Abstract—Two \( \beta \)-carbolines, methyl \( \beta \)-carboline-3-carboxylate (\( \beta \)-CCM) and ethyl \( \beta \)-carboline-3-carboxylate (\( \beta \)-CCE), caused the parallel shift of the dose-response curve for cholecystokinin (CCK) in isolated guinea-pig gallbladder muscle. The Schild plot regarding the parallel shift in the dose-response curves had a regression line with a slope of 1.03 and a \( \text{pA}_2 \) value of 5.17 for \( \beta \)-CCE, while the method of van Rossum gave a \( \text{pA}_2 \) value of 5.24 for \( \beta \)-CCE and 5.53 for \( \beta \)-CCM. Both the \( \beta \)-carbolines protected CCK receptors in the gallbladder muscle from alkylation by dibenamine, but \( \beta \)-CCM did not protect acetylcholine receptors from dibenamine alkylation. These results suggest that \( \beta \)-CCM and \( \beta \)-CCE, so-called inverse agonists of benzodiazepines (BZP), antagonize the CCK action in the gallbladder muscle in a competitive manner, and the antagonism takes place at CCK receptor sites. No spare receptors for CCK were found in the guinea-pig gallbladder muscle.

The antagonism between CCK and BZP have been first reported by Kubota et al. (1) in 1983. Later, they showed that the antagonism could be observed not only in the contractile response of the guinea-pig gallbladder muscle (2) but also in the various central effects induced by CCK (3, 4), and the antagonism probably took place at the CCK receptor sites, at least in the gallbladder, because BZP caused the parallel shift of the dose-response curve for CCK (2). In addition, we also showed (5) that BZP could protect CCK receptors from alkylation by dibenamine, which has been used as a receptor alkylating agent (6, 7), while Bradwejn and De Montigny (1984) (8) electrophysiologically demonstrated the BZP-CCK antagonism in rat hippocampal neurons.

\( \beta \)-Carbolines such as \( \beta \)-CCE and \( \beta \)-CCM are well known to reverse the benzodiazepine action in the central nervous system (9–11). Recently, Hunkeler et al. (12) claimed that \( \beta \)-carbolines act on the BZP receptor as inverse agonists in the central nervous system.

In this paper, we provide several lines of evidence that \( \beta \)-CCM and \( \beta \)-CCE also act as receptor antagonists of CCK in the guinea-pig gallbladder muscle.

Materials and Methods

Male guinea pigs weighing 350 to 450 g were killed by a blow on the head, and the gallbladder was removed. A 4 mm wide helical strip was prepared from a gallbladder, and it was cut into three segments, each of which had the length of 10 mm.

Effect of \( \beta \)-carbolines on the dose-response curve for CCK: The strips were suspended in Locke-Ringer solution which contained \( 3 \times 10^{-9} \) M of atropine sulfate, kept at 32°C and bubbled with air. The Locke-Ringer solution had the following composition in mM: NaCl, 154.0; KCl, 5.6; CaCl\(_2\), 2.2; MgCl\(_2\), 2.1; NaHCO\(_3\), 5.9; and dextrose, 2.8. The strips were connected via cotton thread to a force displacement transducer for recording the isometric force of the smooth muscle. The gallbladder muscle strips were applied repeatedly with cumulative doses of sulfated CCK octapeptide (CCK8) until the magnitude of the muscle response to
CCK8 reached a constant level. The dose-response curve for CCK8 was obtained from the last application of cumulative CCK8. About a 1.5 hr rest was given for washing the muscle between the sequential, cumulative CCK8 applications. Another cumulative application of CCK8 was made after the last one in the presence of β-CCE or β-CCM that was applied 5 min before the CCK8 application.

Effect of β-carbolines on the inhibition of CCK response by dibenamine: The gallbladder muscle strips were suspended in Locke-Ringer solution and equilibrated for 60 min. An agonist, CCK8 (10^{-8} M) or ACh (10^{-4} M), was repeatedly applied to the muscle strip. When the response to the agonists became constant, the muscle was exposed to 5 \times 10^{-5} M dibenamine for 30 min. After washing the muscle with Locke-Ringer solution, the dibenamine treatment was given one more time. Agonists at the same concentrations as those given before the dibenamine treatment were applied to the muscle which was washed for 90 min after the second dibenamine treatment. The contractile responses of the gallbladder to each agonist before and after the dibenamine treatment were compared to each other. In order to protect receptors from the dibenamine alkylation, β-carbolines were applied to the muscle strips for 15 min prior to the dibenamine treatment and during the dibenamine treatment.

CCK8-induced contractile response of the gallbladder incubated preliminarily with β-CCM: The gallbladder muscle showing stable responsiveness to 10^{-8} M CCK8 after repeated applications of CCK8 was incubated with 5 \times 10^{-5} M β-CCM for a total of 75 min (15, 30 and 30 min each) in accordance with the procedure for the receptor protection experiments and then washed for 90 min with Locke-Ringer solution. The magnitudes of the gallbladder response to CCK8 applied before and after the β-CCM treatment were compared to each other.

Examination of the presence of receptor reservation to CCK8: The gallbladder strips showing stable responsiveness to CCK8 were treated with 5 \times 10^{-5} M dibenamine for 30 min and the dibenamine-treatment was repeated one more time. The strips were then washed with Locke-Ringer solution for 90 min and received a cumulative application of CCK8. The dose-response curves for CCK8 before and after the dibenamine treatment were compared.

CCK8 was purchased from Protein Research Foundation (Osaka), dibenamine hydrochloride from Tokyo-Kasei Co. (Tokyo), β-CCE from Aldrich Chem. Co. and β-CCM from Research Biochemicals, Inc. (U.S.A.)

Significant differences between data were assessed by Student’s t-test for paired samples.

Results

Effect of β-carbolines on the dose-response curve for CCK: The dose-response curve for CCK8 was concentration-dependently shifted in parallel to the right by β-CCE and β-CCM as shown in Figs. 1 and 2. The degree of the shift was greater in β-CCM than in β-CCE. When a higher concentration such as 5 \times 10^{-5} M was used, β-CCM also produced a non-competitive action in addition to its competitive action in that it caused a decrease in the maximum response in the dose-response curve for CCK8. The Schild analysis (13) of the data shown in Figs. 1 and 2 gave regression lines with

Fig. 1. Effect of β-CCE on the dose-response curve for CCK8 applied to the muscle strip from the guinea-pig gallbladder. CCK8 was applied cumulatively to the organ bath fluid containing 3.0 \times 10^{-6} M of atropine sulfate in the absence (○) (n=12) or presence of 10^{-6} M (●) (n=6) and 5 \times 10^{-5} M (□) (n=6) of β-CCE. Each point represents the mean ± S.E. β-CCE was applied 5 min before the cumulative application of CCK. Asterisks denote a significant difference in the comparison of ○ vs. ● or □ (Student’s t-test, **P<0.01).
slopes of 1.03 and 1.45 for β-CCE and β-CCM, respectively, and 5.17 and 5.36 as the pA$_2$ value for the respective β-carboline. On the other hand, the calculation of pA$_2$ value according to the method of van Rossum (14) gave values of 5.24±0.99 (n=12) and 5.53±0.16 (n=6) for β-CCE and β-CCM, respectively. In the case of β-CCM, only the dose-response curve obtained in the presence of a lower concentration, 10$^{-5}$ M, of β-CCM was used for the calculation of the pA$_2$, since 5×10$^{-5}$ M of β-CCM also exerted nonspecific inhibition of the CCK8 response as noted above.

**Protection against dibenamine alkylation by β-carboline:** As shown in Table 1, β-CCM failed to protect the ACh response of the gallbladder muscle against the inhibition caused by the dibenamine treatment. On the other hand, both β-CCE and β-CCM significantly protected the CCK8 response from the inhibition by dibenamine (Table 2).

A 75 min incubation of the gallbladder by 5×10$^{-5}$ M β-CCM resulted in apparent irreversible inhibition of the gallbladder response to CCK8 even after 90 min washing. That is, the response to CCK8 after the β-CCM treatment decreased to 80.0±2.8 (%) (n=3) of the control response before β-CCM treatment.

**Receptor reservation of CCK8:** The dibenamine treatment did not cause a parallel shift of the dose-response curve for CCK8, but did affect the suppression of the maximum response in the dose-response curve for CCK8 (Fig. 3).

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**Table 1.** Effect of β-CCM on the dibenamine (5×10$^{-5}$ M)-induced inhibition of the contractile response of the guinea-pig gallbladder to ACh (10$^{-4}$ M)

| Treatment                        | Contractile response (%) |
|----------------------------------|--------------------------|
| Dibenamine (control)             | 6.66±1.22                |
| Dibenamine+β-CCM (10$^{-5}$ M)   | 6.51±1.02                |
| Dibenamine+β-CCM (5×10$^{-5}$ M) | 6.78±1.17                |

The contractile response of the gallbladder to ACh before dibenamine treatment was taken as 100%. The values are given as the mean±S.E. (n=6).

**Table 2.** Effects of β-CCE and β-CCM on the dibenamine (5×10$^{-5}$ M)-induced inhibition of the contractile response of the guinea-pig gallbladder to CCK8 (10$^{-8}$ M)

| Treatment                        | Contractile response (%) |
|----------------------------------|--------------------------|
| Dibenamine (control)             | 54.17±3.71               |
| Dibenamine+β-CCE (10$^{-5}$ M)   | 69.20±5.73*              |
| Dibenamine (control)             | 61.97±3.13               |
| Dibenamine+β-CCM (10$^{-5}$ M)   | 72.80±3.53**             |

The contractile response of the gallbladder to CCK8 before dibenamine treatment was taken as 100%. The values are given as the mean±S.E. (n=6). **P<0.01, *P<0.05, significant difference from the control by Student’s $t$-test.
Effect of dibenamine on the contractile response of the gallbladder to CCK8. CCK8 was cumulatively applied to the gallbladder muscle strips before and after dibenamine treatment which was described in the text. (O) Dose-response curve (n=6) for CCK8 before dibenamine treatment and (●) (n=6) after dibenamine treatment. ***P<0.005, *P<0.05, significant difference from the control by Student's t-test.

Discussion

In the previous paper (2), the contractile response of the gallbladder muscle to CCK8 was found to be antagonized by BZP in a competitive manner. The competition was presumed to take place at the CCK receptor site on the smooth muscle cell membrane since the dose-response curve for CCK8 was shifted in parallel to the right by BZP, the slope of the regression line in the Schild analysis on the parallel shift was close to 1.0; and in addition, the inhibition of the contractile response of the gallbladder to CCK8 by dibenamine could be protected in the presence of BZP (5). Quite similar results were obtained with caerulein (15) which is closely related to CCK in terms of chemical structure as well as physiological properties.

β-Carbolines have been purported to act as inverse agonists with regard to BZP receptors (12) since it has been evidenced to bind to these receptors (9, 11), to reverse BZP action (9–11, 16, 17) and to be antagonized by a BZP antagonist, Ro 15-1788 (18, 19). Therefore, β-carbolines and BZP are expected to have a certain common feature from the structural point of view on the binding to the BZP receptor.

In the present experiments, both β-CCM and β-CCE were found to have characteristics very similar to those of BZP in regards to the competition to CCK8 action. That is, they caused the parallel shift of the dose-response curve for CCK8 and protected CCK receptors from alkylation by dibenamine. The fact that the slope of the regression line in the Schild plot was close to 1.0 in the case of β-CCE and the fact that there was significant protection of the dibenamine alkylation by both the β-carbolines suggest that β-CCM and β-CCE also act as CCK receptor antagonists like BZP. There was some discrepancy between the pA2 value for β-CCM as determined by the Schild method, 5.36, and that obtained by the van Rossum method, 5.53. However, this is due to the nonspecific inhibitory action of β-CCM in the higher concentrations and resulting suppression of the maximum response to CCK8. This is also reflected in the value of the slope obtained in the Schild analysis, 1.45, which is slightly larger than 1.0. Thus we consider that the pA2 value of 5.53 obtained by the method of van Rossum should be more valid than that obtained by Schild analysis as far as β-CCM is concerned. On the basis of the pA2 values, β-CCM is stronger as a CCK antagonist than β-CCE, but a little weaker than diazepam (2).

In the previous paper (5) on the CCK receptor protection, atropine failed to protect CCK receptors from alkylation by dibenamine, but protected against ACh receptor alkylation, while BZP failed to protect ACh receptors from alkylation by dibenamine. In the present work, 10⁻⁵ M β-CCM also failed to protect ACh receptors from alkylation by dibenamine (Table 1), but significantly protected the CCK receptors against alkylation by dibenamine.

Dibenamine suppressed the response of the gallbladder to ACh more strongly than the one to CCK8 (Tables 1 and 2). The discrepancy results from the characteristics of the receptors since the susceptibility of receptors to dibenamine alkylation is well-known to be variable according to the characteristics of receptors, and receptors such as β-adrenoceptors and ACh receptors are susceptible to dibenamine attack (6, 7).

Since β-CCM left a partial (20%), irreversible inhibition of the gallbladder muscle
response to CCK8 at higher concentrations such as $5 \times 10^{-5} \text{ M}$ after a 75 min incubation, a less significant protection against dibenamine alkylation was apparently obtained with $5 \times 10^{-6} \text{ M}$ of $\beta$-CCM, although the data were not shown here.

The dose-response curve for CCK8 was not shifted in parallel, but the maximum height of the curve was suppressed after the dibenamine treatment, suggesting the absence of spare receptors for CCK in the guinea-pig gallbladder muscle cells. In the experiments showing the parallel shift of the CCK dose-response curve by $\beta$-carbolines, we added a low concentration of atropine to the Locke-Ringer solution in order to block the contractile effect of endogenous ACh that would be released by CCK from the cholinergic neurons (20–22). This procedure also implied that in the parallel shift experiments, the receptors involved in the CCK response are those on the smooth muscle cell membrane and excludes those on the neurons, although the contribution of the neuronal CCK receptors is essentially a minor one in the CCK-induced contraction of the guinea-pig gallbladder as compared with those on the smooth muscle cells according to the previous paper (2).

Since it was previously observed that the central effects of CCK8 such as antinociception (3), hypothermia (23) and satiety (4) were all antagonized by BZP, it is also highly probable that the two $\beta$-carbolines tested here antagonize such central effects of CCK. Experiments for testing whether this is true are under way. The facts that the specific binding of CCK to mouse brain homogenate could be displaced by BZP and a peripheral CCK receptor antagonist, proglumide, also antagonized the satiety effect of CCK in mice (K. Sugaya et al., unpublished data), the BZP-CCK antagonism reported previously (5) and the present results, when taken all together, suggest that CCK may be closely involved in the central effects of BZP.

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