substituted for the standard medium 18 min before depolarization. Superfusate fractions were collected into vials containing acetic acid (final concentration 1 M) and boiled to ensure peptidase inactivation. At the end of superfusion, synaptosomes were sonicated in distilled water and the extracts were boiled in 1 M acetic acid. All superfusate fractions and synaptosomal extracts were freeze dried before assay.

Radioimmunoassay

The freeze dried samples were reconstituted in phosphate buffer (50 mM; pH 7.4) and radioimmunoassayed essentially according to Emson *et al.* (1980). The antiserum used (Ab 2609) had been characterized in detail by Rehfeld (1978). The assay sensitivity was 1 pg per tube and the calibration curve was linear between 1.5 and 100 fmol with [¹²⁵I]-CCK-8 (sulphated) used as a tracer.

Calculation

The amount of CCK-LI released in each fraction and that remaining in the synaptosomes at the end of the superfusion was expressed as fmol mg⁻¹ protein. The depolarization-evoked overflow was estimated by subtracting the CCK-LI content of the basal release from the release evoked in the 6-min fraction collected during and after the depolarization period. The drug effects were evaluated as the ratio of the depolarization-evoked overflow calculated in the presence of the drugs vs. that calculated under control conditions. The two-tailed Student's *t* test was used for statistical comparison of the data.

Drugs

[¹²⁵I]-cholecystokinin-8 and cholecystokinin-8 were obtained from Amersham Radiochemical Centre (UK) and from NOVA Biochem (Switzerland), respectively. 5-Hydroxytryptamine was purchased from Sigma Chemical Co (St. Louis, MO, U.S.A.) and 1-phenylbiguanide from RBI (Natick, MA, U.S.A.). The following drugs were kindly given from the companies indicated: methiothepin (Hoffman-La Roche, Basel, Switzerland); ICS 205-930 (3 α -tropanyl)-1H-indole-3-carboxylic acid ester, Sandoz AG, Basel, Switzerland); MDL 72222 (1 α H,3 α ,5 α H-tropan-3yl-3,5-dichlorobenzoate, Merrell Dow Research Institute, Strasbourg, France); ondansetron (Glaxo, Ware, U.K.).

Results

Depolarization-evoked release of cholecystokinin-like immunoreactivity

The basal CCK-LI release from rat cerebrocortical synaptosomes was 2.9 ± 0.13 fmol mg⁻¹ protein min⁻¹, corresponding to $0.2 \pm 0.01\%$ of the total synaptosomal content ($n = 20$). Increasing concentrations of KCl in the superfusion medium (from 15 to 35 mM) resulted in a concentration-dependent increase in CCK-LI release (Figure 1). The K⁺-stimulated overflow was found to be largely calcium-dependent. Results similar to those shown for the cerebral cortex were obtained with synaptosomes from n. accumbens (data not presented). In the subsequent experiments 15 mM K⁺ was chosen as the depolarizing stimulus because this submaximal concentration of KCl allowed for an increase or decrease in release following drug exposure.

Figure 1 K⁺-evoked overflow of cholecystokinin-like immunoreactivity (CCK-LI) from rat brain cortex synaptosomes and its Ca²⁺-dependence. Synaptosomes were prepared and superfused as described in the Methods section. A 90 s pulse of 15, 25 or 35 mM KCl was applied after 39 min of superfusion. The Ca²⁺-free medium, when used, was introduced 18 min before the depolarizing stimuli. Shown are means with s.e.mean (vertical bars) of 3 experiments in triplicate.

Effect of 5-hydroxytryptamine

When added to the superfusion medium concomitantly with 15 mM K⁺, 5-HT (0.1–100 nM) produced concentration-dependent increases in the depolarization-evoked CCK-LI release (Figure 2). The concentration-response curve for 5-HT obtained with cerebrocortical synaptosomes was superimposable on that obtained with synaptosomes from n. accumbens. The maximal effect (about 60% increase of CCK-LI release) was produced by 10 nM of the indoleamine. The EC₅₀ values were 0.40 ± 0.045 nM and 0.48 ± 0.053 nM, respectively, in the cortex and in the n. accumbens.

Figure 2 Effect of 5-hydroxytryptamine (5-HT) or 1-phenylbiguanide on the K⁺ (15 mM)-evoked overflow of cholecystokinin-like immunoreactivity (CCK-LI) from rat cortical or n. accumbens synaptosomes. 5-HT or 1-phenylbiguanide was added to the superfusion medium concomitantly with the depolarizing stimulus. See Methods for other technical details. Each point represents the mean with s.e.mean (vertical bars) of 3–6 experiments in triplicate: (●) 5-HT (cortex); (△) 5-HT (n. accumbens); (○) 1-phenylbiguanide (cortex).

Characterization of the 5-HT receptor involved

The releasing effect of 5-HT (at the concentration of 10 nM corresponding to the maximal effect) was insensitive to 300 nM methiothepin. In contrast it was antagonized by 100 nM MDL 72222, in both brain regions (Figure 3). Figure 4 shows that the effect of 10 nM 5-HT in cortical synaptosomes was concentration-dependently antagonized by ICS 205-930 ($IC_{50} = 3.56 \pm 0.42$ nM) or by ondansetron ($IC_{50} = 8.15 \pm 0.73$ nM). Data superimposable on those in the cortex were obtained in the n. accumbens with ICS 205-930 ($IC_{50} = 3.90 \pm 0.50$).

Figure 3 Antagonism by methiothepin or MDL 72222 of the enhancement by 5-hydroxytryptamine (5-HT) of cholecystokinin-like immunoreactivity (CCK-LI) release from cortical (a) or n. accumbens (b), synaptosomes. Synaptosomes were exposed to superfusion with 5-HT at the same time as the depolarizing stimulus; methiothepin or MDL 72222 was added 8 min before 5-HT. See Methods for other technical details. Values represent means with s.e.mean (vertical bars) of 3-4 experiments run in triplicate. Open columns: 10 nM 5-HT; hatched columns: 10 nM 5-HT + 300 nM methiothepin; solid columns: 10 nM 5-HT + 100 nM MDL 72222. * $P < 0.01$ vs. 10 nM 5-HT.

Figure 4 Antagonism by ICS 205-930 or ondansetron of the enhancement by 5-hydroxytryptamine (5-HT) of cholecystokinin-like immunoreactivity (CCK-LI) release from cortical or n. accumbens synaptosomes. 5-HT (10 nM) was added to the superfusion medium at the same time as the depolarizing stimulus was applied; antagonists 8 min before 5-HT. For other technical details see Methods. Each point of the curve represents the mean with s.e.mean (vertical bars) of 3-5 experiments in triplicate. Concentrations of antagonists higher than 0.1 nM antagonized 5-HT significantly ($P < 0.001$): (●) 5-HT plus ICS 205-930 (cortex); (○) 5-HT plus ondansetron (cortex); (□) 5-HT plus ICS 205-930 (n. accumbens).

The effect of 5-HT on the K^+ -evoked CCK-LI release from cortical synaptosomes was mimicked by 1-phenylbiguanide (0.1–100 nM). This drug was slightly (although not significantly) less potent than 5-HT ($EC_{50} = 0.64 \pm 0.071$) (Figure 2).

Discussion

The first result of this work shows that nanomolar concentrations of 5-HT enhanced the release of CCK-LI, produced by exposure to high- K^+ , from crude synaptosomal preparations of rat cerebral cortex or nucleus accumbens. The data suggest a functional connection between the 5-HT and the CCK systems in the rat brain.

The origin of the released CCK-LI is most probably neuronal since CCK-LI has been reported to be concentrated in CNS nerve terminals (Pinget *et al.*, 1978). Possible 'glial' contamination of our nerve ending preparations should not contribute significantly to the released peptide inasmuch as CCK has not been detected in glial cells (Loren *et al.*, 1979).

The experimental set up employed in this study (a thin layer of synaptosomes up-down superfused; Raiteri *et al.*, 1974) prevents or minimizes indirect effects. Thus it can be assumed that the site of action of 5-HT is on the CCK-releasing nerve terminals suggesting that the 5-HT and the CCK systems are directly interconnected in the rat brain.

Mammalian 5-HT receptors are heterogeneous and have been divided into three major types: 5-HT₁-like, 5-HT₂ and 5-HT₃ (Bradley *et al.*, 1986). The CCK-releasing effect of 5-HT was insensitive to methiothepin, a non-selective 5-HT₁/5-HT₂ receptor antagonist, while it was sensitive to nanomolar concentrations of the 5-HT₃ receptor antagonists MDL 72222 (Fozard, 1984), ICS 205-930 (Richardson *et al.*, 1985), and ondansetron (Butler *et al.*, 1988). Moreover, the effect of 5-HT on CCK-LI release was mimicked by 1-phenylbiguanide, a selective 5-HT₃ receptor agonist (Ireland & Tyers, 1987). These results suggest that the enhancement of CCK-LI release by 5-HT is mediated by receptors of the 5-HT₃ type.

Involvement of the recently proposed 5-HT₄ receptor type (Dumuis *et al.*, 1989) seems unlikely for a number of reasons: (a) the 5-HT₄ receptor is totally insensitive to MDL 72222 and to ondansetron; (b) the 5-HT₄ receptor can be blocked by ICS 205-930, but only at micromolar concentrations; (c) 5-HT shows relatively low potency towards the 5-HT₄ receptor while the receptor mediating CCK-LI release seems to be activated by extremely low concentrations of 5-HT.

If the pharmacological characteristics of the 5-HT receptor situated on CCK-releasing terminals are most compatible with its classification as a 5-HT₃ type, it should be noted that the 5-HT₃ receptors classically found in the periphery require micromolar concentrations of 5-HT or 1-phenylbiguanide for activation (Richardson *et al.*, 1985; Richardson & Engel, 1986; Ireland & Tyers, 1987). The present finding that 5-HT and 1-phenylbiguanide were almost 1000 times more potent than expected for a 5-HT₃-mediated effect may have various explanations. In a thin layer of superfused synaptosomes the agonists reach the receptor very rapidly, thus minimizing factors (tissue uptake, metabolism, receptor access etc.) which may influence estimates of agonist potency in more intact tissues. A high 5-HT₃ receptor reserve may be present on CCK-releasing nerve endings. A receptor coupling which is more efficient in brain than in other tissues may also contribute. Finally, the possibility that the central receptor under study represents a subtype of the 5-HT₃ receptor cannot be excluded.

Receptors of the 5-HT₃ type mediate depolarizing actions and are probably coupled to membrane cation channels (Derkach *et al.*, 1989), properties which are particularly well-suited to a release-regulating receptor. Evidence from radioligand binding studies (Kilpatrick *et al.*, 1987; Peroutka & Hamik, 1988; Waeber *et al.*, 1989) and behavioural tests

(Costall *et al.*, 1987; Jones *et al.*, 1988) have shown that 5-HT₃ receptors exist not only in the periphery but also in the central nervous system.

5-HT₃ receptors appear most promising as targets for novel drugs. Antagonists at these receptors are indeed under clinical development in a variety of important indications including emesis in cancer chemotherapy, schizophrenia and anxiety (for a review see Fozard, 1987). The recent finding that 5-HT₃ receptors modulate acetylcholine release in rat entorhinal cortex suggests that 5-HT₃ receptor antagonists might also be useful in conditions of impaired cholinergic function (Barnes *et al.*, 1989).

A relationship between central 5-HT₃ receptors and anxiety is supported by a wealth of data. As already mentioned, the prevailing view points to a reduction in abnormally elevated 5-HT neuronal activity as the basis of anxiolysis. In keeping with this view, 5-HT_{1A} receptor agonists which cause a generalized depression of impulse flow by activating somatodendritic autoreceptors on raphe neurones (Sprouse & Aghajanian, 1987) are effective novel anxiolytics (Goa & Ward, 1986; Fozard, 1987). However, 5-hydroxytryptaminergic transmission can also be blocked more distally, i.e. at the axon terminal synapses. Considering that 5-HT is released onto a variety of receptor types and subtypes, antagonists at 5-HT₃ receptors may be more selective anxiolytics than those acting in a more generalized way.

The finding that the release of CCK can be enhanced by 5-HT₃ receptor activation deserves some comments. CCK binding sites are widely distributed throughout the brain (Innis & Snyder, 1980; Saito *et al.*, 1980). It was shown in electrophysiological experiments that CCK was selectively

antagonized by low doses of benzodiazepines (Bradwejn & De Montigny, 1984) and it was predicted that CCK receptor activation might induce anxiety. This idea has been supported by experiments in the laboratory animal (Deupree & Hsiao, 1988) and by recent clinical studies (De Montigny, 1989) in which i.v. injection of CCK provoked panic attacks in normal volunteers and reproduced the symptoms of spontaneous panic in anxious patients. On the basis of these observations, CCK receptor antagonists have been tested and found to possess anxiolytic properties in animal models of anxiety (Dourish *et al.*, 1990; Hughes *et al.*, 1990), suggesting that CCK antagonists may be useful anxiolytic drugs (see for review, Ravard & Dourish, 1990).

In conclusion, we would like to propose that 5-HT and CCK may strictly cooperate in the biochemistry of anxiety. According to this idea, in some yet unidentified brain regions, but possibly in the areas examined in the present study, abnormally elevated 5-HT transmission activates release-enhancing 5-HT₃ receptors situated on CCK nerve terminals and the released CCK contributes to the expression of anxiety. This picture would represent a rationale for the clinical development of both 5-HT₃ and CCK receptor antagonists as novel antianxiety drugs.

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