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In vivo release of CCK-8 from the dorsal horn of the rat: inhibition by DAGOL

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There is evidence that CCK-8 may interact with opioids and that both systems are probably implicated in pain modulation. In order to elucidate this relationship we sought to examine factors governing the movement of CCK-8 from the spinal cord into the extracellular space. We report that CCK-8 like immunoreactivity, as measured by RIA, is released from the spinal cord of the rat in vivo, following potassium stimulation and by direct activation of high threshold peripheral afferents by stimulation of the sciatic nerve. Also, we show that CCK-8 release is inhibited by the μ-selective opioid receptor agonist DAGOL. Naloxone totally reversed the effect produced by DAGOL, implying an opiate mediated mechanism.

Hormone release; Cholecystokinin-8; Dorsal horn; Enkephalin, [D-Ala^2-MePhe^4, Gly-ol^5]-

1. INTRODUCTION

Cholecystokinin octapeptide (CCK(26–33); CCK-8) is the most abundant peptide in the human central nervous system (CNS) and this octapeptide is present throughout the CNS of a number of mammalian species [1–3]. The physiological actions attributed to CCK are as diverse as its distribution. The physiological action of CCK-8 in the gastrointestinal tract is excitatory. It is a potent activator of digestion, pancreatic secretion, and gall-bladder contraction, as well as increasing gut motility. In the CNS, the physiological actions attributed to CCK include regulation of satiation [4], modulation of catecholaminergic activity [5] and regulation of hypothalamic peptides [6]. A variety of effects have been observed after central administration of CCK-8. These include hypothermia [7], hyperglycemia [8], and sometimes analgesia [9]. Also, electrophysiological data have shown both excitatory and inhibitory actions of CCK in the CNS [10,11].

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At the present time there is supporting evidence that CCK-8 may interact with opioid systems. It is known that CCK-8 physiologically increases respiration [12], whereas a well-known side effect of morphine is respiratory depression and, probably the most important point is the fact that many of the CCK-containing loci are areas observed to contain high concentrations of endogenous opioids, with many of these areas implicated in pain modulation.

Besides, the distribution of enkephalins and CCK are strikingly similar. Hence, a functional relationship may exist between them, though sulfated CCK-8 does not bind to opiate receptors in the brain [13]. At present, however, the results concerning the CCK-8/opiate relationship are controversial [9]. Utilizing a spinal superfusion procedure, we sought to examine factors governing the movement of CCK-8 from the spinal cord into the extracellular space. Hence, we report here that CCK-8-like immunoreactivity, as measured by RIA, is released from the spinal cord of the rat in vivo, following potassium stimulation (50 mm), and by direct activation of high threshold peripheral afferent by stimulation of the sciatic nerve. Also, we show that this effect is inhibited by

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the μ-selective opiate agonist [D-Ala^{2}MePhe^4,Gly-ol^5]enkephalin (DAGOL). Naloxone totally reversed the effect produced by DAGOL, implying an opiate mediated mechanism.

2. MATERIALS AND METHODS

2.1. *Animals*

Experiments were performed in male Wistar rats weighing 500–700 g under urethane (1.25 mg/kg) anaesthesia. The animals were artificially ventilated through a tracheal cannula. The heart rate and blood pressure were continuously monitored, and the central temperature was kept constant by means of an homeothermic blanket system.

2.2. *Spinal perfusion procedures*

To perfuse the spinal space an exposure of the cisterna magna was made by a midline incision and blunt dissection, followed by incision of the dura and arachnoid membranes. A piece of 10 cm of polythene tubing (0.28 mm i.d., 0.61 mm o.d., PORTEX) was advanced down the subarachnoid space to the lumbar area. The tip of this tubing served as an inflow cannula and ACSF was pumped in at a rate of 110 μl/min with a Gilson peristaltic pump. The ACSF was withdrawn with another polyethylene tube positioned at the opening of the cisterna magna at a rate of 100 μl/min using a second peristaltic pump. Before entering the animal's body, the perfusion fluid was warmed (37 ± 5°C) and was continuously oxygenated (95% O, 5% CO_2). Normal ACSF medium consisted of (in mM): 134 NaCl, 5 KCl, 1.25 KH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 16 $NaHCO_3$, 10 glucose. Experiments were conducted by first flushing the subarachnoid space with ACSF for 1 h to allow stabilization of the preparation, and then collecting 5 min (500 μl) fractions. Statistical significance of the results was assessed by Student's t-test ($P \leq 0.05$).

2.3. *Radioimmunoassay*

Perfusate samples were collected in plastic tubes and shell frozen on dry-ice. Samples were lyophilized and stored at −20°C until assayed. For assay, samples were reconstituted to 1/3 of their original volume with assay buffer prior to their determination. The radioimmunoassay procedure for CCK employed in the present experiments is the same as that previously published by other authors [14,15]. We have used synthetic CCK rabbit antiserum (no.3440) as our CCK antibody source. This antiserum recognized the carboxyl-terminal of CCK and gastrin equally, so radiolabeled gastrin is used as a tracer in our RIA procedure and all data, unless otherwise indicated, are expressed in femtomoles of $gastrin_{17}$ equivalent. The sensitivity of the assay is 0.2 fmol/tube with 6% intraassay and 10% in interassay variation. Reconstituted lyophilized samples were chromatographed on 1 × 100 cm columns of Sephadex G-50 superfine at 4°C and 0.1 ml/min flow rate. Columns were equilibrated and samples eluted with 0.01 M potassium phosphate buffer, pH 7.6. Blue dextran 2000 (Pharmacia) and ^{125}I (New England Nuclear) were used to characterize the void volume and salt peaks, respectively. Fractions of 1 ml were collected, and assayed for CCK using the radioimmunoassay procedure described above.

2.4. *Stimulation procedures*

The concentration of KCl in the ACSF was raised to produce a final concentration of 50 mM K^+ while a corresponding amount of NaCl was removed from the ACSF solution to minimize undesirable osmotic effects.

To stimulate somatosensory input into the lumbar spinal cord of the rat, the sciatic nerve was exposed and prepared for stimulation and recording of the compound action potential. Stimulation of the nerve was performed with rectangular pulses 3–4 V, 0.05 ms, 50 Hz for activation of Aα and Aβ fibers and 40–50 V, 0.05 ms, 50 Hz for recruitment of Aδ and C fibers.

3. RESULTS AND DISCUSSION

CCK-8-like immunoreactivity was observed to be present in the resting superfusate of the spinal cord of the anaesthetised rat. The mean resting levels in these samples were above the absolute sensitivity of the RIA used (0.55 ± 0.12 fmol/ml per 5 min) (n = 20), which is in accordance with other authors [16]. The addition of potassium (50 mM in excess) to the infused artificial CSF resulted in a 160% increase in CCK-8 above baseline in the spinal superfusate (n = 5, fig.1). The increase was significant ($P \leq 0.05$, Student's t-test). Upon substitution of normal CSF, CCK-8 levels fell within limits of baseline variability. The localization of CCK-8 in synaptosomal fractions [17,18] and the in vitro experiments demonstrating an increase following depolarization suggest that CCK-8 may exist within releasable pools.

To examine whether CCK-8 is released from the spinal cord following activation of specific afferent fiber populations, we stimulated the rat sciatic nerve bilaterally, which resulted in a 282% increase in CCK-8 release (after Aδ/C fiber stimulation). This provides direct evidence that CCK-8 is released from rat spinal cord in vivo by activating nociceptive primary afferents.

We have also examined whether the spinal application of the μ-selective opiate agonist, DAGOL, at concentrations known to produce analgesia (1 nM) can affect CCK-8 release from rat spinal cord in vivo. Following superfusion with 1 nM DAGOL, the resting release of CCK-8 was substantially decreased (fig.2). Furthermore, bilateral stimulation of the sciatic nerve at intensities that clearly caused the release of CCK-8 before the addition of DAGOL now failed to increase the release of CCK-8 (fig.2). Following subcutaneous injection of naloxone hydrochloride (0.1 mg/kg), stimulation of the sciatic nerve at the

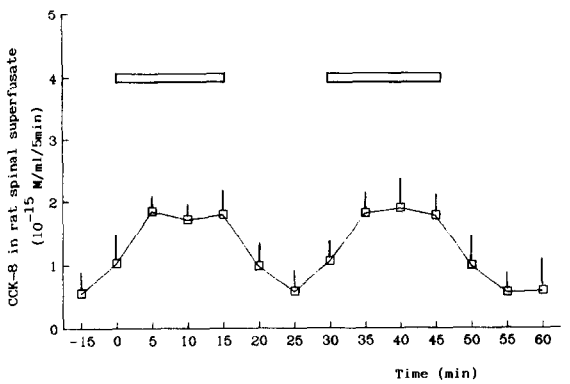

Fig.1. Release of CCK-8 from superfused rat spinal cord in response to high potassium concentrations (50 mM) (open bars). The figure shows the amount (in fmol/ml per 5 min fractions) of CCK-8 released in the perfusing medium. The baseline, as observed 15 min prior to the administration of 50 mM K^+ is shown to indicate the basal levels of CCK-8 release in the perfusate. The results shown are the mean ± SE from 5 animals. The K^+-induced effect was statistically significant as assessed by Student's t-test ($P \leq 0.05$).

same intensity in the continued presence of intrathecal DAGOL fully restored the evoked release of CCK-8, suggesting that the effect observed is opiate mediated and selective for the μ-receptor type.

The addition of capsaicin (3×10^{-4} M) to the superfusion medium failed to have any significant effect on CCK-8 levels. The actions of capsaicin in the spinal cord seem to be restricted to nociceptive primary sensory neurons [19]. In our experiments direct superfusion of capsaicin into the spinal cord failed to increase the release of CCK-8 from basal levels. On the other hand, chemical stimulation with potassium (50 nM) or electrical stimulation of the sciatic nerve produced a significant release of CCK-8. This suggests that we are measuring intrinsic CCK-8 rather than CCK-8 from primary afferents. It is possible that the external stimuli that we have used activate interneurons, from which CCK-8 is released through an interaction with the μ-opioid receptor.

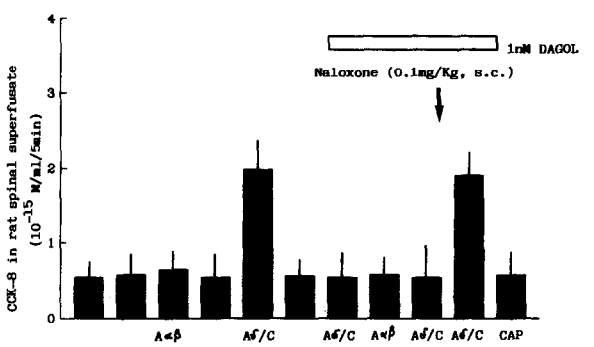

Fig.2. Release of CCK-8 from superfused rat spinal cord in response to sciatic nerve stimulation and stimulation with capsaicin (CAP). Each value is the mean ± SE from five experiments. Statistical significance was assessed by Student's t-test ($P \leq 0.05$). To examine whether CCK-8 is released from the spinal cord following activation of specific afferent fiber populations, we stimulated the rat sciatic nerve bilaterally, while superfusing segments of the spinal cord were receiving sensory input from the sciatic nerve. During stimulation the compound action potential was monitored to determine the stimulus intensity required to activate A$\alpha\beta$ fibers alone, and that required to recruit Aδ/C-fibers.

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