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Influences of cholecystokinin octapeptide on phosphoinositide turnover in neonatal-rat brain cells

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Cholecystokinin octapeptide (CCK-8) has been shown to be coupled to phosphoinositide turnover in pancreatic acini as well as in a kind of neuroblastoma cell and a human embryonic cell line. Little is known, however, about its link with phosphatidylinositol breakdown in the brain. The brains (minus cerebella) from 1–2-day-old neonatal rats were enzymically dissociated into single cells. The intact cells were prelabelled by incubation with *myo*-[^{3}H]inositol for 3 h, and were then stimulated with agonists in the presence of 10 mM-LiCl. Carbachol at 1 mM induced an increase in InsP_3 labelling in brain cells (peak at 30 min, and then a gradual decrease), and a static accumulation of Ins$P$ with time, whereas the labelling of InsP_2 remained essentially unchanged. A very similar time–response curve was obtained for 10 nM-CCK-8 in stimulating phosphoinositide turnover. The dose–response curve for incubated brain cells revealed that the formation of InsP_3 increased when the concentration of CCK-8 was increased from 0.1 to 10 nM. A further increase in CCK-8 concentration to 100–1000 nM resulted in a gradual decrease in InsP_3 formation. Ins$P$ and InsP_2 levels stayed relatively stable. The production of InsP_3 stimulated by 10 nM-CCK-8 was dose-dependently suppressed by the CCK-A antagonist Devazepide in the concentration range 1–10 nM; the effect declined when the concentration was further increased to 100–1000 nM. In contrast, the CCK-B antagonist L365,260 showed a sustained suppression of InsP_3 production at concentrations above 0.1 nM, i.e. in the range 1–1000 nM. The results provide evidence that CCK-8 stimulates the turnover of phosphoinositide and increases InsP_3 labelling in dissociated neonatal-rat brain cells, in which both CCK-A and CCK-B receptors seem to be involved.

INTRODUCTION

Cholecystokinin (CCK), which was originally discovered in the gastrointestinal tract, has been shown to be the major hormonal regulator of the pancreatic acinar cells. It is composed of a group of structurally related peptides, among which the *C*-terminal octapeptide (CCK-8) has all the known activities of this hormone group. It has been demonstrated that CCK exerts its effects in the pancreatic acini via receptor-mediated phosphodiesteratic breakdown of phosphatidylinositol bisphosphate, producing two intracellular messengers, 1,2-diacylglycerol (DAG) and Ins(1,4,5)P_3, which are involved in protein kinase C activation and intracellular Ca^{2+} mobilization respectively (Berridge, 1985; Williamson *et al.*, 1985; Willems *et al.*, 1986).

CCK-8 has recently been found to be abundantly and widely distributed in the central nervous system of many species as a neurotransmitter or neuromodulator (Vanderhaeghen *et al.*, 1975; Beinfeld, 1983). In fact, the absolute quantity of this hormone is greater in the central nervous system than in the entire gut (Rehfeld, 1978). Binding sites for CCK have been characterized in brain tissues from various species (Barrett *et al.*, 1989*b*; Gut *et al.*, 1989; Hill *et al.*, 1990; Hruby *et al.*, 1990; Zelles *et al.*, 1990), and these binding sites are known to couple to nucleotide-regulatory proteins (G-proteins) (Wennogle *et al.*, 1988). To our knowledge, central CCK-recognition sites have not yet been proved to link to any second-messenger system in the brain, including the phosphoinositide system, although phosphoinositide metabolism has been shown to be affected by CCK in neuroblastoma cells (Barrett *et al.*, 1989*a*) and an embryonic pituitary cell line (Lo & Hughes,, 1988).

With enzymically dispersed neonatal-rat brain cells, we investigated the potential influence of CCK-8 on phosphoinositide turnover and the receptor mechainisms thereof.

MATERIALS AND METHODS

Materials

[^{3}H]Inositol (15.4 Ci/mmol) was obtained from NEN/Du Pont. Dowex AG1X8 resin (200–400 mesh, formate form) was the product of Bio-Rad. Trypsin was from Merck, Dulbecco's modified Eagle's medium (DMEM) from J. R. Scientific, and DNAase I from Sigma. CCK-8 was generously given by Squibb (Princeton, NJ, U.S.A.), and the CCK-8 antagonists Devazepide and L365,260 were given by MSD Neuroscience Research Centre (Harlow, Essex, U.K.).

Single-cell preparation and prelabelling with [^{3}H]inositol

Methods for cell preparation were a modification of those of Ransom *et al.* (1977) and Muraoka & Takahashi (1989). Brains (minus cerebella) were taken quickly from 1–2-day-old neonatal Wistar rats and washed in DISGH solution (made up of 50 ml of 20 × concentrated Puck's D1 salt solution/l, 3 g of glucose/l, 7.5 g of sucrose/l and 10 mM-Hepes buffer, adjusted to 320–330 mosM, pH 7.3). After removal of meninges, the brains were minced into 2–4 mm^3 small pieces, and digested with 0.125% trypsin and 0.01% DNAase I with gentle agitation at 36.5 °C for 15 min. The nutrient medium (DMEM) containing 10% (v/v) heat-inactivated (56 °C for 30 min) calf serum and 10% (v/v) fetal-calf serum was added to the supernatant to terminate the digestion. The mass of tissue at the bottom was triturated by gentle pipetting in and out of a Pasteur pipette until it appeared almost gelatinous. After adding DMEM plus serum, the resulting suspension was merged with the supernatant and filtered with a 200-mesh stainless-steel net. After centrifugation (1000 *g*, 15 °C, 5 min), the cells were resuspended in the nutrient medium. The viability, estimated by Trypan Blue exclusion with a haemocytometer, was over 95%.

Abbreviations used: CCK, cholecystokinin; CCK-8, *C*-terminal octapeptide of CCK; DAG, 1,2-diacylglycerol; DMEM, Dulbecco's modified Eagle's medium.

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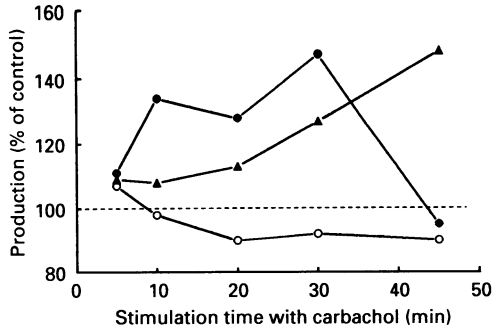

Fig. 1. Formation of inositol phosphates in dissociated neonatal-rat brain cells stimulated by 1 mM-carbachol in the presence of 10 mM-LiCl

The stimulation time was 30 min. Points on the curves are averages of triplicate tubes. Symbols: ●, InsP_3; ○, InsP_2; ▲, Ins$P$.

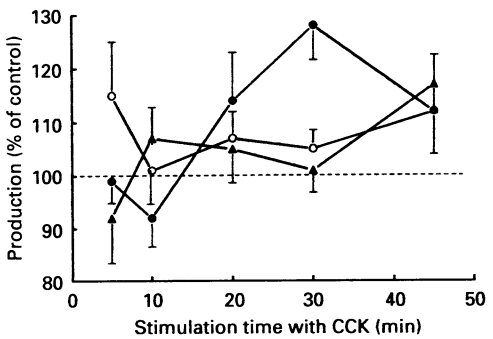

Fig. 2. Formation of inositol phosphates in dissociated neonatal-rat brain cells stimulated by 10 nM-CCK-8 (in the presence of 10 mM-LiCl)

Results were means ± S.E.M. for four separate experiments, with triplicate tubes in each experiment. The stimulation time was 30 min. Symbols: ●, InsP_3; ○, InsP_2; ▲, Ins$P$.

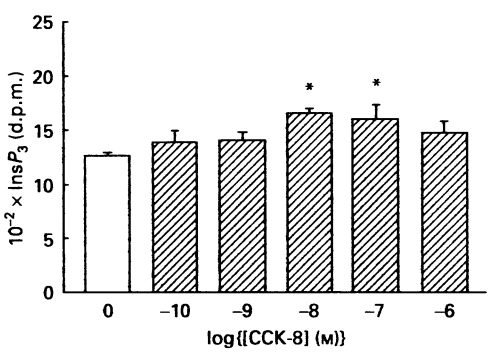

Fig. 3. Dose–response of stimulation of InsP_3 formation in dissociated neonatal-rat brain cells by CCK-8

Results are means ± S.E.M. for four separate experiments, with triplicate tubes in each experiment: *$P < 0.05$ compared with control group (ANOVA followed by Duncan's test). The stimulation time was 30 min.

The cells were prelabelled in an air/CO_2 (19:1) incubator for 3 h with 12.5 μCi of [^{3}H]inositol/0.75 ml of nutrient medium per brain. After centrifugation (1000 *g*, 15 °C, 5 min), the cells were resuspended in modified Krebs–Ringer buffer (KRB; 108 mM-NaCl, 4.7 mM-KCl, 24.8 mM-$NaHCO_3$, 1.18 mM-KH_2PO_4, 1.18 mM-$MgSO_4$, 0.75 mM-$CaCl_2$, 10 mM-LiCl, 11 mM-glucose, adjusted to pH 7.4).

Measurement of inositol phosphates

The procedure was a modification of the method of Monsma *et al.* (Schoepp & Johnson, 1988; Ohmori & Kuriyama, 1989; Dyck, 1990; Monsma *et al.*, 1990). The total volume of reaction mixture was 500 μl in each plastic vial (5 ml capacity), containing (2–5) × 10^5 cells in modified KRB, 10 mM-LiCl and 50 μl of chemicals. After preincubation at 36.5 °C for 10 min in a shaking water bath, the cells were stimulated by chemicals at different concentrations and time periods. The incubations were terminated by the addition of 1 ml of chloroform/methanol (1:2, v/v), followed by shaking and putting into ice-cold water. Then 330 μl of chloroform and 330 μl of distiled water were added to each tube, which was then centrifuged at 3000 *g* for 25 min for phase separation. A 1.3 ml sample of the upper aqueous phase was removed and diluted to 6.3 ml before being applied to 1 ml Dowex columns (AG1X8; 200–400 mesh; formate form). The columns were washed with 7 ml of 5 mM-inositol to remove free [^{3}H]inositol and then with 7 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate to remove [^{3}H]glycerophosphoinositol and inositol 1:2-cyclic phosphate. The various inositol phosphates were eluted with 5 ml each of increasing concentrations of ammonium formate, as follows: 0.2 M-ammonium formate/0.1 M-formic acid for Ins$P$, 0.4 M-ammonium formate/0.1 M-formic acid for InsP_2 and 1.0 M-ammonium formate/0.1 M-formic acid for InsP_3. The eluates were counted for radioactivity in 11 ml of Triton scintillation fluid in an automatic β-counter with efficiency of 12%. Data were analysed by one-way analysis of variance (ANOVA), and comparisons were performed by Duncan's test.

RESULTS

Effects of carbachol and CCK-8 on phosphoinositide turnover

With stimulation by 1 mM-carbachol, there was a 34% increase in InsP_3 labelling in brain cells 10 min after stimulation, which reached a peak at 30 min (47% increase), and then fell to the baseline level at 45 min. In the meantime, there was a constant accumulation of Ins$P$, to reach a 48% increase at 45 min after stimulation (Fig. 1). A very similar time–response curve was obtained with 10 nM-CCK-8 to stimulate phosphoinositide turnover. InsP_3 labelling tended to increase at 20 min (114 ± 9.1%) after stimulation, reached a peak at 30 min (128 ± 6.4%), and decreased thereafter, to approach the baseline at 45 min (112 ± 8.1%); Ins$P$ accumulated with time, being 117 ± 5.5% of control at 45 min; InsP_2 fluctuated in the range of 101% and 115% (Fig. 2).

The dose–response curve revealed that the formation of InsP_3 increased when the concentration of CCK-8 was increased from 0.1 to 100 nM. The labelling of InsP_3 increased from a baseline level of 1261 ± 32 d.p.m. to 1646 ± 42 d.p.m. (ANOVA followed by Duncan's test, $P < 0.05$) and 1594 ± 128 d.p.m. ($P < 0.05$) in the presence of 10 nM- and 100 nM-CCK-8 respectively. A further increase in CCK-8 concentration to 1 μM resulted in a decreased production of InsP_3 (Fig. 3). InsP_2 and Ins$P$ stayed at relatively stable levels.

Effect of receptor antagonists on CCK-8-stimulated phosphoinositide turnover

The formation of InsP_3 with stimulation by 10 nM-CCK-8 was dose-dependently suppressed by the CCK-A antagonist

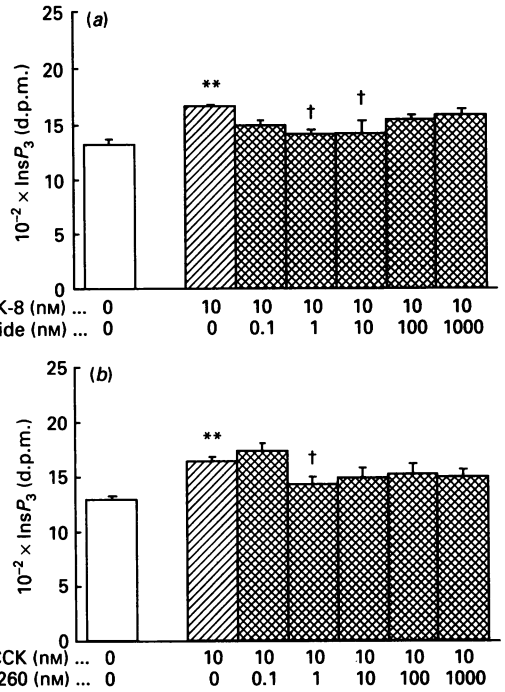

Fig. 4. Stimulation of InsP_3 formation in dissociated neonatal-rat brain cells by CCK (10 nM, 30 min) and its reversal by the CCK-A-receptor antagonist Devazepide (*a*) and by the CCK-B antagonist L365,260 (*b*)

Results are means ± S.E.M. for four separate experiments, with triplicate tubes in each experiment: $^{**}P < 0.01$ compared with blank control group; $\dagger P < 0.05$ compared with CCK control group without antagonist (ANOVA followed by Duncan's test).

Devazepide in the concentration range 1–10 nM ($P < 0.05$); the effect declined when the concentration was further increased to 100 and 1000 nM (Fig. 4*a*). In contrast, the CCK-B antagonist L365,260 showed a sustained suppression of InsP_3 formation at concentrations above 0.1 nM, i.e. in the range 1–1000 nM (Fig. 4*b*).

DISCUSSION

It is well known that CCK-receptor occupation activates phospholipase C via G-protein, leading to production of two second messengers, DAG and InsP_3, in peripheral tissues such as the pancreas. Besides, in the human embryonic pituitary cell line Flow 9000 and in CHP212 neuroblastoma cells, CCK has been documented to stimulate phosphoinositide turnover (Lo & Hughes, 1987, 1988; Barrett *et al.*, 1989*a*). However, little is known about the post-receptor signal transduction of CCK-8 in the brain.

To study the influences of certain neurotransmitters or neuromodulators on the phosphoinositide turnover in brain, one could use brain slices or dissociated brain cells. In cerebral-cortical slices prelabelled with [^{3}H]inositol, the muscarinic-receptor agonist carbachol was shown to cause rapid accumulation of [^{3}H]phosphoinositide, a reaction effectively reversed by muscarinic-receptor blockade (Brown *et al.*, 1984; Batty *et al.*, 1985; Batty & Nahorski, 1989). Enzymically dissociated neonatal-rat brain cells, which have been used in neuroscience research (Gonzales *et al.*, 1985; Dildy & Leslie, 1989; Muraoka & Takahashi, 1989), were used in the present study. We were able to demonstrate unequivocally that CCK-8 accelerated the turnover of phosphoinositide in a manner almost identical with that of carbachol. Whereas the labelling of Ins*P* increased steadily with time, that of InsP_3 reached a peak at 30 min and decreased thereafter. The latter may result from continuous hydrolysis of a limited amount of [^{3}H]phosphatidylinositol bisphosphate available, and/or a decreased sensitivity to CCK-8 (as well as to carbachol) after prolonged receptor activation. It should be pointed out that the changes induced by carbachol observed in the present study are not as rapid and dramatic as were seen in brain-slice studies (Batty & Nahorski, 1989), which may have been attributed to putative damage to the cell membrane by the enzyme trypsin.

A bell-shaped dose–response curve is very often seen in the pharmacology of CCK-8. An established phenomenon of CCK's effect in pain modulation is that it antagonizes the opiate effect at a low dose, and mimics the opioid effect at a high dose (Faris *et al.*, 1983; R. G. Hill *et al.*, 1987). In the dose–response curve for CCK-8 stimulation of production of InsP_3 in dissociated brain cells, we also see a bell-shaped curve with a peak at 10 nM and a clear-cut decrease at 1 μM. The simplest explanation would be that a high-affinity site is responsible for the effect at low concentrations, whereas a low-affinity site is responsible for the opposite effect at high concentrations. Although we had no evidence to demonstrate the presence of high- and low-affinity sites in our experimental model, we did have means to analyse whether these two effects are mediated by two different types of CCK receptor, type A and type B. Using the specific CCK-A antagonist Devazepide and the CCK-B antagonist L365,260, we were able to construct the dose–response curves for the relevant antagonists to affect the production of [^{3}H]InsP_3 evoked by CCK-8 at 10 nM. It was interesting to show that both CCK-A and CCK-B antagonists worked at the same concentration (1 nM), although the CCK-B antagonist seemed to have a broader effective dose range (1–1000 nmol) as compared with the CCK-A antagonist (1–10 nM). Although the data were straightforward in their own right, they are difficult to reconcile with the existing information that in adult rat brain most of the CCK receptors are of the B type (Moran *et al.*, 1986; D. R. Hill *et al.*, 1987). Further experiments are needed to clarify this phenomenon: (*a*) a radioreceptor assay to see whether CCK-A and CCK-B receptors are equally abundant in the rat brain tissue at the neonatal stage of development; (*b*) a brain-slice study using adult rat brain to see whether the effect of CCK-8 could be preferentially blocked by CCK-B antagonist.

In conclusion, this is the first demonstration that in neonatal-rat brain cells one of the post-receptor events of CCK-8 is to increase the production of InsP_3. The potential importance of InsP_3 in mediating the anti-opioid effect of CCK-8 in pain modulation can be derived from the two following established facts (1) that InsP_3 accelerated the Ca^{2+} release from the intracellular Ca^{2+}-storage pool, resulting in an increase in intracellular [Ca^{2+}], and (2) that a cardinal mechanism of the opiate effect was to decrease intracellular [Ca^{2+}] via blockade of Ca^{2+} influx (MacDonald & Werz, 1986; Tort *et al.*, 1989; Wang *et al.*, 1989). In connection with this, it should be pointed out that a decrease in the InsP_3-stimulating effect at higher concentrations of CCK-8 in the experiment *in vitro* may have some relevance to the finding *in vivo*, where the anti-opioid effect of CCK-8 tended to diminish and shift to an opio-mimetic effect along with an increase in dosage.

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