Interaction of β -carboline inverse agonists for the benzodiazepine site with another site on GABA_A receptors

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1 We examined the effects of methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate (DMCM), a β -carboline inverse agonist for the benzodiazepine site, on γ -aminobutyric acid (GABA)-induced Cl[−] currents in several cloned rat GABA_A receptor subtypes expressed in human embryonic kidney cells. The Cl[−] currents were measured in the whole cell configuration of patch clamp techniques.

2 DMCM at low concentrations (<0.5 μ M) occupying only the benzodiazepine site decreased GABA-induced Cl[−] currents in the α 1 β 2 γ 2 and α 3 β 2 γ 2 subtypes as expected from an inverse agonist, but produced no change in the α 6 β 2 γ 2 subtype (perhaps a neutral antagonist). The drug at higher concentrations (>0.5 μ M) enhanced Cl[−] currents in all the subtypes, with a half maximal concentration of 6 to 20 μ M, depending on the α isoform. In the α 1 β 2 subtype, which is without the benzodiazepine site, DMCM monophasically increased Cl[−] currents with a half maximal concentration of 1.9 μ M.

3 Ro 15-1788 (a classical benzodiazepine antagonist) had no effect on Cl[−] current enhancement by DMCM and, in fact, increased the current level through blocking current inhibition by DMCM via the benzodiazepine site. Also, Cl[−] current enhancement by pentobarbitone or by 3 α , 21-dihydroxy-5 α -pregnan-20-one was additive to that by DMCM at saturating doses. It appears that the agonist site for DMCM is distinct from those for benzodiazepines, barbiturates and neurosteroids.

4 Among β -carboline analogues, methyl- β -carboline-3-carboxylate and propyl- β -carboline-3-carboxylate markedly enhanced GABA-induced Cl[−] currents in the α 1 β 2 γ 2 subtype, while N-methyl- β -carboline-3-carboxamide and 1-methyl-7-methoxy-3,4-dihydro- β -carboline did not. It appears that the 3-carboxyl ester moiety is necessary for β -carbolines to interact with a novel site on GABA_A receptors as agonists.

Keywords: β -Carbolines; cloned GABA_A receptors; methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate (DMCM); agonist action of DMCM

Introduction

β -Carbolines are known to interact with the benzodiazepine site on GABA_A receptors, the chloride channel-receptor complex consisting of multiple subunits (Sieghart, 1992). Several β -carboline derivatives inhibit the receptor function (inverse agonists) (Braestrup *et al.*, 1982). Methyl 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylate (DMCM), in particular, has been frequently employed as a prototype inverse agonist and shown to induce convulsions in experimental animals (Braestrup *et al.*, 1982). However, in our preliminary studies DMCM at micromolar concentrations marginally decreased GABA-induced Cl[−] currents in cloned GABA_A receptors (unpublished observations). This led us to examine full dose-response profiles for DMCM in several subtypes of cloned rat GABA_A receptors. The drug at low concentrations (<0.5 μ M) inhibited GABA-induced Cl[−] currents as expected from an inverse agonist, but at higher concentrations (>0.5 μ M) enhanced Cl[−] currents. A further competition study indicated that the low affinity site for DMCM appears to be distinct from those for benzodiazepines, barbiturates and neurosteroids, and accommodates various β -carbolines containing the 3-carboxylate moiety.

Methods

The stable cell-lines expressing α 1 β 2, α 1 β 2 γ 2, α 3 β 2 γ 2 and α 6 β 2 γ 2 subtypes of GABA_A receptors were derived by transfection of plasmids containing cDNA for respective GABA_A receptor subunits and a plasmid encoding G418 resistance into the human kidney cells (HEK 293 cells) as described elsewhere (Hamilton *et al.*, 1993). GABA-induced Cl[−] cur-

rents were recorded in the whole cell configuration of patch clamp techniques (Hamill *et al.*, 1981), using the pipette and extracellular solutions as detailed elsewhere (Im *et al.*, 1993). Currents were recorded at a holding potential of −60 mV at room temperature, by use of an Axopatch 1D amplifier, a CV-4 headstage (Axon Instrument Co.) and a Gould Recorder 220. A submaximal concentration of GABA, at which allosteric ligands are highly effective, varied for each subtype of GABA_A receptors. In the α 1 β 2 γ 2 and α 3 β 2 γ 2 subtypes, we chose 5 μ M GABA at which diazepam (5 μ M) increased Cl[−] currents by 125 ± 20 and 441 ± 96%, respectively. In the α 1 β 2 and α 6 β 2 γ 2 subtypes (diazepam-insensitive subtypes), we chose 1 μ M GABA at which U-92813 (5 μ M), a substituted pyrazinone (Im *et al.*, 1993), increased Cl[−] currents by 160 ± 27 and 350 ± 47%, respectively.

Results

Biphasic effect of DMCM on GABA-induced Cl[−] currents in the α 1 β 2 γ 2 subtype

We examined the effect of DMCM at various concentrations on GABA-induced Cl[−] currents in the α 1 β 2 γ 2 subtype of GABA_A receptors expressed in HEK 293 cells (Figure 1). DMCM displayed a biphasic effect; the drug at low concentrations (<0.2 μ M) reduced Cl[−] currents (−33 ± 8% at 0.1 μ M), but at higher concentrations (>0.5 μ M) enhanced the currents (+45 ± 18% at 20 μ M). From analysis of the latter phase with a logistic equation ($E = E_{\max} \cdot [DMCM]^n / (K_{0.5}^n + [DMCM]^n)$), we obtained a half maximal DMCM concentration ($K_{0.5}$) of 6 ± 1.2 μ M, a slope factor (n) of 0.9 ± 0.2, and a maximal net current increase ($E_{\max}$) of 115 ± 19%. Furthermore, as shown in Figure 2, the current

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Figure 1 Dose-response profile for the action of DMCM on GABA-induced Cl⁻ in the $\alpha 1\beta 2\gamma 2$ subtype of GABA_A receptor expressed in HEK293 cells. The traces (a) represent GABA (5 μ M)-induced Cl⁻ currents in the whole cell configuration at a holding potential of -60 mV, in the presence of a symmetrical Cl⁻ gradient. DMCM was applied at the indicated concentration with 5 μ M GABA for 10 s. The amplitude of the currents was normalized to that observed with 5 μ M GABA alone. The data fit the logistic equation given in the text (b) and represent the mean $\pm$ s.e. from three experiments. The horizontal calibration bar represent 30 s, and the vertical bar 500 pA.

Figure 2 Effect of Ro 15-1788 on DMCM-induced potentiation of Cl⁻ currents in the $\alpha 1\beta 2\gamma 2$ subtype. The traces (a) represent Cl⁻ currents induced by 5 μ M GABA in the presence of 10 μ M Ro 15-1788, 5 μ M DMCM, or the combination of the two drugs. Panel (b) shows similar traces obtained with 20 μ M DMCM. The average increase in the current response in the presence of Ro 15-1788 was $34 \pm 7\%$, regardless of DMCM concentration. The horizontal calibration bar represents 30 s, and the vertical bar 500 pA.

amplitude observed with DMCM increased upon addition of Ro 15-1788, a classical benzodiazepine antagonist which has been shown to block the inhibitory effect of DMCM. For instance, DMCM at 5 and 20 μ M increased GABA-induced Cl⁻ currents to 105 and 174% of control (5 μ M GABA alone), respectively, but in the presence of Ro 15-1788 (ethyl-8-fluoro-5, 6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a] [1,4] benzodiazepine-3-carboxylate; 10 μ M) these were increased to 136 and 204%, respectively. The average increase in the presence of Ro 15-1788 was $34 \pm 7\%$, regardless of DMCM concentration (i.e., 5, 10, 20 and 40 μ M), which coincided with the amount blocked by DMCM at low concentrations (0.2 μ M or less) via the benzodiazepine site. Thus, the two opposing effects of DMCM on Cl⁻ currents appears to be additive. These results indicate that DMCM at low concentrations acts as an inverse agonist via the benzodiazepine site, but at higher concentrations interacts with another independent site on the GABA_A receptor as an agonist.

Effect of DMCM on GABA-induced currents in the $\alpha 3\beta 2\gamma 2$, $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2$ subtypes

The $\alpha 1\beta 2\gamma 2$ subtype examined above represents Type 1 GABA_A receptors, the most wide-spread subtype in mammalian brains. We also examined DMCM action in Cl⁻ currents in the $\alpha 3\beta 2\gamma 2$ subtype (Type 2 receptor), the $\alpha 6\beta 2\gamma 2$ subtype (the cerebellar granule cell-specific receptor), and the $\alpha 1\beta 2$ subtype (without the benzodiazepine site) (Figure 3). In the $\alpha 3\beta 2\gamma 2$ subtype, DMCM also displayed a biphasic effect; the drug at low concentrations (<0.5 μ M) decreased GABA

(5 μ M)-induced Cl⁻ currents ($-35 \pm 9\%$ at 0.5 μ M), but at high concentrations (>0.5 μ M) enhanced the currents with a maximal current increase of $346 \pm 28\%$, a half maximal concentration of 20 ± 4 μ M, and a slope factor of 1.3. In the $\alpha 6\beta 2\gamma 2$ subtype, on the other hand, DMCM at concentrations up to 1 μ M showed no appreciable effect, but at higher concentrations enhanced the currents with a maximal current increase of $96 \pm 47\%$, a half maximal concentration of 8.5 ± 3 μ M, and a slope factor of 1.2. No detectable functional effect of DMCM at 1 μ M suggests the drug may be a neutral antagonist at the benzodiazepine site on the $\alpha 6\beta 2\gamma 2$ subtype. DMCM has been reported to inhibit [³H]-Ro 15-4513 binding with a K_i value of 210 ± 50 nM (Luddens *et al.*, 1990). In the $\alpha 1\beta 2$ subtype, which is without a benzodiazepine site (Prichett *et al.*, 1989), DMCM monophasically increased GABA (1 μ M)-induced currents (Figure 6) with a maximal current increase of $113 \pm 8\%$, a half maximal concentration of 1.9 ± 0.3 μ M, and a slope factor of 1.2. This indicates that the γ subunit is not an integral part for the second site for DMCM, unlike the benzodiazepine site (Prichett *et al.*, 1989). Nevertheless, quaternary interactions involving γ seem to influence the second site judging from the decrease in the half maximal DMCM concentration, for 6 to 1.9 μ M, upon removal of the subunit.

The agonist site for DMCM being distinctive from those for barbiturates and neurosteroids

Besides the benzodiazepine site, there are another two major agonist modulatory sites on GABA_A receptors, the barbiturate and the neurosteroid sites. DMCM appeared not to interact with these sites, because enhancement of GABA-induced Cl⁻ currents by pentobarbitone or 3 α , 21-dihydroxy-5 α -pregnan-20-one (5 α -THDOC) was additive to that by DMCM at saturating concentrations (i.e. 20 μ M) (Figure 4).

Figure 3 Dose-dependent potentiation of GABA-induced Cl⁻ currents by DMCM in the $\alpha 3\beta 2\gamma 2$, $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2$ subtypes of GABA_A receptors. The traces represent Cl⁻ currents evoked by 5 μ M GABA in the $\alpha 3\beta 2\gamma 2$, and by 1 μ M GABA in the $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2$ subtypes in the whole cell configuration. The holding potential was -60 mV under a symmetrical Cl⁻ gradient. DMCM was applied at the indicated concentrations with GABA. The horizontal calibration bar represents 30 s, and the vertical bar 500 pA.

For example, pentobarbitone at 5 μ M and 5 α -THDOC at 20 nM increased Cl⁻ currents by 75 ± 18 and $121 \pm 33\%$, respectively, as normalized to the control (5 μ M GABA alone). A combination of pentobarbitone (5 μ M) and DMCM (20 μ M) enhanced the current response by $76 \pm 20\%$ above the level observed with DMCM alone. Similarly, a combination of 5 α -THDOC (20 nM) and DMCM (20 μ M) produced an increase of $133 \pm 35\%$ above the level observed with DMCM alone. This additiveness indicates that the agonist site for DMCM (low affinity site) is distinctive from that for barbiturates or neurosteroids.

Effect of various β -carboline analogues on GABA-induced Cl⁻ currents in the $\alpha 1\beta 2\gamma 2$ subtype

We examined the effects of methyl- β -carboline-3-carboxylate (β -CCM), propyl- β -carboline-3-carboxylate (β -CCP), N-methyl- β -carboline-3-carboxamide (FG-7142), and 1-methyl-7-methoxy-3,4-dihydro- β -carboline (harmaline) on GABA-induced Cl⁻ currents in the $\alpha 1\beta 2\gamma 2$ subtype (Figure 5). β -CCM at 0.5 μ M reduced the currents to $69 \pm 6\%$ of control, but at higher concentrations enhanced currents, i.e., at 40 μ M by nearly 300%. β -CCP at 0.05, 0.1 and 0.2 μ M, on the other hand, produced no appreciable effect on Cl⁻ currents, but at higher concentration increased the currents, i.e., at 10 μ M by nearly 400%. FG-7142 monophasically reduced the currents to $67 \pm 7\%$ of control at concentrations ranging from 0.1 to 40 μ M. Harmaline had no appreciable effect on Cl⁻ currents (91 to 100% of control) at concentrations ranging from 0.1 to 20 μ M. The dose-response profiles for β -CCM and β -CCP were analysed with the logistic equation (Figure 6). For β -CCM, we obtained a half maximal concentration of 22.6 ± 3 μ M, a slope factor of 0.9, and a maximal net current increase of $342 \pm 26\%$. For β -CCP, the corresponding values were 4.3 ± 1.4 μ M, 1.4 and $388 \pm 48\%$, respectively.

Figure 4 Potentiating effect of pentobarbitone and 5 α -THDOC on GABA-induced Cl⁻ currents being additive to that of DMCM. (a) Shows the current traces obtained in the presence of 5 μ M GABA without or with 20 μ M DMCM, 5 μ M pentobarbitone or the combination of the two. Similar traces obtained with 20 nM 5 α -THDOC instead of pentobarbitone are shown in (b). Pentobarbitone at 5 μ M and 5 α -THDOC at 20 nM increased GABA-induced Cl⁻ currents by 75 ± 1 and $121 \pm 33\%$, respectively, and their level of potentiation was not altered in the presence of DMCM at 20 μ M, a saturating concentration. The horizontal calibration bar represents 30 s, and the vertical bar 1000 pA.

Figure 5 Effect of various β -carbolines on GABA-induced Cl^- currents in the $\alpha 1\beta 2\gamma 2$ subtype. The current traces represent 5 μM GABA-induced Cl^- currents in the $\alpha 1\beta 2\gamma 2$ subtype of cloned GABA_A receptor in the presence of β -CCM (a), β -CCP (b), FG-7142 (c), and harmaline (d) at various concentrations. β -CCM and FG-7142 at 0.5 μM or less reduced GABA-induced Cl^- currents by about 32%, but β -CCP and harmaline at 0.05 or 0.1 μM had no appreciable effect on the currents. Furthermore, only β -CCM and β -CCP at micromolar concentrations markedly enhanced the currents. The horizontal calibration bar represents 30 s, and the vertical bar 1000 pA for (a) and (b), 500 pA for panel (c) and (d).

Discussion

In this study, we have shown a biphasic effect of DMCM on GABA-induced Cl^- currents; the drug at low concentrations acted as an inverse agonist via the benzodiazepine site (its most well known action), but at higher concentrations interacted with another site on the GABA_A receptor as an agonist. The agonist site for DMCM appears to be distinct from that for benzodiazepines, barbiturates and neurosteroids (the three major positive modulatory sites on GABA_A receptors), on the basis of several observations presented in this study. (1) Ro 15-1788, a classical benzodiazepine antagonist, did not block the agonist action of DMCM at high concentrations, but rather boosted the agonist action by abolishing the inhibitory effect of DMCM via the benzodiazepine site. (2) DMCM potentiated GABA-induced Cl^- currents in the $\alpha 1\beta 2$ subtype which is without a

Figure 6 Plots showing concentration-dependent enhancement of Cl^- currents by β -CCM (O) and β -CCP (●) in the $\alpha 1\beta 2\gamma 2$ subtype; effects of FG-7142 (Δ) and harmaline ($\blacktriangle$) are also shown. The amplitude of Cl^- currents obtained in the presence of GABA (5 μM) and β -CCM or β -CCP at indicated concentrations was normalized to that for GABA alone (dotted line). The data for β -CCM and β -CCP were fitted with the logistic equation as described in the text (solid lines). For β -CCM, the half maximal concentration was $22.6 \pm 3 \mu\text{M}$, with a maximal current enhancement of $442 \pm 26\%$ and a slope factor of 0.9. The corresponding values for β -CCP were $4.3 \pm 1.4 \mu\text{M}$, $488 \pm 48\%$, and 1.4, respectively. Each data point represents the mean $\pm$ s.e. from three separate measurements.

benzodiazepine site. (3) The agonist action of DMCM at a saturating concentration was additive to that of the barbiturate and neurosteroid (5 α -THDOC). We propose that DMCM at high concentrations interacts with a novel site on GABA_A receptors, which could possibly be exploited as a novel therapeutic target.

The current study with several key analogues of β -carbolines showed that the 3-carboxyl ester moiety is necessary for their agonist action. For example, β -CCM and β -CCP containing the 3-methyl or propyl ester group, respectively, markedly enhanced GABA-induced Cl^- currents, while FG-7142, containing the 3-carboxamide group, and harmaline, a 3,4 dihydro β -carboline without a C3 carboxyl substituent, did not appreciably enhance the currents. Among the analogues containing the 3-carboxyl ester moiety, β -CCP showed the highest potency and intrinsic efficacy, suggesting a key interaction of the alkyl chain of the ester group with GABA_A receptors.

In view of its newly discovered agonist activity, we need to re-evaluate the use of DMCM as a prototype inverse agonist for GABA_A receptors. So far, central nervous system depressant activity of DMCM has not been reported in experimental animals. Even at high doses of DMCM, its depressant activity could be transient and easily overlooked because of the subsequent appearance of its powerful convulsant activity, as drug occupancy of the low affinity site gradually decreases. Careful examination of animal behaviours will be needed following DMCM administration. *In vitro*, DMCM at concentrations less than 0.5 μM behaves as an inverse agonist for the benzodiazepine site, but at concentrations greater than 1 μM will not be effective as an inverse agonist. FG-7142 appears to be a more reliable inverse agonist because of its failure to interact with the low affinity site for DMCM. In summary, DMCM and several β -carbolines containing the 3-carboxyl ester moiety interact with a novel, low affinity site on GABA_A receptors as an agonist besides its interaction with the benzodiazepine site as an inverse agonist.

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