Diazepam Potentiates the Corticoidogenic Response of Bovine Adrenal Fasciculata Cells to Dibutyryl Cyclic AMP

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Abstract—To provide a possible role of peripheral type benzodiazepine receptors in the regulation of glucocorticoid biosynthesis. We have examined the effect of diazepam on the corticoidogenic response to dibutyryl cyclic AMP in isolated bovine adrenal fasciculata cells. Diazepam alone (up to 100 μ M) had no effect on the corticoidogenesis. Diazepam caused a dose-dependent potentiation of dibutyryl cyclic AMP-induced corticoidogenesis. However, diazepam had no effect on the corticoidogenic response to ACTH and a high concentration of KCl. The potentiating effect by diazepam was clearly detected after 90 min-incubation, and it was blocked by YM-684 (diazepam antagonist) and ML-236B (cholesterol *de novo* synthesis inhibitor). Diazepam caused no significant decrease of intracellular content of cholesteryl esters during the corticoidogenic response to dibutyryl cyclic AMP. When the cells were incubated in the presence of (+)-PN200-110, a potent voltage-dependent Ca channel inhibitor, the potentiating effect by diazepam was not affected in spite of a significant inhibition of dibutyryl cyclic AMP-induced corticoidogenesis. These results indicate that the potentiating effect of diazepam on dibutyryl cyclic AMP-induced corticoidogenesis is due in part to the activation of the intracellular cholesterol supply system (cholesterol *de novo* synthesis) without any change of voltage-dependent Ca channels.

It has been established that benzodiazepines, anxiolytic and anticonvulsant drugs, bind to at least two classes of binding sites termed central and peripheral type benzodiazepine receptors, respectively (1, 2). Central type benzodiazepine receptors are closely linked to GABA receptors (3–5) and modulate the GABA-regulated chloride channels (6–8). In contrast, peripheral type benzodiazepine receptors are not linked to GABA-receptors (9, 10). These receptors have been identified in many peripheral tissues (11–13), in which the greatest receptor density is observed in the adrenal cortex (11, 13). However, the precise physiological function of these peripheral receptors is still not clear. Shibata et al. demonstrated that the prototypic benzodiazepine diazepam inhibited potassium-induced aldosterone secretion in adrenal glomerulosa cells

by acting on voltage-dependent Ca channels (14). Recently, we reported the purification of a novel protein from bovine adrenal cortex that is rapidly synthesized by ACTH in a dose-dependent manner and plays an essential role during the corticoidogenic response of adrenal fasciculata cells (15, 16). (K. Yanagibashi et al., unpublished data). Structural analysis of this protein reveals identity to the endogenous benzodiazepine-like substance (endozepine) from bovine brain (17) except the loss of two amino acids from the carboxy terminus (18). These observations suggest the possibility that peripheral type benzodiazepine receptors may also play an important role in the regulation of glucocorticoid biosynthesis in adrenal fasciculata cells.

In the present study, we have examined whether or not diazepam, a potent agonist to

peripheral type benzodiazepine receptors, modulates the corticoidogenic response to dibutyryl cyclic AMP (Bt₂cAMP) in bovine adrenal fasciculata cells. Our results demonstrate that peripheral type benzodiazepine receptors are indeed involved in the potentiating action of diazepam on Bt₂cAMP-induced corticoidogenesis.

Materials and Methods

Preparation of dispersed bovine fasciculata cells: Bovine adrenocortical fasciculata cells were prepared by tryptic digestion by the method described previously (19, 20).

Incubation: The cell suspensions (approximately 10⁵ cells per each incubation volume of 0.5 ml) were incubated at 37°C under 95% O₂–5% CO₂ as the gas phase with or without test substances. Incubation was

routinely continued for 1 to 3 hr in a dark room. Diazepam and (+)-PN200-110 were dissolved in ethanol, and other chemicals were dissolved in the ordinary buffer for cell suspensions as described elsewhere (19, 20). The final concentration of ethanol in the incubation medium was less than 0.2%, a concentration which was shown to exert no demonstrable effect on corticoidogenesis.

Analytical methods: Corticoids were extracted from whole cell suspensions with dichloromethane and measured fluorometrically by the method of Silber et al. using cortisol as the standard (21). Cellular free cholesterol and cholesteryl esters were assayed by the enzymatic method of Gamble et al. (22).

Chemicals: The sources of the various chemicals employed in this study were as fol-

Fig. 1. Effect of diazepam on the corticoidogenic response to Bt₂cAMP, KCl and ACTH. Dispersed cells prepared from bovine adrenal fasciculata were preincubated at 37°C for 10 min with the indicated concentrations of diazepam, and the reaction was started by adding the steroidogenic agents shown. The concentrations of the agents used were 0.5 mM Bt₂cAMP, 10 mM KCl and 100 pM ACTH. After 3 hr-incubation, corticoids were extracted with dichloromethane from the cell suspensions and estimated fluorometrically. Values are the mean±S.E. of triplicate determinations.

lows: Dibutyl cyclic AMP ($N^6,2'$ -O-dibutyladenosine 3',5'-cyclicmonophosphate) was from Sigma Chemical Co.; Diazepam was from Wako Pure Chemical Industry, Japan; (+)-PN200-110 was kindly donated by Drs. R. Viret and F. Mitterhofer (Sandoz); ML-236B and YM-684 (Ro 15-1788) were gifts from Sankyo Co., Japan and Yamanouchi Pharmaceutical Co., Japan, respectively. All other chemicals were reagent grade or better.

Results

Effect of diazepam on the corticoidogenic response of bovine adrenocortical cells to Bt_2 cAMP, KCl and ACTH: As shown in Fig. 1, diazepam potentiated the Bt_2 cAMP-induced corticoidogenesis in a dose-dependent man-

ner, but was without effect on the corticoidogenesis induced by either ACTH (100 pM) or a high concentration of KCl (10 mM). In the absence of steroidogenic agents, no effect by diazepam alone (up to 100 μ M) on the corticoidogenesis was observed in these experiments.

Effect of diazepam on Bt_2 cAMP-induced corticoidogenesis: It can be seen from Fig. 2 that diazepam (100 μ M) potentiates the dose-dependent stimulation of the corticoidogenic response to Bt_2 cAMP in bovine adrenal fasciculata cells.

Figure 3 shows the time-course of the corticoidogenic response to 0.5 mM Bt_2 cAMP with or without 100 μ M diazepam. Bt_2 cAMP caused a linear increment of the corticoid production until 90 min, and after then, the Bt_2 -

Fig. 2. Effect of diazepam on Bt_2 cAMP-induced corticoidogenesis. Fasciculata cells were preincubated at 37°C for 10 min with (●) or without (○) diazepam (100 μ M), and this was followed by a 2 hr-incubation with Bt_2 cAMP (indicated concentrations). After incubation, corticoids were extracted with dichloromethane from cell suspensions and estimated fluorometrically. Each value represents the mean $\pm$ S.E. of triplicate determinations of two separate experiments.

Fig. 3. Time-course of corticoidogenic response to Bt_2cAMP with or without diazepam. Fasciculata cells were preincubated at $37^\circ C$ for 10 min with (●) or without (○) $100 \mu M$ diazepam, and the reaction was started by adding $0.5 mM$ Bt_2cAMP . Samples of cell suspensions were taken at the indicated time for determination of corticoids. Values are the mean $\pm$ S.E. of triplicate determinations of two separate experiments.

cAMP-induced corticoidogenesis was prevented. However, in the presence of $100 \mu M$ diazepam, the corticoidogenic response to Bt_2cAMP continued to increase even after 90 min incubation.

Effect of diazepam on intracellular content of cholesteryl esters: Figure 4 shows that diazepam ($100 \mu M$) does not cause a significant decrease of intracellular content of cholesteryl esters under the same experimental condition as shown in Fig. 3. Similarly, no significant difference of the free cholesterol content was observed in the presence or absence of diazepam (data not shown).

Influence of several antagonists on poten-

tiating effect of diazepam: The experiments shown in Fig. 5 were designed to examine whether or not the potentiation by diazepam on Bt_2cAMP -induced corticoidogenesis is due to the interaction of diazepam with its specific receptor. The potentiating effect by diazepam is blocked by YM-684, a diazepam receptor antagonist (23).

ML-236B is known to be potent inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase), which is the rate limiting enzyme in cholesterol *de novo* synthesis (24). As shown in Fig. 6, this inhibitor abolished the potentiating effect of diazepam on Bt_2cAMP -induced corticoido-

Fig. 4. Effect of diazepam on intracellular content of cholesteryl esters during Bt_2cAMP -induced corticoidogenesis. Fasciculata cells were preincubated at $37^\circ C$ for 10 min with (●) or without (○) $100 \mu M$ diazepam, and the reaction was started by adding $0.5 \text{ mM } Bt_2cAMP$. Samples of cell suspensions were taken at the indicated time for determination of cholesteryl esters as described under "Materials and Methods". Values are the mean $\pm$ S.E. of triplicate determinations of three separate experiments.

genesis.

When the cells were incubated with (+)-PN200-110, a potent inhibitor of voltage-dependent Ca channels (25), Bt_2cAMP -induced corticoidogenesis was inhibited in a dose-dependent manner. However, as shown in Fig. 7, the potentiation by diazepam was not affected by this antagonist.

Discussion

In the present study, we have investigated the possible role of peripheral type benzodiazepine receptors in the regulation of adrenal glucocorticoid synthesis. Although the tissue distribution and biochemical properties of the peripheral type receptor have been examined, the precise physiological function of this receptor has not yet been elucidated. Peripheral type benzodiazepine receptors have

Fig. 5. Effect of YM-684 on the potentiating effect of diazepam during corticoidogenic response to $0.5 \text{ mM } Bt_2cAMP$. Fasciculata cells were preincubated at $37^\circ C$ for 10 min with (●) or without (○) diazepam ($100 \mu M$) and YM-684 (indicated concentrations), and the reaction was started by adding $0.5 \text{ mM } Bt_2cAMP$. Corticoids were extracted with dichloromethane from the cell suspensions after a 2 hr incubation and measured fluorometrically. Each value represents the mean $\pm$ S.E. of triplicate determinations of two separate experiments.

been distinguished according to their ligand specificities (1, 26). Certain benzodiazepines (e.g., Ro 5-4864 and PK11195) are high affinity ligands for the peripheral type benzodiazepine receptor (11–13), but have no pharmacological activity in the modulation of the $GABA_A$ -regulated chloride channels linked to central type benzodiazepine receptor (9, 10). By using [3H]Ro 5-4864, peripheral type benzodiazepine receptors have been detected in a number of tissues, especially in the adrenal cortex (13). The binding of these specific ligands is displaced by dihydropyridine calcium channel blockers, suggesting that one of the possible functions of the peripheral type receptor is to be coupled to voltage-dependent calcium channels (27–29). In adrenal glomerulosa cells, Shibata et al. reported that peripheral type benzodiazepine

Fig. 6. Effect of ML-236B on the potentiating effect of diazepam during corticoidogenic response to 0.5 mM Bt_2cAMP . Fasciculata cells were preincubated at 37°C for 10 min with (●) or without (○) diazepam (100 μM) and ML-236B (indicated concentrations), and the reaction was started by adding 0.5 mM Bt_2cAMP . Corticoids were extracted with dichloromethane from the cell suspensions after a 2 hr-incubation and measured fluorometrically. Each value represents the mean ± S.E. of triplicate determinations of two separate experiments.

Fig. 7. Effect of (+)-PN200-110 on the potentiating effect of diazepam during corticoidogenic response to 0.5 mM Bt_2cAMP . Fasciculata cells were preincubated at 37°C for 10 min with (●) or without (○) diazepam (100 μM) and (+)-PN200-110 (indicated concentrations), and the reaction was started by adding 0.5 mM Bt_2cAMP . After a 2 hr-incubation, corticoids were extracted with dichloromethane from the cell suspensions and estimated fluorometrically. Each value represents the mean ± S.E. of triplicate determinations of two separate experiments.

pine receptors have an inhibitory role in the regulation of aldosterone synthesis by interacting with voltage-dependent calcium channels (14). However, the experiments shown in Fig. 7 indicate that in adrenal fasciculata cells, voltage-dependent calcium channels are not involved in the potentiating effect of diazepam on Bt_2cAMP -induced corticoidogenesis.

It is obscure at present why diazepam is not able to potentiate the corticoidogenic responses to ACTH and KCl as shown in Fig. 1. However, a possible explanation for these discrepancies is as follows: Especially in "bovine" adrenal fasciculata cells, either 100 pM ACTH or 10 mM KCl appears to stimulate corticoidogenesis via a mechanism dependent on calcium ions but not on cyclic AMP (19, 20). Indeed, ACTH (100 pM) increases an intracellular cyclic AMP also in the "bovine"

cells, but the increased concentration of cellular cyclic AMP is one-thousand fold less than that by Bt_2cAMP used in the present study (20). Thus, the stimulatory mechanism of exogenously added Bt_2cAMP on corticoidogenesis might be different from those of ACTH and KCl.

In an attempt to elucidate the mechanism of action of diazepam on the potentiating effect, the intracellular cholesterol dynamics under the corticoidogenic response to Bt_2cAMP plus diazepam were observed. We have purified a novel protein termed des-endozepine whose synthesis is tightly coupled to acute adrenal steroidogenesis (15, 16), (K. Yanagibashi et al., unpublished data). This protein appears to bind to the adrenal peripheral type benzodiazepine receptor (30)

and stimulate cholesterol delivery to the inner mitochondrial membrane (15). Studies are now being undertaken in this laboratory to determine the precise mechanism of des-endozepine on cholesterol mobilization and also the interrelation between this protein and benzodiazepines. Our results shown in Figs. 4 and 6 suggest that the potentiating effect of diazepam is due in part to the activation of the intracellular cholesterol supply system (cholesterol *de novo* synthesis). This is in good agreement with the results reported by Sekimoto et al. (31) that the corticoidogenic action of Bt_2cAMP in bovine adrenal fasciculated cells is mediated by the activation of cholesteryl ester hydrolase, and the derived free cholesterol from the cholesteryl esters is one of the essential sources for the substrate of corticoidogenesis within the 90 min-incubation. By contrast, it is likely that the activation of cholesterol *de novo* synthesis is a possible site of the potentiating action of diazepam (Fig. 6).

In order to determine whether or not the potentiating action of diazepam is mediated by a specific interaction with benzodiazepine receptors, we evaluated the effect of the diazepam antagonist YM-684 (Ro 15-1788) on the diazepam-induced potentiation. As shown in Fig. 5, the potentiating effect of diazepam is inhibited by YM-684 in a concentration range ($\approx \mu M$) that is consistent with its pharmacological effect on the rat hippocampal slice (32, 33). These results suggest that the antagonist not only binds to the central type receptor with high affinity but may also interact less strongly or non-selectively with the peripheral type receptor (23, 34).

The present experiments indicate that a possible role of peripheral type benzodiazepine receptors on the corticoidogenic response to Bt_2cAMP is in the activation steps of the intracellular cholesterol supply system without any functional coupling to voltage-dependent calcium channels.

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