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Depletion of cortical cholecystokinin levels after excitotoxin injection into the nucleus basalis: sensitivity to MK-801

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Abstract

The release of cholecystokinin (CCK) in vitro has been shown to be influenced by NMDA receptors. In this study we have investigated whether excitotoxin-induced seizure activity affects the release and tissue content of CCK. Excitotoxin injection caused a significant decrease in CCK in ipsilateral frontal, parietal and temporal cortex by (30–54%) at 8 h compared to contralateral cortex and sham-operated controls and the effect was reversed by 24 h. No change was detected in occipital cortex, hippocampus and nucleus accumbens. The effect in frontal and temporal cortex was maximal at 8 h and could be completely prevented by treatment with MK-801(3 mg/kg i.p.). Anaesthesia (pentobarbital) alone or in combination with MK-801 did not affect peptide levels at 8 h. CCK mRNA levels were also studied quantitatively by slot-blot analysis but were unaffected at 6, 8 and 24 h after excitotoxin injection. The decrease in CCK tissue levels indicated that seizure activity stimulated CCK release which was confirmed in ex vivo experiments where K^+-evoked (34 mM) CCK release was significantly enhanced in ipsilateral cerebral cortex at 6 h compared to contralateral cortex.

Keywords: CCK (cholecystokinin); Cerebral cortex; Glutamate; NMDA receptor; Seizure

1. Introduction

Cholecystokinin octapeptide (CCK) is widely distributed throughout the mammalian central nervous system, being most abundant in the cerebral cortex where it is thought to act as a neurotransmitter (Rehfeld, 1985), although the nature of its interactions with other neurotransmitters are largely unknown. There is some evidence in the literature suggesting that CCK levels can be influenced in vivo by persistent excitation in experimental models of chemically or electrically induced seizures. CCK is severely depleted in mossy fibre endings in the mouse 4 days after electrolytic lesion of the dentate gyrus or intracerebroventricular (i.c.v.) kainate administration (Gall, 1988) and is also significantly depleted in the rat cortex, striatum and amygdala 3 h after intraperitoneal (i.p.) kainate injection. The latter effect is followed by a significant increase in CCK levels at 10 days (Meyer et al., 1986). Levels of preprocholecystokinin mRNA have been reported to be either increased in cortex at 7–10 days and striatum at 10 days or decreased in the hippocampus 2–10 days after seizures induced by a single intraperitoneal injection of kainic acid (Olenik et al., 1989). Different regional effects complicate interpretation of results obtained from i.p. injection. Further, the mechanism by which seizure activity influences CCK release in vivo is not established, but we have recently shown that depolarisation-induced release of CCK from rat frontal cortex in vitro can be significantly enhanced by glutamate receptor agonists, namely *N*-methyl-D-aspartate (NMDA), and the effect can be blocked by NMDA receptor antagonists, suggesting the involvement of this receptor in this process (Bandopadhyay and De Belleroche, 1991).

In this study we have investigated the effect of excitotoxin-induced seizures on levels of CCK peptide and CCK mRNA in cerebral cortex in order to determine whether CCK neurones are influenced by glutamate in vivo. The experimental model that we have used is a well-characterised one in which excitotoxin (kainic acid) injection into nucleus basalis causes a

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significant cholinergic deficit in cerebral cortex ipsilateral to lesion (De Belleroche et al., 1985) and transiently activates glutamate neurotransmission through NMDA receptors in cerebral cortex with the development of seizure activity (Reed and De Belleroche, 1990). Similarly the involvement of NMDA receptors in regulating CCK levels was investigated by the use of the antagonist MK-801. The mechanisms underlying changes in peptide levels were further investigated by ex vivo experiments in which CCK release from cortical slices was studied at various times after in vivo injection of kainate.

2. Materials and methods

2.1. Excitotoxin injection procedure

Male CFY rats (260 ± 15 g at the time of surgery) were anaesthetised with sagatal (pentobarbitone) and placed in a stereotaxic frame. Unilateral lesion of the right nucleus basalis was carried out by injection of kainate (1 μg in 0.5 μl water, pH 7.0) into the nucleus basalis (coordinates: anteroposterior +1.2, lateral −2.7, with respect to bregma: incisor bar at +5 mm above the intra-aural line at a depth of 8.8 mm below the skull surface over a period of 1 min). The needle was left in place for 5 min to reduce reflux of kainate. At the dose of barbiturate used animals regained consciousness within 2 h and seizure activity was apparent from 2 to 8 h. Initial behavioural features of this activity were unilateral limb jerking and turning. Maximal seizure activity was seen at 4–6 h after injection of kainate. Sham-operated animals were treated as above using saline vehicle for injection. Rats were killed by stunning and cervical dislocation at 4 h, 6 h, 8 h, 1 day and 7 days after kainate injection. Samples from four cortical regions (frontal, parietal, temporal and occipital), hippocampus and nucleus accumbens were dissected and collected in ice-cold 90% methanol for CCK-8 extraction or frozen immediately in liquid nitrogen and stored at −70°C for RNA extraction.

2.2. CCK radioimmunoassay

CCK immunoreactivity was measured by radioimmunoassay using a well characterised antibody, Ab2717, raised in rabbits against gastrin-1–17 (a kind gift of Professor J. Rehfeld) at a dilution of 1:600 000. Iodinated gastrin-(2–17) (purchased from Amersham International PLC) was used as a tracer. The assays were performed at 0°C in 20 mM barbital-0.1% bovine serum albumin, pH 8.4 and incubated at 2°C for a period of 72 h. Bound tracer was separated from free by use of a plasma-activated charcoal (Norit PN5) suspension containing 3 g/100 ml charcoal in 20 mM sodium phosphate buffer (pH 7.4).

2.3. CCK release protocol

Tissue slices of frontal cortex from kainate injected rats were cut (0.3 mm thick) using a McIlwain tissue chopper and immediately immersed in Krebs bicarbonate medium of the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4.7H_2O$,1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2; $CaCl_2$ 2.5: glucose 11.1; and 0.1% bovine serum albumin, pH 7.4, gassed with 95% O_2-5% CO_2. Separated slices were transferred (3/vial) to a vial containing 1 ml of incubation medium maintained at 37°C in a water bath for a preincubation period of 20 min. Tissue slices were then transferred to another vial containing 1 ml Krebs-bicarbonate medium and incubated for 10 min at 37°C to measure basal release, and transferred to fresh medium containing 34 mM K^+ for an additional 5 min incubation period to measure evoked release. At the end of incubation, tissue slices were transferred into ice-cold 90% methanol (0.5 ml) and the incubation media from the basal and K^+-evoked periods were stored at −20°C prior to analysis of CCK content by radioimmunoassay. Release is expressed as % tissue levels released/min.

2.4. Protein estimation

Protein was measured by the method of Lowry et al. (1951), using Folin Ciocalteau's reagent and bovine serum albumin as standard.

2.5. RNA extraction

Total RNA was extracted from tissue samples by acid guanidinium thiocyanate phenol-chloroform extraction using a modified version of the method of Chomczynski and Sacchi (1987). Frozen samples were homogenised for 10 s with a polytron blender in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. Sequentially, 2 M sodium acetate, phenol and chloroform: isoamyl alcohol (49:1) were added to the tubes which were then centrifuged for 20 min at 8000 rpm and the aqueous layer removed. Samples were precipitated twice by isopropanol and once by 70% ethanol and the resulting pellet was resuspended in diethylpyrocarbonate-treated water for Northern-blot and slot-blot analysis. Concentrations were determined from absorbance values (260 and 280 nm).

2.6. Slot-blot analysis

RNA samples were serially diluted (8–2 μg), denatured in 7.7% formaldehyde, 6 × saline sodium citrate for 10 min at 65°C, rapidly cooled on ice and applied to a Hybond-N membrane by vacuum filtration using a slot-blot apparatus (Schleicher and Schuell). The filter was irradiated for 2 min on an UV transilluminator.

Filters were preincubated in polythene bags in hybridisation buffer containing 50% formamide, 5 × SSPE (saline sodium phosphate ethylenediaminetetraacetic acid) (0.9 M NaCl, 0.05 M sodium phosphate, pH 7.4, 5 mM EDTA), 5 × Denhardt's solution (0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone), 0.5% (w/v) sodium dodecyl sulphate (SDS), 100 μg/ml salmon sperm DNA for 2 h at 42°C. Hybridisation of filters took place overnight at 42°C in hybridisation buffer containing 3–5 × 10^7 dpm [^{32}P]preproCCK cDNA (535 bp cDNA fragment complementary to the mRNA of rat preproCCK – a kind gift of Dr. Jack Dixon) labelled with [^{32}P]dCTP by the oligolabelling method of Feinberg and Vogelstein (1983, Feinberg and Vogelstein 1984). Blots were washed under stringent conditions to abolish non-specific signals with 3 × saline sodium citrate, 0.1% SDS for 30 min at 65°C followed by 0.5 × saline sodium citrate, 0.1% SDS for 30 min at 65°C and exposed to Hyperfilm (Amersham International PLC) with intensifying screens for 48 h at −70°C. Each blot was stripped of labelled probe by washing in 0.1% SDS for 30 min and then reprobed with [^{32}P]β-tubulin cDNA as a reference probe. After autoradiography mRNA signals were quantified by densitometric scanning (Joyce Loebl Chromoscan 111). Results were expressed as a ratio of CCK mRNA/β-tubulin mRNA.

2.7. Statistical analysis

The Student's paired *t*-test was used to evaluate the significance of changes between ipsilateral and contralateral cortex in the same animal whilst the unpaired *t*-test was used to evaluate significance between different groups of animals. A *P* value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Region-specific depletion of CCK following unilateral injection of kainate into nucleus basalis: time course

Levels of CCK peptide were measured by radioimmunoassay in frontal, parietal, temporal and occipital cortex, hippocampus and nucleus accumbens at 8 h, 1 day and 7 days after unilateral injection of kainate into the nucleus basalis. Significant decreases in CCK were seen in frontal, parietal and temporal cortex ipsilateral to kainate injection compared to contralateral cortex or sham-operated controls (Fig. 1a,b,c). The maximal decrease seen at 8 h was in frontal cortex (54%: $P < 0.001$) followed by parietal (41%; $P < 0.04$) and temporal cortex (30%; $P < 0.03$). No change in peptide levels was detected in occipital cortex, hippocampus or in the nucleus accumbens (Table 1) at 8 h after kainate injection. No significant differences between ipsilateral and contralateral cortical regions were detected in sham-operated animals measured at 8 h after vehicle injection. By 24 h, the CCK levels had returned to control levels with no significant differences between contralateral and ipsilateral cortical areas (Fig. 1). The CCK levels in ipsilateral cortex of kainate-treated animals at both 1 and 7 days were significantly greater than those at 8 h when the maximum depletion of CCK occurred.

At 7 days after kainate injection, CCK levels in sham-treated animals or in control (contralateral) cortex were not significantly different from levels at 8 h under comparable conditions but in frontal cortex were significantly increased compared to levels at 1 day. This latter effect may be a delayed non-specific response due to the operation procedure manifest bilaterally and not related to the presence of kainate.

3.2. Effect of treatment of MK-801 on CCK changes at 8 h post-kainate injection

In order to test the involvement of excitatory amino acids in the process of CCK depletion from cortical areas 8 h after kainate injection into nucleus basalis, rats were injected with MK-801 (3 mg/kg i.p.) 30 min after excitotoxin injection. Administration of MK-801 completely prevented the CCK peptide loss from frontal and parietal cortex, two cortical regions showing significant peptide depletion (Fig. 2). Pentobarbital anaesthesia (90 mg/kg i.p.) alone or in conjunction with MK-801 (3 mg/kg i.p.) did not affect peptide concentration in any of the four cortical regions.

Table 1
CCK levels in contralateral and ipsilateral areas of nucleus accumbens, hippocampus and occipital cortex 8 h after vehicle (sham) and kainate injection

	Sham (pg CCK/μg protein)		Kainate injection (pg CCK/μg protein)	
Area	Contralateral	Ipsilateral	Contralateral	Ipsilateral
Nucleus accumbens	2.3 ± 0.40 (3)	2.3 ± 0.17 (3)	2.4 ± 0.44 (6)	1.8 ± 0.91 (6)
Hippocampus	2.6 ± 0.23 (3)	2.4 ± 0.17 (3)	2.1 ± 0.36 (7)	1.7 ± 0.20 (7)
Occipital cortex	2.9 ± 0.48 (3)	2.5 ± 0.01 (3)	3.5 ± 0.42 (7)	2.5 ± 0.57 (7)

Values (pg CCK/μg protein) are means ± S.E.M. from the number of experiments shown in parentheses.

3.3. PreproCCK mRNA levels after unilateral nucleus basalis lesion by kainate

To test whether CCK decreases in cortical regions were accompanied by changes in CCK mRNA levels were quantitated by slot-blot analysis at 6 h, 8 h and 1 day after kainate injection. Northern analysis revealed one major band of 0.75 kb for CCK mRNA as previously reported (De Belleroche et al., 1990) which was found to be abundantly expressed in the cortical region in the rat. Levels of CCK mRNA were quantitated densitometrically by reference to β-tubulin mRNA levels. No significant changes were detected in CCK mRNA/β-tubulin mRNA ipsilateral to the injection compared to contralateral cortex or sham-operated controls at any of the three time points investigated (Fig. 3). However, small increases in CCK mRNA in ipsilateral cortex were noticed at 4 h in kainate-treated animals and at 6 h in sham-operated animals.

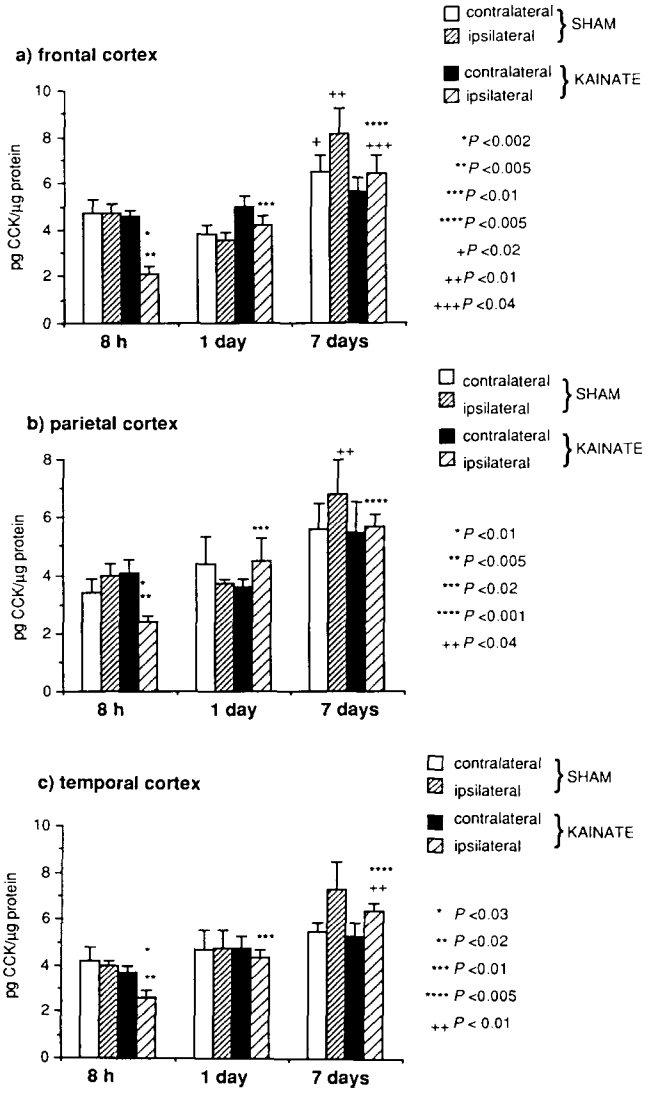

Fig. 1. (a–c). Time course of CCK depletion in different regions of rat cerebral cortex following unilateral kainate injection into the nucleus basalis. Values are expressed as pg CCK/μg protein and are means ± S.E.M. shown by error bars from three to seven experiments. * and ** indicate that CCK levels were significantly decreased in ipsilateral cortex of kainate-treated animals compared to ipsilateral cortex of sham-operated animals and contralateral cortex of kainate treated animals respectively for the P values indicated. *** and **** indicate significant reversal of the reduction at 1 and 7 days, respectively for the P value indicated. $^{+}$, $^{++}$ and $^{+++}$ indicate that levels of CCK were significantly elevated at 7 days compared to 1 day for ipsilateral sham-, contralateral sham- and contralateral kainate-treated animals respectively.

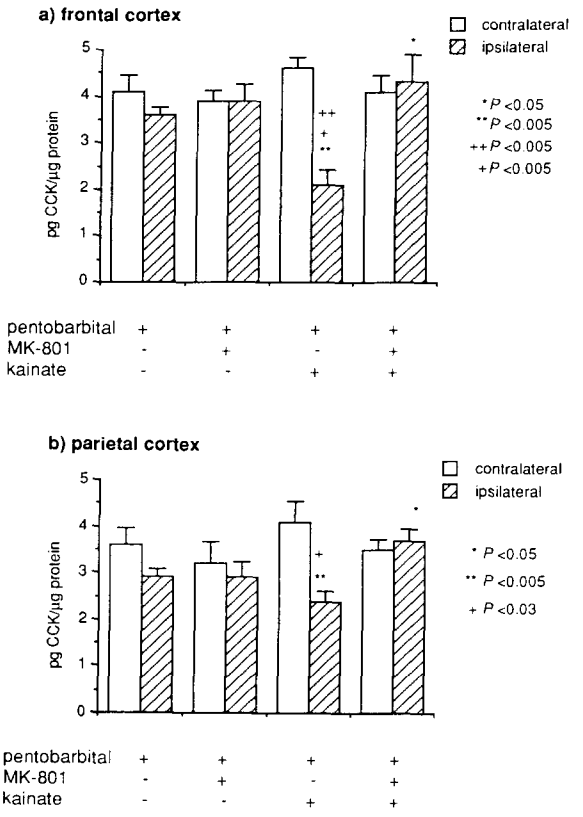

Fig. 2. (a,b). Effect of four different treatments on the levels of CCK in (a) frontal and (b) parietal cortex. All animals were anaesthetised with pentobarbital (90 mg/kg). The effect of MK-801 (3 mg/kg i.p.; 30 min post operation) treatment was tested with and without unilateral injection of kainate into the nucleus basalis. Values are means ± S.E.M. shown by error bars. **, $^{+}$, $^{++}$ indicate that CCK levels were significantly reduced at 8 h after kainate injection compared to CCK levels in contralateral cortex receiving kainate injection, anaesthetised animals receiving no kainate and vehicle injection, MK-801-treated anaesthetised animals, while * indicates CCK levels in ipsilateral cortex were significantly reversed by MK-801 compared to CCK levels in ipsilateral cortex of kainate-treated animals for the P values shown.

3.4. Time-dependent changes in CCK release from frontal cortical slices following unilateral injection of kainate into right nucleus basalis

The effect of persistent stimulation of cerebral cortex caused by unilateral injection of kainate into right

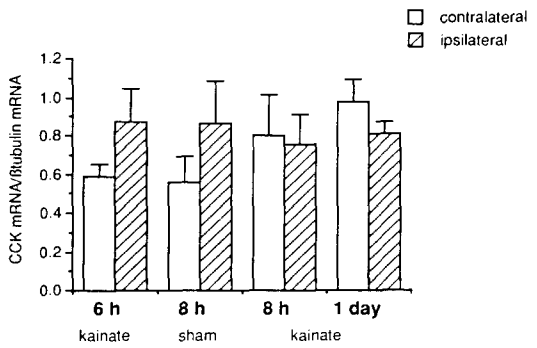

Fig. 3. Effect of kainate injection on preprocholecystokinin mRNA in the fronto-parietal cortex. Values are means (CCK mRNA/β-tubulin mRNA)±S.E.M. shown by error bars from three to six experiments. No statistically significant changes were detected between sham- and kainate-treated animals.

nucleus basalis was studied ex vivo on the depolarisation-induced release of CCK from slices of fronto-parietal cortex at 3 times after kainate injection. Depolarisation-induced (34 mM K^+) release of CCK was assayed at 4 h, 6 h and 1 day after injection of kainate. These time points were chosen to cover the period of maximum seizure activity (4–6 h) and recovery to normal behaviour and control levels of CCK (1 day). The K^+-evoked release of CCK (as a percentage of tissue CCK stores) was significantly elevated ($P < 0.001$) in ipsilateral cortex compared to contralateral cortex at 6 h after kainate injection (Fig. 4). CCK release was not related to tissue protein and thus will not reflect overall decreases in CCK tissue levels. No significant difference in CCK release was observed between ipsilateral and contralateral cortex at 4 h or at 1 day after kainate injection. There were no significant differences in the release of CCK in contralateral cortex compared to controls at any of the time points investigated.

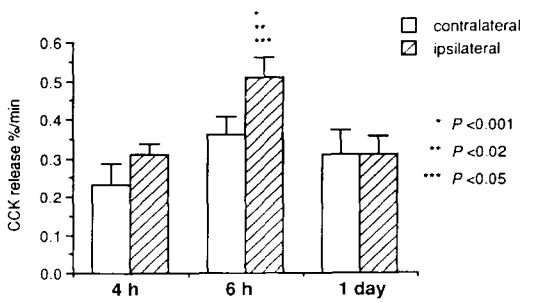

Fig. 4. Ex vivo release of CCK at 4, 6 and 24 h after kainate administration into the nucleus basalis. Tissue slices of ipsilateral and contralateral fronto-parietal cortex were incubated in vitro in Krebs medium at 37°C at various times after kainate injection. Values are means (CCK release %/min)±S.E.M. indicated by error bars for three to four experiments. *, ** and *** indicate CCK release in ipsilateral cortex at 6 h was significantly greater compared to 6 h contralateral cortex, 4 h ipsilateral cortex and 1 day ipsilateral cortex.

4. Discussion

The present experiments demonstrate that the CCK content of rat cortical neurones is strongly influenced by changes in physiological activity that accompany unilateral kainate injection into the nucleus basalis. The most striking effect was seen at 8 h when CCK was observed to be significantly decreased in three cortical regions, frontal, parietal and temporal cortex ipsilateral to the injection (Fig. 1). The effect was transient and peptide levels returned to normal by 1 day. No significant differences in CCK peptide levels were observed in occipital cortex or in hippocampus and nucleus accumbens (Table 1). Sham-operation (where saline vehicle was injected) did not affect peptide levels. Specific decreases of CCK in ipsilateral cortical areas (target tissues for afferent input) rather than in the contralateral cortex, hippocampus or nucleus accumbens indicate a clear association between activation of a specific pathway and the activity of potential target CCK neurones. Kainate injection into the nucleus basalis has been shown to activate glutamate transmission in cerebral cortex transiently with an associated induction of ornithine decarboxylase enzyme activity, ornithine decarboxylase mRNA, c-fos, c-jun mRNA (Reed and De Belleroche, 1990; Wood and De Belleroche, 1990, Wood and De Belleroche, 1991; Collaço-Moraes and De Belleroche, 1992), which effects are reversed by MK-801. The source of glutamate is speculated to be from cortical glutamatergic neurones stimulated by the release of acetylcholine from the nucleus basalis-cortex pathway following introduction of excitotoxin into the nucleus basalis. The decrease in CCK levels at 8 h was also prevented by the NMDA receptor antagonist MK-801, thus indicating that NMDA receptors are involved. When MK-801 was administered alone or in conjunction with pentobarbital anaesthesia CCK levels did not show any alteration, indicating that the anticonvulsant properties of these compounds did not affect peptide levels (Fig. 2).

The reduction in tissue levels of CCK may be attributed either to an increased release followed by rapid degradation of the peptide, or a reduction in peptide synthesis or a combination of these effects as a result of seizure activity caused by kainate. Studies on CCK release from cortical slices show that indeed there is a time-dependent enhancement of 34 mM K^+-evoked CCK release from fronto-parietal cortex which is maximal at 6 h after kainate injection. A similar effect was not noticed at 4 h or at 1 day after kainate injection.

The decrease of CCK tissue levels at 8 h was not sustained and returned to normal levels by 24 h, implying that these neurones are not destroyed by this process. The restoration of peptide levels at 24 h was not due to decreased release since the ex vivo CCK

release experiments showed no significant decrease in 34 mM K^+-evoked CCK release between ipsilateral and contralateral cortex at 24 h. Although peptide levels had rapidly returned to normal by 24 h no marked changes in CCK mRNA levels at 8 h or 24 h could be detected. Overall it is likely that kainate injection into nucleus basalis stimulates the release of glutamate, which is either associated with seizure activity or induced by spreading depression, and acting via NMDA receptors possibly situated on CCK interneurones influences CCK release. These results suggest that a major functional interaction between CCK and glutamate neurones exists in vivo.

These results are in line with those reported by Meyer et al. (1986), who showed that a single i.p. injection of kainic acid caused a significant decrease in CCK levels in cortex at 3 h. Levels were also decreased in amygdala and pyriform cortex and returned to normal by 3 days. The authors hypothesise that the initial decrease was due to increased CCK release which was reversible in all regions studied except amygdala where the peptide levels remained lower than control levels up to 10 days. At the later period of 10 days, CCK levels in cortex were significantly elevated above control levels. In the present study the peptide levels in cortex measured 7 days after kainate injection were increased relative to levels at 1 day, but this increase was also observed in the sham-operated animals. Increases of this type may reflect a non-specific response to the surgery of the operation. Small increases in CCK mRNA in ipsilateral cortex of both kainate and sham-operated animals were also noted in this study but these did not reach significance. These changes may reflect the sensitivity of CCK neurones in cerebral cortex to minor injury at surgery which is consistent with the findings of Olenik and Meyer (1990) and Olenik et al. (1991). Increases in CCK mRNA levels in frontal cortex following i.p. injection of kainic acid have been observed by Olenik et al. (1989) at 2 and 10 days, returning to control levels by 30 days. As no such increase was seen in the present study by intracerebral injection of kainate, it must be assumed that the peripheral route of administration produces a more long-lasting effect.

In conclusion, our results suggest that excitotoxin-induced seizures are associated with CCK depletion in cortical regions at 8 h which is accompanied by increased release of CCK. The decrease was only transient and peptide levels were restored by 24 h, indicating that enhanced synthesis had occurred to replenish peptide stores. However, no significant change in CCK mRNA was noticed at any time point. The involvement of NMDA receptors, and hence glutamate, is implicated in this seizure model since CCK depletion was sensitive to MK-801.

4.1. Physiological consequences of this interaction

CCK is present in a subpopulation of cortical interneurones that also synthesise γ-aminobutyric acid (GABA) (Hendry et al., 1984). Any physiological challenge that might alter CCK in the cortex might also reflect activity of the respective GABA interneurones. CCK is also known to be an anticonvulsant (Zetler, 1981). Caerulein (a CCK analogue) has been found to increase GABA release (Sheehan and De Belleroche, 1983) which may account for the anticonvulsant action of the peptide. Thus increased release of CCK after unilateral lesion of nucleus basalis may underlie changes in seizure susceptibility. Further evidence that CCK-8 is involved in seizures comes from experiments where rats with congenital audiogenic seizures have lower CCK-8 concentrations in cerebral cortex and hippocampus compared to normal rats (Zhang et al., 1993).

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