Title: **GENERAL ANESTHETICS AND ETHANOL INHIBIT ENZYMATIC ACTIVITY OF CNS-DERIVED PROTEIN KINASE C.**

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INTRODUCTION: Protein kinase C (calcium/phospholipid-dependent kinase, PKC) is the intracellular effector for a multiplicity of central neuronal cell surface receptors (1). PKC also modulates synaptic transmission, by phosphorylating neurotransmitter receptors, ion channels, and/or synaptic proteins involved in neurotransmitter release (2). Since PKC activity depends upon membrane association and availability of its phospholipid-derived activating ligand, diacylglycerol (DAG), it is a likely target for general anesthetic (GA) action. This hypothesis is supported by our previous reports that GAs inhibit binding of an analog of DAG, 3H-phorbol bibutyrate (PDBU), with potencies paralleling clinical potencies (3,4). To test whether GAs also affect the functional properties of the enzyme, we measured protein phosphorylation catalyzed by PKC in the presence of several volatile agents, using a 32P-incorporation assay adapted from Bell (5).

METHODS: PKC was derived from fresh rat brain homogenates as described previously (4). Membranes were treated with calcium chelators to release PKC, ultracentrifuged, and after supernatants were passed over DEAE-Sephacel, the enzyme was eluted by ionic gradient. The amount of PKC in eluates was quantified via 3H-PDBU assay (4). Reaction mixtures consisted of aliquots of dilute enzyme, lipid (phosphatidylserine-DAG) dispersion, calcium/magnesium chloride (final Ca^{++} 20 μM; Mg^{++} 10 mM), substrate protein (histone III-S, Sigma Chemicals), $\pm$ GAs diluted in buffer. After equilibration to 30°C in gas-tight vials, reactions were initiated by addition of 10 μM (final) γ-32P-ATP (Amersham), allowed to incubate for 2.5 minutes, then quenched with excess iced 25% trichloroacetate. Following filtration through GF/C glass fiber (Whatman), filters were dried and counted in scintillation cocktail. 32P-incorporation was corrected for other kinase activities, determined in the absence of added lipids. Concentrations of GAs were confirmed by gas chromatography.

RESULTS: 32P-incorporation was linear during the time-course of assays, and exquisitely calcium-dependent. All GAs tested significantly inhibited specific calcium/lipid-dependent 32P-incorporation. For example, compared to controls, halothane 500 μM diminished incorporation by 30 $\pm$ 4%; diethylether 24 mM, 36 $\pm$ 4%; and, ethanol 50 mM, 45 $\pm$ 4%. Inhibition was reversible, and concentration-dependent for all agents, but could not be overcome by increasing calcium concentration.

DISCUSSION: These data demonstrate that PKC's enzymatic activity is sensitive to perturbation by physiologic concentrations of several GAs. This is consistent with previously reported effects assessed by equilibrium binding of an activating cofactor (3,4). However, further kinetic studies will be required to elucidate the mechanism of enzymatic inhibition.

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Supported by UACCMF.

A707

DIETHYLETHER'S ENHANCEMENT OF ^{36}Cl$^-$ FLUX THROUGH THE GABA-GATED CHANNEL IS NOT AGONIST-DEPENDENT

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Introduction: The GABA receptor chloride channel macromolecule ("GABA-R") mediates synaptic inhibition for the predominant inhibitory neurotransmitter in the mammalian CNS, GABA, by increasing membrane permeability to chloride which hyperpolarizes the neurons.[1] GABA-R also mediates the effects of two classes of major sedatives, the benzodiazepines and the barbiturates. In addition to these, volatile general anesthetics (GAs) can also enhance chloride permeability via GABA-R, as measured by ^{36}Cl$^-$ flux assays in vitro.[2,3] However, whether GAs enhance flux *directly*, or alternatively, require the presence of the agonist GABA, remains unclear. This is because the studies which detected direct effects of GAs did not control for the presence of endogenous GABA[2], which is usually present in significant quantity in freshly prepared brain membranes.[4] Thus we assessed the ability of diethylether (DEE) to enhance transmembrane ^{36}Cl$^-$ flux via GABA-R in the presence, and absence, of the specific GABA antagonist, bicuculline.

Methods: Rat brain membrane microvesicles were prepared by differential centrifugation.[3] Fresh membrane suspensions (200 μl, 14 mg protein/ml by Hartree-Lowry assay[5]) were incubated in glass test tubes in a water bath at 34°C. ^{36}Cl$^-$ (770 μCi/mmol, Amersham, diluted in buffer to 2 μCi/ml), $\pm$ DEE, $\pm$ bicuculline 37.5 μM, $\pm$ GABA 200 μM (total volume of 200 μl) was added. At 3 sec, reaction mixtures were quenched with 4 ml of ice-cold buffer containing 100 μM picrotoxin (a specific GABA-channel blocker), and then rapidly vacuum filtered over glass-fiber filters (Whatman GF-C) in a Hoeffer manifold. Filters were washed with 8 ml of cold buffer containing picrotoxin, dissolved in scintillation cocktail, and counted (100% efficiency) over the entire energy spectrum of ^{36}Cl. Flux values were expressed as dpm (mean $\pm$ standard deviation), after nonspecific binding to the filter was subtracted. Statistical analysis was by two-tailed t-test. Concentrations of DEE present in reaction mixtures at the moment of quenching were confirmed by gas chromatography.

Results: In the absence of exogenous GABA, specific chloride flux amounted to 693 $\pm$ 50 dpm, and this was unchanged by bicuculline. The response to GABA 200 μM was 1526 $\pm$ 72. DEE (30 mM) increased flux to 909 $\pm$ 94 ($p < 0.002$, compared with baseline), but bicuculline had no effect on DEE-induced flux.

Discussion: Clinically relevant concentrations of DEE can produce GABA-independent chloride flux via the GABA-R. These data demonstrate that, analogous to barbiturates[6], and in contrast to benzodiazepines[7], DEE can directly gate the chloride channel of GABA-R.

References: 1. Edelman, Gall, Cowan, (Eds.), Synaptic Function, pp. 257-71, 1988. 2. J Neurochem 51: 1386-93, 1988. 3. J Pharmacol Exp Ther 242:963-9, 1987. 4. Life Sci 46: 527-41, 1990. 5. Analyt Biochem 48:422-7, 1972. 6. J Neurosci 5: 2963-2970, 1985. 7. J Neurochem 50:302-306, 1988.

Supported by University Anesthesiology/CCM Foundation

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