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DEPOLARIZING ACTION OF CHOLECYSTOKININ ON RAT SUPRAOPTIC NEURONES *IN VITRO*

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SUMMARY

1. Cholecystokinin is co-localized within the oxytocin- and, to a lesser extent, vasopressin-synthesizing magnocellular neurones in the hypothalamic supraoptic and paraventricular nuclei. These nuclei are also prominent binding sites for cholecystokinin. In the present study we used intracellular current- and voltage-clamp recordings from fifty-seven supraoptic nucleus cells, maintained in superfused explants of rat hypothalamus, to assess their membrane responses to exogenous cholecystokinin and define the nature of their cholecystokinin receptors.

2. In a majority of the fifty-seven cells tested, bolus infusions into the superfusion media of cholecystokinin fragments (maximum concentrations estimated at 0·3–15 μM) were followed within 1–5 s by a transient and reversible membrane depolarization. Active peptides included sulphated cholecystokinin octapeptide (26–33) (28 of 33 cells responded), non-sulphated cholecystokinin octapeptide (26–33) (21 of 25 cells responded), cholecystokinin tetrapeptide (30–33) (20 of 24 cells responded and caerulein (4 of 4 cells responded). None of five cells responded to cholecystokinin (26–28). Depolarizing responses to cholecystokinin analogues persisted in the presence of tetrodotoxin (0·2–0·4 μM), and in Ca^{2+}-free solutions containing $MnCl_2$ (2·5 mM).

3. Under voltage clamp, cholecystokinin fragments evoked an inward current accompanied by an increase in membrane conductance. The amplitude of the inward current varied linearly as a function of membrane voltage, with an extrapolated reversal potential of ≈ -15 mV. Reversal potentials were not altered by chloride injection. These features suggest that cholecystokinin activates a non-selective cationic conductance.

4. Active cholecystokinin analogues were approximately equipotent in their depolarizing actions, a feature that supports the activation of cholecystokinin-B type receptors. Moreover bath application of 200 nM L-365,260, an antagonist with a high affinity for cholecystokinin-B receptors, reversibly attenuated the cholecystokinin-induced responses in four of six cells tested.

5. These observations indicate that cholecystokinin can directly influence the excitability of rat supraoptic nucleus neurones and provide evidence for an

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additional site where this peptide may act within the hypothalamo-neurohypophysial axis.

INTRODUCTION

Cholecystokinin (CCK) was initially identified as a gastrointestinal hormone (Ivy & Oldberg, 1928; Mutt & Jorpes, 1971). Subsequent clarification that its C-terminal octapeptide, (CCK(26–33) or CCK-8) was the source for the 'gastrin-like' material in rat brain (Vanderhaegen, Signeau & Gepts, 1975; Dockray, 1980; Beinfeld, 1981) has led to the recognition that this neuropeptide is one of the most abundant of the biologically active peptides in the central nervous system. CCK-synthesizing neurones are widely yet selectively distributed at various levels of the neural axis (Hökfelt, Herrera-Marschitz, Seroogy, Ju, Staines, Holets, Schalling, Ungerstedt, Post, Rehfeld, Frey, Fischer, Dockray, Hamaoka, Wash & Goldstein, 1988; Ingram, Krause, Baldino, Skeen & Lewis, 1989; Hökfelt, Cortés, Schalling, Ceccatelli, Pelto-Huikko, Persson & Villar, 1991). Moreover, the brain contains numerous binding sites for CCK (Saito, Sankaran, Goldfine & Williams, 1980) and contains at least two types of CCK receptors, referred to as 'peripheral' or CCK-A receptors and 'central' or CCK-B receptors (Moran, Robinson, Goldrich & McHugh, 1986).

It would appear that CCK can influence specific behaviours (see Crawley, 1985; Ravard & Dourish, 1990). In addition, its exogenous application at the cellular level alters the excitability of central neurones (reviewed in Boden & Hill, 1988*a*). These features, in combination with the evidence for localization and synthesis of CCK within central neurones (see Vanderhaeghen, 1985; Hökfelt *et al.* 1988; Ingram *et al.* 1991), its presence in synaptic vesicles (Rehfeld, Gottermann, Larsson, Emson & Lee, 1979) and its calcium-dependent release (e.g. Meyer & Krause, 1983) offer support for a neurotransmitter role for this peptide.

The hypothalamic magnocellular neurosecretory neurones are a target for CCK, achieved through independent peripheral and central sites of action. A peripheral vagally mediated site of action is observed in the rat where systemic injections of CCK-8 induce a selective activation of oxytocin-secreting neurones (Renaud, Tang, McCann, Stricker & Verbalis, 1987; Verbalis, Stricker, Robinson & Hoffman, 1991). Central (intracerebroventricular) administration of CCK induces release of vasopressin (Marley, Lightman, Forsling, Todd, Goedert, Rehfeld & Emson, 1984), although the site of action remains undetermined. The presence of CCK-immunoreactive axons in the vicinity of magnocellular neurones (Hökfelt *et al.* 1988), the high-affinity binding for [^{3}H]pentagastrin and ^{125}I-CCK-8 in the hypothalamic paraventricular and supraoptic nuclei (Gaudreau, St Pierre, Pert & Quirion, 1985; Akesson & Micevych, 1986), and an increase in the latter during conditions which stimulate the hypothalamo-neurohypophysial axis (Day, Hall & Hughes, 1989) suggest that the magnocellular cells may be directly influenced by centrally applied (or endogenously released) CCK.

The present study was undertaken to determine the presence and nature of CCK receptors on rat supraoptic nucleus neurones. Intracellular current- and voltage-clamp recordings obtained from supraoptic neurones were used to evaluate their responses to exogenously applied CCK agonists and antagonists. Results indicate that CCK acts directly on CCK-B receptors on supraoptic neurones to induce

membrane depolarization, presumably mediated by a non-selective cationic conductance. A portion of these results has been reported briefly (Jarvis, Bourque & Renaud, 1988, 1991).

METHODS

Preparation of hypothalamic explants

Experiments used explants of basal hypothalamus prepared as described elsewhere (Bourque, 1990). Briefly, unanaesthetized male Long Evans rats (150–300 g) were decapitated; $8 \times 8 \times 2$ mm explants of basal hypothalamus were pinned to a Sylgard base in a humidified recording chamber, maintained at 32–34 °C and superfused (0·5–1·0 ml min^{-1}) with an oxygenated (95 % O_2, 5 % CO_2) artificial cerebrospinal fluid (ACSF; pH 7·4) comprising (mM): NaCl, 106; KCl, 3; $MgCl_2$, 1·3; $NaHCO_3$, 25·9; $CaCl_2$, 2·5; glucose, 10. Covering membranes were removed to expose the supraoptic nucleus to the ACSF, and to provide access for microelectrodes. Explants were allowed to stabilize for 3 h before recording was begun.

Electrophysiology

Intracellular recordings were obtained with micropipettes filled with 3 M potassium acetate/0·15 M potassium chloride (tip resistance 50–100 MΩ). The reference electrode consisted of a chlorided silver wire embedded in a tube containing 10 % agar gel in contact with the recording solution. Microelectrodes were connected to the input stage of an Axoclamp-2A preamplifier (Axon instruments) for current- and voltage-clamp recording. Measurements of membrane current were obtained using the discontinuous single electrode voltage-clamp mode; the switching frequency (1·3–2·6 kHz) was adjusted to allow a complete stabilization of the continuously monitored headstage voltage prior to the acquisition of each sample. All signals were either stored on a FM recorder (Racal) or digitized by a pulse code modulator (Neurodata Corp.) and recorded on videotape. On- and off-line recordings were displayed on a Gould RS 3200 chart recorder.

Drugs

Test peptides (from Bachem) included the C-terminal sulphated CCK octapeptide (26–33) (CCK-8S), nonsulphated CCK octapeptide (26–33) (CCK-8NS), CCK tetrapeptide (30–33) (CCK-4), CCK (26–28) (CCK-2) and caerulein, a structurally related decapeptide. All were dissolved in ACSF at concentrations of 10–100 μM and injected as a 10–50 μl bolus into the superfusion line just proximal to the explant. Their final concentrations (0·3–15 μM) are estimates based on a technique described elsewhere (Yang, Bourque & Renaud, 1991). It should be noted that the quoted concentrations reflect the transient peak concentration of the drug in media flowing over the supraoptic nucleus; effective concentrations surrounding a cell are likely to be lower due to diffusion and metabolism within the tissue. In addition bath applications were made of a CCK agonist, CCK-4 (100 nM), and a CCK-B receptor antagonist, L-365,260 (3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-*N'*-(3-methyl-phenyl)urea) (200 nM; kindly provided by Merck, Sharp and Dohme). Tetrodotoxin (TTX, 0·2–0·4 μM; Sigma) was added to the bathing medium as required and nominally calcium-free solutions were prepared by replacing $CaCl_2$ with $MnCl_2$ (2·5 mM).

RESULTS

The data presented below were obtained from fifty-seven neurones which displayed resting membrane potentials (V_m) more negative than −55 mV, input resistances ranging between 70 and 466 MΩ (181·7 ± 10·5) and action potentials of 68 to 110 mV. All cells displayed features deemed typical of supraoptic neurones, i.e. frequency-dependent action potential broadening and transient outward rectification during depolarizing pulses applied from hyperpolarized levels (Bourque & Renaud, 1990). Spontaneous firing in a phasic pattern, a feature of a majority of cells displaying vasopressin-like immunoreactivity (Cobbett, Smithson & Hatton, 1986) was present in fifteen cells at resting potentials.

Response to CCK fragments

In twenty-eight of thirty-three cells tested, infusions of CCK-8S (0·3–15 μM) were followed by dose-dependent and reversible membrane depolarizations (range 2–26 mV) (Fig. 1). The remaining five cells showed no response. Under voltage

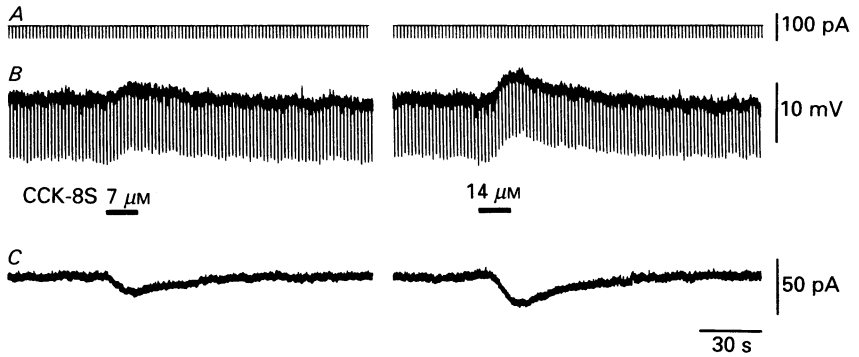

Fig. 1. Dose-dependent responses to bolus infusions (bar) of sulphated CCK-8 (CCK-8S) in a supraoptic neurone recorded under current ($V_m = -66$ mV) (*B*) and voltage clamp (holding potential $V_h = -70$ mV) (*C*). Traces shown in *B* illustrate voltage responses recorded during consecutive applications of distinct concentrations of CCK-8S (7 μM in left panel and 14 μM in right panel). Note that these concentrations reflect the maximum concentration estimated over the supraoptic nucleus; actual concentrations reaching the neurone are likely to be lower (see Methods). Negative deflections in *B* are electrotonic responses to injection of constant current pulses (*A*). The traces shown in *C* represent responses to similar applications of CCK-8S recorded under voltage clamp ($V_h = -70$ mV). Although the responses observed under current and voltage clamp were recorded at different times, the time course and amplitude of the responses mirror each other, indicating that the depolarizing effect of CCK-8S is probably due to the induction of an inward current.

clamp, CCK-8S evoked an inward membrane current ($n = 6$ cells) whose amplitude and time course mirrored subthreshold depolarizing responses recorded under current clamp (Fig. 1). When CCK-8S was applied at resting membrane potentials near spike threshold (≈ -58 mV), the ensuing membrane depolarization triggered a burst of action potentials (Fig. 2). With intervals between applications $\geqslant 5$ min, successive responses were highly reproducible. Figure 2 also illustrates that similar responses followed the application of CCK-8NS (21 of 25 cells tested), CCK-4 (20 of 24 cells tested) and the structural analogue caerulein (4 of 4 cells tested), whereas none of five cells responded to CCK-2, even with concentrations up to 100 μM. When applied at the same concentrations (range 0·3–15 μM), each of the CCK fragments (CCK-8S, CCK-8NS, CCK-4 and caerulein) appeared to be roughly equipotent in depolarizing supraoptic neurones (e.g. Fig. 2). Moreover, bath application of 100 nM CCK-4 to two cells evoked small depolarizing responses which, despite a variable amount of sag, could be maintained over a period of 1–2 min (Fig. 3). Based on agonist affinity profiles (see Discussion), these results are consistent with an action at CCK-B receptors.

To determine whether the responses described above were mediated by post-synaptic receptors, CCK fragments were applied during superfusion with Ca^{2+}-free

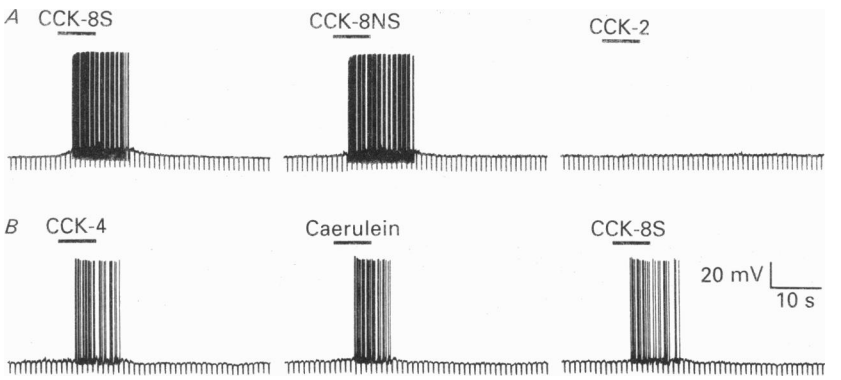

Fig. 2. *A*, current-clamp recordings from a single supraoptic neurone illustrate events associated with bolus infusion (bar) of three different CCK fragments, at a maximum concentration of 7 μM. Note the induction of membrane depolarization and action potential generation by sulphated CCK-8 (CCK-8S) and non-sulphated CCK-8 (CCK-8NS), but not by CCK-2. *B*, another neurone displays similar responses when exposed to CCK-4, caerulein and CCK-8S at a maximum concentration of 7 μM. Resting membrane potential for each neurone was −60 mV. Negative deflections represent electrotonic responses to 50 pA, 80 ms hyperpolarizing current pulses.

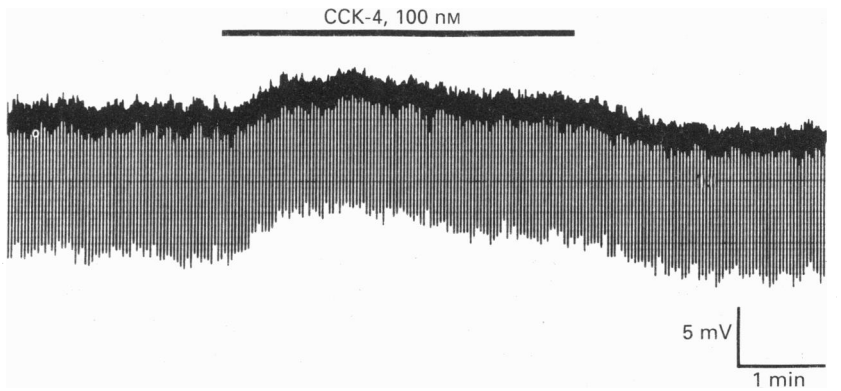

Fig. 3. Current-clamp recording in a supraoptic neurone during bath application of 100 nM CCK-4 reveals a gradual 2–3 mV membrane depolarization, accompanied by an apparent decrease in input resistance. Downward voltage deflections correspond to hyperpolarizing pulses of constant current (100 pA amplitude and 80 ms duration). $V_m = -67$ mV.

ACSF containing Mn^{2+} (2·5 mM). In each of four cells tested, infusion of CCK receptor agonists induced reversible membrane depolarizations comparable to those recorded under control conditions (Fig. 3). Depolarizing responses or inward currents were also evoked by CCK agonists in ACSF containing 0·2–0·4 μM TTX (see Fig. 4). These results indicate that the CCK-evoked responses were not dependent on presynaptic transmitter release, nor on the occurrence of Na^+-dependent impulses, suggesting that the receptors involved were located directly on the soma and/or dendrites of these cells.

Current-clamp analysis of CCK-evoked responses

Changes in input conductance associated with CCK effects recorded under current clamp were strongly influenced by the presence or absence of evoked firing. Subthreshold depolarizing responses were usually devoid of any obvious conductance

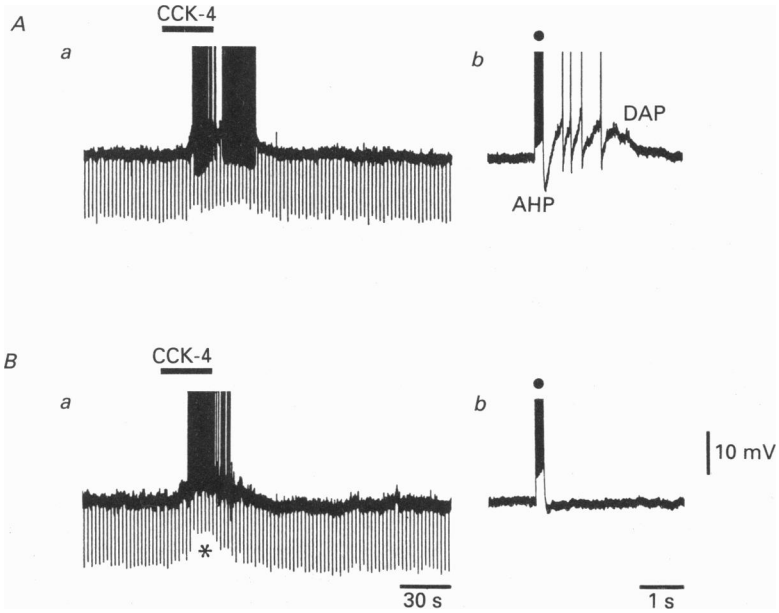

Fig. 4. Current-clamp data illustrate membrane depolarizations and a burst of action potentials (left panel, traces labelled *a*) in a single supraoptic neurone following bolus infusions (bar) of CCK-4 (maximum concentration of 15 μM) in standard ACSF (*A*) and 25 min after switching to media containing zero Ca^{2+} and 2·5 mM $MnCl_2$ (*B*). Downward deflections are voltage responses to intracellular hyperpolarizing current pulses (30 pA); note the attenuation in their amplitude in trace *Ba* (asterisk; see text). In the right panel (traces labelled *b*), a depolarizing current pulse (0·15 nA; 200 ms in duration) (●) triggering 8 action potentials is followed by an after-hyperpolarizing potential (AHP) and a depolarizing after-potential (DAP) (see Bourque & Renaud, 1990) in standard ACSF (*Ab*). However, these post-burst Ca^{2+}-dependent events (AHP and DAP) are absent in media containing zero Ca^{2+} and 2·5 mM $MnCl_2$ (*Bb*) as are Ca^{2+}-dependent hyperpolarizing after-potentials following individual spikes in *Ba*. Illustrated action potentials are truncated. $V_m = -62$ mV.

change (e.g. Fig. 1), or were associated with an apparent *increase* of input conductance. By contrast, apparent *decreases* of input conductance were generally found to accompany suprathreshold responses (e.g. Fig. 2). However, given the array of spike after-currents which are expressed by supraoptic neurones (Bourque & Renaud, 1990), it is likely that assessments of impedance change under current clamp are influenced by the presence or absence of spike discharge, or by changes in firing frequency. In particular, the voltage-dependent properties of the current which underlies depolarizing after-potentials (I_{DAP}) may confound impedance measurements made using current pulses. Indeed, the hyperpolarization associated with

negative pulses applied during activity may be sufficient to de-activate a proportion of inward I_{DAP}. The decreased conductance generated by I_{DAP} de-activation causes an apparent increase of the electronic voltage transient compared to that recorded in the absence of I_{DAP} (Bourque, 1986). In agreement, constant-current pulses

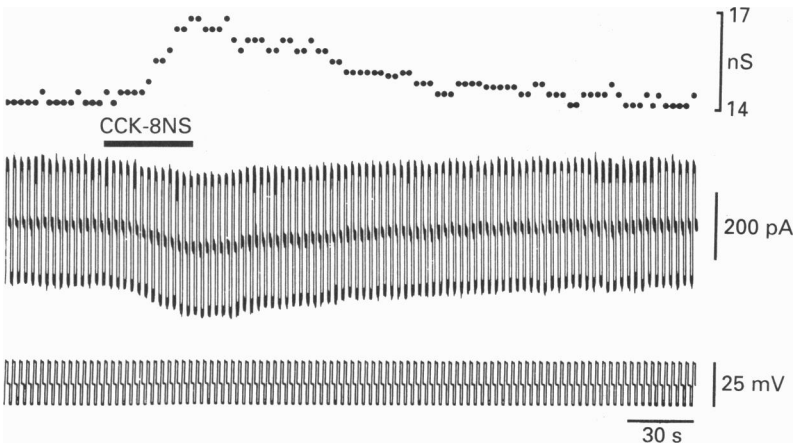

Fig. 5. Voltage-clamp data obtained from a supraoptic neurone during bolus infusion of non-sulphated CCK-8 (CCK-8NS, 10 μM) in the presence of TTX (0·4 μM). As shown in the middle trace, CCK-8NS induced a reversible inward current that was associated with an increase in the amplitude of the current response (I) at successive voltage (V) steps ($V_h = -43$, -54, -64 mV) (lower trace). Top trace reflects chord conductance, computed as ($\Delta I/\Delta V$). Note that the change (increase) in chord conductance coincides with the rise and fall of the CCK-induced inward current.

applied during CCK-evoked responses recorded in Ca^{2+}-free solutions, where I_{DAP} is absent, consistently yielded smaller amplitude electrotonic responses (Fig. 4), suggesting that the response was associated with an *increase* in input conductance.

Voltage-clamp analysis of CCK-evoked responses

To ascertain the nature of the conductance change underlying the response, the effects of CCK receptor agonists were examined under voltage clamp ($n = 11$ cells). As shown in Fig. 5, a reversible inward current was induced by application of CCK agonists (I_{CCK}). Repetitive application of a multiple level staircase protocol (100–500 ms per level) permitted a monitoring of I_{CCK} amplitude (ΔI) as a function of membrane potential (ΔV) during single CCK applications. The amplitude of I_{CCK} (range 17–89 pA) measured during such experiments was found to be linearly related to voltage, becoming greater when measured at progressively more negative holding potentials (Fig. 5). Values of chord conductance calculated as $\Delta I/\Delta V$ during such experiments revealed that the rise and fall of I_{CCK} coincided with a reversible increase of input conductance ($n = 6$ cells; range 7–21%) (Fig. 5). Extrapolation of I_{CCK}–voltage relationships measured in five cells revealed a mean ($\pm$S.D.) reversal potential (E_{CCK}) of -15 ± 7 mV (Fig. 6). Estimates of E_{CCK} made from two other cells

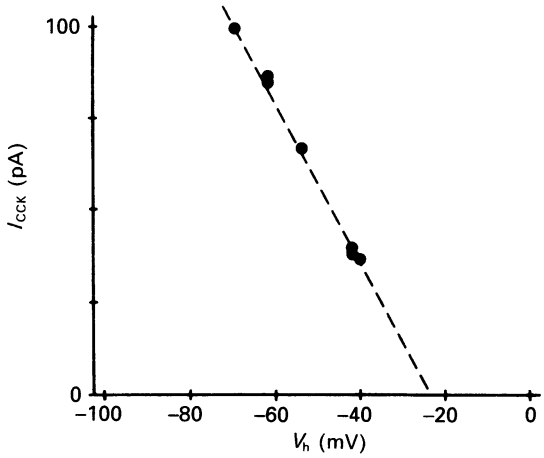

Fig. 6. The peak amplitude of several CCK-evoked responses measured at different holding potentials in the supraoptic cell whose data are illustrated in Fig. 5. The dashed line connecting the points indicates that the CCK-induced current is linear at voltages between −70 and −40 mV, and shows reversal at −22 mV. Mean reversal potential from five cells was 15±7 mV (not illustrated).

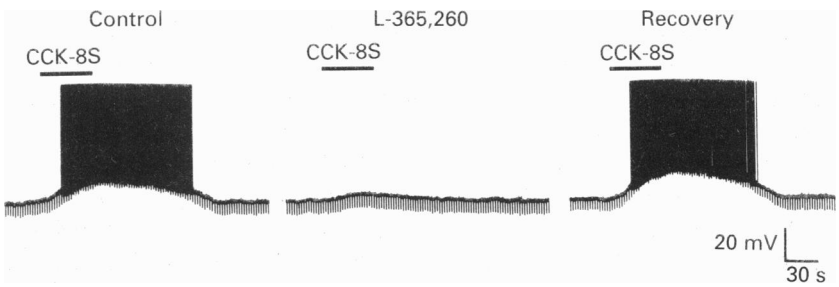

Fig. 7. L-365,260, a CCK antagonist, reversibly attenuates responses to sulphated CCK-8 (CCK-8S). The left panel illustrates data from a supraoptic neurone where membrane depolarization is evoked by bolus infusion of CCK-8S (10 μM) in standard ACSF (Control). As shown in the centre panel, this response is markedly attenuated 10 min after superfusion with ACSF containing 200 nM L-365,260. The panel on the right illustrates recovery of the response 35 min after returning to standard ACSF (Recovery). Negative pulses represent voltage deflections to 50 pA hyperpolarizing pulses, 100 ms in duration. $V_m = -70$ mV.

impaled with KCl-filled microelectrodes yielded similar values, suggesting that chloride ions were not involved in the genesis of the CCK-evoked response. These results suggest that the activation of a mixed cationic conductance is responsible for the depolarizing effects of CCK.

Effects of a CCK antagonist

When L-365,260, a benzodiazepine derivative with high affinity for CCK-B receptors (Lotti & Chang, 1989), was bath applied at 200 nM it reversibly attenuated CCK-evoked responses in four out of six cells (Fig. 7). In 2/2 of these cells, the blockade by L-365,260 was selective for CCK and had no effect on quisqualate-

induced excitation. By itself, L-365,260 had no consistent effect on membrane potential nor on input resistance.

DISCUSSION

The present results indicate that supraoptic neurones possess functional CCK receptors whose occupation induces membrane depolarization and increases in cellular excitability. The source of endogenous ligands for these receptors remains to be defined. Recent immunocytochemical data reveal that a modest number of fibres with CCK-like immunoreactivity are present in the area of the supraoptic nucleus (Hökfelt *et al.* 1988). Thus CCK may act as an excitatory transmitter in one or more afferent pathways to these neurosecretory neurones. It is interesting that CCK is also co-localized within oxytocin-containing (Vanderhaeghen, Lostra, Vandesande & Dierickx, 1981) and (to a lesser extent) vasopressin-containing magnocellular neurones (Meister, Villar, Ceccatilli & Hökfelt, 1990). Given the evidence that oxytocin is released locally within the magnocellular nuclei (reviewed in Richard, Moos & Freund-Mercier, 1991), it seems appropriate to propose that CCK may also be released locally from axon collaterals, or possibly from soma-dendritic membranes as has been proposed for vasopressin (see Pow & Morris, 1990). If so, locally released CCK might function in a paracrine manner to influence cell excitability, to modulate other transmitter systems, or to induce the plasticity described in supraoptic nucleus under certain circumstances (see e.g. Theodosis, Montagnese, Rodriguez, Vincent & Poulain, 1986). Thus the CCK input to magnocellular neurones may be derived from intrinsic and/or extrinsic sources.

While excitatory responses to exogenous CCK have been reported in a variety of other CNS regions, few studies have described their ionic mechanisms. Available reports suggest that the ionic mechanism underlying CCK-evoked responses may vary according to the cell type under study. CCK-induced increases in input resistance have been reported in spinal (Rogawski, Beinfeld, Hays, Hökfelt & Skirboll, 1985), hippocampal (Boden & Hill, 1988*b*) and hypothalamic ventromedial nucleus neurones (Boden & Hill, 1988*a*). These actions may result from the suppression of a potassium conductance. For example, in ventromedial nucleus neurones studied under voltage-clamp conditions, CCK produces an inward current with a reduction in a voltage-dependent outward current (Boden, 1991). In supraoptic neurones CCK induces an inward current with an increase in a voltage-independent (between -70 and -40 mV) non-selective cationic conductance. Interestingly, our findings are similar to observations in rat pancreatic acinar cells, where CCK also increases a non-selective cationic conductance (see Petersen, 1987).

Currently available pharmacological tools suggest the existence of at least two distinct types of CCK receptors. While CCK-B receptors predominate centrally, a number of brain regions also contain CCK-A receptors, i.e. similar to those found in the periphery (Moran *et al.* 1986; Hill, Campbell, Shaw & Woodruff, 1987; Hill & Woodruff, 1990). CCK-B receptors have a similar affinity for CCK-8S ($IC_{50} \approx 2$ nM), CCK-8NS ($IC_{50} \approx 9$ nM), and CCK-4 ($EC_{50} \approx 90$ nM) whereas CCK-A receptors have a much lower affinity for CCK-8NS ($EC_{50} \approx 700$ nM) and CCK-4 ($EC_{50} > 100$ μM) (Moran *et al.* 1986). The testing of CCK agonists in our experiments indicates that all

agonists (CCK-8S, CCK-8NS, CCK-4 and caerulein) were approximately equipotent with respect to their ability to evoke depolarizing responses from supraoptic neurones. In particular supraoptic neurones were found to be remarkably sensitive to nanomolar concentrations of CCK-4. These findings suggest that CCK-evoked responses were mediated by CCK-B receptors. Such a classification is in agreement with the binding of the non-sulphated ligand [^{3}H]pentagastrin in the supraoptic nucleus (Gaudreau *et al.* 1985). In addition, bath application of the non-peptide CCK antagonist L-365,260 (200 nM) significantly attenuated CCK-evoked responses in the present study. This antagonism, however, was incomplete at a concentration which should have saturated CCK-B receptor sites (Lotti & Chang, 1989). Therefore, although supraoptic neurones express functional CCK receptors that resemble CCK-B type, they may represent a subtype of CCK-B receptor, differing slightly from cortical CCK-B receptors, or a distinct type of CCK receptor which has not yet been defined pharmacologically. The isolation of cDNA clones coding novel forms of CCK receptors and the development of new and more selective CCK ligands should help resolve this issue.

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