charcoal-stripped medium, that unsaturated fatty acids were growth stimulatory and that linoleic acid reversed the antiproliferative effect of antiestrogens. Fatty acids may thus influence lymphoid cell proliferation, in part, by decreasing binding of an endogenous cytostatic ligand(s) to the antiestrogen-binding site.

Although the choice of lymphoid cells for these studies may appear unusual, antiestrogens have been reported to alter circulating lymphocyte concentrations, to modify a variety of lymphocyte functions such as natural killer activity and Fc receptors for IgG, and to suppress lectin-induced mitogenesis[23-31]. The changes induced, however, have been inconsistent and there is little agreement on what they signify. Nonetheless, two case reports of human lymphomas that responded to tamoxifen treatment[32,33] suggest that the present findings may have wider implications for this class of drugs. On a more fundamental level, they raise new and potentially interesting questions about the molecular mechanisms of antiestrogen action in nonestrogen target cells.

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Effects of a new cholecystokinin antagonist (GE 410) on the smooth muscle of the guinea pig ileum

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Summary. Suc-Tyr-(SE)-Met-Gly-Trp-Met-Asp-β-phenethylamide (GE 410) competitively antagonized the contractions of smooth muscle strips from guinea pig ileum ($pA_2 = 7.6$, $n = 0.95$) induced by cholecystokinin-octapeptide (CCK8). GE 410 inhibited the electrically-induced cholinergically mediated contractile responses and the [^{3}H]ACh release in the ileum, as well as the CCK-stimulated electrical contractile responses and the [^{3}H]ACh release in the cholinergic nerve terminals. The results suggest the existence of CCK-receptors not only in the smooth muscles but also on the neurons.

Key words. Cholecystokinin octapeptide; cholecystokinin antagonist (GE 410); [^{3}H]ACh release; ileac smooth muscle.

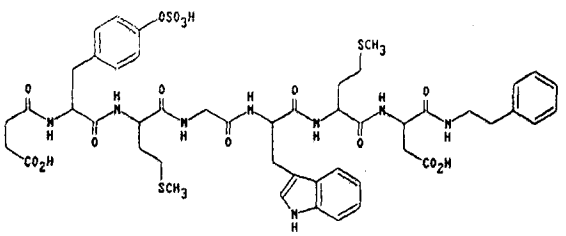

Figure 1. Amino acid sequence of cholecystokinin antagonist GE 410.

A large body of research has shown that peptides of the CCK group stimulate the contractile activity of gastrointestinal and biliary smooth muscles both through a direct action[1-4] and indirectly through an increase of ACh release in the cholinergic nerve terminals[5-7]. The direct myogenic effect of CCK is produced by specific receptors located on the smooth muscle cells[8,9]. According to some authors, CCK-receptors are located not only on the smooth-muscle cells[8] but also in the cholinergic nerve terminals[10-15]. In earlier studies[16] we found that the CCK antagonist Suc-Tyr-(SE)-Met-Gly-Trp-Met-Asp-β-phenethylamide, which has the code name GE 410, a derivative of the amino acids chain shown in figure 1, manifested selectivity and high binding affinity to CCK-receptors in the stomach and gall-bladder[4]. GE 410 injected i.v. in dogs, which were awake, selectively and dose-dependently antagonized the stimulant effects of CCK8 and CCK7 on gall-bladder motility[16].

The present work was undertaken to examine the effect of GE 410 on a) spontaneous contractions and those induced by electric field stimulation, and [^{3}H]ACh release, in guinea pig ileum, and b) on the electrically- and CCK-stimulated contractile responses and [^{3}H]ACh release in the cholinergic terminals of the guinea pig ileum.

Materials and methods

Recording of spontaneous activity in vitro. Muscle strips from the ileum of guinea pig were cut out 5–10 cm proximal to the ileocecal sphincter. Ten of the strips were mounted in separate 20-ml organ baths with Krebs solution. The strips were suspended under 1 g tension. There was a 60-min equilibration period before any measurements were made. The contractile activity was recorded by means of mechanoelectrical force transducers and a direct recorder (Linsis-6). GE 410 or CCK8 or CCK7 were administered in increasing concentrations. Cumulative dose-response curves for CCK8 or CCK7 prior to or in the presence of increasing concentrations of GE 410 were plotted to evaluate the antagonistic effect of GE 410. The affinity of cholecystokinins (CCKs), pD_2, was determined according to Van Rossum[17] and the affinity of GE 410 (pA_2) after Schild[18].

Recording of contractile responses to field electrical stimulation. Electrical field stimulation (square pulses of 1 ms duration, 0.2 Hz, 50 V, train duration of 5 s) was applied at 4–5-min intervals by means of two platinum ring electrodes. The contractile responses were recorded before the addition of, and in the presence of CCK8, CCK7 or GE 410.

The Krebs solution contained (mmol): Na^+ 137; K^+ 5.9; Ca^{++} 2.5; Mg^{++} 1.2; Cl^- 124; HCO_3^- 15.5; $H_2PO_4^-$ 1.2; glucose 11.5; equilibrated with 95% CO_2 at 36 °C, pH 7.4.

Determination of [^{3}H]ACh release. Fifteen strips were used to determine the electrically-stimulated [^{3}H]ACh release. Muscle preparations (60 mg each) were isolated from the segments. The preparations were incubated for 60 min in oxygenated modified Krebs solution[19] containing 8.3 Ci/mmol of [^{3}H]-methyl choline chloride (0.1 µM sp. act. 80 Ci/mmol). After washing in Krebs solution the preparations were transferred into a perfusion chamber of 2 ml capacity[20] and were perfused with Krebs solution by a peristaltic pump at a rate of 0.5 ml/min. The Krebs solution contained (mmol): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25.0, glucose 11.7, pH 7.4. Hemicholinium-3-bromide (10 µM) and physostigmine (5 µM) were used to block the neuronal reuptake of [^{3}H]ACh originating from the hydrolysis of released [^{3}H]ACh and for the inhibition of cholinesterase activity. During the first 90 min effluent was not collected. From the 91st min on, perfusion fluid was collected continuously to give a total of 23 aliquots. Electrical field stimulation (square pulses with a frequency of 2 Hz, pulse duration of 1 ms, 20 V, train duration of 3 min) was applied at the time of collection of the 3rd, 11th and 19th fractions. The perfused liquid was collected in 5-min fractions. At the end of the collection period the muscle preparations were extracted for 30 min at room temperature with 0.5 ml acidic ethanol (95% ethanol, 5% 0.1 N HCl) to release the residual intracellular ^{3}H-radioactivity.

Radioactivity was counted in a Beckman liquid scintillation spectrometer at a counting efficiency of 50%. The electrically-induced [^{3}H]ACh was expressed as percentage of the total cellular content of ^{3}H-radioactivity present in the tissue at the onset of stimulation. The effect of the substances tested was determined by calculating the ratio of radioactivity (as a percentage) released during the first and third (S_3/S_1) or the second and third (S_3/S_2) stimulations. The first stimulation was taken as the control. In some of the experiments we used Ca^{++}-free Krebs solution, which led to the abolition of the electrically-stimulated release of tritium. TTX (µM) applied 20 min before electrical and CCK8 stimulation significantly inhibited [^{3}H]ACh release.

The data were analyzed statistically and Student's t-test was used to determine the significance of differences in the effect. P values less than 0.05 were considered significant. The means ± SEM are shown.

Substances used: Acetylcholine (Lematte-Boinot), atropine sulfate (Cascan), methyl-[^{3}H]-choline chloride (Amersham), synthetic desaminocholecystokinin octapeptide (CCK7), synthesized by P. Henklein (DDR), cholecystokinin octapeptide, CCK8 (Sigma), dibutyryl

cyclic GMP (Sigma), hemicholinium-3-bromide (Sigma), histamine hydrochloride (Sigma), GE 410, synthesized by P. Henklein (DDR), physostigmine sulfate (Sigma), tetrodotoxine, TTX (Sankyo).

Results

Specificity of the effect of GE 410. GE 410 at the concentration of 5×10^{-6} M inhibited the tonic activation produced by CCK8 and CCK7 in all muscle strips used. The means are presented in figure 2 showing that the CCK8- and CCK7-, but not histamine- and acetylcholine-produced contractions were significantly depressed by GE 410.

Contractile responses to cholecystokinins were reproducible in Krebs GE 410-free solution at the end of the experiments.

Effect of GE 410 on spontaneous and CCK-stimulated contractions. GE 410 at concentrations of 10^{-8} M to 10^{-5} M slightly inhibited both the tone and the phasic activity of ileac muscle strips, which were restored after three-fold washing with Krebs solution at 15-min intervals. Figure 3 shows the cumulative concentration-effect curves for CCK8 before (control) and after the addition of increasing concentrations of GE 410 (10^{-9} M to 10^{-6} M). It is seen that, with increasing concentration, the antagonist shifted the dose-response curves to the right without changing the maximum. This type of shift is characteristic of competitive antagonism[17,21]. The pA_2 value for GE 410 was 7.6 and the Schild plot did not differ from 1 (n = 0.95). A similar antagonistic effect on the dose-response curves for CCK8 was observed with dibutyryl cyclic GMP (dbcGMP) in concentrations of 10^{-5} M to 5×10^{-4} M. The pA_2 value for dbcGMP was 7.2.

Effect of GE 410 on contractions induced by field electrical stimulation. Electrical field stimulation with a frequency of 0.1 Hz elicited atropine-sensitive contractile responses. Atropine (10^{-6} M) decreased CCK-induced contractions by 70%. GE 410 (10^{-7} M to 10^{-5} M) dose-dependently inhibited the amplitude of both the electrically- and the CCK-induced contractile responses of the guinea pig ileum. The contractile responses to electrical field stimulation were restored to normal after three-fold washing with Krebs solution at 15-min intervals. Figure 4a shows the averaged values for the effect of GE 410 on the electrically-induced contractile responses. GE 410 in concentrations of 10^{-7} M, 10^{-6} M and 10^{-5} M reduced the contractile responses by 10, 20 and 30%, re-

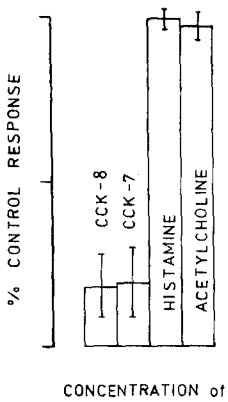

Figure 2. Effect of GE 410 on the contractions produced by 5×10^{-9} M CCK8, 5×10^{-9} M CCK7, 5×10^{-7} M histamine and 2.5×10^{-7} M acetylcholine on the muscle strips of guinea pig ileum (n = 5).

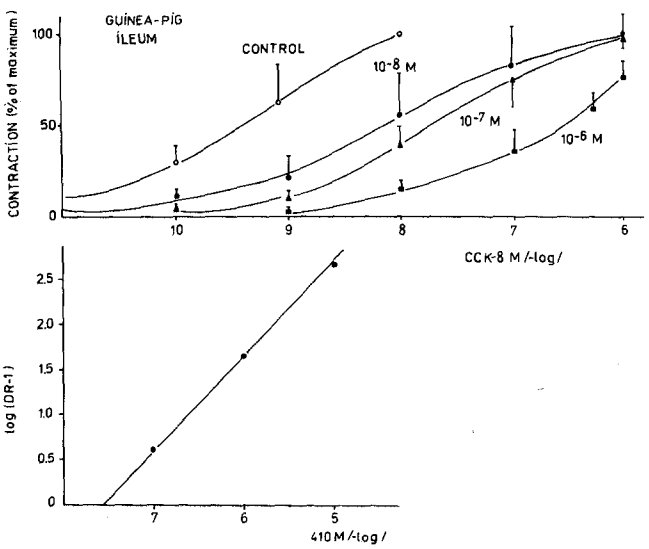

Figure 3. Dose-response curves of guinea pig ileum, longitudinal muscle strips to CCK8 (10^{-10} M to 10^{-6} M) before (control) and in the presence of different concentrations of GE 410. Progressive shift of the concentration-effect curves for CCK8 to the right by increasing concentrations of GE 410 (10^{-8} M, 10^{-7} M and 10^{-6} M). The Schild plot below was derived from these curves with a slope of 0.95. Averaged results of 12 experiments.

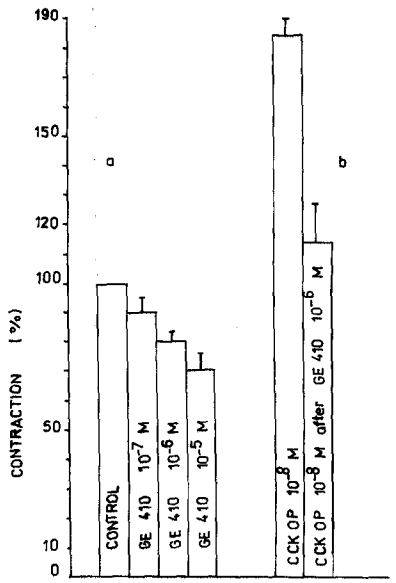

Figure 4. *a* Effect of GE 410 (10^{-7} M, 10^{-6} M and 10^{-5} M) on the electrical field stimulation-induced contractions in guinea pig ileum muscle strips. *b* Effect of CCK8 (10^{-8} M) before and in the presence of GE 410 (10^{-6} M); (n = 6).

spectively ($n = 10$, $p < 0.05$) as compared to the control, which was taken to be 100%. The stimulant effect of CCK8 (10^{-8} M), which was greater by 85% than the control, and the antagonistic effect of GE 410 (10^{-6} M) as compared to the effect of CCK8, are presented in figure 4b.

Effect of GE 410 on electrically-stimulated [^{3}H]ACh release. After a 60-min incubation with tritium choline and 90-min washing with perfusion Krebs fluid the efflux of ^{3}H-radioactivity, expressed as fraction released, was relatively constant in the different experiments. The amount of ^{3}H-radioactivity, measured during the second stimulation (S_2) was similar to that determined during the first period of stimulation (S_1), (fig. 5). With the frequency used (2 Hz) the S_2/S_1 ratio was close to unity (0.85 ± 0.05, $n = 10$, $p < 0.05$). GE 410 at a concentration of 10^{-5} M applied 15 min before the third period of stimulation (S_3) exerted no effect on the spontaneous efflux of [^{3}H]ACh but reduced the electrically-stimulated release of [^{3}H]ACh (fig. 5). The S_3/S_2 ratio was 0.5 ± 0.07 ($n = 9$, $p < 0.05$). CCK8 stimulated the electrically-induced release of [^{3}H]ACh by 56% as compared to the control, which was taken to be 100%. This effect

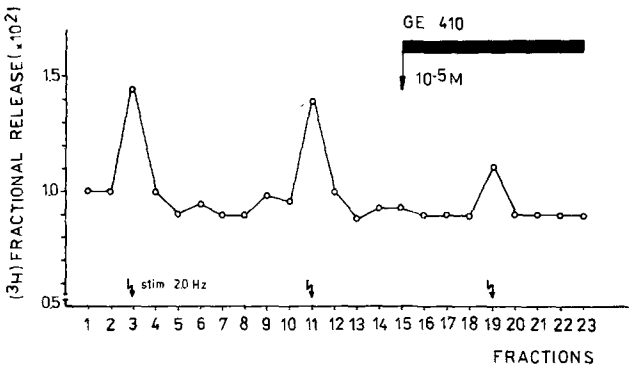

Figure 5. Effect of GE 410 on the [^{3}H]ACh release in guinea pig ileum muscle strips. Electrical field stimulation (2 Hz for 3 min) was applied at 3rd (S_1), 11th (S_2) and 19th (S_3) fractions. GE 410 (10^{-6} M) is administered 15 min before the third (S_3) stimulation. The release of radioactivity [^{3}H] is expressed as fractional release ($\times 10^2$).

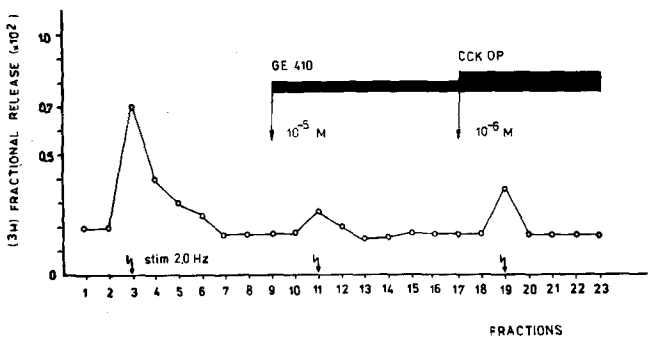

Figure 6. Effect of GE 410 on the [^{3}H]ACh release in guinea pig ileum muscle strips. GE 410 (10^{-6} M) is applied before the second (S_2) stimulation. The electrically-induced release of [^{3}H]ACh during the third period of stimulation (S_3) in the presence of CCK8 did not reach the values obtained in the first period of stimulation (S_1-control).

of CCK8 was antagonized by GE 410 (10^{-6} M). The electrically-induced release of [^{3}H]ACh during the third period of stimulation (S_3) in the presence of CCK8 did not reach the values obtained in the first period of stimulation (S_1-control); its values were similar to those obtained during the second period of stimulation (S_2) in the presence of GE 410 ($S_3/S_2 = 1.13 \pm 0.09$, $n = 6$, $p < 0.05$), (fig. 6).

Discussion

The present results showed that the antagonistic effect of GE 410 on the CCK-induced contractions in guinea pig ileum was manifested both at the smooth muscle level and at the level of cholinergic neurotransmission. In smooth muscle, GE 410 selectively and reversibly antagonized the contractile effect of CCKs. The Schild plot proved the existence of competitive antagonistic effect produced through CCK-receptors. The binding affinity of GE 410 to these receptors was higher than of dbcGMP to the same receptors [22]. At the level of cholinergic neurotransmission, GE 410 inhibited the electrically-induced cholinergically-mediated contractile responses and [^{3}H]ACh release in the ileum, as well as the CCK8-stimulated contractions and the [^{3}H]ACh release in the cholinergic nerve terminals. Since cholecystokinins are located in the myenteric plexus neurons [23] and since exogenous cholecystokinins stimulate the ileac smooth muscle by activating the cholinergic nerves [6, 22] it is possible that endogenous cholecystokinins and cholinergic neurons are responsible for the transmission at the synaptic level. The exact level of these interactions is still a matter for speculation. The fact that one and the same antagonist (GE 410) could inhibit the effect of CCK8 both at the myogenic and at the neurogenic level suggests the existence of identical CCK-receptors. Activation or blockade of these receptors by cholecystokinins or CCK-antagonists could regulate cholinergic neurotransmission.

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Further evidence for the dissociation of digoxin-like immunoreactivity from Na^+, K^+-ATPase inhibitory activity

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Summary. The effects of adrenalectomy or nephrectomy, carried out one hour previously, on the levels of endogenous digitalis-like factors were determined in rat plasma. Factors were assayed by digoxin-like immunoreactivity and direct Na^+, K^+-ATPase inhibitory activity. Digoxin-like immunoreactivity significantly decreased one hour after bilateral ablation of adrenals, while Na^+, K^+-ATPase inhibitory activity remained unaltered. There were no changes in either activity one hour after bilateral nephrectomy. These results suggest that digoxin-like immunoreactivity may be derived from the adrenal gland or under adrenal control and the major substances detected by digoxin-like immunoreactivity and direct Na^+, K^+-ATPase inhibitory activity may be different.
Key words. Endogenous digitalis-like factor; digoxin-like immunoreactivity; Na^+, K^+-ATPase inhibitor; adrenalectomy.

The notion that endogenous inhibitors of the sodium pump exist, and bind to the cardiac glycoside binding site on Na^+, K^+-ATPase, has been a source of much controversy[1, 2]. Although considerable work has been carried out, the exact nature, structure and production site of the inhibitors are not as yet known. Moreover, because of the lack of specific assay methods, a variety of different procedures have been used to detect such endogenous digitalis-like factors (EDLF). It is possible that each procedure may detect a completely different substances. Indeed, we have indicated that the major substances detected by digoxin-like immunoreactivity and direct Na^+, K^+-ATPase inhibitory activity are totally different at least in rat plasma[3].

Recent findings have pointed to the possibility that digoxin-like immunoreactivity is closely associated with the adrenal gland[4–8]. In the present study, we determined the effects of adrenalectomy or nephrectomy carried out only one hour before assay on plasma levels of EDLF, assayed by digoxin-like immunoreactivity and Na^+, K^+-ATPase inhibitory activity, to gain further insight into the tissue source of EDLF.

Materials and methods

Male Sprague-Dawley rats under pentobarbital anesthesia (40 mg/kg b.wt, i.p.) were used in this experiment. Bilateral adrenalectomy was performed through dorsal incisions in 8 rats. Bilateral nephrectomy was performed in another 8 rats, also through dorsal incisions. They were compared to 8 sham-operated controls.

A PE-50 catheter was inserted to the right carotid artery and direct blood pressure was recorded. A blood sample was obtained from the catheter into a heparinized syringe 60 min after the completion of the operation. Arterial blood was immediately chilled and centrifuged at 3000 rpm for 5 min. 5 ml of plasma was mixed with 10 ml of methanol and the mixture was kept at 4 °C for 16 h. After filtration through filter paper, the filtrate was evaporated and lyophilized. The resulting residue was dissolved in 8 ml of distilled water and the solution was applied to Amberlite XAD-2 (3 ml). After washing with 30 ml of distilled water, EDLF was eluted with 8 ml of methanol. The eluent was evaporated and the residue was redissolved in 0.5 ml of distilled water. EDLF was determined by digoxin-like immunoreactivity and Na^+, K^+-ATPase inhibitory activity according to the methods described in detail previously[3]. The data are expressed as mean ± SE. Group comparisons were made by analysis of variance and differences between two groups were analyzed by the unpaired Student's t-test.

Results

Body weight, mean blood pressure and hematocrit were not different among the three groups (334 ± 8, 329 ± 9