0026-895X/94/051004-09$03.00/0

MOLECULAR PHARMACOLOGY, 45:1004-1012

Ethanol Inhibits the Function of 5-Hydroxytryptamine Type 1c and Muscarinic M_1 G Protein-Linked Receptors in *Xenopus* Oocytes Expressing Brain mRNA: Role of Protein Kinase C

ENRICO SANNA, J. E. DILDY-MAYFIELD, and R. ADRON HARRIS

Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262 (E.S., J.E.D.-M., R.A.H.), and Denver Veteran Administration Medical Center, Denver, Colorado 80220 (R.A.H.)

Received September 3, 1993; Accepted February 2, 1994

SUMMARY

Effects of ethanol on the function of Ca^{2+}-activated Cl^- channels activated by G protein-coupled serotonin (5-hydroxytryptamine, $(5\text{-HT})_{1c}$) and muscarinic M_1 cholinergic receptors were studied in *Xenopus* oocytes expressing mouse whole-brain mRNA. Ethanol (25–200 mM) inhibited currents evoked by both 5-HT and acetylcholine (ACh), in a concentration-dependent manner. The maximal effect was obtained with 150 mM ethanol, which produced 65 and 49% inhibition of 5-HT and ACh responses, respectively. In the presence of 100 mM ethanol, the EC_{50} values for both 5-HT and ACh were increased about 4-fold. In contrast, in oocytes expressing rat cerebellar mRNA, metabotropic glutamate receptor responses were much less sensitive to ethanol. To examine potential postreceptor sites for ethanol inhibition, guanosine-5′-*O*-(3-thio)triphosphate and *myo*-inositol-1,4,5-trisphosphate were injected intracellularly. Ethanol (100 mM) did not significantly inhibit the currents produced by either guanosine-5′-*O*-(3-thio)triphosphate or *myo*-inositol-1,4,5-trisphosphate. Activation of protein kinase C (PKC) by phorbol-12-myristate-13-acetate markedly inhibited 5-HT-induced responses. Both the PKC inhibitor peptide and staurosporine prevented ethanol inhibition of 5-HT-induced responses. Moreover, ethanol, similarly to phorbol-12-myristate-13-acetate and opposite to PKC inhibitors, enhanced the rate of Ca^{2+}-activated Cl^- current desensitization induced by repeated applications of 5-HT. These results indicate that certain types of receptor-G protein interactions are more susceptible than others to uncoupling by ethanol and that ethanol inhibition of 5-HT_{1c} receptors requires PKC-mediated phosphorylation. We suggest that ethanol may activate PKC, which phosphorylates the receptors, resulting in inhibition of the responses.

Compared with voltage- and receptor-gated ion channels, few studies have investigated the effects of ethanol on the function of ion channels regulated by receptors linked to second messenger systems. However, there is evidence that ethanol modulates the coupling between neurotransmitter receptors and intracellular second messenger production. For example, acute ethanol exposure increases β-adrenergic receptor-stimulated adenylate cyclase activity in brain and cultured cell preparations (1, 2). Moreover, activation of PIP_2 hydrolysis, a second messenger pathway coupled to neurotransmitter receptors (3–5), by 5-HT, carbachol, or norepinephrine was inhibited by concentrations of ethanol ranging from 100 to 500 mM (6–8). The effect of ethanol was found to be dependent upon the type of stimulus and the brain area used, indicating that ethanol selectively affects the coupling of certain receptors to the PLC-dependent pathway.

Although the mechanism of action of ethanol on these systems has not been elucidated, a number of reports suggest that ethanol might interact with a G protein that couples the neurotransmitter receptor to PLC (9–12). It is noteworthy that a similar mechanism of action has also been proposed for the inhibitory effects of several anesthetic agents on receptor-mediated PIP_2 hydrolysis (13, 14).

On the basis of these data, we predicted that ion channels linked to the activation of second messenger systems would be sensitive to ethanol. To address this question, we used *Xenopus laevis* oocytes as an experimental model. These cells possess an endogenous Cl^- conductance that is activated by a rise in the

This work was supported by funds from the Department of Veterans Affairs and the National Institutes of Health/National Institute on Alcohol Abuse and Alcoholism (Grant AA06399) and by National Research Service Award Fellowship AA05334 to J.E.D.-M.

ABBREVIATIONS: PIP_2, phosphatidylinositol-4,5-bisphosphate; DAG, diacylglycerol; ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; *t*-ACPD, (*trans*)-1-amino-1,3-cyclopentanedicarboxylic acid; GPTγS, guanosine-5′-*O*-(3-thio)triphosphate; IP_3, *myo*-inositol-1,4,5-trisphosphate; PKCI, protein kinase C inhibitor peptide; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMA, phorbol-12-myristate-13-acetate; MBS, modified Barth's solution; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PLC, phospholipase C; PKC, protein kinase C; ANOVA, analysis of variance; LSD, least significant difference; TPA, 12-O-tetradecanoylphorbol 13-acetate.

intracellular Ca^{2+} concentration (15–17). After the injection of brain mRNA, the oocyte expresses several metabotropic receptors, such as the 5-HT$_{1c}$, muscarinic M$_1$, and glutamate receptors (18–20), all of which are linked to a common second messenger system (20, 21). Stimulation of these receptors leads to the activation of PLC, a process that is mediated by a G protein, resulting in the formation of IP_3 and DAG. IP_3 promotes, through its interaction with IP_3 receptors, the release of Ca^{2+} from endoplasmic reticulum, and this, in turn, triggers the opening of Cl^- channels, an event that can be monitored electrophysiologically. DAG, on the other hand, activates PKC, which initiates and regulates a number of cellular functions through phosphorylation of proteins, including some receptors, whose function is then down-regulated (22–25).

In the present report, we describe the effect of acute ethanol treatment on Ca^{2+}-activated Cl^- currents evoked by the stimulation of 5-HT$_{1c}$ and muscarinic M$_1$ receptors expressed in *Xenopus* oocytes after the injection of mouse whole-brain mRNA. We investigated the possible site of the action of ethanol, with respect to the different steps of the PIP_2 hydrolysis cascade. An account of this work has appeared in abstract form (26).

Experimental Procedures

Materials. Adult *Xenopus laevis* female frogs were purchased from Xenopus I (Ann Arbor, MI). ACh, 5-HT, quisqualic acid, EGTA, BAPTA, GTPγS, staurosporine, and PMA were purchased from Sigma Chemical Company (St. Louis, MO). *t*-ACPD was from Tocris Neuramin (Essex, England), IP_3 was purchased from Calbiochem (La Jolla, CA), and PKCI [PKC(19–36)] was obtained from GIBCO BRL (Gaithersburg, MD). Ethanol and all other reagents used were of analytical grade and were obtained from commercial sources.

Drugs used for bath perfusion were dissolved in MBS (see below), whereas those injected intracellularly were suspended in distilled water. All drug solutions were prepared fresh daily before each experiment.

Isolation of mRNA. Poly(A)$^+$ mRNA was isolated from adult (6–8-week-old) male C57BL/6 mice or Sprague-Dawley rats by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA), following the procedure described elsewhere (27). The poly(A)$^+$ mRNA was then purified by phenol/chloroform extraction, precipitated with 0.15 volumes of 2 M sodium acetate and 2 volumes of absolute ethanol, and stored at −80°. The amount of extracted poly(A)$^+$ mRNA was subsequently determined spectrophotometrically.

Postmortem human cortical tissue was obtained from The Schizophrenia Research Center of The Denver Veteran Affairs Medical Center, and poly(A)$^+$ mRNA was isolated as described by Lin *et al.* (14).

Isolation of *Xenopus* oocytes and microinjection of brain poly(A)$^+$ mRNA. Stage V and VI oocytes were isolated from a section of ovary by manual dissection, using fine surgical forceps, and then exposed to collagenase type 1A as described elsewhere (27). mRNA was reconstituted in diethyl pyrocarbonate-treated water, and each oocyte was injected with 100–150 ng of mRNA/50 nl, using a sterile glass pipette (tip diameter, 20–25 μm), and was cultured at 16–19° in sterile MBS [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, pH 7.5] supplemented with 10 mg/liter streptomycin, 10,000 units/liter penicillin, 50 mg/liter gentamycin, 2 mM sodium pyruvate, and 0.5 mM theophylline. Oocytes were then used for electrophysiological recording on days 3–10 after injection (27).

Whole-cell voltage-clamp of injected oocytes. Oocytes were placed in a rectangular 100-μl recording chamber and perfused with MBS at a flow rate of 2 ml/min, at room temperature. Recording and clamping microelectrodes (0.5–5 MΩ) were pulled from 1.2-mm (outside diameter) capillary tubing and filled with 3 M KCl. Electrodes were impaled in the animal pole and voltage-clamped at −70 mV, using an Axoclamp IIA amplifier (Axon Instruments, Inc., Burlingame, CA). Resting membrane potentials of mRNA-injected oocytes usually ranged between −30 and −70 mV. The drugs were perfused for 20 sec (about 10 sec were required to reach equilibrium in the recording chamber). At least 20-min intervals were allowed between applications of 5-HT, ACh, quisqualic acid, or *t*-ACPD, to reduce rundown of current amplitude due to desensitization. Ethanol was preapplied for 30–60 sec before being coapplied with 5-HT, ACh, quisqualic acid, or *t*-ACPD.

Intracellular injections of stock solutions of EGTA (20 mM), BAPTA (20 mM), GTPγS (1 mM), IP_3 (10 μM), and PKCI (300 ng/30 nl) were performed using a digital microdispenser (Drummond Scientific, Broomall, PA), according to the method of Lin *et al.* (14). PMA (10 or 25 nM) was prepared in dimethylsulfoxide (final concentration in MBS, 0.05–0.1%). PMA was bath-perfused for 5 min, and 5-HT (500 nM) or quisqualic acid (10 μM) responses were measured at 1 and 25 min after PMA application.

Statistical analysis. Currents were expressed as percentages of control responses due to variability in oocyte expression. The control responses were measured before and after each drug application, to take into account possible shifts in the control currents as recording proceeded. Each oocyte represents a single determination, and oocytes from many different frog donors were used for each experiment. One-way ANOVA, followed by Fisher's LSD *post hoc* test, was used for statistical analysis. Curve-fitting and estimation of EC_{50} values for concentration-response curves were performed using ALLFIT.

Results

Effect of ethanol on membrane currents induced by 5-HT and ACh. In agreement with other studies (18, 19), bath perfusion of 5-HT or ACh produced inward Cl^- currents with a slow onset (5–10 sec after perfusion) that were characterized by a transient inward current component followed by a long-lasting oscillatory component. Both of these components were shown to be Ca^{2+} dependent, because they were prevented by the intracellular injection of 400 pmol of EGTA or BAPTA (data not shown). Pharmacological characterization of the receptor subtypes mediating these responses in oocytes demonstrated that the actions of 5-HT and ACh are mediated through selective interactions with 5-HT$_{1c}$ and muscarinic M$_1$ receptors, respectively (14). Currents elicited by both 5-HT and ACh showed the expected desensitization (14, 21, 28); responses produced by the first application of 5-HT or ACh could never be achieved during subsequent drug applications. However, by allowing at least 20 min between drug applications it was possible to diminish rundown and to obtain reproducible responses during the recording period.

Ethanol inhibited 5-HT responses in a reversible (Fig. 1A) and concentration-dependent (Fig. 1B) manner. The minimal ethanol concentration required to significantly inhibit 5-HT-gated currents was 50 mM (36 ± 5% inhibition), and the maximal inhibition (65 ± 4%) was reached with 150 mM ethanol. A higher ethanol concentration (200 mM) produced a similar effect. The IC_{50} value for ethanol inhibition of 5-HT-induced currents was calculated to be about 46 mM.

Similar results were obtained when the action of ethanol was evaluated on currents induced by 10 μM ACh (Fig. 2). Ethanol at concentrations of 50 and 150 mM produced 30 and 49% inhibition of ACh responses, respectively (Fig. 2B). As in the case of 5-HT, 200 mM ethanol did not induce further inhibition of these currents. The IC_{50} value for ethanol inhibition of ACh-induced currents was also 46 mM.

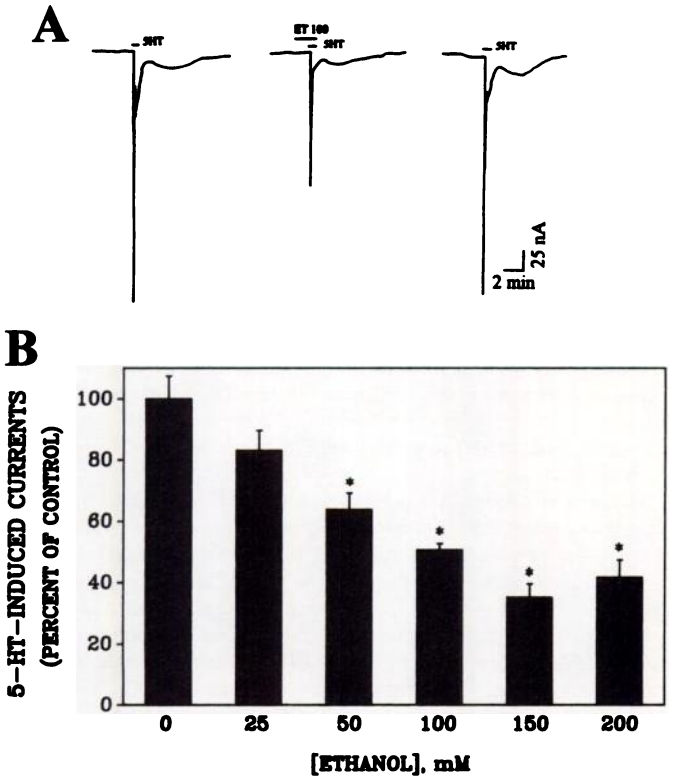

Fig. 1. Ethanol inhibition of 5-HT-induced Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing mouse brain mRNA. A, *Tracings* were obtained from a single cell and show the effect of 100 mM ethanol (*ET*) on currents elicited by 100 nM 5-HT. *Horizontal bars*, time of drug application. B, The graph shows the concentration-dependent inhibition by ethanol of 5-HT responses. Ethanol (25–200 mM) was bath perfused for 60 sec before being coapplied with 100 nM 5-HT for 20 sec. Values represent the mean ± standard error of 12 different oocytes. *, $p < 0.05$ versus control response with 100 nM 5-HT (one-way ANOVA followed by Fisher's LSD *post hoc* test).

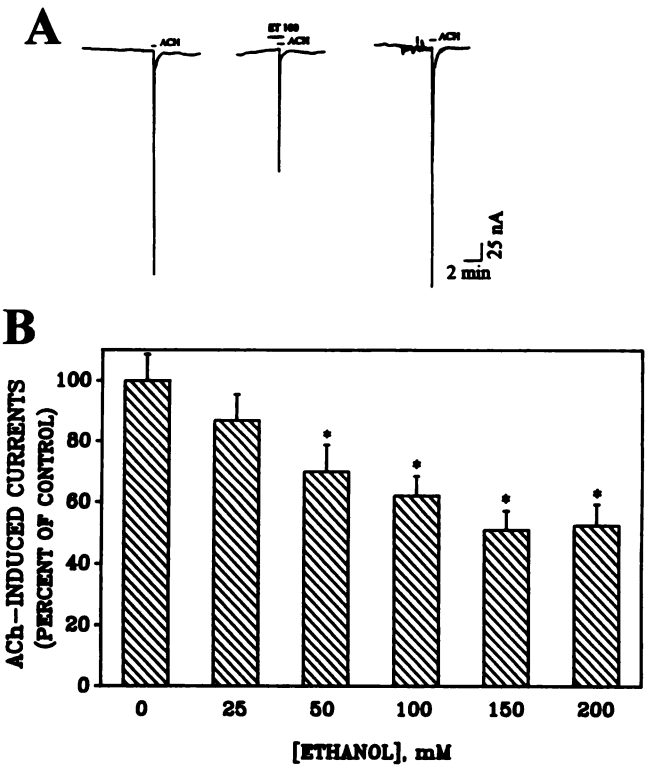

Fig. 2. Ethanol inhibition of ACh-induced Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes expressing mouse brain mRNA. A, *Tracings* were obtained from a single cell and show the effect of 100 mM ethanol (*ET*) on membrane currents induced by 10 μM ACh. *Horizontal bars*, time of drug application. B, The graph shows the concentration-dependent inhibition by 25–200 mM ethanol of ACh responses. Drugs were bath perfused as described for Fig. 1. Values represent the mean ± standard error of eight different oocytes. *, $p < 0.05$ versus control response with 10 μM ACh (one-way ANOVA followed by Fisher's LSD *post hoc* test).

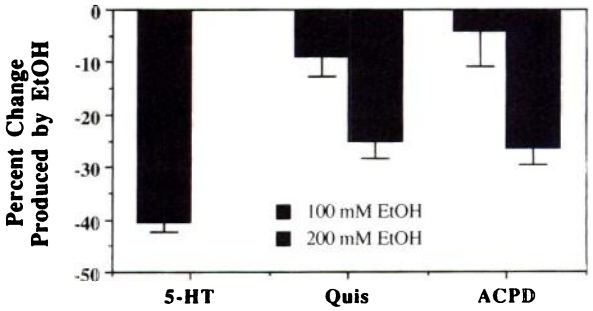

Fig. 3. Effects of ethanol (*EtOH*) on 5-HT_{1c} and metabotropic glutamate receptor-mediated responses. The effects of 100 or 200 mM ethanol on 100 nM 5-HT-, 10 μM quisqualic acid (*Quis*)-, or 100 μM *t*-ACPD-mediated responses were compared in oocytes expressing mRNA from rat cerebellum. Values represent percentage change from control responses produced by ethanol (mean ± standard error of six to 12 different oocytes).

The microinjection of postmortem human cortical mRNA into oocytes produced measurable ACh-induced currents that were attenuated by ethanol in a reversible manner (data not shown).

Ethanol and metabotropic glutamate receptor-mediated membrane currents. To evaluate whether ethanol affected Ca^{2+}-activated Cl^- currents elicited via the activation of other G protein- and PIP_2 turnover-linked receptors, we investigated its effect on metabotropic glutamate receptors. These receptors are expressed in oocytes after the injection of brain mRNA and can be activated by quisqualic acid or the more selective agonist *t*-ACPD (29). Fig. 3 shows that, in oocytes expressing rat cerebellar mRNA, 100 mM ethanol inhibited membrane currents elicited by 100 nM 5-HT by 40% but failed to significantly modify those produced by the application of 10 μM quisqualic acid or 100 μM *t*-ACPD. However, when the ethanol concentration was increased to 200 mM, currents evoked by both quisqualic acid and *t*-ACPD were inhibited by about 25%.

Ethanol alteration of the concentration-response curves for 5-HT- and ACh-mediated membrane currents. To characterize further the effect of ethanol on 5-HT and ACh responses, concentration-response curves for 5-HT or ACh were obtained in the absence and presence of 100 mM ethanol. 5-HT was applied at concentrations ranging from 1 nM to 10 μM (Fig. 4A). The threshold 5-HT concentration was about 10 nM and the EC_{50} value was estimated to be 124 nM. In the presence of 100 mM ethanol, the threshold 5-HT concentration was increased to about 100 nM, the EC_{50} value was 443 nM, and the maximal response induced by 10 μM 5-HT was inhibited by 28%. The magnitude of the inhibition by ethanol was dependent upon the 5-HT concentration, with the ethanol inhibition being greater at lower concentrations of 5-HT (Fig. 4A). Similar results were obtained with ACh (Fig. 4B). Perfu-

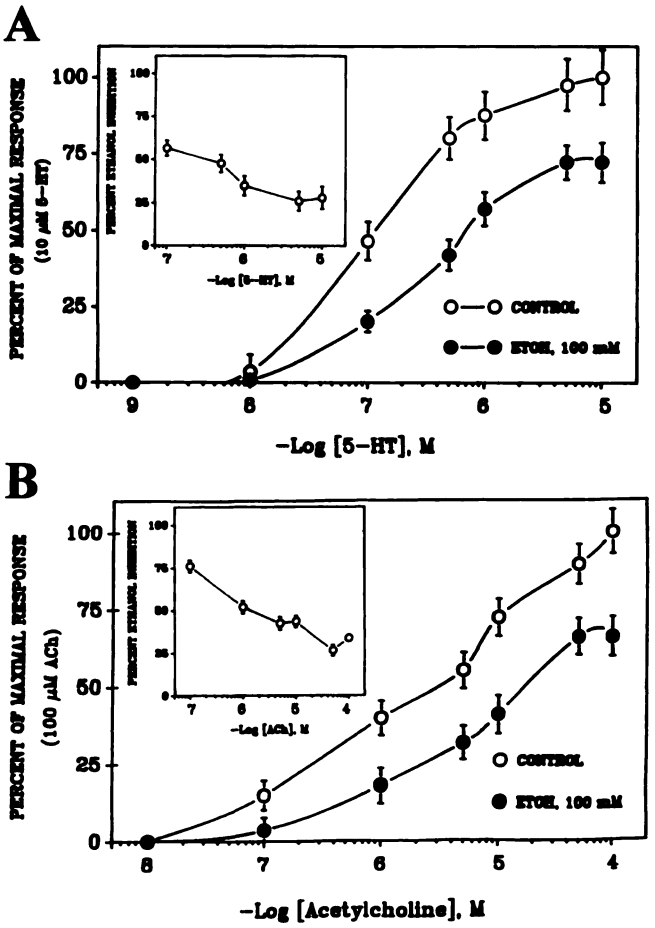

Fig. 4. Ethanol alteration of the concentration-response curve for 5-HT- and ACh-mediated Ca^{2+}-activated Cl^- currents. Increasing concentrations of 5-HT (1 nM to 10 μM) (A) and ACh (10 nM to 100 μM) (B) were applied in the absence or presence of 100 mM ethanol (*ETOH*). Values represent the percentage of the maximal response obtained with 10 μM 5-HT (A) or 100 μM ACh (B) (mean ± standard error of four to six different oocytes). *Insets* (A and B), percentage ethanol inhibition as a function of increasing 5-HT or ACh concentrations. Drugs were bath perfused as for Fig. 1.

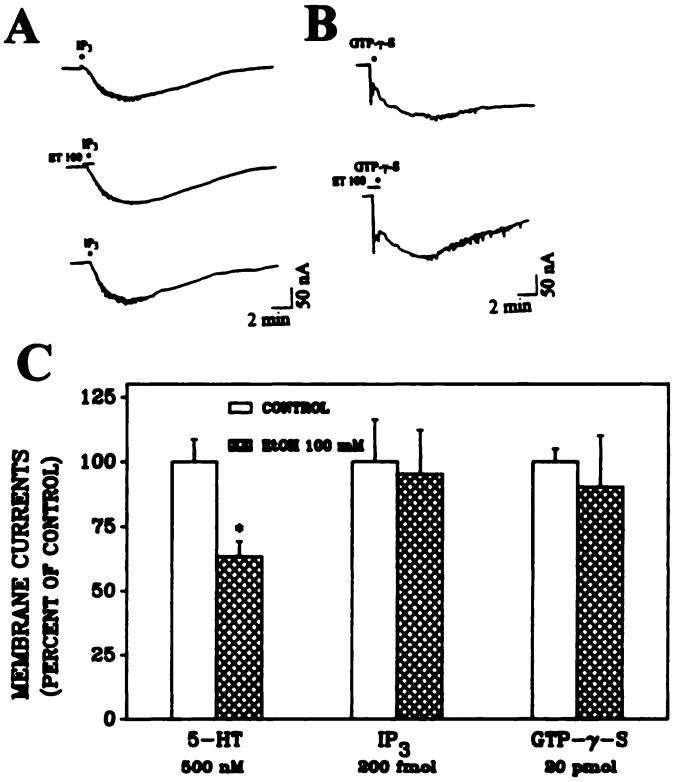

Fig. 5. Effect of ethanol on Ca^{2+}-activated Cl^- currents elicited by the intracellular injection of IP_3 or GTPγS. A and B, *Tracings* show the effect of 100 mM ethanol (*ET*) on currents induced by the intracellular injection of 200 fmol of IP_3 or 20 pmol of GTPγS. Tracings were obtained using a single oocyte in A, but two different oocytes were used in B because only a single GTPγS response could be obtained in each oocyte. C, Summary of the effect of 100 mM ethanol (*EtOH*) on currents induced by 5-HT, IP_3, and GTPγS. Values represent percentage of control responses (mean ± standard error of six to 14 different oocytes).

sion of ACh concentrations between 10 nM and 100 μM induced increasing inward currents, with an EC_{50} value of 2.3 μM. In the presence of 100 mM ethanol, the EC_{50} value was increased by about 4-fold (9.6 μM) and the maximal response induced by 100 μM ACh was inhibited by 34%. As for 5-HT, the ethanol inhibition was dependent upon the concentration of ACh, with lower concentrations of ACh showing higher sensitivity to inhibition by ethanol (Fig. 4B).

Ethanol and currents induced by intracellular injection of IP_3 or GTPγS. Based on previous reports that indicated that ethanol has differential actions on receptor-G protein interactions, we attempted to clarify the site of action of ethanol. First, we studied the effect of ethanol on the Ca^{2+}-activated Cl^- currents produced by the intracellular injection of GTPγS (20 pmol), a nonhydrolyzable activator of G proteins, or IP_3 (200 fmol). As shown in Fig. 5, 100 mM ethanol did not significantly alter the currents produced by either compound, whereas, by comparison, it inhibited by about 40% the currents elicited by the bath application of 500 nM 5-HT. Increasing the ethanol concentration to 200 mM also did not affect the GTPγS or IP_3 responses (data not shown).

Ethanol inhibition of 5-HT$_{1c}$ receptor-mediated currents and the role of PKC. Activation of 5-HT$_{1c}$, M$_1$, and metabotropic glutamate receptors leads to the activation of PKC, which in turn is believed to play a crucial role in the regulation of the function of these receptors in *Xenopus* oocytes (23, 24, 28, 30, 31). We also found that application of the phorbol ester PMA (10 or 25 nM) for 5 min inhibited 5-HT-evoked currents measured 1 and 25 min after PMA treatment by 60 and 80%, respectively (Fig. 6). Although the quisqualic acid (10 μM) response was similarly inhibited 25 min after PMA treatment, the inhibition at 1 min after PMA treatment was only 13%. However, due to the large variability of quisqualic acid responses immediately after PMA exposure, the difference between the effects of 5-HT and quisqualic acid was not statistically significant.

On the basis of these findings, we investigated whether the effect of ethanol on Ca^{2+}-activated Cl^- currents evoked by 5-HT could be altered by modulating the activity of PKC. As shown in Fig. 7, the inhibition by 150 mM ethanol of 5-HT responses was almost completely abolished after the intracellular injection of 300 ng of PKCI. We should note that the effect of PKCI was not observed in every cell tested. Variability of responses to this inhibitor was more marked among oocytes obtained from different frog donors, compared with oocytes from the same batch. To test another PKC inhibitor, oocytes were exposed to 400 nM staurosporine for 12–18 hr. In agree-

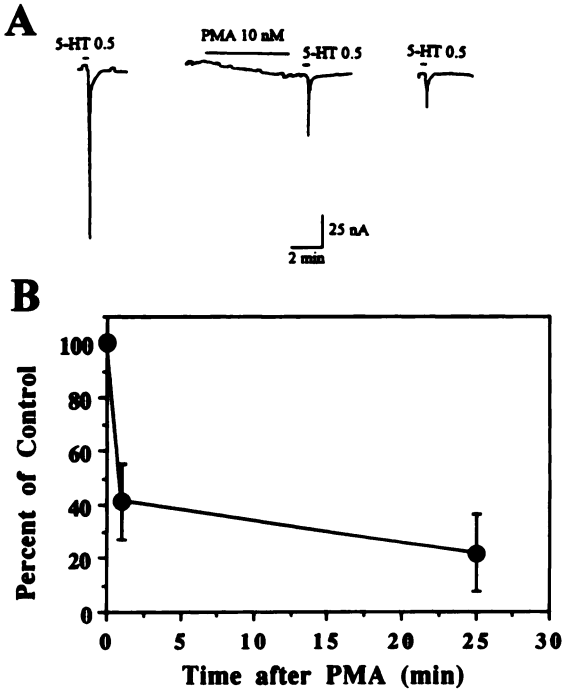

Fig. 6. PMA-mediated inhibition of Ca^{2+}-activated Cl^- currents evoked by 5-HT. A, *Tracings* were obtained from a single oocyte and show 500 nM 5-HT-evoked responses before and after 10 nM PMA perfusion for 5 min. B, Summary of the effect of 10 or 25 nM PMA treatment for 5 min on 5-HT (500 nM) responses. Values represent percentage of control (mean ± standard error of five different oocytes).

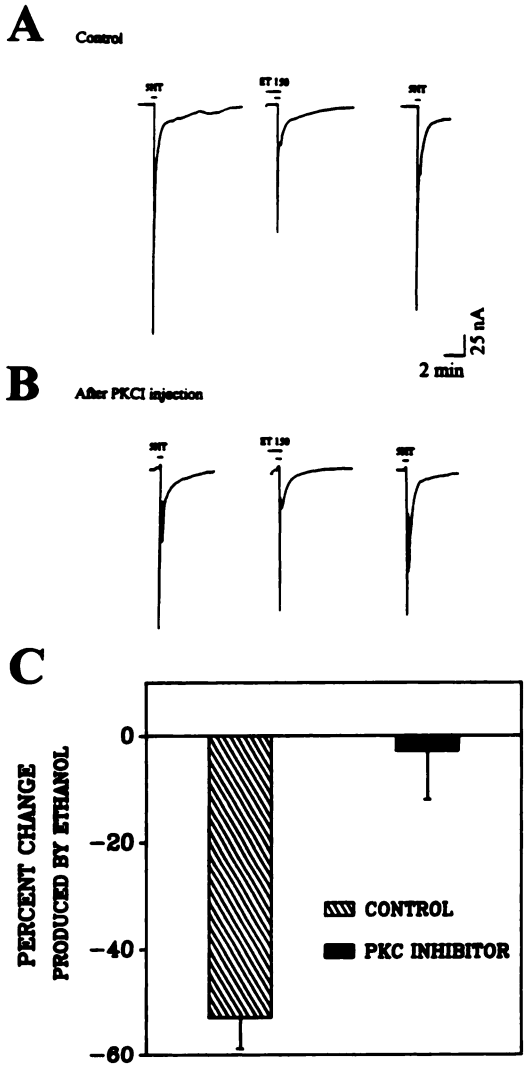

Fig. 7. PKCI prevention of ethanol inhibition of 5-HT-induced Ca^{2+}-activated Cl^- currents. A and B, Effects of 150 mM ethanol (*ET*) on currents induced by 500 nM 5-HT in a control oocyte (A) and in an oocyte that was injected intracellularly with 300 ng of PKCI/30 nl, 40 min before recording (B). C, Summary of the effect of PKCI on ethanol inhibition of 5-HT responses. Values are percentage change from control responses (mean ± standard error of four different oocytes).

ment with the findings of Sakuta *et al.* (31), we found that such treatment resulted in a decrease of desensitization and a potentiation of the oscillatory responses to 5-HT, as well as to ACh. It is also worth noting that ethanol, in some staurosporine-treated oocytes, was able to activate chloride currents that were abolished by the intracellular injection of 400 pmol of EGTA or by changing the membrane potential to −20 mV (data not shown). Induction of Ca^{2+}-activated Cl^- currents by intracellular injection of ethanol was previously reported by Wafford *et al.* (32). This effect is believed to occur through the release of intracellular Ca^{2+}. Thus, it is possible that staurosporine treatment sensitizes some oocytes to this ethanol-induced effect.

Fig. 8 shows that staurosporine treatment, similarly to PKCI treatment, completely prevented the inhibition of 5-HT responses produced by 150 mM ethanol. Note that, in the tracings in Fig. 8A, bath application of 150 mM ethanol alone failed to elicit any measurable membrane current.

These findings indicate that inhibition of PKC activity prevents or reduces the depressant effect of ethanol, and they suggest at least two possible mechanisms, i.e., that 1) ethanol directly activates PKC, promoting phosphorylation of the receptor and resulting in inhibition of receptor function, or 2) ethanol requires a receptor in a phosphorylated state to exert its inhibitory effect.

Ethanol and Ca^{2+}-activated Cl^- current desensitization. To investigate further the role of PKC in the inhibitory modulation by ethanol of 5-HT_{1c} receptor-mediated responses, desensitization studies were performed. Others have shown that PKC plays an important role in the desensitization of these receptors (25, 28, 31). Based on the results described above, we predicted that ethanol, like PKC activators and in contrast to PKC inhibitors, would promote receptor desensitization.

Desensitization of 5-HT_{1c} receptors was studied by using repeated 5-HT applications, at 3-min intervals (Fig. 9). The current amplitude gradually decreased and, in accordance with other reports (28), the transient component of the 5-HT response was more susceptible to desensitization, compared with the long-lasting component. Fig. 9A shows that either the continuous perfusion of 150 mM ethanol or pretreatment with 10 nM PMA accelerated desensitization of 5-HT responses. In agreement with other reports (25, 31), staurosporine treatment decreased current desensitization. The rates of desensitization calculated from regression analysis (Fig. 9B) were as follows: control, 0.0368/min; ethanol, 0.0636/min; PMA, 0.0486/min; staurosporine, 0.0203/min.

Discussion

In the present study we used an electrophysiological approach to demonstrate, for the first time, that acute exposure to ethanol

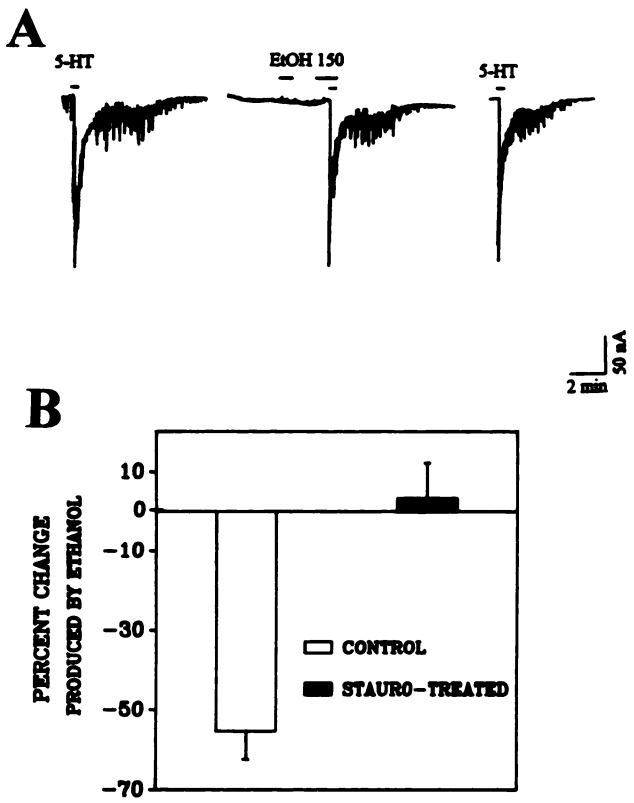

Fig. 8. Effect of ethanol on 5-HT-induced Ca^{2+}-activated Cl^- currents in oocytes treated with staurosporine. Oocytes were incubated with 400 nM staurosporine for 12–18 hr. A, *Tracings* obtained from a single cell show the effects of 150 mM ethanol (*EtOH*) on currents evoked by 500 nM 5-HT. B, Summary of the effects of 150 mM ethanol in control and staurosporine (*STAURO*)-treated oocytes. Values represent percentage change from control responses (mean ± standard error of 10 different oocytes).

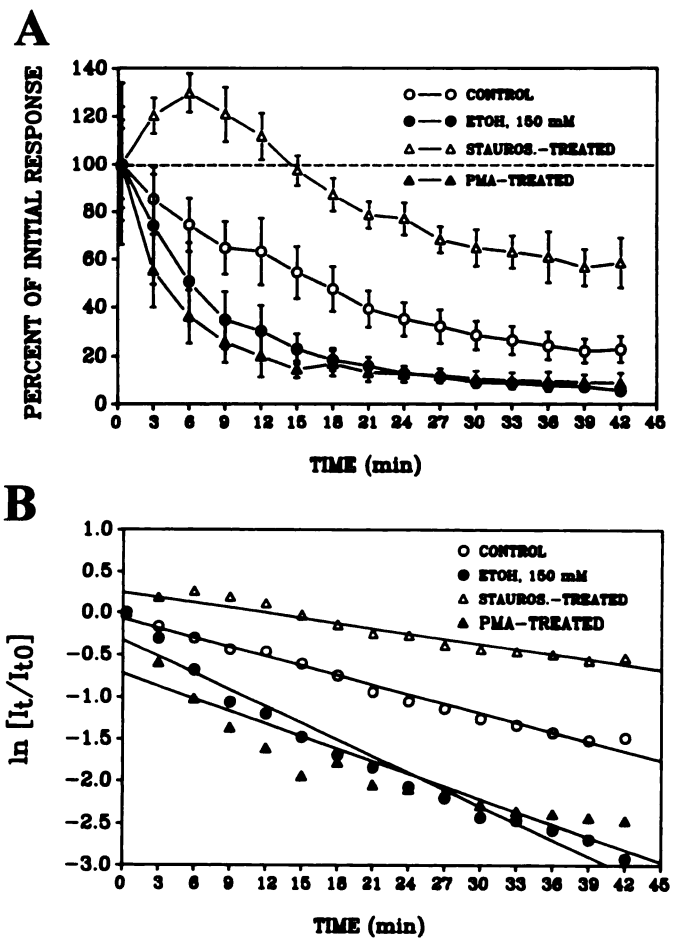

Fig. 9. Desensitization of Ca^{2+}-activated Cl^- currents induced by repeated applications of 5-HT and effects of ethanol (*ETOH*), staurosporine (*STAUROS.*), and PMA. Currents produced by repeated (every 3 min) 20-sec bath applications of 500 nM 5-HT were recorded in oocytes perfused in the absence or in the continued presence of 150 mM ethanol or in oocytes preincubated with 400 nM staurosporine for 12–18 hr or with 10 nM PMA for 1 hr. A, *Values on the abscissa*, time (min) after the first 5-HT application; *values on the ordinate*, percentage of the initial response produced by 5-HT (mean ± standard error of seven to 15 different oocytes). B, *Values on the ordinate*, same as in A but expressed as $\ln[I_t/I_{t0}]$, where I_t and I_{t0} represent the currents measured at a given time t and at time 0, respectively.

inhibits the function of an ion channel activated by second messenger-linked receptors. *Xenopus* oocytes represented a convenient model system for this study, because they express a large number of endogenous Ca^{2+}-activated Cl^- channels (15, 17). The physiological role of Ca^{2+}-activated Cl^- channels is likely to involve mediation of the responses of native muscarinic receptors present in the cells of the follicular layer, which encloses the oocytes (33, 34). These endogenous muscarinic receptor-activated responses, which are mediated by muscarinic M_3 receptors (35) and linked to phospholipid metabolism, are abolished after treatment of oocytes with collagenase (19). However, the same signaling pathway can be operated by heterologous receptors expressed from brain mRNA, including serotonin 5-HT$_{1c}$ receptors (18, 36), muscarinic M_1 receptors (19, 36), and metabotropic glutamate receptors (20). The occurrence of Ca^{2+}-activated Cl^- currents has also been described in mammalian nervous system, such as in cultured mouse spinal neurons (37) and cultured rat dorsal root ganglia (38).

Our experiments showed that Ca^{2+}-activated Cl^- currents evoked by 5-HT, by ACh, and, to a much lesser extent, by quisqualic acid or *t*-ACPD, were decreased by ethanol. The inhibitory effect on 5-HT and ACh responses was reversible and concentration dependent, occurred at a concentration of ethanol as low as 50 mM, and was maximal at 150 mM for both receptors. We found that 100 mM ethanol increased by about 4-fold the EC_{50} value for both 5-HT and ACh responses and also decreased the maximal effect of both agonists. Ethanol also inhibited ACh responses expressed from human brain mRNA, which is consistent with the study of Lin *et al.* (14) showing that the anesthetic enflurane produced a similar inhibition of mouse and human ACh responses. In addition, we also have evidence that the rat 5-HT$_{1c}$ receptor clone is ethanol sensitive.

In earlier studies, Hoffman *et al.* (8) showed that much higher concentrations of ethanol (250–500 mM) were needed to inhibit carbachol-stimulated PIP_2 breakdown in different mouse brain areas, and 500 mM ethanol caused a 2–3-fold increase in the EC_{50} value for the carbachol response. Similar concentrations (500 mM) of ethanol also inhibited muscarinic receptor-mediated PIP_2 hydrolysis in the striatum, but not in other brain areas (6). However, Rabe and Weight (39) demonstrated that lower concentrations (25–100 mM) of ethanol were sufficient to decrease muscarine-stimulated and Ca^{2+}-dependent release of norepinephrine in PC12 cells. Moreover, Balduini and Costa

(40) found that ethanol (150–500 mM) inhibition of muscarinic receptor-mediated PIP_2 metabolism was age dependent, in that it could be detected in cortical slices from 7-day-old but not adult rats. Ethanol (50–500 mM) was also found to decrease PIP_2 metabolism stimulated through α-adrenergic, bradykinin, and neurotensin receptors in brain membranes and cultured cells (6, 41).

The question arises as to why most studies to date required large concentrations of ethanol (up to 500 mM) to produce only modest inhibition of ACh responses but our system is much more sensitive. It is important to note that the two studies using either single cells or cells in culture, namely ours and that of Rabe and Weight (39), demonstrated effects of lower concentrations of ethanol. Thus, it is possible that different types of cells vary in their sensitivity to ethanol (perhaps due to kinase or phosphatase differences; see below) and the experiments with brain tissue measure the average of ethanol-sensitive and -resistant cells.

Our experiments provide some information about the molecular mechanism by which ethanol affects receptor-mediated PIP_2 hydrolysis. Although we do not have direct evidence, at least three considerations seem to exclude the neurotransmitter binding site of the 5-HT_{1c} and M_1 receptors as the site of action for ethanol. Firstly, the concentration-response studies showed that ethanol increased by about 4-fold the EC_{50} value for both 5-HT and ACh responses and decreased the maximal response induced by both compounds; this suggests that this action is uncompetitive in nature. The classical explanation for uncompetitive inhibition is that the inhibitor alters the ligand-receptor complex after binding of the ligand has occurred. Secondly, both receptor-mediated responses had identical sensitivity to ethanol (IC_{50}, 46 mM). Thirdly, receptor binding studies on muscarinic receptors in brain membranes and cultured cells did not reveal any significant effect of acute ethanol treatment, even at very high concentrations (39, 40, 42). Thus, if ethanol does not affect the binding of agonist to the receptors, then some other step(s), downstream from the receptor, must be altered by this drug. To investigate other sites, we injected GTPγS or IP_3 into the oocyte to bypass the receptor and selectively activate different steps of the second messenger pathway. The lack of effect of ethanol on Ca^{2+}-activated Cl^- currents evoked by IP_3 indicates that ethanol does not interfere with IP_3-induced Ca^{2+} release. Moreover, this finding also shows that ethanol does not directly inhibit the Ca^{2+}-activated Cl^- channel.

Direct activation of G proteins by injection of GTPγS was also not altered by ethanol. Thus, it is unlikely that ethanol directly interferes with the binding of guanine nucleotides to G proteins or with the coupling between G proteins and PLC. However, this result does not exclude the possibility of an action of ethanol on the coupling between the receptor and the G protein.

Although native oocytes possess their own G proteins, it has been shown that the 5-HT_{1c} and M_1 receptors expressed in oocytes from brain mRNA are coupled to newly synthesized pertussis toxin-sensitive G_o and G_i, respectively (43, 44). The stimulatory G protein G_s, coupled to the adenylate cyclase second messenger system, appears to be an ethanol-sensitive target, whereas other G proteins seem to be much less sensitive to this drug (10). This is consistent with the findings of Gonzales and Crews (7), who showed that guanine nucleotide- and calcium-dependent PIP_2 hydrolysis in cortical membranes was not affected by concentrations of ethanol as high as 500 mM. Moreover, Candura *et al.* (9) reported that 250 and 500 mM ethanol induced a slight inhibitory effect on GTPγS- and fluoride-stimulated PIP_2 turnover in cortical membranes of 7-day-old rats, but this effect could not account for the extent of ethanol inhibition of carbachol-stimulated PIP_2 turnover observed in the same preparation. Our data further support the idea that G_o and G_i are resistant to direct actions of ethanol.

General anesthetic agents also affect muscarinic and 5-HT receptor-stimulated PIP_2 turnover by altering receptor-G protein coupling (13, 14). However, Lin *et al.* (14), using *Xenopus* oocytes expressing mouse brain mRNA, found that enflurane directly suppresses GTPγS-induced Ca^{2+}-activated Cl^- currents. Thus, this result suggests that enflurane and ethanol alter 5-HT_{1c} and M_1 receptor function through different mechanisms of action.

Stimulation of PKC activity by Ca^{2+} and DAG is thought to be a crucial molecular event for the regulation of 5-HT_{1c} and M_1 receptors (24, 28). Pharmacological manipulation of PKC activity with activators, such as the phorbol ester derivatives, or with inhibitors led to the conclusion that the stimulation of PKC produces a down-regulation of 5-HT and ACh responses in oocytes as well as in other systems (23, 45–47). Using PMA, we verified that the 5-HT response is inhibited immediately after activation of PKC. This process represents a physiological negative feedback mechanism by which the intracellular response to receptor stimulation is terminated. Conversely, inhibition of PKC activity with the use of different PKC inhibitors blocked this process and reduced the inactivation of the responses (25, 31). On the basis of this body of knowledge, we tested the effect of ethanol on 5-HT responses in oocytes treated with the PKC inhibitors staurosporine and PKCI. Our results indicated that both compounds were able to abolish the inhibition by ethanol of 5-HT-induced Ca^{2+}-activated Cl^- currents. Moreover, we found that ethanol increased current desensitization produced by repeated stimulations with 5-HT. This effect was similar to that induced by PMA and opposite to that of staurosporine. These data clearly suggest that the action of ethanol on these receptors involves PKC modulation.

One possible mechanism to explain our results is that ethanol stimulates PKC, which, in turn, down-regulates the 5-HT_{1c} receptor-mediated response. In support of this hypothesis, there are several reports showing that ethanol activates PKC by promoting its translocation from the cytosol to the membrane fraction (48–50). It is interesting to note that in these studies the effective ethanol concentrations ranged from 2 to 200 mM, the ethanol effect on PKC occurred within 30 sec, and the effect was reversible. Thus, these parameters are very similar to those used in our study. In addition, we have recent experimental evidence that ethanol, like PMA, increases protein phosphorylation in oocytes, in an "*in vivo*" assay.[1] However, ethanol does not directly stimulate the activity of isolated PKC (51, 52), and the mechanism by which ethanol may activate PKC *in vivo* has not been clarified. It is, however, likely that *in vitro* assays with purified PKC and substrates might lack some factors that would be present *in vivo* and that would be important for ethanol-mediated activation of PKC.

Thus, assuming that ethanol acts through PKC, what com-

[1] C .F. Valenzuela, E. Sanna, and R. A. Harris, unpublished observations.

ponent(s) of the 5-HT$_{1c}$ and M$_1$ receptor-linked metabolic pathway might be phosphorylated by PKC? Kato *et al.* (23), using *Xenopus* oocytes expressing rat brain mRNA, proposed that the site of TPA-stimulated PKC phosphorylation is upstream from G proteins and is likely the 5-HT$_{1c}$ receptor. Similar findings were reported by Maruyama (53), who demonstrated, using pancreatic cells, that PKC activation does not affect the activity of G proteins but uncouples the muscarinic receptor and the G protein. Because those authors found that TPA did not significantly alter GTPγS-induced responses, those results suggested that TPA stimulated phosphorylation of the receptor. However, it has been proposed that PKC can phosphorylate both the receptor (53, 54) and the coupled G protein (55, 56). Although there is evidence that the metabotropic glutamate receptor couples through a pertussis toxin-sensitive G protein similar to that used by the 5-HT$_{1c}$ and M$_1$ receptors, likely of the G$_o$ or G$_i$ type (20, 57), and, similarly to these receptors, its function is inhibited after PKC activation, quisqualic acid or *t*-ACPD responses were less sensitive to the inhibitory action of ethanol. However, when comparing the time courses of the effects of PMA on 5-HT$_{1c}$ and metabotropic glutamate receptors we found that, at 1 min after PMA treatment, the metabotropic glutamate receptor response was variably affected, compared with the marked inhibition of the 5-HT$_{1c}$ receptor. The weaker inhibition immediately after activation of PKC observed in some oocytes may explain why the metabotropic glutamate receptors are less sensitive to the acute actions of ethanol. Molecular cloning of the metabotropic glutamate receptor revealed that, although this protein contains seven transmembrane regions, no amino acid sequence homology or conserved amino acids were found between the glutamate receptor and other G protein-linked receptors (57). In addition, this receptor contains a large amino-terminal extracellular domain that is common to large hormone molecule G protein-linked receptors but that is absent in receptors for small molecule neurotransmitters (58). Thus, it is tempting to speculate that these amino acid sequence and structural differences, in addition to the differences in the acute effects of PKC between the metabotropic glutamate receptor and other G protein-linked receptors, may be the molecular basis for the lower sensitivity of the metabotropic glutamate receptor to the effects of ethanol.

Based on our results and those of others, ethanol may activate PKC, leading to phosphorylation of the 5-HT$_{1c}$ and M$_1$ receptors and inhibition or desensitization of the responses. An alternative hypothesis is that ethanol acts on the receptor proteins but this action requires that the receptor be phosphorylated. These two hypotheses are similar to those proposed for the actions of ethanol on ligand-gated ion channels. PKC-mediated phosphorylation appears to be important for ethanol enhancement of γ-aminobutyric acid type A responses (59), for ethanol inhibition of *N*-methyl-D-asparate action (60), and (under conditions of augmented calcium conductance) for ethanol inhibition of kainate responses.[2] Thus, some of the pleiotropic actions of ethanol may be explained by a common mechanism involving activation of PKC.

In summary, this is the first report on the acute effects of ethanol on PIP$_2$ hydrolysis stimulated through 5-HT$_{1c}$ and metabotropic glutamate receptors. We have shown that concentrations of ethanol that can be reached in brain during intoxication inhibit the function of 5-HT$_{1c}$ and M$_1$ receptors expressed in *Xenopus* oocytes from mRNA isolated from mouse, rat, and human brain. The metabotropic glutamate receptor was only weakly inhibited by ethanol, suggesting that not all PIP$_2$ hydrolysis-linked receptors represent targets for the action of this drug. Our results, together with reports demonstrating that 5-HT$_{1c}$ receptor function is altered during withdrawal from chronic ethanol treatment (61–63) and that the 5-HT$_{1c/1b}$ agonist 1-(3-chlorophenyl)piperazine HCl substitutes for ethanol in a drug discrimination paradigm (64), support the idea that some of the metabotropic receptors linked to the PIP$_2$ hydrolysis pathway are inhibited by ethanol and this action may be important in the pharmacology of this drug of abuse. In addition, ethanol has been shown to inhibit long term potentiation in the hippocampus (65), and the recent report by Maeda *et al.* (66) showing an involvement of M$_1$ receptors in long term potentiation in hippocampal slices, together with the present data, suggests that these receptors may have a role in the inhibition of long term potentiation by ethanol. We propose that a possible mechanism of action of ethanol involves activation of PKC, resulting in uncoupling of the receptor from the G protein. Finally, our data suggest that some G protein-coupled receptors may be as sensitive to ethanol as are the ligand-gated ion channels (67).

Acknowledgments

The authors wish to thank Drs. W. R. Proctor and T. V. Dunwiddie for many helpful discussions and technical advice.

References

1. Saito, T., J. M. Lee, and B. Tabakoff. Ethanol's effects on cortical adenylate cyclase activity. *J. Neurochem.* **44**:1037–1044 (1985).
2. Bode, D. C., and P. B. Molinoff. Effects of ethanol *in vitro* on the *beta* adrenergic receptor-coupled adenylate cyclase system. *J. Pharmacol. Exp. Ther.* **246**:1040–1047 (1988).
3. Berridge, M. J., and R. F. Irvine. Inositol phosphates and cell signalling. *Nature (Lond.)* **341**:197–205 (1989).
4. Nishizuka, Y. Turnover of inositol phospholipids and signal transduction. *Science (Washington D. C.)* **225**:1365–1370 (1984).
5. Rana, R. S., and L. E. Hokin. Role of phosphoinositides in transmembrane signalling. *Physiol. Rev.* **70**:115–164 (1990).
6. Gonzales, R. A., C. Theiss, and F. T. Crews. Effects of ethanol on stimulated inositol phospholipid hydrolysis in rat brain. *J. Pharmacol. Exp. Ther.* **237**:92–98 (1986).
7. Gonzales, R. A., and F. T. Crews. Ethanol effects on receptor activated lipid hydrolysis in brain and liver. *Alcohol Alcohol.* **1**(suppl.):567–571 (1987).
8. Hoffman, P. L., F. Moses, G. R. Luthin, and B. Tabakoff. Acute and chronic effects of ethanol on receptor-mediated phosphatidylinositol 4,5-bisphosphate breakdown in mouse brain. *Mol. Pharmacol.* **30**:13–18 (1986).
9. Candura, S. M., L. Manzo, and L. G. Costa. Inhibition of muscarinic receptor- and G protein-dependent phosphoinositide metabolism in cerebrocortical membranes from neonatal rat by ethanol. *Neurotoxicology* **13**:281–288 (1992).
10. Hoffman, P. L., and B. Tabakoff. Ethanol and guanine nucleotide binding proteins: a selective interaction. *FASEB J.* **4**:2612–2622 (1990).
11. Hoek, J. B., A. P. Thomas, R. Rubin, and E. Rubin. Ethanol-induced mobilization of calcium by activation of phosphoinositide specific phospholipase C in intact hepatocytes. *J. Biol. Chem.* **262**:682–691 (1987).
12. Rubin, R., and J. B. Hoek. Alcohol-induced stimulation of phospholipase C in human platelets requires G protein activation. *Biochem. J.* **254**:147–153 (1988).
13. Anthony, B. L., R. L. Dennison, and R. S. Aronstam. Disruption of muscarinic receptor-G protein coupling is a general property of liquid volatile anesthetics. *Neurosci. Lett.* **99**:191–196 (1989).
14. Lin, L.-H., S. Leonard, and R. A. Harris. Enflurane inhibits the function of mouse and human brain phosphatidylinositol-linked acetylcholine and serotonin receptors expressed in *Xenopus* oocytes. *Mol. Pharmacol.* **43**:941–948 (1993).
15. Miledi, R. A calcium-dependent transient outward current in *Xenopus laevis* oocytes. *Proc. R. Soc. Lond. B Biol. Sci.* **215**:491–497 (1982).
16. Miledi, R., and I. Parker. Chloride current induced by injection of calcium into *Xenopus* oocytes. *J. Physiol. (Lond.)* **357**:173–183 (1984).
17. Barish, M. E. A transient calcium-dependent chloride current in the immature *Xenopus* oocyte. *J. Physiol. (Lond.)* **342**:309–325 (1983).
18. Lubbert, H., T. P. Snutch, N. Dascal, H. A. Lester, and N. Davidson. Rat

[2] J. E. Dildy-Mayfield and R. A. Harris. Submitted for publication.

brain 5-HT_{1c} receptors are encoded by a 5–6 kbase mRNA size class and are functionally expressed in injected *Xenopus* oocytes. *J. Neurosci.* **7**:1159–1165 (1987).
19. Sugiyama, H., Y. Hisanaga, and C. Hirono. Induction of muscarinic cholinergic responsiveness in *Xenopus* oocytes by mRNA isolated from rat brain. *Brain Res.* **338**:346–350 (1985).
20. Sugiyama, H., I. Ito, and C. Hirono. A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature (Lond.)* **325**:531–533 (1987).
21. Dascal, N., C. Ifune, R. Hopkins, T. P. Snutch, H. Lubbert, N. Davidson, M. I. Simon, and H. A. Lester. Involvement of a GTP-binding protein in mediation of serotonin and acetylcholine responses in *Xenopus* oocytes injected with rat brain messenger RNA. *Mol. Brain Res.* **11**:201–209 (1986).
22. Boton, R., D. Singer, and N. Dascal. Inactivation of calcium-activated chloride conductance in *Xenopus* oocytes: roles of calcium and protein kinase C. *Pfluegers Arch.* **416**:1–6 (1990).
23. Kato, K., S. Kaneko, and Y. Nomura. Phorbol ester inhibition of current response and simultaneous protein phosphorylation in *Xenopus* oocytes injected with brain mRNA. *J. Neurochem.* **50**:766–773 (1988).
24. Moran, O., and N. Dascal. Protein kinase C modulates neurotransmitter responses in *Xenopus* oocytes injected with rat brain RNA. *Mol. Brain Res.* **5**:193–202 (1989).
25. Tan, Y. P., and A. Marty. Protein kinase C-mediated desensitization of the muscarinic response in rat lacrimal gland cells. *J. Physiol. (Lond.)* **433**:357–371 (1991).
26. Sanna, E., and R. A. Harris. Inhibition by ethanol of metabotropic responses to serotonin and acetylcholine in *Xenopus* oocytes. *Soc. Neurosci. Abstr.* **18**:476 (1992).
27. Dildy-Mayfield, J. E., and R. A. Harris. Comparison of ethanol sensitivity of rat brain kainate, DL-α-amino-3-hydroxy-5-methyl-4-isoxalone propionic acid and *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. *J. Pharmacol. Exp. Ther.* **262**:487–494 (1992).
28. Singer, D., R. Boton, O. Moran, and N. Dascal. Short- and long-term desensitization of serotonergic responses in *Xenopus* oocytes injected with brain RNA: roles for inositol 1,4,5-trisphosphate and protein kinase C. *Pfluegers Arch.* **416**:7–16 (1990).
29. Manzoni, O., L. Fagni, J. P. Pin, F. Rassendren, F. Poulat, F. Sladeczek, and J. Bockaert. (*trans*)-1-Aminocyclopentyl-1,3-dicarboxylate stimulates quisqualate phosphoinositide-coupled receptors but not ionotropic glutamate receptors in striatal neurons and *Xenopus* oocytes. *Mol. Pharmacol.* **38**:1–6 (1990).
30. Manzoni, O. J. J., F. Finiels-Marlier, I. Sassetti, J. Blockaert, C. le Peuch, and F. A. J. Sladeczek. The glutamate receptor of the Q_p-type activates protein kinase C and is regulated by protein kinase C. *Neurosci. Lett.* **109**:146–151 (1990).
31. Sakuta, H., M. Sekiguchi, K. Okamoto, and Y. Sakai. Oscillatory muscarinic acetylcholine responses of *Xenopus* oocytes are desensitized by protein kinase C and sensitized by protein phosphatase 2B. *Eur. J. Pharmacol.* **208**:297–305 (1991).
32. Wafford, K. A., T. V. Dunwiddie, and R. A. Harris. Calcium-dependent chloride currents elicited by injection of ethanol into *Xenopus* oocytes. *Brain Res.* **505**:215–219 (1989).
33. Dascal, N., and E. M. Landau. Types of muscarinic response in *Xenopus* oocytes. *Life Sci.* **27**:1423–1428 (1980).
34. Dascal, N., B. Gillo, and Y. Lass. Roles of calcium mobilization in mediation of acetylcholine-evoked chloride currents in *Xenopus laevis* oocytes. *J. Physiol. (Lond.)* **366**:299–313 (1985).
35. Van Wezenbeek, L. A. C. M., J. A. D. M. Tonnaer, and G. S. F. Ruigt. The endogenous muscarinic acetylcholine receptor in *Xenopus* oocytes is of the M_3 subtype. *Eur. J. Pharmacol.* **151**:497–500 (1988).
36. Nomura, Y., S. Kaneko, K. Kato, S. Yamagishi, and H. Sugiyama. Inositol phosphate formation and chloride current responses induced by acetylcholine and serotonin through GTP-binding proteins in *Xenopus* oocytes after injection of rat brain messenger RNA. *Mol. Brain Res.* **2**:113–123 (1987).
37. Owen, D. G., M. Segal, and J. L. Barker. Voltage-clamp analysis of a Ca^{2+}- and voltage-dependent chloride conductance in cultured mouse spinal neurons. *J. Neurophysiol.* **55**:1115–1135 (1986).
38. Mayer, M. L. A calcium-activated chloride current generates the after-depolarization of rat sensory neurones in culture. *J. Physiol. (Lond.)* **364**:217–239 (1985).
39. Rabe, C. S., and F. F. Weight. Effects of ethanol on neurotransmitter release and intracellular free calcium in PC12 cells. *J. Pharmacol. Exp. Ther.* **244**:417–422 (1988).
40. Balduini, W., and L. G. Costa. Developmental neurotoxicity of ethanol: *in vitro* inhibition of muscarinic receptor-stimulated phosphoinositide metabolism in brain from neonatal but not adult rats. *Brain Res.* **512**:248–252 (1990).
41. Smith, T. L. Selective effects of acute and chronic ethanol exposure on neuropeptide and guanine nucleotide stimulated phospholipase C activity in intact N1E-115 neuroblastoma. *J. Pharmacol. Exp. Ther.* **258**:410–415 (1991).
42. Smith, T. L., H. I. Yamamura, and L. Lee. Effect of ethanol on receptor-stimulated phosphatidic acid and polyphosphoinositide metabolism in mouse brain. *Life Sci.* **39**:1675–1684 (1986).
43. Kaneko, S., K. Kato, S. Yamagishi, H. Sugiyama, and Y. Nomura. GTP-binding proteins G_i and G_o transplanted onto *Xenopus* oocyte by rat brain messenger RNA. *Mol. Brain Res.* **3**:11–19 (1987).
44. Kaneko, S., H. Takahashi, and M. Satoh. Metabotropic responses to acetylcholine and serotonin of *Xenopus* oocytes injected with rat brain mRNA are transduced by different G protein subtypes. *FEBS Lett.* **299**:179–182 (1992).
45. Llano, I., and A. Marty. Protein kinase C activators inhibit the inositol trisphosphate-mediated muscarinic current responses in rat lacrimal cells. *J. Physiol. (Lond.)* **394**:239–248 (1987).
46. Richardson, R. M., J. Ptasienski, and M. M. Hosey. Functional effects of protein kinase C-mediated phosphorylation of chick heart muscarinic cholinergic receptors. *J. Biol. Chem.* **267**:10127–10132 (1992).
47. Vincentini, L. M., F. Di Virgilio, A. Ambrosini, T. Pozzan, and J. Meldolesi. Tumor promoter 12-myristate 13-acetate inhibits phosphoinositide hydrolysis and cytosolic Ca^{2+} rise induced by the activation of muscarinic receptors in PC12 cells. *Biochem. Biophys. Res. Commun.* **127**:310–317 (1985).
48. DePetrillo, P. B., and R. M. Swift. Ethanol exposure results in a transient decrease in human platelet cAMP levels: evidence of a protein kinase C mediated process. *Alcohol. Clin. Exp. Res.* **16**:290–294 (1992).
49. Skwish, S., and W. Shain. Ethanol and diolein stimulate PKC translocation in astroglial cells. *Life Sci.* **47**:1037–1042 (1990).
50. Tuominen, R. K., S. Lustig, P. T. Mannisto, and M. Viluksela. Ethanol activates protein kinase C (PKC) in cultured bovine adrenal medullary (BAM) chromaffin cells. *Soc. Neurosci. Abstr.* **18**:283 (1992).
51. Machu, T. K., R. W. Olsen, and M. D. Browning. Ethanol has no effect on cAMP-dependent protein kinase-, protein kinase C-, or Ca^{2+}-calmodulin-dependent protein kinase II-stimulated phosphorylation of highly purified substrates *in vitro*. *Alcohol. Clin. Exp. Res.* **15**:1040–1044 (1991).
52. Slater, S. J., K. J. A. Cox, J. V. Lombardi, C. Ho, M. B. Kelly, E. Rubin, and C. D. Stubbs. Inhibition of protein kinase C by alcohols and anaesthetics. *Nature (Lond.)* **364**:82–84 (1993).
53. Maruyama, Y. Activation and desensitization mechanisms of muscarinic current response in single pancreatic acinar cells of rats. *J. Physiol. (Lond.)* **417**:343–359 (1989).
54. Wojcikiewicz, R. J. H., A. B. Tobin, and S. R. Nahorski. Desensitization of cell signalling mediated by phosphoinositidase C. *Trends Pharmacol. Sci.* **14**:279–285 (1993).
55. Katada, T., A. G. Gilman, Y. Watanabe, S. Bauer, and K. H. Jakobs. Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur. J. Biochem.* **151**:431–437 (1985).
56. Yatomi, Y., Y. Arata, S. Tada, S. Kume, and M. Ui. Phosphorylation of the inhibitory guanine-nucleotide-binding protein as a possible mechanism of inhibition by protein kinase C of agonist-induced Ca^{2+} mobilization in human platelet. *Eur. J. Biochem.* **205**:1003–1009 (1992).
57. Masu, M., Y. Tanabe, K. Tsuchida, R. Shigemoto, and S. Nakanishi. Sequence and expression of a metabotropic glutamate receptor. *Nature (Lond.)* **349**:760–765 (1991).
58. Tanabe, Y., M. Masu, T. Ishii, R. Shigemoto, and S. Nakanishi. A family of metabotropic glutamate receptors. *Neuron* **8**:169–179 (1992).
59. Wafford, K. A., and P. J. Whiting. Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the *gamma* 2 subunit. *FEBS Lett.* **313**:113–117 (1992).
60. Snell, L. D., B. Tabakoff, and P. L. Hoffman. Involvement of protein kinase C in ethanol induced inhibition of NMDA receptor function in cerebellar granule cells. *Alcohol. Clin. Exp. Res.* **18**:81–85 (1994).
61. Lal, H., P. L. Prather, and S. M. Rezazadeh. Potential role of $5HT_{1c}$ and/or $5HT_2$ receptors in the mianserin-induced prevention of anxiogenic behaviors occurring during ethanol withdrawal. *Alcohol. Clin. Exp. Res.* **17**:411–417 (1993).
62. Pandey, S. C., M. P. Dubey, M. Piano, D. W. Schwertz, J. M. Davis, and G.N. Pandey. Effect of chronic ethanol consumption on $5HT_{1c}$ receptors and phosphoinositide signalling system in rat choroid plexus. *Alcohol. Clin. Exp. Res.* **17**:499 (1993).
63. Rezazadeh, S. M., P. L. Prather, and H. Lal. Sensitization to 5-HT_{1c} receptor agonist in rats observed following withdrawal from chronic ethanol. *Alcohol* **10**:281–283 (1993).
64. Grant, K. A., G. Colombo, and G. J. Gatto. Characterization of 5-HT_1 receptor agonists and the mediation of ethanol mimetic discriminative stimulus effects. *Alcohol. Clin. Exp. Res.* **17**:497 (1993).
65. Blitzer, R. D., O. Gil, and E. M. Landau. Long-term potentiation in rat hippocampus is inhibited by low concentrations of ethanol. *Brain Res.* **537**:203–208 (1990).
66. Maeda, T., S. Kaneko, and M. Satoh. Bidirectional modulation of long-term potentiation by carbachol via M_1 and M_2 muscarinic receptors in guinea pig hippocampal mossy fiber-CA3 synapses. *Brain Res.* **619**:324–330 (1993).
67. Sanna, E., and R. A. Harris. Neuronal ion channels. *Recent Dev. Alcohol.* **11**:169–186 (1993).

Send reprint requests to: Enrico Sanna, Department of Pharmacology, University of Colorado Health Sciences Center, 4200 E. 9th Avenue, Campus Box C236, Denver, CO 80262.