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Potentiation of Proopiomelanocortin Gene Expression in Cultured Pituitary Cells by Benzodiazepines

Kazuhiko Fukuda, M.D., Ph.D., Nobuo Uetsuki, M.D.,† Hisatoshi Uga, M.D.,‡ Mitsuko Hashiguchi, M.D.,† Masami Sato, M.D.,† Taizo Hisano, M.D.,§ Hajime Segawa, M.D., Ph.D.,‖ Yasumasa Iwasaki, M.D., Ph.D.#*

Background: **Benzodiazepines are frequently used not only as a part of general anesthesia but also for the purpose of sedation during regional anesthesia. Effects of these drugs on the hypothalamic-pituitary-adrenal axis activity have been studied, but are still controversial. It is not known whether benzodiazepines affect expression of proopiomelanocortin, precursor protein of adrenocorticotropic hormone and related peptides.**

Methods: **AtT20PL cell line, a clone of AtT20/D16v mouse corticotroph tumor cells stably transfected with approximately 0.7 kilobases (kb) of the rat proopiomelanocortin 5' promoter-luciferase fusion gene, was used. In the presence or absence of diazepam or midazolam, cells were stimulated by corticotropin-releasing hormone (CRH) or forskolin. Proopiomelanocortin gene expression was estimated by measurement of luciferase activity. Furthermore, to study the mechanism of benzodiazepine effects, cyclic adenosine 3',5'-monophosphate (cyclic AMP) efflux was measured by enzymeimmunoassay.**

Results: **Diazepam and midazolam dose-dependently increased the proopiomelanocortin gene expression induced by CRH or forskolin. The potentiating effect was not affected by benzodiazepine receptor antagonists flumazenil and PK11195, but was abolished by a cyclic AMP-dependent protein kinase inhibitor H89. Cyclic AMP efflux induced by CRH or forskolin was also enhanced by diazepam and midazolam. In the presence of isobutylmethylxanthine, a nonspecific phosphodiesterase inhibitor, potentiation of proopiomelanocortin gene expression and enhancement of cyclic AMP efflux by benzodiazepines were not observed.**

Conclusions: **Benzodiazepines potentiate the effect of CRH or forskolin on proopiomelanocortin gene expression. The potentiating effect is not mediated by the benzodiazepine receptors, but its mechanism probably involves inhibition of phosphodiesterase.**

THE hypothalamic-pituitary-adrenal (HPA) axis plays a central role in maintaining the homeostasis of an organism against various stressful stimuli, including surgical noxious stimulations.[1,2] When an organism suffers stressful stimuli, corticotropin-releasing hormone (CRH) is released from the hypothalamus to stimulate the corticotrophs in the anterior pituitary, resulting in secretion of adrenocorticotropic hormone (ACTH). The ACTH, in turn, induces adrenal glucocorticoid release, which exhibits diverse systemic actions against stresses. Among the HPA axis, ACTH secretion is controlled by a variety of secretagogues, and the control mechanism has been extensively studied.[3] At the cellular level, ACTH, stored in intracellular pool is secreted by elevation of cytosolic Ca^{2+} concentration induced by activation of voltage-gated Ca^{2+} channels or intracellular Ca^{2+} release.[4] Moreover, most of the secretagogues are supposed to be involved in the regulation of ACTH production.[5] The ACTH production is regulated by CRH, vasopressin, catecholamines, and cytokines through the modulation of the gene expression of proopiomelanocortin a precursor protein of ACTH and related peptides.[6–9]

Control of excessive responses to surgical stresses is one of the objectives of anesthesia. Effects of general and regional anesthesia on the surgical stress responses, including those mediated by HPA axis, have been investigated.[10,11] Benzodiazepines, including diazepam and midazolam, are widely used in clinical anesthesia for the purpose of premedication, induction and maintenance of general anesthesia, and sedation during regional anesthesia. However, the effect of benzodiazepines on stress responses has been controversial. It was reported that benzodiazepines significantly inhibited the ACTH and cortisol secretion after CRH administration in normal subjects,[12] and attenuated the stress-induced release of corticosterone and catecholamine.[13] In contrast, Vargas *et al.* showed that diazepam can stimulate basal HPA axis activity.[14] Whether benzodiazepines affect ACTH synthesis or secretion in anterior pituitary cells has not been examined at the cellular or molecular level.

In this investigation, to elucidate the effects of benzodiazepines on ACTH synthesis in anterior pituitary cells, we have analyzed the effect of benzodiazepines, diazepam and midazolam, on the proopiomelanocortin gene expression in AtT20PL cells, a clone of AtT20/D16v mouse corticotroph tumor cells stably transfected with approximately 0.7 kilobases (kb) of the rat proopiomelanocortin 5'-promoter-luciferase fusion gene. By the use of these cells, we could analyze the level of proopiomelanocortin gene expression and elucidate the mechanism of its regulation.

Materials and Methods

Materials

Culture medium was purchased from Sigma (St. Louis, MO), and fetal bovine serum was from ICN Biomedicals (Aurora, OH). Rat CRH was purchased from Peptide Institute (Osaka, Japan). Luciferase Assay System and

* Professor and Chair, Department of Anesthesia, ‡ Instructor, Department of Anesthesia, § Clinical Fellow, Department of Anesthesia, ‖ Lecturer, Division of Critical Care Medicine, Kyoto University Hospital, † Graduate Student, Department of Anesthesia, Kyoto University Graduate School of Medicine, Kyoto, # Lecturer, Department of Clinical Laboratory Medicine, Nagoya University School of Medicine, Nagoya, Japan.

From the Department of Anesthesia, Kyoto University Hospital, Shogoin, Sakyo-ku, Kyoto, Japan. Submitted for publication September 11, 2002. Accepted for publication January 7, 2003. Supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Tokyo, Japan.

Address reprint requests to Dr. Fukuda: Department of Anesthesia, Kyoto University Hospital, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Address electronic mail to: kfukuda@kuhp.kyoto-u.ac.jp. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

Reporter Lysis Buffer were from Promega (Pittsburgh, PA). Midazolam and flumazenil were purchased from Yamanouchi (Osaka, Japan), and diazepam was from Takeda (Osaka, Japan). PK11195 was from Calbiochem (San Diego, CA). Cyclic adenosine 3',5'-monophosphate (cyclic AMP) assay kit was from Amersham Pharmacia (Buckinghamshire, England). All the other reagents were obtained from Wako (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Sigma.

Isobutylmethylxanthine was dissolved in ethanol (final concentration 0.1%), and the other drugs were diluted with the culture medium to adjust adding volume to 20 μl.

Cell Culture

Establishment of AtT20PL cell line was previously described.[6] Briefly, AtT20/D16v mouse corticotroph cells were stably cotransfected with the plasmid pA3Luc containing a fragment (approximately 0.7 kb) of the rat proopiomelanocortin gene 5'-promoter fused with luciferase gene and the plasmid pRSV-Neo. Neomycin-resistant clones were screened in the medium containing G418, and a representative clone AtT20PL was selected.

AtT20PL cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, in the humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Culture medium was changed every two days. For all experiments, AtT20PL cells were plated in six-well culture dishes (Becton Dickinson, Franklin Lakes, NJ) with approximately 60% confluency and were cultured in DMEM supplemented with 1% fetal bovine serum (low-serum medium) for four days.

In each experiment, two wells were similarly treated, luciferase activity or cyclic AMP efflux was determined separately from the two wells, and the two values were averaged. Independent experiments, the numbers of which are indicated in the figure legends, were repeated using separately prepared cell cultures.

Luciferase Assay

After 4-day incubation in the low-serum medium, incubation medium was replaced with 2 ml/well of fresh low-serum medium, and the cells were stimulated with CRH or forskolin for 5 h with or without simultaneous addition of benzodiazepines and inhibitors. After stimulation, cells were washed twice with ice-cold phosphate-buffered saline (PBS), and cell lysate was prepared with 200 μl/well of Reporter Lysis Buffer. The luciferase activity in the cell lysate was measured by the use of the Luciferase Assay System and the luminometer Lumat LB9507 (Berthold, Victoria, Australia), and was shown in relative luciferase unit (RLU).

Cyclic AMP Assay

After 4-day incubation in the low-serum medium, incubation medium was replaced with 2 ml/well of fresh serum-free DMEM, and then the cells were stimulated with CRH or forskolin for 3 h with or without simultaneous addition of benzodiazepines. After stimulation, culture medium in each well was collected. The cyclic AMP concentration in the collected medium was determined by the use of the cyclic AMP enzymeimmunoassay system[15] (Amersham Pharmacia), which combines the use of a peroxidase-labeled cyclic AMP conjugate and a specific anticyclic AMP antiserum immobilized to microtiter plates.

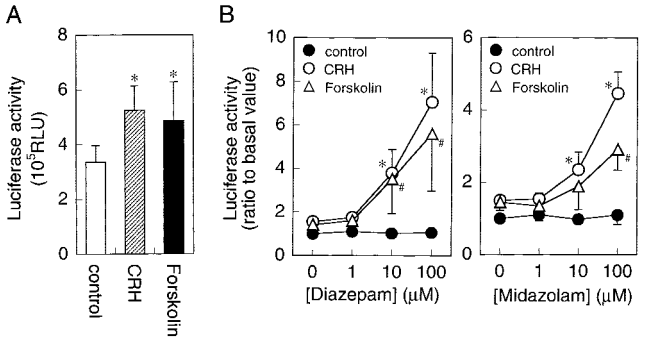

Fig. 1. Effects of benzodiazepines on the proopiomelanocortin gene expression. (*A*) Effects of corticotropin-releasing hormone (CRH) and forskolin. Cells were stimulated with 1 μM CRH or 10 μM forskolin. Luciferase activity is expressed as mean ± SD from eight experiments. *$P < 0.05$ *versus* nonstimulated control. (*B*) Dose-dependent potentiation by benzodiazepines. In the presence of diazepam or midazolam, cells were stimulated with 1 μM CRH or 10 μM forskolin. The ratios of luciferase activity to that of nonstimulated control are expressed as means ± SD from five experiments. *$P < 0.05$ *versus* cells stimulated with CRH without benzodiazepines. #$P < 0.05$ *versus* cells stimulated with forskolin without benzodiazepines.

Statistical Analysis

Data are expressed as means ± SD. Statistical analyses of data were performed by one-way analysis of variance, followed by Fisher's protected least significance difference test. P values < 0.05 were considered statistically significant.

Results

Potentiation of Proopiomelanocortin Gene Expression by Benzodiazepines

As representative secretagogues, we chose CRH, a physiologic stimulus for ACTH secretion, and forskolin, a direct activator of adenylate cyclase. As described previously,[6,16] CRH and forskolin significantly induced proopiomelanocortin gene expression, detected as luciferase activity, in AtT20PL cells (fig. 1A). Stimulation of the cells with 1 μM CRH and 10 μM forskolin for 5 h resulted in elevation of proopiomelanocortin expression by 50 ± 9% (n = 8) and 45 ± 23% (n = 8) of the basal level, respectively. Previous reports[6,16] indicated that these concentrations of the secretagogues produce nearly maximal responses. Therefore, we used CRH and forskolin of these concentrations in the following experiments.

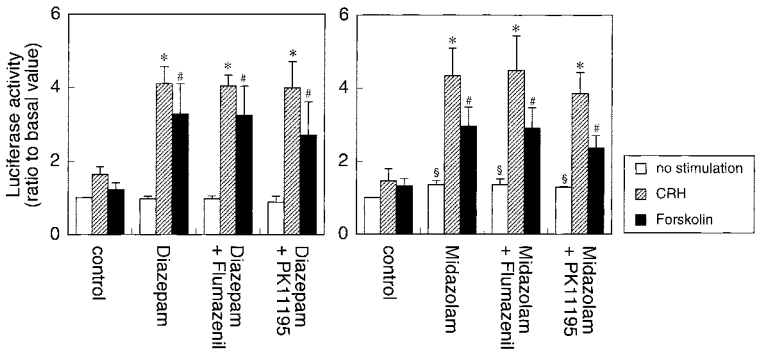

Fig. 2. Effects of benzodiazepine antagonists on proopiomelanocortin gene expression. In the presence or absence of 10 μM flumazenil or 10 μM PK11195, cells were treated with 10 μM diazepam or 100 μM midazolam, and stimulated with 1 μM corticotropin-releasing hormone (CRH) or 10 μM forskolin. The ratios of luciferase activity to that of nonstimulated control are expressed as means ± SD from four experiments. *$P < 0.05$ *versus* cells stimulated with CRH without benzodiazepines. #$P < 0.05$ *versus* cells stimulated with forskolin without benzodiazepines. §$P < 0.05$ *versus* nonstimulated controls.

Figure 1B demonstrates the effect of diazepam and midazolam on the proopiomelanocortin expression in AtT20PL cells. In the absence of CRH or forskolin, the effect of diazepam and midazolam on the proopiomelanocortin expression level was not significant. However, CRH-induced proopiomelanocortin expression was significantly increased by diazepam and midazolam at 10 and 100 μM. Forskolin-induced proopiomelanocortin expression was also enhanced by diazepam (10 and 100 μM) and midazolam (100 μM). Thus, benzodiazepines exhibited dose-dependent potentiating effects on the CRH- or forskolin-stimulated proopiomelanocortin gene expression in AtT20PL cells.

Effects of Benzodiazepine Antagonists

A number of pharmacological effects of benzodiazepines are mediated by the central[17] and the peripheral[18] benzodiazepine receptors. To analyze the involvement of the benzodiazepine receptors in the potentiation of the proopiomelanocortin expression by diazepam and midazolam, we tested the effect of benzodiazepine antagonists on the proopiomelanocortin expression in AtT20PL cells (fig. 2). Flumazenil[19] (10 μM) and PK11195[20] (10 μM), antagonists for the central and the peripheral benzodiazepine receptor, respectively, do not significantly affect the CRH- or forskolin-induced proopiomelanocortin expression in the presence of benzodiazepines. In this experiment, midazolam (100 μM) induced a small but significant increase in luciferase activity in the absence of CRH or forskolin, which also was not affected by benzodiazepine antagonists.

Involvement of Cyclic AMP/Cyclic AMP-dependent Protein Kinase Pathway

It has been demonstrated that CRH and forskolin induce expression of proopiomelanocortin by increasing intracellular cyclic AMP level and activating cyclic AMP-dependent protein kinase (PKA).[6] To explore the possibility that potentiating effect of diazepam and midazolam is mediated by cyclic AMP/PKA pathway, we tested the effect of H89, a specific inhibitor of PKA (fig. 3). As shown previously,[6] the stimulatory effect of CRH on proopiomelanocortin expression was completely abolished by H89 (30 μM). Furthermore, figure 3 demonstrates that proopiomelanocortin expression by CRH was not significantly enhanced by diazepam and midazolam in the presence of H89.

Effect of Benzodiazepines on CRH-stimulated Proopiomelanocortin Expression under Isobutylmethylxanthine Treatment

To further clarify the mechanism of the enhancing effect of benzodiazepines, we tested the effect of diazepam and midazolam on proopiomelanocortin gene expression stimulated by CRH and forskolin in cells treated with isobutylmethylxanthine (200 μM), a nonselective phosphodiesterase inhibitor[21] (fig. 4). Isobutylmethylxanthine itself did not significantly induce proopiomelanocortin expression, but enhanced the response to CRH and forskolin by 7.8 ± 1.9-fold (n = 6) and 9.6 ± 2.2-fold (n = 6), compared with the response in cells without isobutylmethylxanthine treatment, respectively. However, in the presence of isobutylmethylxanthine,

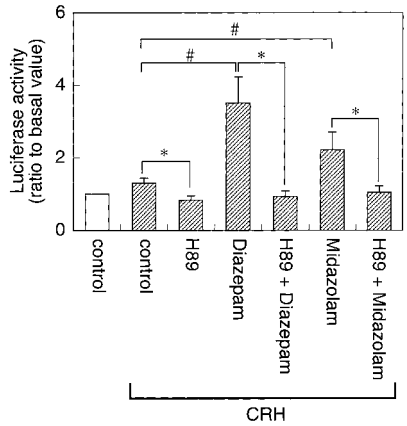

Fig. 3. Effects of H89, a cyclic adenosine 3',5'-monophosphate-dependent protein kinase inhibitor, on the proopiomelanocortin gene expression. In the presence or absence of 30 μM H89, cells were treated with 10 μM diazepam or 10 μM midazolam, and stimulated with 1 μM corticotropin-releasing hormone (CRH). The ratios of luciferase activity to that of nonstimulated control cells are expressed as means ± SD from seven experiments. *$P < 0.05$ *versus* cells stimulated with CRH without H89. #$P < 0.05$ *versus* cells stimulated with CRH without benzodiazepines and H89.

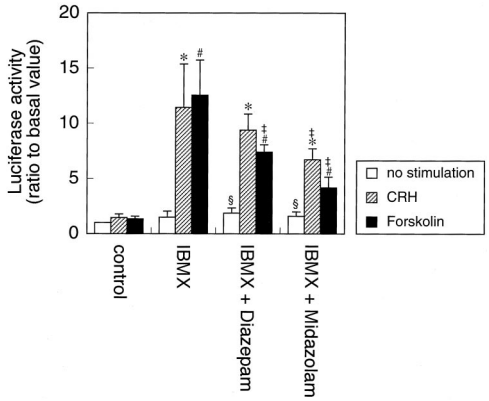

Fig. 4. Effects of isobutylmethylxanthine (IBMX) on the proopiomelanocortin gene expression. In the presence of 200 μM IBMX, cells were treated with 100 μM diazepam or 100 μM midazolam, and stimulated with 1 μM corticotropin-releasing hormone (CRH) or 10 μM forskolin. The ratios of luciferase activity to that of nonstimulated controls are expressed as means ± SD from six experiments. *$P < 0.05$ *versus* cells stimulated with CRH without benzodiazepines and IBMX. #$P < 0.05$ *versus* cells stimulated with forskolin without benzodiazepines and IBMX. §$P < 0.05$ *versus* nonstimulated controls. ‡$P < 0.05$ *versus* cells stimulated by each secretagogue without benzodiazepines in the presence of IBMX.

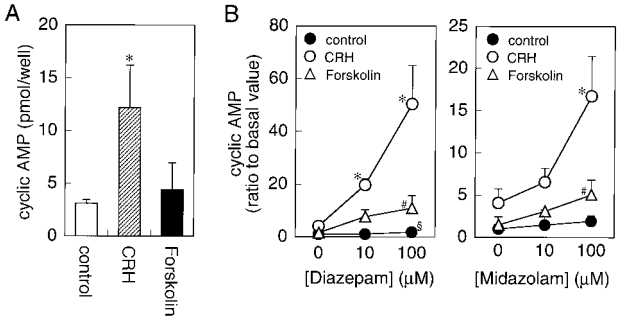

Fig. 5. Effects of benzodiazepines on the cyclic adenosine 3',5'-monophosphate (cyclic AMP) efflux. (*A*) Effects of corticotropin-releasing hormone (CRH) and forskolin. Cells were stimulated with 1 μM CRH or 10 μM forskolin. Cyclic AMP efflux is expressed as means ± SD from four experiments. *$P < 0.05$ *versus* nonstimulated control. (*B*) Benzodiazepines dose-dependently increase cyclic AMP efflux induced by CRH or forskolin. In the presence of diazepam or midazolam, cells were stimulated with 1 μM CRH or 10 μM forskolin. The ratios of cyclic AMP efflux to that of nonstimulated controls are expressed as means ± SD from four experiments. *$P < 0.05$ *versus* cells stimulated with CRH without benzodiazepines. #$P < 0.05$ *versus* cells stimulated with forskolin without benzodiazepines. §$P < 0.05$ *versus* nonstimulated controls.

neither diazepam (100 μM) nor midazolam (100 μM) exhibited potentiating effect on proopiomelanocortin gene expression. Unexpectedly, the CRH- or forskolin-stimulated proopiomelanocortin expression was significantly reduced by benzodiazepines.

Effect of Benzodiazepines on Cyclic AMP Efflux

To clarify the effect of benzodiazepines on the cyclic AMP regulation in AtT20PL cells, we next estimated the cyclic AMP efflux in the presence and absence of benzodiazepines. As shown previously,[6] CRH (1 μM) significantly increased cyclic AMP efflux (fig. 5A). Diazepam (10 and 100 μM) and midazolam (100 μM) significantly enhanced the CRH response (fig. 5B). In the absence of benzodiazepines, forskolin (10 μM) showed marginal increase in cyclic AMP efflux (fig. 5A). However, in the presence of diazepam (100 μM) or midazolam (100 μM), forskolin significantly stimulated the cyclic AMP efflux. Thus, diazepam and midazolam dose-dependently enhance cyclic AMP efflux induced by CRH or forskolin.

Figure 6 demonstrates the effect of isobutylmethylxanthine on the cyclic AMP efflux. Isobutylmethylxanthine (200 μM) increased the cyclic AMP efflux by 7.3 ± 1.4-fold (n = 4), compared with the basal level. CRH and forskolin further enhanced the isobutylmethylxanthine-stimulated cyclic AMP efflux by 23.1 ± 2.5-fold (n = 4) and 5.5 ± 1.2-fold (n = 4), respectively. However, diazepam (100 μM) and midazolam (100 μM) did not further increase CRH- or forskolin-stimulated cyclic AMP efflux in the presence of isobutylmethylxanthine. The forskolin-stimulated cyclic AMP efflux was significantly reduced by benzodiazepines.

Discussion

Although benzodiazepines are frequently used in clinical anesthesia, the effects of benzodiazepines on HPA-mediated stress responses induced by various stimuli, including surgical noxious stimulation, are not completely understood. In this investigation, to elucidate the effects of benzodiazepines on HPA axis, we examined the effects of benzodiazepines on the proopiomelanocortin gene expression in AtT20PL cells. The results show that benzodiazepines potentiate the effect of CRH or forskolin on proopiomelanocortin gene expression.

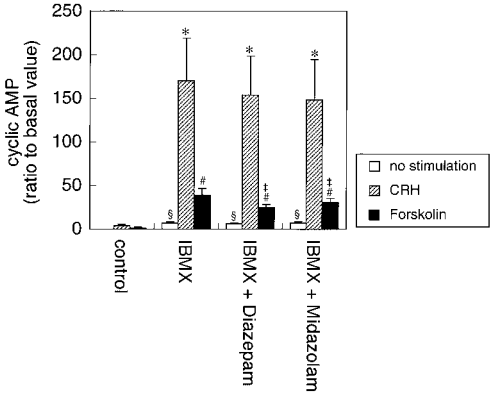

Fig. 6. Effects of isobutylmethylxanthine (IBMX) on the cyclic adenosine 3',5'-monophosphate (cyclic AMP) efflux. In the presence of 200 μM IBMX, cells were treated with 100 μM diazepam or 100 μM midazolam, and stimulated with 1 μM corticotropin-releasing hormone (CRH) or 10 μM forskolin. The ratios of cyclic AMP efflux to that of nonstimulated controls are expressed as means ± SD from four experiments. *$P < 0.05$ *versus* cells stimulated with CRH without benzodiazepines and IBMX. #$P < 0.05$ *versus* cells stimulated with forskolin without benzodiazepines and IBMX. §$P < 0.05$ *versus* nonstimulated controls. ‡$P < 0.05$ *versus* cells stimulated by each secretagogue without benzodiazepines in the presence of IBMX.

The AtT20 cells (mouse anterior pituitary tumor cell line) have been widely used to study regulation of secretion of ACTH and β-endorphin, because these cells faithfully model corticotroph cells.[22] In the field of anesthesiology, it was shown that propofol suppresses β-endorphin secretion from AtT20 cells and was suggested that propofol inhibits neuropeptide exocytosis.[23] We have used AtT20PL cells, a clone of AtT20 cells stably transfected with approximately 0.7 kb of the rat proopiomelanocortin 5'-promoter-luciferase fusion gene.[24] AtT20PL cells have enabled us to easily estimate the level of proopiomelanocortin gene expression by measurement of luciferase activity without assessing the amount of proopiomelanocortin messenger RNA by Northern blotting. Amount of proopiomelanocortin messenger RNA and protein may be affected also by a number of factors other than the promoter activity, including regulatory elements of the gene, degradation of messenger RNA, and posttranslational modifications. However, the luciferase assay is appropriate for assessing promoter activity, which determines the speed of gene transcription and is regulated by various factors. By the use of AtT20PL cells, the mechanism of regulation of proopiomelanocortin gene expression by physiologic secretagogues and cytokines have been investigated.[6,7,16]

It is known that benzodiazepines, including diazepam and midazolam, bind with the central benzodiazepine receptor, which constitutes a part of the γ-aminobutyric acid type A ($GABA_A$) receptor located in the central nervous system, and produce a facilitatory effect on the $GABA_A$ receptor channel, resulting in their sedative, hypnotic and anxiolytic effects.[17] Benzodiazepines also bind with peripheral benzodiazepine receptors, located mainly in peripheral tissues including adrenal gland and kidney, and are suggested to affect mitochondrial cholesterol transport.[18] In this study, we demonstrate that the potentiating effect of diazepam and midazolam on the proopiomelanocortin gene expression was affected by neither flumazenil nor PK11195, antagonists for the central and peripheral benzodiazepine receptor, respectively. These results indicate that the effect of diazepam and midazolam on proopiomelanocortin gene expression is mediated by a mechanism that involves neither the central nor the peripheral benzodiazepine receptors. Biologic effects of benzodiazepines mediated by mechanisms other than the central or the peripheral benzodiazepine receptors have been described. Yamakage *et al.* demonstrated that diazepam and midazolam have inhibitory effects on the activity of voltage-dependent Ca^{2+} channels in canine tracheal smooth muscle cells, which was not affected by flumazenil and PK11195.[25] Furthermore, we have recently reported that midazolam, but not diazepam, induces expression of immediate early gene products c-Fos and EGR-1, by activation of extracellular signal-regulated kinases through a mechanism independent of the central or the peripheral benzodiazepine receptors in PC12 cells.[26] Thus, it is likely that molecules affected by benzodiazepines other than the benzodiazepine receptors exist.

It was previously reported that a PKA inhibitor, H89, completely abolishes proopiomelanocortin gene expression and ACTH secretion induced by CRH and forskolin, suggesting that the mechanism of action of these secretagogues involves adenylate cyclase activation leading to PKA activation.[6] Our data show that potentiation of the proopiomelanocortin expression by diazepam and midazolam was also completely abolished by H89 treatment. Therefore, it is reasonable to speculate that the effect of benzodiazepines on proopiomelanocortin expression involves the cyclic AMP/PKA pathway, consistent with the benzodiazepine-induced increase in CRH- or forskolin-stimulated cyclic AMP efflux. We further demonstrate that diazepam- and midazolam-induced enhancement of the proopiomelanocortin expression and cyclic AMP efflux is not observed in the presence of isobutylmethylxanthine, a phosphodiesterase inhibitor. This result suggests that diazepam and midazolam increase cyclic AMP content by inhibiting phosphodiesterase, rather than by enhancing production of cyclic AMP, leading to PKA activation and potentiation of proopiomelanocortin expression.

Molecular biologic and pharmacologic studies have clearly established the existence of seven families of phosphodiesterase isozymes, with their differences in their tissue distribution.[27] Consistently with our conclusion, it was demonstrated that the type 4 phosphodiesterase inhibitors stimulates the HPA axis at the level of the anterior pituitary.[28,29] However, it was reported that diazepam behaves as a selective type 4 phosphodiesterase inhibitor in cardiac tissue, and that this effect is mediated neither by the central nor the peripheral benzodiazepine receptors.[30] Taking these findings into consideration, it is probable that type 4 phosphodiesterase exists in anterior pituitary cells or AtT20 cells, but the molecular identity remains to be clarified. Furthermore, the precise mechanism of phosphodiesterase inhibition by benzodiazepines should be elucidated.

Our results may suggest that benzodiazepines facilitate ACTH and cortisol secretion evoked by stress-induced CRH release *in vivo*. However, effects of benzodiazepines on CRH release from hypothalamus, which is regulated by a number of factors,[31] should also be investigated. Furthermore, sedative effects of benzodiazepines might indirectly change HPA activity. In the present study, potentiation of proopiomelanocortin gene expression and increase in cyclic AMP efflux by benzodiazepines were significant at concentrations 10–100 μM, which nearly corresponds with the reported concentration range of benzodiazepines inducing pharmacologic effects through the non-GABAergic mechanism.[25,26,32,33] This concentration range seems to be the highest level of the plasma concentration of benzodiaz-

epines obtained when these drugs are systemically administered.[34,35] However, it is possible that the effective concentration of these drugs in plasma is lower, if we take into account the fact that benzodiazepines are highly bound to plasma protein.[36] Therefore, clinical implications of our findings should be explored.

In conclusion, benzodiazepines potentiate the effect of CRH or forskolin on proopiomelanocortin gene expression by inhibiting phosphodiesterase through a mechanism independent of the benzodiazepine receptors. It is expected that our results may give a clue to elucidation of benzodiazepine effects on the HPA axis and mechanism of neuroendocrine responses to stresses.

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