Analysis of the Long-Lasting Antagonistic Effect of Caerulein on Amphetamine Hyperactivity in Rats

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Abstract—Caerulein (CLN), which is chemically related to cholecystokinin octapeptide (CCK-8) and produces a short-lasting pharmacological effect when administered peripherally, caused a long-lasting antagonistic effect on amphetamine (AMP) hyperactivity in rats when given in combination with haloperidol (HLP). Briefly, rats were treated with a combination of CLN (0.3–40 μg/kg, s.c.) and HLP (0.1 mg/kg, s.c.) and exposed to AMP on the first day. The animals became less sensitive to AMP for 24 hr to about 2 weeks depending on the CLN dose, according to measurements of their ambulatory activities in an open field or with an Animex activity meter at low sensitivity. Examination of the properties of this long-lasting effect revealed that: 1) in animals treated with CLN and HLP, but without AMP on the first day, the susceptibility to AMP was not influenced on the next day; 2) in substitution experiments, the antagonistic effect of CLN could be reproduced by higher doses of CCK-8 (160 μg/kg) but not by nonsulfated CLN; 3) in the regimen of the treatment schedule, HLP could be replaced by chlorpromazine or sulpiride, but not by α-blocking agents like phenoxybenzamine or yohimbine; 4) apomorphine, nomifensine and tranylcypromine could not substitute for AMP. Thus, injection of CLN together with HLP and AMP on the first day might be necessary to produce the long-lasting anti-AMP effect. The possible mechanism of this CLN effect is discussed.

Much pharmacological data suggest that the dopamine (DA) system in the brain is influenced by CCK-like peptides (1–4). This fact seems to underlie the clinical effects of the peptides on schizophrenia, but the difference in the duration of action between pharmacological and clinical effects contradicts this. In general, the effect of CCK-8 or caerulein (CLN) in schizophrenia lasts for 1 to 3 weeks following a single injection (5, 6).

In order to find the reason for this difference, we treated animals with the drug administration schedule used in clinical studies and found that CLN given with haloperidol (HLP) produced a long-lasting inhibitory effect on amphetamine (AMP)-induced hyperactivity in rats (7). The present work involved further pharmacological analysis of the nature of this long-lasting effect.

Materials and Methods

Male Wistar rats (Shizuoka Laboratory Animal Center, Japan), weighing 250–300 g at the beginning of the experiments, were used. They were maintained with ad libitum food and water on an 8 a.m.–8 p.m. lighting schedule during the experiments.

Rat locomotor activities were measured with the open field apparatus described in a previous report (8). An Animex activity meter was also employed in some cases.

On the first day, four groups of six rats were pretreated with saline, HLP (0.1 mg/kg), CLN (0.3–40 μg/kg) or the combination of both drugs and then received AMP (2 mg/kg) 60 min later. At 30 min after AMP administration, each rat was placed on the center of the open field apparatus and the
ambulatory activity was measured by counting the number of compartments traversed by the animals during 3 min. On the 2nd, 8th, 15th and 22nd days, all animals of the four groups were treated with AMP (2 mg/kg) alone, and 30 min later, the activity was measured as on the first day. All experiments were carried out between 12:30-4:30 p.m.

In similar experiments, apomorphine (APO), nomifensine or tranylcypromine was used instead of AMP; CCK-8 or nonsulfated CLN instead of CLN; and chlorpromazine (CPZ), sulpiride (SPR), phenoxybenzamine (PBZ) or yohimbine (YHB) instead of HLP. These drugs were dissolved in physiological saline, except for PBZ, which was dissolved in saline and propyleneglycol (1:1, v/v). All drugs were administered subcutaneously with each injection volume at 1 ml/kg body weight except for AMP which was given at 2 ml/kg body weight. The drugs were prepared or obtained as follows: caerulein (ceruletide diethylamine, synthesized in our laboratory), nonsulfated caerulein (synthesized in our laboratory), CCK-8 (Calbiochem-Behrig), dl-amphetamine sulfate (Zedrin, Takeda), apomorphine hydrochloride (Sigma), nomifensine (synthesized in our laboratory), tranylcypromine hydrochloride (Sigma), haloperidol (Shionogi), chlorpromazine hydrochloride (Shionogi), sulpiride (Dogmatyl, Fujisawa), phenoxybenzamine hydrochloride (Nakarai), and yohimbine hydrochloride (Sigma).

The experimental results were initially subjected to one-way analysis of variance (ANOVA). Further statistical comparisons of the means were based on Dunnett’s t-test. Differences between two groups were analyzed with Student’s t-test in some cases.

Results

We previously reported that CLN has a long-lasting antagonistic effect on AMP-induced hyperactivity in rats, when it is given together with haloperidol, and we also briefly mentioned the dose-dependency of the duration of its action (7). For a better understanding of our experimental situation, our results on the dose-dependency will be described first. Four experiments were performed with different doses of CLN given on the first day (0.3, 1, 10 and 40 μg/kg, s.c.); each experiment consisted of four groups, i.e., saline-, HLP-, CLN- and HLP+CLN-treated animals. The anti-AMP activity of CLN alone was observed in the Day 1 test of only the group given 40 μg/kg of the peptide; ambulation scores of the untreated group and the CLN-treated group were 63.2±1.7 and 52.3±3.0, respectively (In Fig. 1-d: ANOVA F=56.87, df 3/20, P<0.01; Dunnett-t P<0.05). This reduction in AMP-hyperactivity is due to an acute effect of CLN and differs in nature from the effect analyzed here. On the second day, there were no significant differences among the four groups given 0.3 μg/kg CLN on the first day (In Fig. 1-a: F=0.74, df 3/20, P>0.05). As for the other three dosage groups, only those treated with HLP+CLN were still less susceptible to AMP (In Fig. 1-b: CLN=1 μg/kg; on Day 2; ANOVA F=13.47, df 3/20, P<0.01; Dunnett-t P<0.01; in Fig. 1-c: CLN=10 μg/kg; on Day 2; ANOVA F=9.93, df 3/20, P<0.01; Dunnett-t P<0.01; and in Fig. 1-d: CLN=40 μg/kg; on Day 2; ANOVA F=16.20, df 3/20, P<0.01; Dunnett-t P<0.01). On Day 8, the animals treated with 1 μg/kg CLN and HLP on the first day showed a normal response to AMP (In Fig. 1-b: ANOVA F=1.27, df 3/20, P>0.05), but those given higher doses of CLN still gave significantly reduced responses (In Fig. 1-c: CLN=10 μg/kg; ANOVA F=4.70, df 3/20, P<0.01; Dunnett-t P<0.01 and in Fig. 1-d: CLN=40 μg/kg; ANOVA F=13.08, df 3/20, P<0.01; Dunnett-t P<0.01). In the tests conducted on Day 15, significant reduction in AMP-hyperactivity was found only in the animals given 40 μg/kg of CLN (In Fig. 1-d: ANOVA F=4.22, df 3/20, P<0.05. Dunnett-t, P<0.05). These rats regained normal susceptibility to AMP by the time of the test on Day 22 (In Fig. 1-d: ANOVA F=0.30, df 3/20, P>0.05). Thus, the duration of the long-lasting reduction in AMP-hyperactivity elicited by combined injections of CLN and HLP clearly depended on the dose of CLN on Day 1.

To further analyze this long-lasting effect, the following experiments were designed to find 1) whether CLN can be replaced by any other peptide, 2) what kind of CNS-acting
drug can substitute for HLP and 3) why AMP-hyperactivity is indispensable for the present experimental paradigm.

1. CLN substitution test
Nonsulfated CLN and CCK-8 were used in the substitution test with the same doses of HLP (0.1 mg/kg) and AMP (2 mg/kg) and administration schedule. A significant reduction in the AMP effect was noted in the group of HLP plus 160 μg/kg of CCK-8 in the Day 2 test (In Fig. 2-b: ANOVA F=5.98, df 3/20, P<0.01; Dunnett-t P<0.01), which disappeared on Day 8 (In Fig. 2-b: ANOVA F=1.01, df 3/20, P>0.05). A dose of 40 μg/kg of CCK-8 could not produce the long-term effect (In Fig. 2-a: ANOVA F=2.27, df
3/20, P>0.05). CCK-8 alone did not suppress the AMP effect on Day 1 even at 160 μg/kg (Fig. 2-b). Nonsulfated CLN (40 μg/kg) did not produce the long-lasting effect (In Fig. 2-c: ANOVA F=0.57, df 3/20, P>0.05).

2. Haloperidol substitution test
DA receptor antagonists (CPZ and SPR) and norepinephrine (NE) receptor antagonists (YHB and PBZ) were used, and the results clearly revealed that CPZ and SPR as well as HLP could produce long-lasting effects, but not NE blockers. The long-lasting anti-AMP effect of CPZ (2 mg/kg) was similar to that of HLP in potency and duration (In Fig. 3-a, on Day 2: ANOVA F=18.21, df 3/20, P<0.01; Dunnett-t P<0.01; on Day 8: ANOVA F=29.76, df 3/20, P<0.01; Dunnett-t P<0.01; on Day 15: ANOVA F=5.32, df 3/20, P<0.01; Dunnett-t P<0.05; and on Day 22: ANOVA F=0.04, df 3/20, P>0.05). SPR was also active at 100 mg/kg; significant reduction in the AMP effect was noted until the test on Day 8 (In Fig. 3-b, on Day 2: ANOVA F=8.72, df 3/20, P<0.01; Dunnett-t P<0.01; on Day 8: ANOVA F=8.31, df 3/20, P<0.01; Dunnett-t P<0.01; and on Day 15: ANOVA F=0.84, df 3/20, P>0.05). Animals given CLN and either PBZ (10 mg/kg) or YHB (2.5 mg/kg) showed normal responses to AMP on Day 2 (Fig. 3-c and d). These results suggest that some modulatory changes in DA systems may play an important role in producing the long-lasting effect of CCK-family peptides.

3. Amphetamine substitution test
Withdrawal of AMP on Day 1: The experiments depicted in Fig. 4 were conducted on the basic drug administration schedule except that AMP was eliminated from the treatment on Day 1. Thus, the activity of the saline group represented the normal spontaneous motor activity (In Fig. 4, on Day 1, group A: 30.3±2.2). On Day 2, no reduction in AMP-hyperactivity occurred in the group treated with HLP and CLN (In Fig. 4: ANOVA F=2.82, df 3/20, P>0.05). Therefore, AMP treatment on Day 1 is obviously essential for producing the long-lasting effect.

Substitution by apomorphine: As DA system stimulation may be involved in AMP-induced hyperactivity, APO which causes hyperactivity by directly stimulating DA receptors in the brain, was also tested. However, it could not substitute for AMP in the present experimental paradigm. When APO (0.25 mg/kg) was given instead of AMP on Day 1, no reduction was noted in
APO hyperactivity (In Fig. 5-a: ANOVA F=1.3, df 3/20, P>0.05) and also in AMP induced hyperactivity (In Fig. 5-b: Student-t P>0.05) of the CLN+HLP group in the Day 2 tests. Furthermore, there was no decrease in APO activity on Day 2, when the animals were treated with AMP on Day 1 (In Fig. 5-c: Student-t, P>0.05).

Effects of nomifensin and tranylcypromine:
AMP causes central nervous system stimulation in several ways, i.e., releasing catecholamines from nerve terminals, blocking uptake processes of released catecholamines and inhibiting monoamine oxidase. To determine which activity of AMP is involved, the effects of nomifensin, a DA uptake blocker, and tranylcypromine, a monoamine oxidase inhibitor, were tested. As shown in Fig. 6-a and b, they did not cause long-term suppression of the hyperactivities.

Fig. 3. Haloperidol substitution test for chlorpromazine, sulpiride, phenoxybenzamine or yohimbine. On Day 1, groups received the following at 60 min prior to amphetamine (2 mg/kg, s.c.): group A, saline; group B. (a) chlorpromazine (2 mg/kg, s.c.), (b) sulpiride (100 mg/kg, s.c.), (c) phenoxybenzamine (10 mg/kg, s.c.), (d) yohimbine (2.5 mg/kg, s.c.); group C, caerulein (40 μg/kg, s.c.); group D, caerulein and chlorpromazine (a), sulpiride (b), phenoxybenzamine (c) or yohimbine (d) (doses the same as for C and B). Abbreviations, see Fig. 1.
These data indicate that a modification of DA release from nerve terminals is related to the long-lasting effect of CLN.

4. On the timing of haloperidol injection

Since the acute pharmacological effects of CLN were short-lasting and those of HLP longer in duration, changes in the long-lasting anti-AMP effect need to be studied in connection with the time-lag between HLP and CLN injections. In the basic drug administration schedule, HLP was simultaneously given with CLN. Interestingly, no obvious reduction in the AMP effect was produced on Day 2, when CLN had been given more than 30 min before HLP. Two groups of animals, treated first with 40 μg/kg of CLN or saline and then, with a simultaneous injection of HLP and AMP 60 min after CLN, exhibited almost the same AMP-hyperactivity on the next day (59.3±4.6 and 60.5±1.7, Student-t, P>0.05). On the other hand, administration paradigms where HLP was injected 0 to 60 min prior to CLN always gave the long-lasting effect. The ambulation scores of HLP-treated and HLP+CLN-treated groups were as follows: 62.0±0.8 vs. 38.2±1.7 (P<0.01) in the 0 min group, 59.0±2.5 vs. 38.5±2.4

Fig. 4. Lack of the antagonistic effect of caerulein with haloperidol on amphetamine-induced hyperactivity when amphetamine was eliminated from the treatment on Day 1. On Day 1, groups received the following at 60 min prior to saline: group A, saline; group B, haloperidol (0.1 mg/kg, s.c.); group C, caerulein (40 μg/kg, s.c.); group D, caerulein and haloperidol (doses the same as for B and C). At 30 min after saline administration, the open field test was conducted for 3 min. On Day 2, each group was treated with amphetamine (2 mg/kg, s.c.) alone. Abbreviations, see Fig. 1.

Fig. 5. Amphetamine substitution test for apomorphine. (a) On Day 1, groups received the following at 60 min prior to apomorphine (0.25 mg/kg, s.c.): group A, saline; group B, haloperidol (0.1 mg/kg, s.c.); group C, caerulein (40 μg/kg, s.c.); group D, caerulein and haloperidol (doses the same as for B and C). At 30 min after apomorphine administration, the open field test was conducted for 3 min. On Day 2, each group was treated with apomorphine alone. Results are expressed as the mean±S.E. of 6 animals. Statistical comparisons between group A and the other groups were based on Dunnett's t-test (***P<0.01). (b) On Day 1, groups received the following at 60 min prior to apomorphine (0.25 mg/kg, s.c.): group A, saline; group D, caerulein (40 μg/kg, s.c.) and haloperidol (0.1 mg/kg, s.c.). At 30 min after apomorphine administration, the open field test was conducted for 3 min. On Day 2, each group was treated with amphetamine (2 mg/kg, s.c.) alone. Results are expressed as the mean±S.E. of 6 animals. Statistical comparisons between group A and B were based on Student's t-test (**P<0.01). (c) In the experimental schedule of (b), the administration order of apomorphine on Day 1 and amphetamine on Day 2 was reversed. Abbreviations, see (b).
(P<0.01) in the 15 min group and 65.2±1.9 vs. 42.8±1.0 (P<0.01) in the 60 min group. However, HLP given 300 min prior to CLN did not produce the long-term effect (60.8±1.7 vs. 63.2±2.2, P>0.05). These data indicate that the coincidence of the peak effect of CLN with that of HLP may be necessary for a long-term reduction in DA activities.

5. Instrumental measurements

For objective observations, we used an Animex activity meter to measure the locomotor activities of the rats. Although no reduction in AMP activity on Day 2 was found when the sensitivity of the activity meter was set at the usual levels (AMP gave a score of 274.2±10.7/3 min for the saline-treated group), measurements of HLP+CLN-treated animals at one-third to one-fourth sensitivity showed a significant reduction in the AMP effect lasting until the test on Day 15 (In Fig. 7, Day 2: ANOVA, F=4.54, df 3/20, P<0.05, Dunnett-t, P<0.01; Day 8: ANOVA, F=3.51, df 3/20, P<0.05, Dunnett-t, P<0.05; Day 15: ANOVA, F=3.95, df 3/20, P<0.05, Dunnett-t, P<0.05; and Day 22: ANOVA, F=1.29, df 3/20, P>0.05). These experiments imply that the observed long-term behavioral changes are restricted to some types of gross motor behavior such as moving from place to place.

Discussion

Administration of CLN with HLP caused long-lasting reduction of AMP-induced hyperactivity in the open field test, and this effect lasted for 24 hr to 2 weeks according to the CLN dosages on the first day. The AMP response is known to be strongly attenuated 6 days after infusion of D-Ala-Met-enkephalinamide to the ventral tegmental area, returning to normal only at 14 days; this suggests that the long-lasting modification reflects a release of inhibition of DA-A10 neurons (9). As these findings agreed with our results, the experiments were done to further analyze the long-lasting effect of CLN.

The antagonistic effect could be produced on Day 2 only by a high dose of CCK-8 (160 µg/kg) and not a small one (40 µg/kg). This agrees with the fact that the pharmacological potencies of CCK-8 are lower than those of CLN (10, 11). The reason for the superiority of CLN over CCK-8 in biological activities may be that CLN is metabolized slower or has a higher affinity for the CCK-8 receptor. Furthermore, the missing effect of nonsulfated CLN in this study agreed with the observations that desulfation of CCK-8 or CLN resulted in loss of the central pharmacological potency (12-14). Thus, the sulfated tyrosine
group is essential for the pharmacological effects. The enhanced locomotor activity elicited by AMP is generally assumed to be due to the release of DA from DA nerve terminals in the central nervous system (15–18). Also, the AMP-induced increase in motor activity has been suggested to be mediated by changes in NE neurons in the brain (19). However, α-NE receptor blocking agents, YHB and PBZ, when used instead of HLP, did not cause the long-term effect of CLN, while DA receptor blocking agents, CPZ and SPR, did. Therefore, one of the sites of this long-lasting action of CLN may be located in the DAergic neuronal system.

When AMP was eliminated from the treatment on Day 1, no reduction occurred in AMP hyperactivity on Day 2. HLP is known to block DA receptors and increase DA turnover, while AMP decreases DA metabolism and the firing of DA cells. In addition, AMP alone produces an increase in total DA content in contrast to its effect on newly synthesized DA. However, when administered together with HLP, AMP produced a significant increase in total DA content compared to HLP alone (20). Therefore, the high neuronal activity of DA neurons induced by the combination of HLP and AMP seems to be essential for producing the effect.

AMP acts presynaptically on catecholaminergic synapses, blocking reuptake of released amines in synaptic clefts and inhibiting monoamine oxidase. In order to determine which activity of AMP is involved, apomorphine, nomifensine and tranylcypromine were used in substitution experiments for AMP, respectively. No antagonistic effects of CLN on each drug-induced hyperactivity were observed, indicating that the modification of the DA release for nerve terminals is involved in the long-term effect of CLN.

The acute pharmacological effect of CLN is short-lasting (10, 11, 21, 22), while that of HLP is notably a long-term one (23). In our administration paradigms, the effect lasted longer when HLP was injected 0 to 60 min prior to CLN. This suggests that coincidence of the peak effect of CLN with that of HLP may be essential for long-term reduction in DA activity.

This long-lasting effect of CLN could also be detected using the Animex activity meter when the instrumental sensitivity was adjusted to one-third to one-fourth of that for customary usages. The reason for this may be that the Animex activity meter count at higher sensitivity includes other behavior such as rearing, grooming or preening (8, 24).

Repeated administration of AMP or methamphetamine results in a gradual augmentation in locomotion (reverse tolerance) (25–30). However, in the present study, reverse tolerance did not develop although AMP was administered five times at intervals of 1 or 6–7 days. The development
of behavioral sensitization to AMP or methamphetamine depends on the interval, dose or duration of drug administration, and the length of the drug-free period (29, 30). Moreover, reverse tolerance to behavioral effects of AMP or methamphetamine is found only in animals repeatedly exposed to a corresponding experimental situation during an acute drug effect (25, 26). The 3-min observation period used here may have been insufficient to elicit an interaction between the experimental situation and the drug effect.

In summary, our study showed that both injection of CLN together with a DA antagonist and AMP on the first day and administration of AMP after the second day are necessary to produce the long-term anti-AMP effect. The mechanism producing this effect remains to be elucidated, but some possible ones are decreased DA synthesis or inhibition of DA release at DA terminals. Our earlier studies also showed that CLN affected AMP-induced behaviors without affecting APO-induced syndromes (22). Thus, the long-lasting effect may result from some actions of CLN on DA presynaptic terminals on which AMP also acts. As CLN is considered to be metabolized rapidly (31, 32), it is difficult to consider CLN itself acting on the DA neuronal system to produce the long-lasting effect. Another important factor to be considered is that CLN increases β-endorphin, ACTH and substance P levels in the plasma and the cerebrospinal fluid in man (33–35). Thus, an indirect action via some endogenous hormones may occur. DA appears to interact with central β-endorphin axons, which originate from perikarya located in the mediobasal hypothalamus to innervate a discrete neuronal system, including the nucleus accumbens region (36), and a variety of behavioral effects following central infusion of β-endorphin appear to be mediated by interaction with the DA system, especially motor phenomena (37). Accordingly, it is tempting to hypothesize that β-endorphin directly released by CLN indirectly modulates DA release induced by AMP in the nucleus accumbens.

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