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BRIEF COMMUNICATION

Ethanol Enhancement of GABA-Induced $^{36}Cl^-$ Influx Does not Involve Changes in Ca^{2+}

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MEHTA, A. K. AND M. K. TICKU. *Ethanol enhancement of GABA-induced $^{36}Cl^-$ influx does not involve changes in Ca^{2+}*. PHARMACOL BIOCHEM BEHAV 47(2) 355–357, 1994.—The effect of changes in intracellular Ca^{2+} on ethanol enhancement of GABA-mediated $^{36}Cl^-$ influx was investigated in mammalian cortical neurons in culture. Ethanol potentiated the effect of submaximal concentrations of GABA on the $^{36}Cl^-$ influx, and at 50 mM ethanol directly activated $^{36}Cl^-$ influx in these neurons. Pretreatment of the neurons with dantrolene (an agent that depletes intracellular Ca^{2+}) and the Ca^{2+} ionophore, A 23187, did not alter the effect of GABA or ethanol enhancement of GABA-mediated $^{36}Cl^-$ influx. Together, these results suggest that changes in intracellular Ca^{2+} are not involved in ethanol modulation of $GABA_A$ergic responses in cortical neurons.

GABA receptor complex Ethanol $^{36}Cl^-$ influx Ca^{2+} Cortical neurons

THE $GABA_A$ receptor complex is a site of drug action for a variety of centrally acting drugs including ethanol (13,19). Behavioral (4,20), electrophysiological (3,17), and biochemical (1,8,18) studies support the notion that some of the effects of ethanol may be mediated by facilitation of the $GABA_A$ receptor-mediated responses. We have previously demonstrated that ethanol enhancement of GABAergic transmission in cultured spinal cord neurons involves $GABA_A$ receptor-gated chloride channels (8). Further, chronic ethanol treatment produces changes in $GABA_A$ receptor gene expression (10,12). Together, these observations implicate $GABA_A$ergic transmission in the actions of ethanol. However, the exact mechanism(s) by which ethanol enhances GABAergic responses have yet to be elucidated. Binding studies have failed to identify a site on the oligomeric $GABA_A$ that may be involved in this effect (20). Recent molecular biological studies have suggested an involvement of the γ_2L-subunit in ethanol's effects on $GABA_A$ receptor-mediated responses (22). However, the exact subunit combination and stoichiometry needed in GABA-mediated responses and ethanol enhancement of these responses is yet to be established. Because ethanol is an anesthetic at high concentrations, and anesthetics have been shown to induce changes in intracellular Ca^{2+} (6,11), we have examined the effect of Ca^{2+} on GABA-induced $^{36}Cl^-$ influx in mammalian cortical neurons in culture. We examined the effect of dantrolene sodium, an agent that inhibits intracellular Ca^{2+} release (21), and A 23187, a Ca^{2+} channel-ionophore, on GABA and ethanol's effect on GABA-induced $^{36}Cl^-$ influx in cortical neurons.

Cerebral hemispheres were dissected from 15-day-old C57 Bl/6CR mouse embryos, dissociated by trituration, and the cells plated on poly L-lysine-coated coverslips, as described previously (8,9). The cells were incubated for 24 h (95% air : 5% CO_2). After this time, the growth medium was replaced with 1 ml medium containing 10% heat-inactivated horse serum (MEM-10) and a mixture of sterile 5-fluoro-2′-dioxyuridine plus uridine (2 mg/ml 5-fluro-2′dioxyuridine and 5 mg/ml uridine) at a final concentration of 10 μg/ml was added. A portion (1 ml) of the medium was replaced with MEM-10 after 3 days and again 24 h before the experiment.

$^{36}Cl^-$ influx was measured as described previously for spinal cord and cortical neurons (8,9). Briefly, coverslips were

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TABLE 1

EFFECT OF DANTROLENE ON ETHANOL'S EFFECT ON GABA$_A$ERGIC RESPONSES IN MAMMALIAN CORTICAL NEURONS

Treatment	^{36}Cl-Influx (nmol/mg protein)	^{36}Cl-Influx (% Increase Over Basal)	
Basal	1.499 ± 0.062		
+20 μM GABA	1.971 ± 0.058	34 ± 4	(4)
+15 μM dantrolene	1.495 ± 0.057	2 ± 2	(4)
+20 μM GABA + 15 μM dantrolene	1.879 ± 0.190	31 ± 6^{NS}	(4)
+20 μM GABA + 20 mM ethanol	2.495 ± 0.066	64 ± 7*	(3)
+20 μM GABA + 20 mM ethanol + 15 μM dantrolene	2.378 ± 0.015	56 ± 7*	(3)
+50 mM ethanol	1.971 ± 0.058	31 ± 5	(3)
+50 mM ethanol + 15 μM dantrolene	1.824 ± 0.062	28 ± 7^{NS}	(3)

Cultured cortical neurons were processed as described in the text. The values (nmol/mg protein) represent mean ± SE of a typical experiment. ^{36}Cl-Influx (% increase over basal) represents mean ± SD of number of experiments indicated in the parentheses.

*$p < 0.01$ as compared to GABA alone; NS refers to not significant as compared to GABA alone or ethanol (50 mM) alone.

rinsed at room temperature in HEPES-buffered saline, pH 7.4 (millimolar: NaCl 136; KCl, 5.4; Mg Cl_2, 1.4; $CaCl_2$, 1.2; NaH_2 PO_4, 1; and HEPES, 20, adjusted to pH 7.4 with Tris base) for ~ 5 s. All experiments were performed on neurons after 8 days in culture. The coverslips were rinsed three times prior to transfer to 2 ml HEPES-buffered saline containing ^{36}Cl (2 μci/ml) in the absence and presence of various drugs. GABA-mediated $^{36}Cl^-$ influx was measured for 5 s, followed by rapid transfer of the coverslips to 1,000 ml ice-cold stop solution, as described previously (8). Protein was estimated by BCA protein assay. All values for $^{36}Cl^-$ influx were expressed per milligram of cellular protein.

In studies involving dantrolene, the coverslips were incubated with 15 μM for 10 min prior to the measurement of the $^{36}Cl^-$ influx. For A 23187 studies, the coverslips were incubated with 20 μM for 4 min of the ionophore prior to the measurement of $^{36}Cl^-$ influx. These concentrations of dantrolene (11) and A 23187 (15,16) were chosen based on previously published reports. Statistical analysis was done using Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

The exposure of cortical neurons to dantrolene and A 23187 did not produce any significant effect on cell morphology or cell viability or cellular protein content. Cortical neurons used in the present study contain all the components of the GABA$_A$-benzodiazepine receptor complex (9). GABA produced a concentration-dependent increase in $^{36}Cl^-$ influx in the cortical neurons, with an EC_{50} value of 10 ± 3 μM and E_{max} value of 84 ± 6% (n = 3). The effect of GABA was potentiated by diazepam (1 μM) and blocked by GABA antagonists like bicuculline (10 μM) and picrotoxin (100 μM). Further, ethanol (20 mM) enhanced the effect of submaximal concentrations of GABA (Table 1). Higher concentrations of ethanol (50 mM) produced a direct influx of $^{36}Cl^-$ influx in the absence of added GABA (Table 1). These observations are similar to what we have observed previously in cultured spinal cord neurons (8).

Elevation of intracellular Ca^{2+} has been reported to inhibit

TABLE 2

EFFECT OF A 23187 ON ETHANOL'S EFFECT ON GABA-INDUCED ^{36}Cl-INFLUX IN MAMMALIAN CORTICAL NEURONS

Treatment	^{36}Cl-Influx (% Increase Over Basal)	
+20 μM GABA	33 ± 5	(3)
+20 μM GABA + 20 nM ethanol	65 ± 5*	(3)
+20 μM A 23187	5 ± 3	(3)
+20 μM GABA + 20 μM A 23187	44 ± 5†	(3)
+20 μM GABA + 20 mM ethanol + 20 μM A 23187	75 ± 6*	(3)
+50 mM ethanol	33 ± 5	(3)
+50 mM ethanol + 20 μM A 23187	42 ± 6^{NS}	(3)

Values are mean ± SD of number of experiments indicated in the parentheses. Each experiment was performed in triplicate.

*$p < 0.05$ as compared to GABA alone.

†$p < 0.01$ as compared to GABA alone; NS refers to not significant as compared to 20 mM ethanol.

GABA-mediated responses in dorsal root ganglion cells (2,5). The effects of changes in Ca^{2+} homeostasis in the anesthetic action are controversial. Ethanol is an anesthetic, and anesthetics have been reported to change neuronal excitability by altering intracellular concentrations of Ca^{2+} (6,11). Further, halothane has been reported to enhance GABAergic responses by elevating intracellular Ca^{2+} (11). These authors have suggested that halothane's effect on $GABA_A$-mediated inhibition involves changes in intracellular Ca^{2+}. Based on these observations, we investigated the effect of dantrolene (an agent that inhibits intracellular Ca^{2+} release) and A 23187 (which increases intracellular Ca^{2+}) on GABA and ethanol enhancement of GABA-induced $^{36}Cl^-$ influx in cortical neurons.

Table 1 shows that dantrolene (15 μM) pretreatment did not alter either the effect of GABA or ethanol's enhancing effect on GABA-induced $^{36}Cl^-$ influx. Further, dantrolene did not affect the direct effect of ethanol on $^{36}Cl^-$ influx. These results are in agreement with a recent study that demonstrated that changes in intracellular Ca^{2+} were not involved in general anesthetics, including alcohol, potentiation of GABA responses in *Xenopus* oocytes (7). In contrast, the same concentration of dantrolene was reported to decrease halothane's effect on GABA-mediated spontaneous inhibitory postsynaptic currents in the hippocampal slices (11).

The Ca^{2+} ionophore, A 23187, increases intracellular Ca^{2+} by translocating Ca^{2+} into the cytoplasm. Table 2 shows that the pretreatment of the cortical neurons with the Ca^{2+} channel ionophore A 23187 also did not alter the effect of GABA or ethanol's enhancing effect on GABA-gated $^{36}Cl^-$ influx. In fact, A 23187 slightly potentiated the effect of GABA and ethanol enhancement of GABA, inducing $^{36}Cl^-$ influx. These data also rule out the involvement of changes in intracellular Ca^{2+} in ethanol enhancement of GABA-mediated responses.

In summary, ethanol enhances GABA-induced $^{36}Cl^-$ influx in the cortical neurons in culture. This effect of ethanol does not appear to involve changes in intracellular Ca^{2+}. Thus, the exact molecular mechanism by which ethanol potentiates $GABA_A$ergic transmission remains to be elucidated.

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