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Pharmacology of a cholecystokinin receptor on 5-hydroxytryptamine neurones in the dorsal raphe of the rat brain

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1 The effect of bath application of sulphated cholecystokinin octapeptide (CCK-8) was studied on neurones in slices containing rat raphe nucleus.

2 Intracellular recordings were made from neurones in the dorsal raphe nucleus. Some of the neurones with the characteristics of 5-hydroxytryptamine (5-HT)-containing cells which were inhibited by 5-HT and excited by noradrenaline were excited by cholecystokinin. The response to cholecystokinin was dose-dependent over the range 10 to 1000 nM.

3 The response to CCK-8 persisted in the presence of tetrodotoxin. Either reduction of extracellular calcium or addition of 25 mM magnesium did not block the CCK response, suggesting it was mediated by receptors located on the membrane of the raphe neurones.

4 The agonist and antagonist specificity of the CCK response was determined. The CCK_B selective agonist, pentagastrin, was inactive when applied at concentrations up to 10 μM. the CCK_A receptor antagonist L-364,718 (1 to 100 nM) blocked the response to cholecystokinin. Much higher (1–10 μM) concentrations of the CCK_B receptor antagonist L-365,260 were required for inhibition of the CCK response.

5 These data support the existence of a CCK receptor, located on raphe neurones in the rat, with a pharmacological profile very similar to that described for the CCK_A type.

Introduction

The dorsal raphe nucleus is the largest of the raphe nuclei and contains the highest density of 5-hyroxytryptamine (5-HT) containing neurones in the rat brain. It is found at the rostral portion of the tegmentum pontis at the border of the pons and mesencephalon, in the midline between the medial longitudinal fasciculi and the aqueduct. 5-HT receptors are present on the soma and dendrites of raphe 5-HT neurones and activation of these receptors by stimulating 5-HT containing nerve terminals or bath application of 5-HT agonists causes an increase in an inwardly rectifying potassium conductance (Williams *et al.*, 1988; Pan *et al.*, 1989) leading to hyperpolarization of the neuronal membrane and a reduction in action potential firing. The same neurones receive a direct synaptic noradrenergic input (Baraban & Aghajanian, 1981) and are excited by noradrenaline and phenylephrine (Aghajanian, 1985).

Although the mechanism of action of monoamines on dorsal raphe neurones appears well understood, nothing is known of the possible modulation of brain stem raphe neurones by peptides, such as cholecystokinin (CCK). No data are available concerning the distribution of CCK receptors within the rat raphe nuclei but CCK-8-like immunoreactive neurones are present in rat dorsal raphe (Vanderhaegen *et al.*, 1980; Van Der Koy *et al.*, 1981). Autoradiographic and binding methods have shown that the majority of CCK receptors in the brain are the B subtype (Hill *et al.*, 1987), thus determination of the receptor type involved in any functional response monitored *in vitro* is essential. Previous electrophysiological studies have relied heavily on the agonist selectivity difference between the two types of CCK receptor identified so far, to confirm that functional CCK_B receptors exist in rat hippocampus (Dodd & Kelly, 1981; Boden & Hill, 1986) and rat ventromedial hypothalamus (VMH) (Boden & Hill, 1988). However, the availability of potent, selective antagonists for the two types of CCK receptor makes it possible now to identify clearly the CCK receptor type. In the present study the effects of sulphated cholecystokinin octapeptide (CCK-8) were examined on neurones which responded to 5-HT and phenylephrine in an *in vitro* slice preparation of the raphe and the selective CCK antagonists, L-364,718 and L-365,260 were used as tools to characterize the receptor involved.

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Methods

Preparation of slices and recording from neurones

Intracellular recordings were made with electrodes fabricated from fibre filled glass (Clark GC120F-10) and filled with 1 M potassium acetate. The resistance of such electrodes measured in physiological saline varied between 80 and 140 MΩ. Current-clamp recordings from dorsal raphe neurones in the slice preparation from rat pons-mesencephalon were made with an Axoclamp-2a amplifier in bridge mode. Current pulses were of sufficient length (200 ms) to ensure full saturation of membrane capacitance. Male rats (50–100 g) were killed by cervical dislocation and the brain rapidly removed. Coronal slices (350 μm) were cut with a Vibratome in cold (4°C) physiological saline. A single slice was placed in a tissue bath through which flowed physiological artificial cerebrospinal fluid (ACSF) (2 ml min^{-1}) at 35°C. The content of the ACSF was as follows (concentrations mM): NaCl 126, KCl 5.0, NaH_2PO_4 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.4, glucose 11 and $NaHCO_3$ 25; the solution was gassed with 95%O_2, 5%CO_2 at 35°C. Magnesium chloride (10–20 mM) was substituted for calcium chloride to obtain calcium-free ACSF. Drugs dissolved in ACSF were applied by superfusion. L-364,718 (3S(−)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-1H-indole-2-carboxamide) and L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepine-3-yl)-N'-(3-methylphenyl)urea) were synthesized in Medicinal Chemistry, Parke-Davis, Cambridge, U.K. CCK-8 was obtained from either Peninsula Laboratories or Cambridge Research Biochemicals. All other drugs were obtained from either Sigma or Aldrich.

Analysis of data

Dose-response curves were constructed from firing rates or the depolarization of the membrane potential of the neurones in the presence of tetrodotoxin (TTX). The number of action potentials occurring during a CCK response was analyzed on-line through a CED 1401 interface and IBM AT with a specially designed suite of software (MRATE, Cambridge Electronic Design, Cambridge, UK). Best-fit curves for dose-response curves were obtained by fitting a simple hyperbolic function ($f\{x\} = (B.x)/(x + a)$) using RS1 version 12.10E software (BBN Software, Cambridge, Massachusetts, U.S.A.). The equilibrium constant was then calculated from the equation, Dose-ratio $- 1$ = [antagonist]/equilibrium constant. These data were expressed as mean $\pm$ s.e.mean. Analyses of significance was by Student's t test, $P = 0.05$ being taken as significant.

Results

Neuronal types in the raphe

In agreement with previous studies (Williams *et al.*, 1988), two populations of neurones were observed in the raphe. One population of neurones did not respond to either 5-HT, or phenylephrine. Neurones which did not respond to 5-HT and phenylephrine did not respond to CCK-8 ($n = 21$). The second group of neurones ($n = 56$) were characterized by the presence of large, long-lasting after-hyperpolarizations and broad action potentials. These neurones were hyperpolarized by a 1 min application of 5-HT (1–10 μM), in agreement with previously reported data (Aghajanian & Lakoski, 1984; Yoshimura & Higashi, 1985), identifying them as 5-hydroxytryptaminergic raphe neurones. They were used subsequently in studies of the action of CCK-8 on raphe neurones.

CCK-8 effects on raphe neurones

Of the 41 neurones which responded to CCK-8 (10–1000 nM), most were excited ($n = 32$), while a few were inhibited ($n = 9$). Fifteen neurones did not respond to CCK-8 application. On neurones which were excited by CCK-8, a 60 s application of CCK-8 produced a fully reversible membrane depolarization which lasted for between 2 and 5 min. The amplitude of the excitatory CCK-8 response was not diminished when a subsequent application of the peptide was made some 10 min after the first, indicating that little or no desensitization had occurred. The effect of CCK-8 was dose-dependent (Figure 1). Depolarizing responses to CCK-8 were not significantly different from control when recorded from neurones in slices treated with TTX (1 μM, 10 min, Figure 2a) which blocked both spontaneous action potentials and electrically evoked potentials, or from neurones in slices perfused with calcium-free ACSF containing 10 to 25 mM magnesium (Figure 2b), indicating a direct action of the peptide on the neuronal membrane.

CCK-agonist studies

The experiments were repeated with pentagastrin (Boc-B-alanine CCK-4), a selective agonist for CCK_B receptors in the rat VMH (Boden & Hill, 1988). Pentagastrin (1 to 10 μM) had no effect on CCK-8-sensitive neurones ($n = 3$).

CCK-antagonist studies

L-364,718 is a selective antagonist of CCK at the CCK_A receptor (Chang & Lotti, 1986). Bath application of L-364,718 (1 to 100 nM on 9 neurones) produced a rapid and potent antagonism of the CCK-8-induced excitation. Figure 3 shows the effect of L-364,718 on responses to CCK-8 recorded from

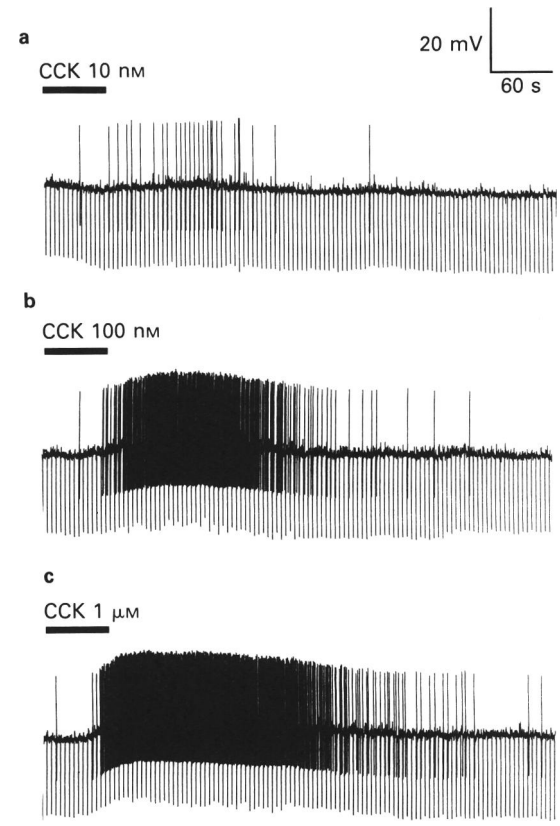

Figure 1 Pen recorder trace from a single neurone in a dorsal raphe nucleus *in vitro* showing dose-dependent excitation by cholecystokinin octapeptide (CCK-8). Resting potential of the neurone was -54 mV. No spontaneous activity was present prior to application of the peptide. Top trace, when CCK-8 (10 nM) was added for a period of 60 s indicated by the black bar, the neurone depolarized and action potentials were elicited. Full recovery from the effects of the peptide were seen on returning the slice to normal, drug-free saline. The depolarizing nature of the response can be seen more clearly when a concentration of 100 nM CCK-8 was applied (b). Finally when 1 μM CCK-8 was applied a much longer lasting effect was seen (c) and some 5 min elapsed before full recovery. Downward deflections are electrotonic voltage pulses resulting from current injection via the microelectrode. The action potentials are somewhat truncated as a result of the limited frequency response of the pen recorder.

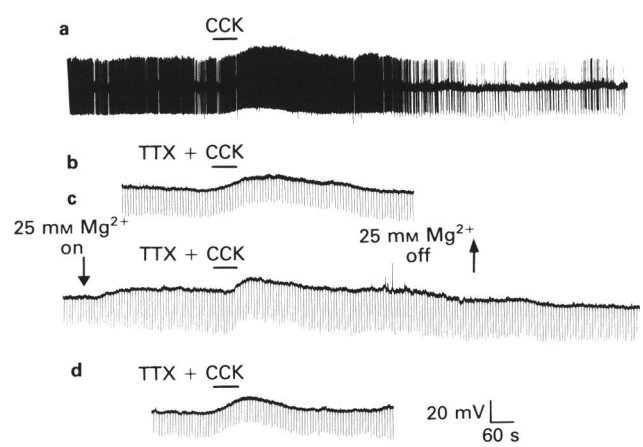

Figure 2 The response to cholecystokinin octapeptide (CCK-8) 100 nM persists in the presence of 25 mM magnesium ions: (a) shows the response to CCK-8 in control conditions; (b) shows that the CCK-8 response is still present during exposure to tetrodotoxin (TTX) 1 μM which abolished the sodium-dependent action potentials. Addition of 25 mM $MgCl_2$ shown in (c) led to a decrease in membrane conductance. The CCK-8 response still occurred in these conditions. The membrane conductance returned to control values on returning to ACSF containing 1.2 mM magnesium. Trace (d) shows that the response to CCK-8 was still present after returning to control ACSF.

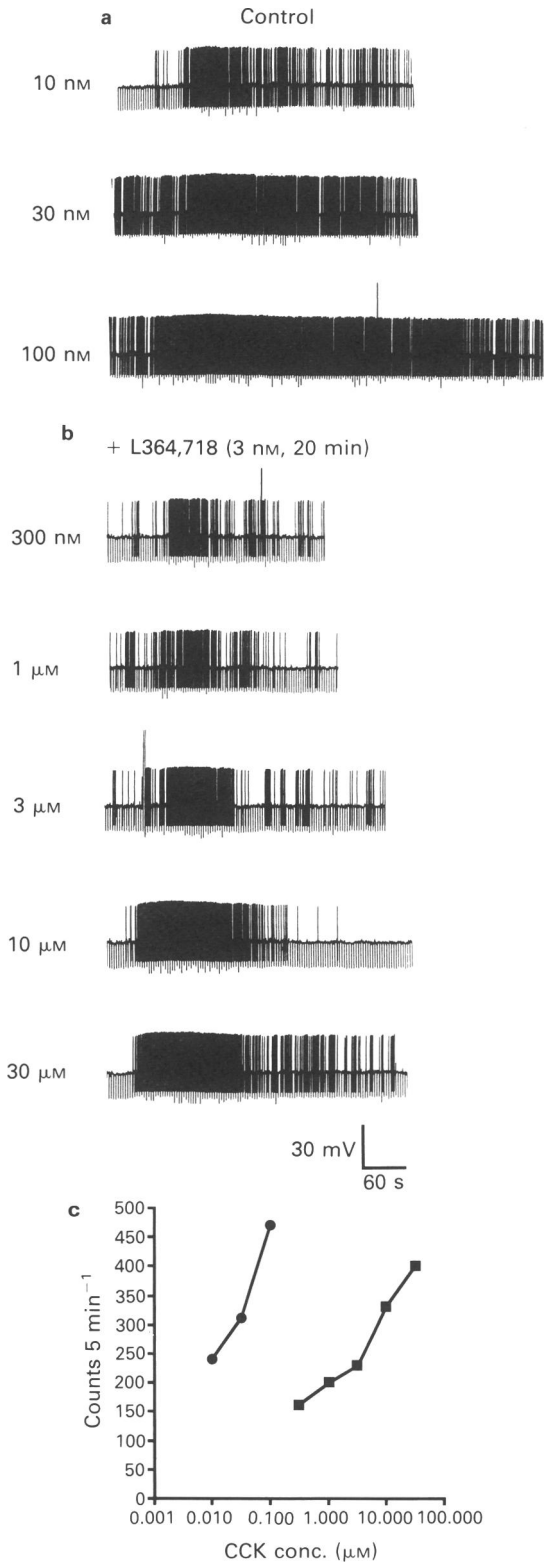

Figure 3 The potent CCK_A antagonist L-364,718 blocks the response to cholecystokinin octapeptide (CCK-8). Traces in column (a) were obtained from a dorsal raphe neurone and show dose-dependent effects of CCK-8 (1 min applications) from 10–100 nM. Pen recorder traces in column (b) were taken from the same neurone after a 20 min incubation in L-364,718 (3 nM) and show that the response to CCK was considerably reduced by the antagonist. The data were used to produce a dose-response curve (c) of the number of action potentials occurring in a 5 min period immediately following peptide application as a function of concentration of CCK-8. The control dose-response curve to CCK (●) was shifted to the right in a parallel fashion in the presence of L-364,718 (3 nM) (■), indicative of a competitive antagonism. The equilibrium constant for L-364,718 was 31 pM in this example.

a rat dorsal raphe neurone. On neurones which were excited by CCK-8, the equilibrium constant for L-364,718 was calculated to be 0.127 ± 0.043 nM ($n = 4$). L-365,260 is a gastrin/CCK_B antagonist, possessing nanomolar affinity for the guinea-pig cortical CCK_B receptor but only micromolar affinity for the guinea-pig pancreatic CCK_A receptor (Chang & Lotti, 1986). Bath application of L-365,260 (10 μM for 15 to 20 min) had a weak antagonist action on the response to CCK-8 (Figure 4) and the equilibrium constant for L-365,260 was calculated to be 724.2 ± 93.8 nM ($n = 4$).

Antagonism of the CCK-8-induced depolarization with either L-364,718 or L-365,260 occurred in the presence of TTX; neither antagonist caused a change in resting membrane potential or resistance.

Actions of other antagonists on the response to CCK

Bath application of the α-adrenoceptor antagonist, prazosin (1 μM) or the 5-HT receptor antagonist spiperone (10 μM), which abolished responses to phenylephrine and 5-HT respectively, had no effect on the response to CCK-8 ($n = 4$). Carbachol (1–10 μM) had no effect on CCK-sensitive neurones ($n = 3$).

Discussion

We have shown for the first time that CCK excites a population of dorsal raphe neurones in a dose-dependent manner. Confirmation that CCK acted directly on the recorded neurone came from experiments using TTX-treated preparations where extracellular calcium had been omitted and replaced by high magnesium. Under these conditions, when sodium-dependent action potentials were blocked and no electrically evoked potential could be seen, we were unable to see any change in the magnitude of the response to CCK. The CCK response was dose-dependent over the range tested (10–1000 nM), similar to that seen in extracellular recordings of CCK_B responses from rat VMH neurones. One major difference in the CCK response recorded from raphe neurones however, was in the lack of effect of CCK_B agonist, pentagastrin.

Secondly, we demonstrated that this effect of CCK occurs via CCK_A type receptors located on the neuronal membrane. The equilibrium constant obtained with L-364,718 was similar to the equilibrium constant obtained in binding studies to CCK_A binding sites in the pancreas (Chang & Lotti, 1986). This suggests that CCK excites a subpopulation of neurones in the dorsal raphe by acting at a CCK_A receptor. This is supported, not only by the lack of effect seen with the CCK_B agonist, pentagastrin, but also by the relatively weak antagonist action of the potent CCK_B receptor antagonist, L-365,260 (Lotti & Chang, 1989). We found that the equilibrium constant for L-365,260 on raphe neurones was similar to the equilibrium constant observed for L-365,260 at CCK_A sites in binding studies on guinea-pig pancreatic membranes. The persistence of the CCK response in the presence of prazosin and spiperone, shows that the mechanism of action of CCK was unlikely to involve either noradrenaline or 5-HT.

Autoradiographic studies indicate that the CCK_B receptor is the predominant type in the rodent central nervous system (Hill *et al.*, 1987). Thus, in the VMH there is a good correlation between a high density of CCK_B binding sites and a biological response which is mediated via CCK_B receptors (Boden & Hill, 1988). Interestingly, in the raphe, carbachol (10 μM), an agonist on muscarinic receptors on ventromedial hypothalamic neurones where the CCK response is mediated via a CCK_B receptor (Boden & Hill, 1988), had no effect on neurones which responded to CCK-8.

Although the majority of CCK receptors in the central nervous system are of the B type, CCK_A receptors in the interpeduncular nucleus have been demonstrated both autoradiographically (Hill *et al.*, 1988) and electrophysiologically (Hill

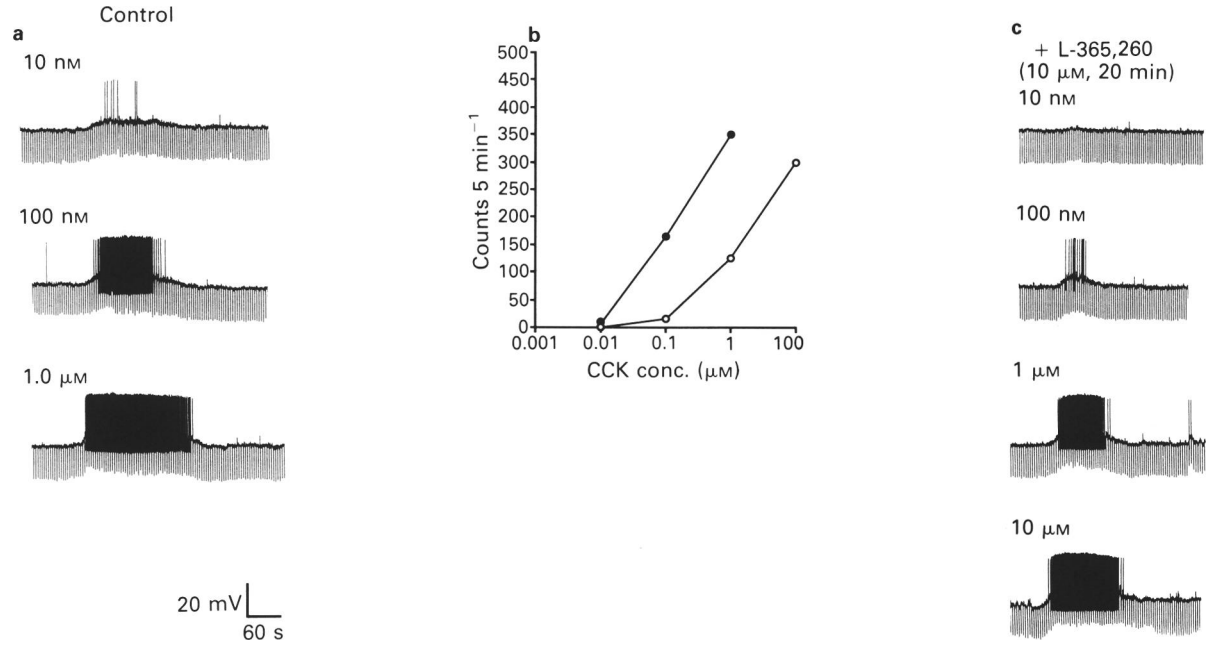

Figure 4 The CCK_B antagonist L-365,260 blocks the response to cholecystokinin octapeptide (CCK-8). Traces in column (a) were obtained from a dorsal raphe neurone and show dose-dependent effects of CCK-8 (1 min applications) from 10–1000 nM. Pen recorder traces from a silent neurone are used for clarity to show the excitatory action of CCK. The traces in column (a) show the effects of CCK before exposure to L-365,260. The prolonged exposure of L-365,260 had no effect on its own on either the membrane potential or activity of the neurone. Traces in column (c) were taken from the same neurone after a 20 min incubation in L-365,260 (10 μM) and show that the response to CCK was reduced by this antagonist. The data were used to produce a dose-response curve (b), with the number of action potentials occurring during the CCK-8 response on the y-axis and the dose of CCK-8 on the x-axis. The control dose-response curve to CCK (○) was shifted to the right in a parallel fashion in the presence of L-365,260 10 μM (●), indicative of a competitive antagonism. The equilibrium constant for L-365,260 was calculated to be 865.8 nM in this example.

& Boden, 1989). Evidence for the existence of CCK_A receptors in other rat brain regions may be hampered by their comparatively low density. For example, in spite of the fact that autoradiographic studies fail to reveal the presence of CCK_A binding sites in the rat nucleus accumbens, it has recently been shown that CCK_A receptors mediating dopamine release are present in this region (Marshall *et al.*, 1990). Our results presented in this paper show clear electrophysiological actions of CCK in the dorsal raphe nucleus and provide further evidence for a central role for CCK_A receptors in rat brain. Studies are in progress to determine the mechanism of action of CCK on raphe neurones.

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