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Down-regulation of benzodiazepine receptors by ethyl β-carboline-3-carboxylate in cerebrocortical neurons

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Abstract

Effect of exposure of primary cultured cerebral cortical neurons to ethyl β-carboline-3-carboxylate (β-CCE) on the function of benzodiazepine receptors was studied. Exposure of neurons to β-CCE (0.1–10 μM) decreased the binding of [^{3}H]flunitrazepam to extensively washed membrane fractions in a dose- and time-dependent manner, whereas the binding of [^{3}H]flunitrazepam to the cytosolic fractions increased (180%) under the same conditions as described above. Ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate (Ro15-1788), an antagonist of the central type of benzodiazepine receptors, completely abolished the β-CCE-induced decrease in [^{3}H]flunitrazepam binding and the IC_{50} value for [^{3}H]flunitrazepam binding to the extensively washed membrane fractions prepared from β-CCE-treated neurons was similar to that from non-treated neurons. Scatchard analysis revealed that only the B_{max} value for [^{3}H]flunitrazepam binding decreased after the exposure to β-CCE (1 μM) for 12 h, although the K_d value was not altered. These results indicate that β-CCE induces the down-regulation of benzodiazepine receptors by an increase in benzodiazepine receptor internalization.

Keywords: β-CCE (ethyl β-carboline-3-carboxylate); Benzodiazepine receptor; Down-regulation; Inverse agonist; Primary cultured neuron

1. Introduction

Chronic exposure of receptor agonists induces down-regulation of receptors (Klein et al., 1989). Down-regulation of receptors is considered to be the removal of receptors from the cell surface and their entry into the cytosolic pool, a process termed internalization or sequestration. The internalized receptors can either be recycled to emerge on the cell surface or be degraded by lysosomal enzymes. In the case of the down-regulation of benzodiazepine receptors, however, controversial data have been reported. Chronic benzodiazepine exposure causes no change (Shibla et al., 1981), increase (Distefano et al., 1979), or decrease (Rosenberg and Chiu, 1981: Miller et al., 1988) in the number of benzodiazepine receptors and/or change in the coupling between γ-aminobutyric acid (GABA) and benzodiazepine receptors (Miller et al., 1988). In addition, recent studies employing cultured neurons have demonstrated that chronic exposure of neurons to GABA results in down-regulation of the GABA/benzodiazepine receptor complex (Maloteaux et al., 1987; Roca et al., 1990; Mehta and Ticku, 1992).

Several lines of evidence reveal that the GABAergic system is a major target of benzodiazepines and β-carbolines to exhibit their pharmacological actions and that the anxiogenic, proconvulsant and convulsant effects of β-carboline, an inverse agonist for benzodiazepine receptors, result from a decrease in the central GABAergic function (Polc et al., 1981; Petersen and Jensen, 1984). Moreover, ethyl β-carboline-3-carboxylate (β-CCE) binds to benzodiazepine receptors, although its binding site is not the same one for benzodiazepine (Chiu and Rosenberg, 1985; Taguchi and Kuriyama, 1990). These data are assumed to lead to a possibility that the activation of benzodiazepine receptors by β-CCE may trigger the decrease in benzodiazepine receptors by allosteric modulation of benzodiazepine binding sites, and in turn, such down-regulation of benzodiazepine receptors causes a reduction of the GABAergic function, although the molecular mechanisms involved in these effects have not been fully clarified. Accordingly, we have examined the effect of long-term exposure of cerebral cortical neurons to β-CCE on benzodiazepine receptors using the [^{3}H]flunitrazepam binding assay.

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2. Materials and methods

2.1. Materials used

[methyl-^{3}H]Flunitrazepam (3.2 TBq/mmol) and Omnifluor were obtained from New England Nuclear (Boston, USA). β-CCE and non-labeled flunitrazepam were purchased from Research Biochemicals (Natick, USA) and Sigma Chemical Co. (St. Louis, USA), respectively. Ro15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazole[1,5-*a*][1,4]benzodiazepine-3-carboxylate) was kindly provided by Nippon Roche Research Center (Kamakura, Japan). Hazalton Research Products was a source of fetal calf and horse serums. Other chemicals used were locally available and of analytical grade.

2.2. Preparation and primary culture of cerebral cortical neurons

The isolation and primary culture of cerebral cortical neurons were carried out as described previously (Ohkuma et al., 1994a). In brief, the neopallium free of meninges was dissected from a 15-day-old fetus of a ddY strain mouse, minced, trypsinized and centrifuged. The cells thus obtained were suspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal calf serum, inoculated on a Falcon 'Primaria' dish (60 mm in diameter) pretreated with poly-L-lysine for 24 h, and cultured at 37°C for 3 days under humidified 95% air-5% CO_2. After the cells were exposed to 10 μM cytosine arabinoside for 24 h to suppress the proliferation of non-neuronal cells, the neurons were continuously cultured in DMEM containing 10% horse serum under the conditions described above and the culture medium was exchanged to a freshly prepared one every 4 days. The neurons of a 13-day-old in culture were used for the experiments. More than 95% of the cells in the primary culture have been confirmed to be neurons by an immunohistochemical approach (Kuriyama et al., 1987).

2.3. Drug treatment

To expose the neurons to β-CCE, 1 μM β-CCE was added into the culture medium and then the neurons were cultured for 3–24 h. Similarly, the neurons were treated with 1 μM flunitrazepam. When examining the effect of Ro15-1788, an antagonist of benzodiazepine receptors of the central type, on β-CCE- and flunitrazepam-induced alterations in benzodiazepine receptors, the antagonist was added to the culture medium immediately before the addition of β-CCE.

2.4. Measurement of [^{3}H]flunitrazepam binding

For the preparation of the extensively washed membrane fractions from the primary cultured neurons (Reynolds et al., 1987; Ohkuma et al., 1994b), the cells were washed 3 times with ice-cold Krebs-Ringer bicarbonate buffer (KRB; pH 7.4), scraped off from the dishes with 0.32 M sucrose, homogenized with a Teflon-glass homogenizer (1000 rpm, 10 strokes) and centrifuged (1000 × *g*, 10 min, 4°C). The resultant supernatant was centrifuged (48 000 × *g*, 4°C, 20 min) again. The pellet thus obtained was suspended in 50 mM Tris-HCl buffer (pH 7.4) with a Polytron homogenizer (set: No. 6, 30 s) and centrifuged as described above. After this procedure was repeated 5 times, the pellet was stored at −80°C for at least 24 h until experimental use.

Before the binding experiment, the frozen pellet was thawed, washed 5 times by centrifugation under the same conditions as described above, and finally resuspended with 50 mM Tris-HCl buffer. The reaction for the binding of [^{3}H]flunitrazepam was initiated by the addition of 1.0 nM [^{3}H]flunitrazepam to the reaction mixture (final assay volume: 1 ml, protein content: 400 μg/assay), and the incubation was carried out at 2°C. After incubation for 60 min, the reaction mixture was filtered through a Whatman GF/B filter followed by washing the filter with ice-cold 50 mM Tris-HCl buffer 4 times under vacuum. The filter was transferred to a scintillation counting vial containing 10 ml Triton/xylene scintillator [Triton X-100 : xylene (containing 4 g of Omnifluor/liter) = 1 : 2] for counting the radioactivity remaining on the filter. The non-specific binding was determined in the presence of 10 μM of unlabeled flunitrazepam. The specific binding was calculated by subtracting non-specific binding from total binding obtained in the absence of unlabeled flunitrazepam. To determine the kinetic parameters for [^{3}H]flunitrazepam binding to the extensively washed membrane fractions, various concentrations of [^{3}H]flunitrazepam ranging from 0.031 to 32 nM were employed. To evaluate a Scatchard plot, we have employed both the LIGAND program (Munson and Rodbard, 1980) and the non-linear regression analysis to evaluate the ligand binding data (Feldman, 1972).

The measurement of internalized (cytosolic) benzodiazepine receptors in the cultured neurons was performed according to the method of Ohmori et al. (1990) with a minor modification. In brief, the neurons were washed 3 times with ice-cold KRB, scraped off from the dishes with 50 mM Tris-HCl buffer, homogenized with a Polytron homogenizer (set: No. 6, 30 s) and centrifuged (108 000 × *g*, 120 min, 4°C), and the resultant supernatant was used as the cytosolic preparation. The reaction for the binding was initiated by the addition of 1.0 nM [^{3}H]flunitrazepam to the reaction mixture (final assay volume: 0.7 ml, protein content: 1 mg/assay), and the incubation was carried out at 2°C for 60 min. After incubation, ice-cold 50 mM Tris-HCl buffer [0.3 ml, containing 50% polyethyleneglycol and γ-globulin (25 mg/ml)] was added to the reaction mixture and allowed to stand for 5 min. The reaction mixture was filtered through a Whatman GF/B filter

followed by washing the filter 4 times with 8% polyethyleneglycol-containing ice-cold 50 mM Tris-HCl buffer under vacuum.

2.5. Protein measurement

The content of protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

2.6. Statistical analysis

The data are expressed as the means ± S.E.M. and statistical significance was analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's or Bonferroni's test as described in the legend to the figures and tables.

3. Results

3.1. Effect of β-CCE on [^{3}H]flunitrazepam binding to the extensively washed membrane fractions from primary cultured neurons

The binding of [^{3}H]flunitrazepam to the extensively washed membrane fractions showed a steep decrease after the addition of β-CCE and reached its minimal value 3 h after the initiation of the exposure to β-CCE (1 μM). Thereafter, the binding remained at this level (Fig. 1). Based on these data, we have employed 12 h as the period for exposing the neurons to β-CCE.

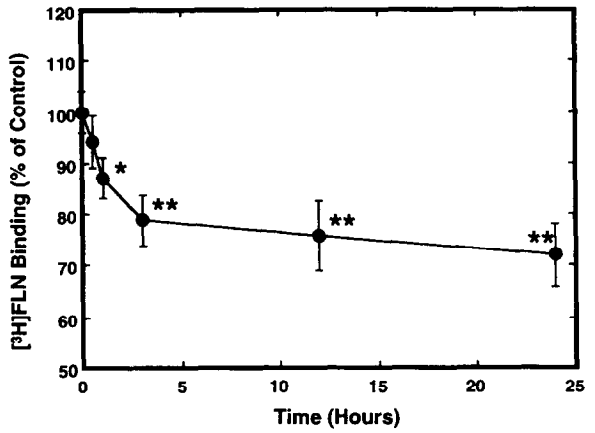

Fig. 1. Time course of changes in [^{3}H]flunitrazepam binding following exposure to β-CCE (1 μM) of primary cultured cerebral cortical neurons. The neurons were cultured with 1 μM β-CCE at 37°C for the period indicated in the figure. The extensively washed membrane fractions obtained from the neurons were incubated with 1 nM [^{3}H]flunitrazepam at 2°C for 60 min. Each point represents the mean ± S.E.M. obtained from 4 separate experiments run in triplicate. The control value for [^{3}H]flunitrazepam binding was 8790 ± 358 dpm/mg protein. * $P < 0.05$ and ** $P < 0.01$, compared with the control value obtained before the exposure to β-CCE (Dunnett's test).

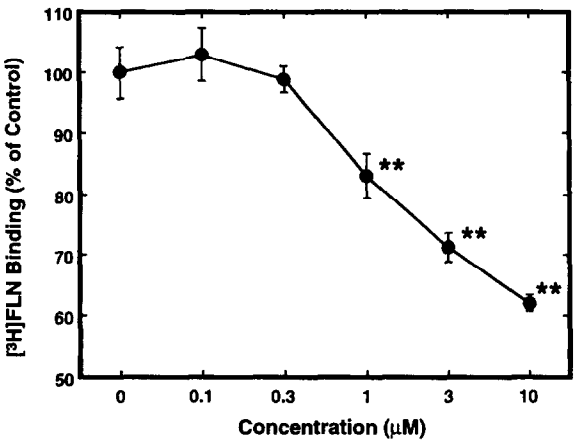

Fig. 2. Effect of β-CCE exposure of primary cultured cerebral cortical neurons on [^{3}H]flunitrazepam binding. The neurons were cultured with 1 μM β-CCE at 37°C for 12 h. The extensively washed membrane fractions obtained from the neurons were incubated with 1 nM [^{3}H]flunitrazepam at 2°C for 60 min. Each point represents the mean ± S.E.M. obtained from 4 separate experiments run in triplicate. The control value for [^{3}H]flunitrazepam binding was 9855 ± 256 dpm/mg protein. ** $P < 0.01$, compared with the control value determined in the membrane fraction from the neurons incubated in the absence of β-CCE (Dunnett's test).

The exposure of the primary cultured neurons to β-CCE (0.1–10 μM) for 12 h significantly decreased [^{3}H]-flunitrazepam binding in a concentration-dependent manner (Fig. 2). The IC_{50} value of flunitrazepam for [^{3}H]flunitrazepam binding to the β-CCE-exposed membrane fractions was almost equal to that of the control (β-CCE non-treated) (the mean IC_{50} values of the control group and β-CCE-treated group were 2.1 nM and 2.8 nM, respectively) (Fig. 3). Similar findings of the IC_{50} values of flunitrazepam for [^{3}H]flunitrazepam binding in both control and β-CCE-treated groups suggest that the decrease in [^{3}H]flunitrazepam binding after the exposure to β-CCE may be attributed to the decrease in the number of binding sites for [^{3}H]flunitrazepam and that β-CCE remaining in the membrane fractions may be negligible.

3.2. Characteristics of [^{3}H]flunitrazepam binding to the extensively washed membrane fractions from primary cultured neurons after β-CCE exposure

In order to confirm that the decrease in [^{3}H]flunitrazepam binding to the membrane fractions prepared from the primary cultured cerebral cortical neurons exposed to β-CCE for 12 h is due to the decrease either in the number of binding sites for [^{3}H]flunitrazepam and/or in the affinity for [^{3}H]flunitrazepam, a Scatchard analysis has been carried out. As shown in Fig. 4, the binding of [^{3}H]-flunitrazepam was saturable and Scatchard analysis by both the LIGAND program (Munson and Rodbard, 1980) and the non-linear regression analysis (Feldman, 1972) revealed that the plot obtained was fitted to a linear line only, that is, the membrane fractions possessed a high

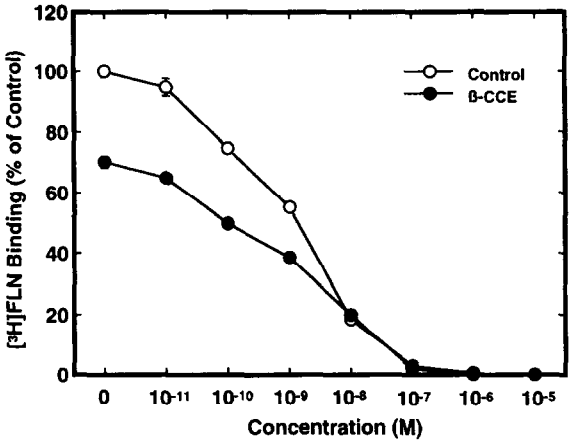

Fig. 3. Displacement of [^{3}H]flunitrazepam binding to the extensively washed membrane fractions from primary cultured cerebral cortical neurons by flunitrazepam. The neurons were cultured with 1 μM β-CCE at 37°C for 12 h. The extensively washed membrane fractions prepared from β-CCE-treated and non-treated neurons were incubated with 1 nM [^{3}H]flunitrazepam at 2°C for 60 min in the presence of various concentrations of flunitrazepam indicated in the figure. Each point represents the mean ± S.E.M. obtained from 3 separate experiments run in triplicate. The control value for [^{3}H]flunitrazepam binding determined in the absence of non-labeled flunitrazepam was 10578 ± 215 dpm/mg protein.

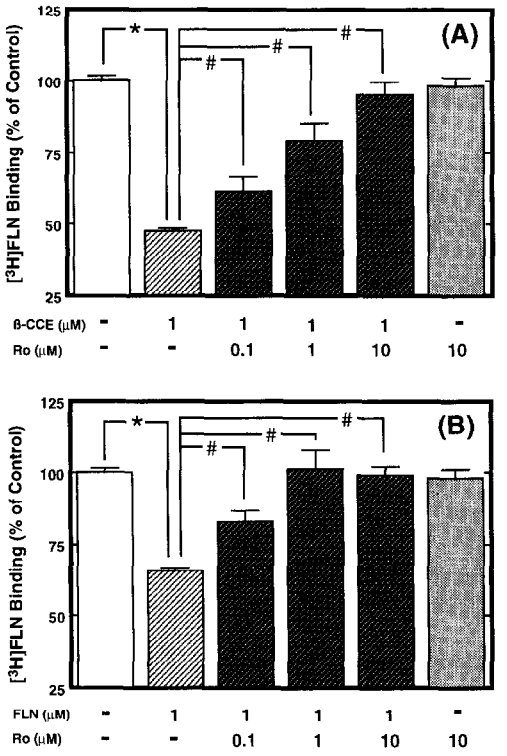

Fig. 4. Effect of Ro15-1788 on [^{3}H]flunitrazepam binding to the extensively washed membrane fractions from primary cultured cerebral cortical neurons following exposure to β-CCE (A) and flunitrazepam (B). The membrane fractions derived from the neurons exposed to β-CCE (1 μM) and flunitrazepam (1 μM) for 12 h were incubated with 1 nM [^{3}H]flunitrazepam at 2°C for 60 min. Each column represents the mean ± S.E.M. obtained from 4 separate experiments run in triplicate. The control values for [^{3}H]flunitrazepam binding were (A) 7653 ± 176 and (B) 8570 ± 351 dpm/mg protein, respectively. * $P < 0.01$ and # $P < 0.01$ (Bonferroni's test).

Table 1
Effect of short-term exposure to β-CCE on [^{3}H]flunitrazepam binding to the extensively washed membrane fractions.

Treatment	B_{max} (pmol/mg protein)	K_d (nM)
Control	1.51 ± 0.10	12.0 ± 0.91
β-CCE (1 μM)	1.06 ± 0.11 [a]	11.2 ± 1.12

The neurons were cultured with 1 μM β-CCE for 12 h. The membrane fractions obtained from the neurons were incubated with 0.031–32 nM [^{3}H]flunitrazepam at 2°C for 60 min. Each point represents the mean ± S.E.M. obtained from 4 separate experiments run in triplicate. [a] $P < 0.01$, compared with the control value obtained from the membrane fractions prepared from the neurons cultured in the absence of β-CCE (Bonferroni's test).

affinity binding site for [^{3}H]flunitrazepam and that the B_{max} value for [^{3}H]flunitrazepam binding decreased after 3 h of exposure to β-CCE (1 μM) with no alteration in the K_d value. (Fig. 4B and Table 1).

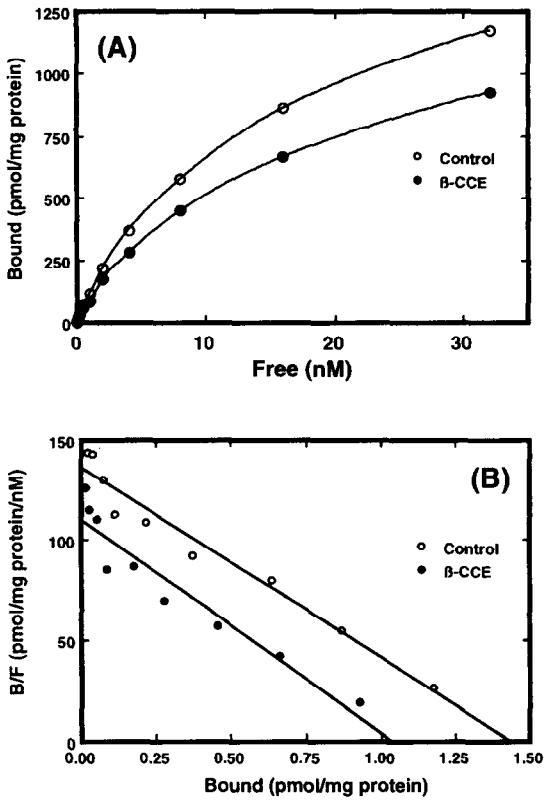

Fig. 5. Characteristics of the binding of [^{3}H]flunitrazepam to the extensively washed membrane fractions prepared from primary cultured cerebral cortical neurons exposed to 1 μM β-CCE for 12 h. (A) Saturation curve of specific binding of [^{3}H]flunitrazepam to the membrane fractions. The membrane fractions were incubated with [^{3}H]flunitrazepam (0.031–32 nM) in the presence or absence of 10 μM flunitrazepam to define non-specific binding. Each point represents the mean obtained from 4 separate experiments run in triplicate. (B) Scatchard plot of the data same as those presented in (A). The plot was analyzed using the LIGAND program (Munson and Rodbard, 1980).

3.3. Effect of Ro15-1788 on β-CCE- and flunitrazepam-induced down-regulation of [^{3}H]flunitrazepam binding to the extensively washed membrane fractions from primary cultured neurons

The β-CCE-induced decrease in [^{3}H]flunitrazepam binding was dose dependently antagonized in the concomitant presence of β-CCE and Ro15-1788 (0.1–10 μM), an antagonist specific to benzodiazepine receptors of the central type and the decrease was completely abolished by 10 μM Ro15-1788 (Fig. 5A). These results indicate that the down-regulation of [^{3}H]flunitrazepam binding induced by the short-term exposure to β-CCE is mediated by the binding of β-CCE to benzodiazepine receptors.

To confirm that the short-term occupation of benzodiazepine receptors by their ligands with agonistic properties is attributed to the decrease in benzodiazepine receptors, we used a typical agonist for the benzodiazepine receptor, flunitrazepam, to induce the down-regulation of benzodiazepine receptors. We also examined whether the down-regulation induced by such pharmacological manipulation was inhibited by Ro15-1788. The short-term (12 h) exposure of the neurons to flunitrazepam induced a decrease in the binding of [^{3}H]flunitrazepam in a time- and dose-dependent manner, which resembled those observed by the short-term exposure to β-CCE (data not shown). The decrease in [^{3}H]flunitrazepam binding induced by the exposure to flunitrazepam was dose dependently inhibited by Ro15-1788 which was concomitantly added with flunitrazepam (Fig. 5B). However, Ro15-1788 alone did not alter the binding of [^{3}H]flunitrazepam even after the neurons were incubated with this agent for 12 h (Fig. 5A and Fig. 5B).

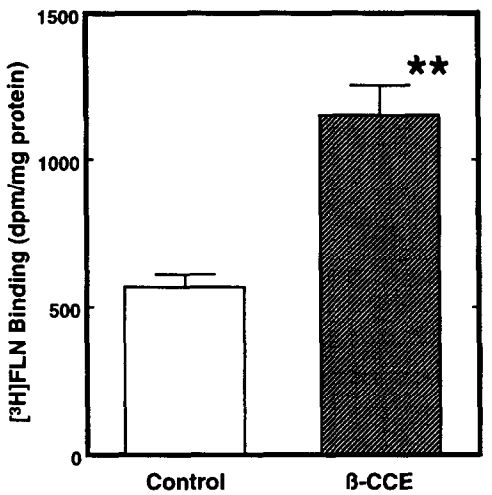

Fig. 6. Effect of β-CCE exposure on [^{3}H]flunitrazepam binding to the cytosolic preparation obtained from primary cultured cerebral cortical neurons. The cytosolic preparation obtained from primary cultured neurons exposed to 1 μM β-CCE for 12 h were incubated with 1 nM [^{3}H]flunitrazepam at 2°C for 60 min. Each column represents the mean ± S.E.M. obtained from 3 separate experiments run in triplicate. The control value for [^{3}H]flunitrazepam binding was 564 ± 44 dpm/mg protein. ** $P < 0.01$, compared with the control value (Bonferroni's test).

3.4. Effect of β-CCE on [^{3}H]flunitrazepam binding to a cytosolic preparation from primary cultured neurons

Using the cytosolic preparation derived from cerebral cortical neurons, [^{3}H]flunitrazepam binding to the cytosolic fraction was investigated after the exposure of the neurons to β-CCE. Fig. 6 shows that the [^{3}H]flunitrazepam binding to the cytosolic preparation was significantly elevated (180%) as compared with the control and this increased binding was dose dependently inhibited by Ro15-1788 (data not shown).

4. Discussion

The present study demonstrates that binding sites for [^{3}H]flunitrazepam are present in the extensively washed membrane fractions derived from the primary cultured neurons. Scatchard analysis of [^{3}H]flunitrazepam binding to the extensively washed membrane fractions revealed the existence of one class of binding sites for [^{3}H]flunitrazepam with a high affinity. In addition, the binding parameters for [^{3}H]flunitrazepam, the K_d and B_{max} values, were 12.0 ± 0.91 nM and 1.51 ± 0.10 pmol/mg protein, respectively, and were in good agreement with those in previous reports where cultured neurons were used (White et al., 1981). Although flunitrazepam, a full agonist for benzodiazepine receptors, has the ability to bind to both central (type I and II) and peripheral benzodiazepine receptor sites, the complete displacement of the binding of [^{3}H]flunitrazepam by Ro15-1788, an antagonist specific to benzodiazepine receptors of the central type, indicates that the binding sites of [^{3}H]flunitrazepam are considered to be of the central type of benzodiazepine receptors in the neurons used in the present study.

The [^{3}H]flunitrazepam binding after the exposure of the neurons to β-CCE (1 μM) for 3 h was reduced by approximately 25% when compared with that in the non-treated neurons. In addition, this decrease in [^{3}H]flunitrazepam binding after the exposure to β-CCE was time- and concentration-dependent. This down-regulation occurred even in the early stage after the exposure, whereas GABA-induced down-regulation of [^{3}H]-flunitrazepam binding in cultured cortical neurons was reported to be induced much more slowly (Mehta and Ticku, 1992). The down-regulation of [^{3}H]flunitrazepam binding induced by β-CCE presented in this study was completely antagonized by the concomitant exposure of the neurons to β-CCE and Ro15-1788 (flumazenil), an antagonist of benzodiazepine receptors of the central type, indicating that the β-CCE-induced decrease in [^{3}H]flunitrazepam binding is certainly mediated through the activation of benzodiazepine receptors of the central

type, and that the exposure to benzodiazepine receptor agonists, including inverse agonists, is involved in the down-regulation of benzodiazepine receptors in the primary cultured cerebral cortical neurons. Similarly, the decrease in [^{3}H]flunitrazepam binding was also observed in the brain prepared from a mouse treated with methyl β-carboline-3-carboxylate (Chapouthier et al., 1991).

Although the K_d value for [^{3}H]flunitrazepam binding was in the 10-nM range, high concentrations (more than 1 μM) of β-CCE were necessary to induce down-regulation of benzodiazepine receptors. These data suggest that the induction of down-regulation of benzodiazepine receptors may occur under the condition that all benzodiazepine receptors are occupied by β-CCE. However, the exact reasons why such high concentrations of β-CCE are necessary to down-regulate benzodiazepine receptors are not clear at present.

Although the data that β-CCE induces down-regulation of benzodiazepine receptors are not available with the exception of our data demonstrated here, and of the report of Chapouthier et al. (1991), the reduction in chloride uptake after acute exposure of cerebrocortical neurons to FG 7142, an inverse agonist of benzodiazepine receptors, has been reported by Miller et al. (1990) who suggested that this effect of FG 7142 was mediated through benzodiazepine receptors. Taken the data reported by Miller and his co-workers and presented in this study together, the effect of FG 7142 is likely to be expressed through the down-regulation of benzodiazepine receptors induced by short-term exposure to the inverse agonist of benzodiazepine receptors, although the exact mechanisms remain to be elucidated.

We have also examined the effect of acute exposure of the neurons to a benzodiazepine receptor agonist, flunitrazepam, and observed that short-term exposure of the neurons to flunitrazepam resulted in a decrease in [^{3}H]flunitrazepam binding, which was reversed by concomitant exposure of the neurons to Ro15-1788 and flunitrazepam. Miller et al. (1988) also reported the down-regulation of benzodiazepine receptors after chronic administration of benzodiazepine.

Scatchard analysis of the [^{3}H]flunitrazepam binding revealed that the reduction of [^{3}H]flunitrazepam binding after exposure to β-CCE was due to a decrease in the B_{max} value with no change in the K_d value. Several mechanisms underlying the reduction of the number of benzodiazepine receptors after short-term exposure to β-CCE are considered. The first explanation is that the down-regulation is mediated by receptor internalization. This process has been observed as a cause of receptor desensitization. Receptor internalization during the development of desensitization has been reported in cases of G-protein-linked receptors such as muscarinic acetylcholine receptors (Harden et al., 1985; Fang et al., 1993), β-adrenoceptors (Valiquette et al., 1993) and 5-HT (Rahman and Neuman, 1993) receptors as well as ion channel-coupled receptors including $GABA_A$ receptors (Mehta and Ticku, 1992). In the present study, therefore, we have examined whether the down-regulation of benzodiazepine receptors induced by β-CCE exposure is due to internalization of benzodiazepine receptors by measuring the binding of [^{3}H]flunitrazepam to the cytosolic preparation derived from the neurons exposed to β-CCE. The binding of [^{3}H]flunitrazepam to the cytosolic preparation after β-CCE exposure was increased as demonstrated in this study, indicating that the down-regulation of benzodiazepine receptors induced by exposure to β-CCE is assumed to be attributed, at least in part, to internalization of benzodiazepine receptors. Another mechanism involved in the down-regulation is considered to be due to an alteration in the expression of receptor mRNA. In fact, short- and long-term exposure of cultured neurons to $GABA_A$ and β-adrenoceptor agonists leads to a reduction in the levels of respective receptor mRNAs (Hadcock and Malbon, 1988; Collins et al., 1989; Montpied et al., 1991). At present, investigations to elucidate such a possibility is underway in our laboratory.

In conclusion, short-term exposure to β-CCE, a benzodiazepine receptor inverse agonist, induced a decrease in the binding of [^{3}H]flunitrazepam to the extensively washed membrane fractions derived from the cerebral cortical neurons in a time- and dose-dependent manner, which was significantly suppressed by Ro15-1788, an antagonist of benzodiazepine receptors of the central type. This decrease in the binding of [^{3}H]flunitrazepam was due to a decrease in the number of [^{3}H]flunitrazepam binding sites without alteration in the affinity for [^{3}H]flunitrazepam. In parallel with the reduction of [^{3}H]flunitrazepam binding to the membrane fractions after the exposure to β-CCE, the binding of [^{3}H]flunitrazepam to the cytosolic preparation was found to be elevated. These results indicate that down-regulation of benzodiazepine receptors induced by short-term exposure to β-CCE is mediated, at least in part, by internalization of benzodiazepine receptors.

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