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Ethanol Effects on Striatal Dopamine Receptor-Coupled Adenylate Cyclase and on Striatal Opiate Receptors

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HOFFMAN, P. L., G. R. LUTHIN, D. THEODOROPOULOS, P. CORDOPATIS AND B. TABAKOFF. *Ethanol effects on striatal dopamine receptor-coupled adenylate cyclase and on striatal opiate receptors.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 355–359, 1983.—The perturbation of neuronal cell membranes by ethanol may result in specific functional changes through modification of the activity of various membrane-bound proteins. In mouse striatum, adenylate cyclase, a membrane-bound enzyme, is coupled to dopamine, as well as to opiate, receptors. Ethanol stimulates striatal adenylate cyclase activity by modifying the regulatory protein ("G-protein")-adenylate cyclase interaction to produce an increased amount of activated enzyme. This action is additive with the effects of dopamine on adenylate cyclase. Ethanol also modifies striatal opiate receptor-effector coupling processes. In the presence of ethanol, opiate receptor affinity is altered, and this alteration is modified by GTP, suggesting that ethanol influences the interaction of the opiate receptor complex with the G-protein. Our results suggest that ethanol can affect receptor-effector coupling, including the binding of opiate agonists to their receptors, through its membrane-disordering capacity, and that particular systems may react in a relatively specific manner with ethanol.

Ethanol Dopamine-sensitive adenylate cyclase Opiate-inhibited adenylate cyclase Opiate receptors
Mouse striatal adenylate cyclase

ETHANOL, like other anesthetics, has the capacity to modify the physical characteristics of cellular membranes, by increasing the "fluidity" of the membrane lipids [6, 15, 26, 27]. Such changes in the properties of neuronal cell membranes in the CNS contribute to the hypnotic effect of ethanol [13], and adaptation of the membranes to ethanol during chronic exposure has been suggested to result in tolerance to the effects of ethanol [7,26]. Cell membranes are integral to neuronal activity and synaptic transmission, and the perturbation of these membranes, by ethanol or other anesthetics, will affect these processes. However, it remains to be seen if changes in membrane lipids, which represent the structural components of cell membranes [20], are, by themselves, responsible for all aspects of the response to ethanol. Since alterations in lipid fluidity can influence the activities of membrane-bound proteins [20], which represent the functional moieties of neuronal cell membranes, it is possible that the translation of ethanol-induced lipid modifications into biochemical and behavioral responses—e.g., intoxication, tolerance, physical dependence—is mediated by effects on membrane-bound proteins, such as receptors and enzymes.

Neuronal membranes are heterogeneous and asymmetrical with respect to lipids and proteins [11, 12, 20], and proteins are often localized in specific areas of the membrane [20]. In the case of enzymes whose activity is subject to modulation by neurotransmitters, e.g., striatal adenylate cyclase, the neurotransmitter receptor is located on the outer leaflet of the membrane, and the catalytic unit on the inner leaflet [11]. In recent years, the proteins involved in the coupling of receptors and enzyme have been at least partially described [10, 18, 29]. Efficient coupling seems to depend on the lateral mobility of these regulatory proteins within the membrane and, therefore, their activity is quite sensitive to the physical state of the membrane lipids [2, 14, 28].

Ethanol's effects on lipid fluidity have been reported to be greatest in the center of the membrane bilayer, which is, under basal conditions, the most "fluid" area [8,17]. This area of the membrane is expected to be critical in modulating the interactions of receptor, regulatory, and catalytic proteins (see Fig. 1). Therefore, these interactions provide a means by which the relatively non-specific, lipid-perturbing effects of ethanol may be transduced into more specific, functional responses.

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We have recently been investigating the effects of ethanol on a mechanistically well-defined membrane-bound system, striatal dopamine (DA)-sensitive adenylate cyclase. Previous work had shown that ethanol stimulates this enzyme activity, and does not act by modifying the binding of DA agonists to the DA receptor [25,30]. We have also begun to investigate the effect of ethanol on striatal opiate receptor binding, and opiate inhibition of striatal adenylate cyclase activity. Although the coupling of opiate receptors to regulatory proteins and effectors has not been described in as great detail as the coupling of DA receptors to adenylate cyclase [1, 9, 19], our results suggest that in each case, ethanol may affect certain aspects of these coupling processes.

METHOD

Striatal Adenylate Cyclase Activity

Male C57Bl mice (22–25 g) were kept in our laboratories under conditions of controlled lighting and temperature for at least seven days prior to being used in an experiment. Mice were killed by decapitation and striatal tissue was removed as previously described [22,30]. Tissue from 10–15 mice was pooled, homogenized in 10 volumes of 2 mM Tris-maleate buffer, pH 7.4, containing 2 mM EGTA (Tris buffer), and the homogenate was centrifuged at 600×g for ten minutes (4°C). The supernatant was centrifuged at 48,000×g for 20 minutes, and the pellet resuspended in Tris buffer to give a concentration of about 1.5 mg/ml of protein [21]. This suspension was used immediately for the adenylate cyclase assays. Adenylate cyclase activity was measured as previously described [30]. In some studies of DA-sensitive adenylate cyclase, enzyme was activated by preincubating the striatal membranes in 25 mM Tris buffer containing 10 mM theophylline, 0.5 mM ATP, 2 mM $MgSO_4$, 10 μM dopamine and 1 μM Gpp(NH)p for five minutes at 30°C [22]. The membranes were washed once by centrifugation, resuspended in Tris buffer, and used immediately for assay.

When opiate inhibition of adenylate cyclase activity was studied, tissue was not preincubated, and all assay mixtures contained 100 mM Na^+ and 100 μM GTP. All assays of opiate inhibition of adenylate cyclase activity also contained a regenerating system consisting of 20 mM phosphocreatine and 25 U of creatine phosphokinase (in 0.1% BSA), and assays were carried out for ten minutes at 30°C.

Opiate Receptor Binding

Binding of ^{3}H-dihydromorphine (DHM) and ^{3}H-[2-D-Ala, 5-D-Leu]enkephalin (ENK) to striatal membranes of male C57Bl mice was carried out as previously described [16,31]. Tissue from 10–25 animals was pooled and homogenized in ice-cold, 0.05 M Tris-HCl buffer (pH 7.7). The homogenate was centrifuged at 49,000×g (4°C) for 20 minutes, and the pellet was resuspended and incubated at 37°C for 60 minutes. After centrifugation, the pellet was resuspended in Tris-HCl buffer to give a concentration of about 1.0 mg protein/ml, and the suspension was used immediately in the binding assays. Each assay contained 1.0 ml of homogenate, ^{3}H-DHM in the presence or absence of 10 μM naloxone, or ^{3}H-ENK in the presence or absence of 1 μM unlabelled [2-D-Ala, 5-D-Leu]enkephalin [17,31]. In some experiments, GTP (100 μM) was added to the binding assay. Specific binding was quantitated by liquid scintillation counting.

Synthesis of Tyr-Pro-Phe-Pro-NH_2 (Morphiceptin) [4]

The peptide was synthesized in solution, and all intermediates were isolated and characterized by thin-layer chromatography on silica gel (BuOH-HOAc-H_2O, 4:1:1; BuOH-pyridine-HOAc-H_2O, 15:10:3:6; BuOH-HOAc-H_2O, 4:1:5, upper phase) and, in some cases, by melting point determination, optical rotation, and amino acid analysis. Synthesis of the protected dipeptide, N-*tert*-butyloxycarbonyl (Boc)-phenylalanylproline methyl ester, was carried out by the mixed anhydride method, using equimolar concentrations of Boc-Phe, isobutylchlorocarbonate and proline methyl ester. The dipeptide ester was N-deprotected using HCl in acetic acid, and Boc-Pro was added with the use of dicyclohexylcarbodiimide and 1-hydroxybenztriazole. The resulting protected tripeptide ester (Boc-Pro-Phe-Pro-OMe) was converted to the amide by treatment for three days with methanol saturated with ammonia, and was N-deprotected by treatment with trifluoroacetic acid. The resulting tripeptide amide (Pro-Phe-ProNH_2) was reacted with a 10% excess of N-benzyloxycarbonyl-Tyr-nitrophenyl ester, and the resulting tetrapeptide was N-deprotected by catalytic hydrogenation. The product (Tyr-Pro-Phe-ProNH_2) was purified by partition chromatography on Sephadex G-10 (BuOH-HOAC-pyridine-H_2O, 15:3:10:12; BuOH-HOAC-H_2O, 4:1:5). All steps produced yields of 80–90%, and all purified intermediates gave single spots (chlorine-tolidine detection) on thin-layer chromatography in all three solvent systems. Amino acid analysis of the final product gave the following ratios: Phe, 1.08; Tyr, 1.02; Pro, 1.90.

1. $DA + R \leftrightharpoons DA\text{-}R^L$
2. $DA\text{-}R^L + GP\text{-}GDP \leftrightharpoons DA\text{-}R^H\text{-}GP$
3. $DA\text{-}R^H\text{-}GP + GTP \leftrightharpoons DA\text{-}R^L\text{-}GP\text{-}GTP$
4. $DA\text{-}R^L\text{-}GP\text{-}GTP \leftrightharpoons DA\text{-}R^L + GP\text{-}GTP$ (EtOH)
5. $GP\text{-}GTP + AC^{IN} \leftrightharpoons GP\text{-}GTP\text{-}AC^A$
6. $GP\text{-}GTP\text{-}AC^A \rightarrow GP\text{-}GDP + AC^{IN}$

FIG. 1. Model for activation of striatal adenylate cyclase by GTP and DA (modified from [22]). R^H, R^L=high- and low-affinity forms of DA receptor. GP=G-protein; AC^{IN}, AC^A=inactive and active forms of adenylate cyclase. In this model, binding of DA to its receptor and formation of the DA-receptor-G-protein complex promotes the displacement of GDP from the G-protein, and results in the high-affinity form of the DA receptor. As GTP binds to this complex, the DA receptor is converted to its low-affinity form. The GTP-G-protein complex activates adenylate cyclase. The cycle is terminated by hydrolysis of GTP (by a GTPase intrinsic to the G-protein) and conversion of adenylate cyclase to its inactive form. Ethanol (EtOH) promotes the interaction of the GTP-G-protein complex with adenylate cyclase.

RESULTS AND DISCUSSION

To determine which step(s) in the coupling sequence (shown in Fig. 1) might be altered when ethanol stimulates striatal adenylate cyclase activity, we first demonstrated that stimulation of enzyme activity by ethanol was dependent on the presence of GTP or Gpp(NH)p, and that ethanol-induced activation increased linearly with guanyl nucleotide concentration [22]. These results suggested that ethanol might facilitate the interaction of the guanyl nucleotide with the regulatory protein ("G-protein"). When striatal membranes were preincubated with Gpp(NH)p, this non-hydrolyzable guanyl nucleotide analog occupied the GTP-binding site on the G-protein, and the adenylate cyclase was no longer susceptible to stimulation by DA or guanyl nucleotides (Table

TABLE 1

EFFECT OF ACTIVATORS ON ADENYLATE CYCLASE ACTIVITY AFTER PREINCUBATION WITH Gpp(NH)p

Additions	Adenylate Cyclase Activity (pmole cAMP/mg protein/min)
None	445 ± 13
1 μM Gpp(NH)p	382 ± 9
10 μM DA	445
1 μM Gpp(NH)p + 10 μM DA	402
500 mM Ethanol	975
500 mM Ethanol + 1 μM Gpp(NH)p	906

Adenylate cyclase activity was determined as described in the text, using tissue which had been preincubated with Gpp(NH)p and DA. Values represent the mean ± SEM of quadruplicate determinations, or the mean of duplicate determinations.

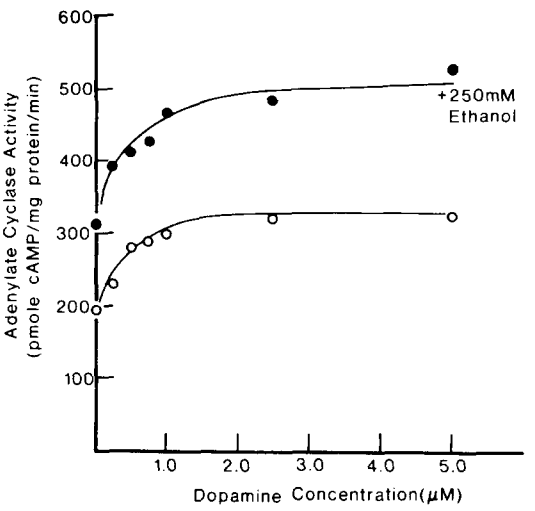

FIG. 2. Effect of ethanol and dopamine (DA) on striatal adenylate cyclase activity. Adenylate cyclase activity in 48,000×g mouse striatal membranes was determined as described in the text, in the presence (●) or absence (○) of 250 mM ethanol, and the indicated concentrations of DA. All assay tubes contained 1 μM Gpp(NH)p; the enzyme preparation was not preincubated with Gpp(NH)p. Assays were carried out at 30°C for three minutes. A Hanes-Woolf plot of the data gave the following parameters: $K_{I(app)}$ for DA in the absence of ethanol=0.53 μM; in the presence of ethanol=0.43 μM.

1). However, under these conditions, ethanol still stimulated activity (Table 1), indicating that ethanol's site of action was beyond the nucleotide-G-protein interaction [22].

These results, in combination with others showing that ethanol did not affect GTPase activity or the affinity of adenylate cyclase for its substrate, Mg-ATP [22], suggested that ethanol influenced the interaction of the nucleotide-G-protein complex with adenylate cyclase, resulting in an increased amount of the activated form of the enzyme [22] (see Fig. 1). Since butanol and propanol were also found to activate enzyme which had been preincubated with Gpp(NH)p, and the potency of the alcohols was proportional to their membrane-buffer partition coefficients, we postulated that the increased membrane fluidity caused by the alcohols promoted lateral diffusion of the guanyl nucleotide-G-protein complex and/or the catalytic unit of the adenylate cyclase, and established an equilibrium favoring formation of the activated enzyme [22]. This hypothesis is supported by data obtained when one determines the effects of ethanol on adenylate cyclase activity in the presence of varying concentrations of DA. In the presence of Gpp(NH)p, ethanol increased basal adenylate cyclase activity, as well as activity stimulated by DA, by a constant factor (Fig. 2). This type of activation by ethanol would be expected if ethanol influences the rate of interaction of the G-protein-guanyl nucleotide complex with adenylate cyclase.

The coupling of DA receptors to adenylate cyclase includes two essential steps which may be common to all "coupled" systems. The agonist-receptor complex interaction with the G-protein (first step, illustrated by Lines 2 and 3, Fig. 1) results in a decrease in receptor affinity for agonist [30]. The activated G-protein interaction with the catalytic unit (second step, illustrated by Line 5, Fig. 1) results in increased enzyme activity, in a stimulatory system. Since ethanol increased striatal adenylate cyclase activity by modulating the second step, ethanol did not alter the affinity of the DA receptor (see Fig. 1). However, we wondered whether all "coupled" systems would react to ethanol in a similar manner, or whether the nature of the coupling process might add specificity to ethanol's actions.

Adenylate cyclase in the striatum is coupled to opiate receptors, as well as to DA receptors [9,19]. However, the opiates inhibit, rather than stimulate, enzyme activity (Fig. 3). It is not clear if the same population of enzyme molecules is coupled to both the DA and opiate receptors, or if identical regulatory proteins mediate stimulation and inhibition of adenylate cyclase activity. There appear to be at least two coupling factors involved in opiate inhibition of adenylate cyclase activity, GTP and sodium ion [1, 9, 19]. Na^+ may prove to contribute to coupling in inhibitory systems, in general [23,24]. Both Na^+ and GTP also reduced the affinity of opiate receptors for agonists. One may postulate that opiate receptor "coupling" includes the two essential steps described above, i.e., interaction of the agonist-receptor complex with the G-protein, resulting in decreased receptor affinity, and interaction (or inhibition of interaction) of the (in)activated G-protein with the adenylate cyclase. Thus, the decrease in opiate receptor affinity which is caused by Na^+ or GTP would reflect coupling of the opiate receptor complex to the regulatory protein [3, 5, 16].

In mouse striatal tissue, both morphine and enkephalin inhibit adenylate cyclase activity. However, morphiceptin, a peptide which selectively interacts with the high-affinity morphine receptor (the "mu" receptor), and has the properties of an agonist at this receptor [4]; and Hoffman, *et al.*, unpublished observations), does not inhibit striatal adenylate cyclase activity (Fig. 3). Thus, in mouse striatum, as has been postulated for the rat striatum [9, 19, 29], adenylate cyclase activity seems to be coupled to the high-affinity enkephalin receptor (the "delta" receptor). While both GTP and Na^+ decrease the affinity for enkephalin of the delta receptor [3,5], GTP has been reported to have a greater influence on the affinity of the mu receptor [3]. The effects of GTP on the affinity of this receptor for agonist probably also reflect a coupling process, although the effector in this case does not appear to be adenylate cyclase (Fig. 3).

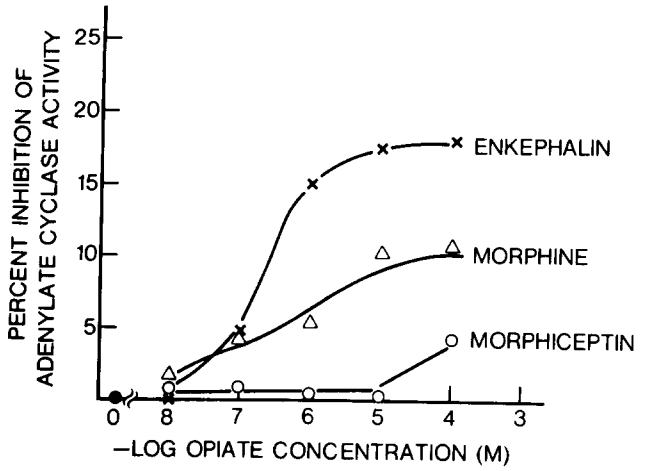

FIG. 3. Effect of opiates on striatal adenylate cyclase activity. Adenylate cyclase activity in 48,000×g mouse striatal membranes was determined as described in the text, in the absence (●) or presence of the indicated concentrations of morphine (△), [2-D-Ala, 5-D-Leu]enkephalin (X) or morphiceptin (○). All assays contained an ATP regenerating system, and were carried out in the presence of 100 mM Na^+ and 100 μM GTP for ten minutes at 30°C.

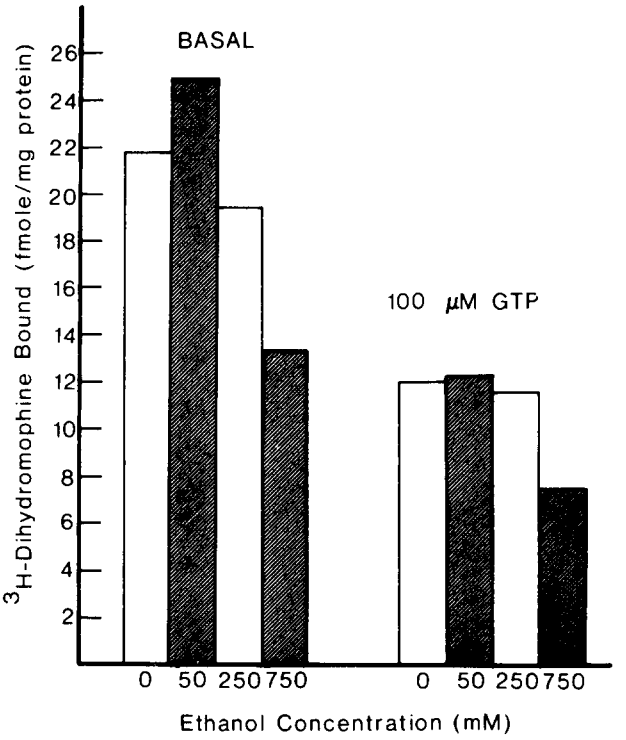

FIG. 4. Effect of ethanol on ^{3}H-DHM binding to striatal membranes in the presence of GTP. ^{3}H-DHM (0.2 nM) binding to striatal membranes of C57Bl mice was determined as described previously [16] at 25°C. Ethanol, at the indicated concentrations, and 100 μM GTP, were added directly to the assay mixtures. Binding in the absence of ethanol, or GTP, was 21.8 fmole/mg protein. GTP alone reduced binding by 42%.

To initiate our investigation of ethanol's effects on opiate receptor-effector coupling processes, we evaluated ethanol's effects on the first essential step in this process, the interaction of the agonist-receptor complex with the regulatory protein. This interaction can be monitored by determining the change in opiate receptor affinity.

When ethanol, at varying doses, was added to assay mixtures containing mouse caudate membranes and a single concentration of ligand for the mu receptor (0.2 nM ^{3}H-dihydromorphine [DHM]) or for the delta receptor (0.5 nM ^{3}H-[2-D-Ala, 5-D-Leu]enkephalin [ENK]), we found that ethanol did alter opiate binding [31]. Ethanol had a biphasic effect on DHM binding: binding was stimulated by low (i.e., 50 mM) ethanol concentrations, and inhibited by high ethanol concentrations. ENK binding was inhibited by all concentrations of ethanol tested. The kinetics of inhibition of binding by high ethanol concentrations showed that ethanol decreased the affinity of the mu receptor for DHM [31], and decreased the affinity for ENK of the delta receptor. The low concentration of ethanol appeared to increase mu receptor affinity. The changes in affinity could, at least in part, be attributed to the membrane-disordering effects of ethanol, since other long-chain alcohols had qualitatively similar effects, and their potency to inhibit binding correlated positively (but not linearly) with alcohol membrane-water partition coefficients [31].

All of the initial binding studies were carried out in the absence of Na^+ and GTP. However, if the changes in opiate receptor affinity caused by ethanol do reflect an influence on the receptor-effector coupling process, then one might expect ethanol's effects to interact with those of Na^+ and/or GTP on opiate receptor binding. As shown in Fig. 4, GTP reduced ^{3}H-DHM binding to the mouse striatal mu receptor, by reducing affinity ([3,5] and Tabakoff and Hoffman, unpublished observations). In the presence of GTP, the pattern of ethanol's effects on DHM binding was altered, in that 50 mM ethanol did not stimulate ^{3}H-DHM binding. The interaction between the effects of GTP and ethanol suggested that ethanol could modify opiate binding to the mu receptor by affecting the interaction of the receptor with the G-protein. Although it is not clear, at present, what final effector is coupled to this particular opiate receptor, our previous work suggests that the striatal mu receptor is involved in the regulation of DA metabolism [16,32]. Regulation of DA metabolism may well reflect a presynaptic opiate action, while inhibition of adenylate cyclase may be a postsynaptic effect which is regulated by delta receptors [1, 9, 19].

In summary, our results show that ethanol can affect receptor-effector coupling processes. In the case of the DA-stimulated striatal adenylate cyclase, a site of action of ethanol has been defined at the level of the activated G-protein-adenylate cyclase interaction. In this system, ethanol does not affect DA receptor binding. Ethanol does affect the affinity of striatal opiate receptors, and seems to do so by modulating the interaction of this receptor with a G-protein. Thus, one may postulate that ethanol, in both cases, influences the interaction of the G-proteins with other membrane-bound proteins, but the aspect of the interaction that is affected by ethanol is specific for each receptor-effector system. Although ethanol's initial effect may be perturbation of neuronal membrane properties, the functional response to this perturbation is complex, and individual membrane-bound systems must be studied in detail if we are to understand the behavioral or neurochemical responses to ethanol.

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