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COMPARISON OF THE ACTION OF CHOLECYSTOKININ, CARBACHOL AND VASOACTIVE INTESTINAL PEPTIDE ON RECEPTOR-ACTIVATED FORMATION OF CYCLIC GMP AND CYCLIC AMP IN THE STRIATUM AND THE PANCREAS

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Abstract—(1) Sulphated cholecystokinin octapeptide (CCK-8S) and sodium nitroprusside (SNP) increased the formation of cyclic GMP in rat striatal slices with no effect on cyclic AMP. (2) CCK-8S, SNP and carbachol increased the formation of cyclic GMP in guinea-pig pancreatic lobules, but had no effect on levels of cyclic AMP. (3) Vasoactive intestinal peptide (VIP) significantly stimulated the formation of cyclic AMP in both striatal and pancreatic tissue without effect on levels of cyclic GMP in these tissues. (4) In rat striatal slices carbachol significantly inhibited the VIP-stimulated increase in cyclic AMP.

Cholecystokinin-like immunoreactivity is present in both the brain [1] and pancreas [2]. CCK-8 is a potent pancreatic secretagogue and also appears to be the naturally occurring fragment in the CNS [3]. In the CNS, endogenous CCK can be released from slices or synaptosomes obtained from various brain regions, including the striatum [4–10]. Depolarization-evoked release in these areas is strictly calcium-dependent, which is in keeping with a role for CCK as a neurotransmitter.

In the pancreas, CCK acts as a local hormone, stimulating enzyme secretion, enhancing secretin-induced bicarbonate secretion from the exocrine pancreas [11, 12] and stimulating insulin and glucagon secretion from the pancreatic islets [13].

The membrane events associated with stimulus–secretion coupling evoked by CCK in the exocrine pancreas are thought to include transient increases in internal calcium concentrations, opening up of ionic pathways permeable to Na^+, Cl^- and K^+ [14, 15]. CCK stimulates phosphatidylinositol turnover [16], and increases cyclic GMP levels both *in vivo* [17] and *in vitro* [18–20]. However, direct CCK-receptor activation of guanylate cyclase in pancreatic membrane or soluble preparations has not been demonstrated [21].

It was unclear whether activation of CCK receptors in the brain and pancreas resulted in similar or different formation of second messengers. The aim of this study was to investigate and compare the action of CCK with carbachol and VIP on the formation of cyclic GMP and cyclic AMP in brain and pancreatic tissue. CCK-like immunoreactivity is abundant in striatum [22], which was the particular brain region chosen. Some of these results have been published in preliminary form [23].

MATERIALS AND METHODS

Preparation of brain slices. Rats were killed by cervical dislocation and striatal tissue dissected out and placed in ice-cold, well-oxygenated Krebs-buffer, pH 7.4 (124 mM NaCl, 5 mM KCl, 26 mM $NaHCO_3$, 0.8 mM $CaCl_2$, 1.3 mM $MgCl_2$, 1.2 mM KH_2PO_4 and 10 mM glucose). Slices were prepared on a McIlwain tissue chopper (cross-chopped 0.26 × 0.26 mm), suspended in a fresh Krebs-buffer, and incubated for 10 min at 37° in a shaking water bath (160 strokes/min) under an atmosphere of 95% O_2 and 5% CO_2. The slices were then carefully teased apart, resuspended in fresh Krebs-buffer, and preincubated for a further 10 min at 37°. Tissue slices were next suspended in Krebs-buffer containing 1 mM 3-isobutyl-1-methyl xanthine (IBMX), 1 mg/ml bacitracin and 0.1% bovine serum albumin (BSA) to give a concentration of 50–100 mg wet wt tissue/ml. 200 μl of well-stirred tissue slices were next placed in soda glass incubation tubes and the preincubation continued for a further 55 min (120 strokes/min, continuously gassed). Prolonged preincubation of brain tissue slices at 37° has been shown to stabilize basal cyclic nucleotide levels, thus facilitating the detection of changes in cyclic nucleotide levels as a result of experimental procedure [24]. At the end of the preincubation period, 100 μl of drug was added to each tube. The reaction was terminated by addition of 25 μl of 100% trichloroacetic acid (TCA) and the samples boiled for 10 min. The tissue was then homogenized and TCA extracted with at least three washes in five volumes of water saturated diethyl ether. Denatured tissue was removed by centrifugation and the supernatant dried overnight, 55°. Residues were reconstituted in

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500 μl assay buffer (50 mM Tris–HCl, pH 7.4 and containing 8 mM theophylline).

Preparation of guinea-pig pancreatic lobules. Guinea-pigs were killed by cervical dislocation. The pancreas was removed, freed of fat and blood vessels, and the lobules gently teased apart. The tissue was incubated for 10 min at 37° in a shaking water-bath, in oxygenated Krebs-medium (containing 121 mM NaCl, 5 mM KCl, 2.6 mM $CaCl_2$, 25 mM $NaHCO_3$, 5.6 mM glucose, 6.1 mM beta-hydroxybutyrate, 3.6 mM sodium glutamate, 3.7 mM sodium pyruvate and 2.7 mM sodium fumarate). The lobules were next divided into 40 mg (wet wt) portions, resuspended in Krebs medium containing 0.5 mM IBMX, 0.1% BSA, 50 μg/ml bacitracin and 260 μg/ml trasylol, and incubated for a further 15 min (total incubation volume 200 μl) under an atmosphere of 95% O_2–5% CO_2; 100 μl of drug was added and incubation continued for a further 2 min. The reaction was terminated by freezing the tissue in a pre-cooled aluminium clamp (cooled in liquid nitrogen). The frozen lobules were placed in 0.5 ml boiling 6 mM theophylline. After homogenization, addition of 0.5 ml acid alcohol (99% absolute alcohol, 1% 1 M HCl) and overnight storage at −20°, the supernatant was collected by centrifugation (1600 *g* for 5 min). The pellet was washed with 0.5 ml acid alcohol, and the combined supernatants evaporated to dryness overnight (55°). The dried residues were reconstituted in 0.5 ml 50 mM Tris–HCl buffer, pH 7.4, containing 8 mM theophylline and assayed for cyclic nucleotides.

Measurement of cyclic AMP. Cyclic AMP was assayed using a competitive protein binding method [25]. These methods are subject to interference from substances which may be present in crude biological samples or incubation media. However, at the sample dilutions routinely used, no interference from Krebs–BSA buffer was observed. Displacement equivalent to 1 pmol of cyclic AMP was observed with: cyclic IMP (10 pmol), cyclic GMP (80 pmol), ATP (>30 nmol), ADP (⩾30 nmol). No displacement was observed using concentrations as high as 30 nmol of AMP, GMP, GDP or GTP.

Measurement of cyclic GMP. Antibodies were raised in a goat [26]. The serum was stored at −20° as lyophillized powder (lyophillization did not affect the cyclic GMP binding capacity). Lyophillized antibody was made up in assay buffer (50 mM sodium acetate, pH 6.0) containing 500 μg/ml gammaglobulin and stored frozen in aliquots. [^{125}I]tyrosine methyl ester of cyclic GMP ([^{125}I]cyclic GMP) was used at a concentration of 4–20 × 10^3 counts per min/100 μl.

To duplicate assay tubes, kept on ice, were added 100 μl acetate buffer, 100 μl standard cyclic GMP (0–14 pmol) or sample, 100 μl of [^{125}I]cyclic GMP and 100 μl antiserum. After vortexing, the tubes were incubated at 4°. Varying the incubation time from 3 to 48 hr had no effect on the assay; a 16-hr incubation was routinely used. Antibody-bound label was separated from free label using a charcoal-separation method with centrifugation for 30 min at 1600 *g* (4°). A concentration of charcoal was chosen which would bind all of the label in the absence of antiserum and none in the presence of excess antiserum (200 μl charcoal solution per assay tube, containing 1 mg/ml BSA and 5 mg/ml charcoal in ice cold sodium acetate buffer, pH 6). Antibody dilution curves were constructed and a concentration was chosen which would bind 40–60% of the label in the absence of exogenous cyclic GMP. Excess antibody bound 85–90% of the label in the absence of exogenous cyclic GMP.

Cross-reactivity studies revealed displacement equivalent to 1 pmol of cyclic GMP in the presence of: dibutyryl cyclic GMP (>100 pmol), cyclic IMP (>100 pmol), cyclic AMP (>1500 pmol). No displacement was observed in the presence of >10,000 pmol guanosine, GMP, GDP, GTP, ADP, ATP or AMP.

Statistical analysis of results. In assessing changes in the levels of cyclic AMP and cyclic GMP, differences between groups were analyzed using Student's *t*-test.

RESULTS

Rat striatal slices

Basal levels of cyclic GMP, after the 90-min preincubation period, reached a steady value of 0.06 pmol/mg (wet wt) tissue. A 10-fold increase in cyclic GMP in the striatal tissue slices was observed in the presence of 100 μM SNP at 30 sec ($N = 3$; $P < 0.05$); and the rise was maintained between 0.4 and 0.5 pmol/mg tissue over a 5-min incubation period. In contrast, CCK-8S and carbachol caused a significant but much smaller increase in cyclic GMP which was apparent after 30 sec (1 μM CCK-8S: 0.085 pmol/mg tissue, $P < 0.02$; 3 μM carbachol: 0.095 pmol/mg tissue, $P < 0.002$) and at 2 min but was no longer observed after 5 min. The dose-dependent increase in cyclic GMP produced by SNP (0.1–10 μM), carbachol (0.03–3.0 μM) and CCK-8S (0.01–1.0 μM) are illustrated in Fig. 1.

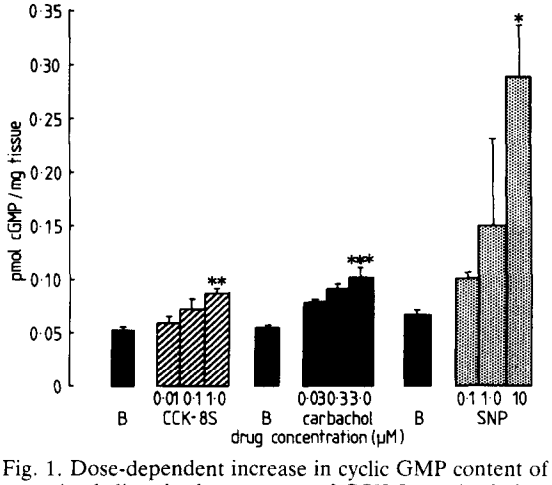

Fig. 1. Dose-dependent increase in cyclic GMP content of rat striatal slices in the presence of CCK-8s, carbachol or SNP. After a 90-min, drug-free preincubation period the slices were exposed to CCK-8S and carbachol for 30 sec, and SNP for 5 min before termination of the reaction, extraction and assay of cyclic nucleotides. Results are expressed as pmol cyclic GMP/mg (wet wt) tissue and are from three incubations assayed in duplicate. B = basal value, Bar = 1 S.E.M., *$P < 0.05$, ** $P < 0.02$, *** $P < 0.002$.

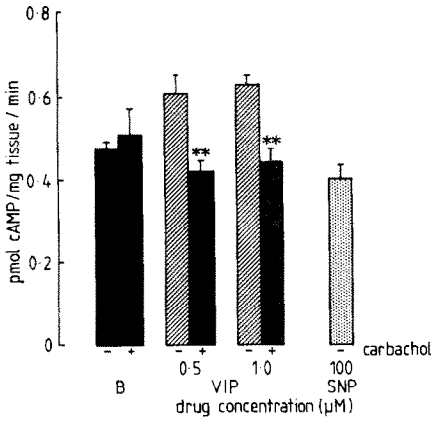

Fig. 2. Effect of carbachol on VIP-induced increase in striatal slice cyclic AMP content. After a 90-min, drug-free incubation period, slices were exposed to VIP in the absence or presence of 100 μM carbachol for a further 5 min. Results are expressed as pmole cyclic AMP/mg (wet wt) tissue/min and are from three incubations assayed in triplicate. Bar = 1 S.E.M., **$P < 0.01$ relative to VIP alone.

After the preincubation period, basal levels of cyclic AMP did not change appreciably with time (up to 30 min). VIP at 1 μM elevated cyclic AMP from 0.2 ± 0.028 to 0.56 ± 0.07 pmol/mg tissue/2 min incubation ($N = 3$; $P < 0.01$). SNP (100 μM) had no effect on basal cyclic AMP levels. CCK-8S (0.001–1.0 μM) had no consistent effect on cyclic AMP using incubation times from 0.5–15 min. Interestingly, carbachol (0.03–3.0 μM) had no observable effect on basal cyclic AMP levels, but in combination with VIP (5 min incubation), 100 μM carbachol inhibited the stimulatory effect of VIP (0.5–1.0 μM; Fig. 2).

Guinea-pig pancreatic lobules

Basal levels of cyclic GMP were 0.03 ± 0.02 pmol/mg wet wt tissue following a 2-min incubation. Tissue levels of cyclic GMP increased 16-fold ($N = 3$; $P < 0.01$) after a 2-min incubation in the presence of 100 μM SNP. In pancreatic lobules, CCK and acetylcholine stimulate the formation of cyclic GMP increasing 2-fold above basal values at 30 sec and 10-fold at 2 min, but rapidly falling to control levels at 5 min [19]. In the present experiments, CCK-8S (0.01–1.0 μM) produced a similar dose-dependent increase in cyclic GMP in the pancreatic lobules (Fig. 3); 0.1 μM CCK-8S brought about significant increase in cyclic GMP ($N = 3$; $P < 0.01$). SNP (100 μM) and CCK-8S (0.001–1.0 μM) had no effect on levels of cyclic AMP in pancreatic lobules, whilst VIP (1.0 μM) produced a 75% stimulation above basal levels ($N = 3$; $P < 0.05$).

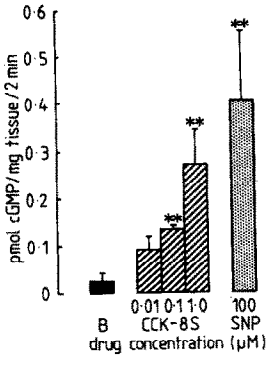

Fig. 3. Dose-related effect of CCK-8S on the cyclic GMP content of guinea-pig pancreatic lobules. After a 25-min preincubation period, the lobules were incubated for 2 min in the presence of CCK-8S and SNP. Results are expressed as pmol cGMP/mg wet wt tissue/2 min and are from three incubations assayed in duplicate. **$P < 0.01$.

DISCUSSION

SNP, which directly activates guanylate cyclase by generating free radicals [27], increased the formation of cyclic GMP in both rat striatum and guinea-pig pancreas, with no effect on the formation of cyclic AMP.

Acetylcholine induces an increase in cyclic GMP in both pancreatic [20] and cerebral tissues [28]. In our experiments, the stable cholinergic agonist, carbachol, enhanced the formation of cyclic GMP in striatal slices. Muscarinic cholinergic receptors are reported to be linked to inhibition of adenylate cyclase [29]. Although it was difficult to observe a fall in cyclic AMP levels in brain slices with carbachol, VIP-stimulated cyclic AMP formation was inhibited by carbachol.

CCK has been shown to increase the formation of cyclic GMP in pancreatic tissue [20, 30] whilst having no effect on cyclic AMP levels [17–19, 29]. Our results are in agreement with these reports. In addition, we demonstrated that CCK produces a similar time- and dose-dependent effect in the rat striatum, namely, an enhanced formation of cyclic GMP. No significant effect of CCK on cyclic AMP levels was observed in striatal slices.

These results suggest that production of the second messengers, cyclic GMP and cyclic AMP, follows a similar pattern in both rat striatal and guinea-pig pancreatic tissues. The inhibition by carbachol of the VIP stimulated cyclic AMP content of the striatum provides credence to the view that neuronal function could be further regulated when transmitters are co-released.

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