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Alcohols increase calmodulin affinity for Ca^{2+} and decrease target affinity for calmodulin

Ichiro Ohashi[a], Roman Pohoreki[b,1], Kiyoshi Morita[a], Paul M. Stemmer[c,*]

[a] Department of Anesthesiology and Resuscitology, Okayama University Medical School, Okayama, Japan
[b] Department of Anesthesiology, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198-6255, USA
[c] Department of Pediatrics, College of Medicine, University of Nebraska Medical Center, Omaha, NE 68198-6255, USA

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Abstract

It has been proposed that alcohols and anesthetics selectively inhibit proteins containing easily disrupted motifs, e.g., α-helices. In this study, the calcineurin/calmodulin/Ca^{2+} enzyme system was used to examine the effects of alcohols on calmodulin, a protein with a predominantly α-helical structure. Calcineurin phosphatase activity and Ca^{2+} binding were monitored as indicators of calmodulin function. Alcohols inhibited enzyme activity in a concentration-dependent manner, with two-, four- and five-carbon *n*-alcohols exhibiting similar leftward shifts in the inhibition curves for calmodulin-dependent and -independent activities; the former was slightly more sensitive than the latter. Ca^{2+} binding was measured by flow dialysis as a direct measure of calmodulin function, whereas, with the addition of a binding domain peptide, measured calmodulin–target interactions. Ethanol increased the affinity of calmodulin for Ca^{2+} in the presence and absence of the peptide, indicating that ethanol stabilizes the Ca^{2+} bound form of calmodulin. An increase in Ca^{2+} affinity was detected in a calmodulin binding assay, but the affinity of calmodulin for calcineurin decreased at saturating Ca^{2+}. These data demonstrate that although specific regions within proteins may be more sensitive to alcohols and anesthetics, the presence of α-helices is unlikely to be a reliable indicator of alcohol or anesthetic potency.

Keywords: Alcohol; Calmodulin; Calcineurin; α-Helix; Protein interaction

1. Introduction

An emerging consensus identifies proteins as critical targets for alcohols and anesthetics [1–9]. The mechanism by which alcohols and anesthetics selectively inhibit specific proteins, however, remains unknown. It has been hypothesized that a determinant of the selective effects of these agents is the presence of easily disrupted structural motifs. Proteins containing such motifs would be selectively affected by alcohols and anesthetics. In support of this hypothesis, anesthetics and alcohols have been shown to preferentially disrupt the structure of some α-helical peptides [10–12]. The molecular target for this effect is the water of hydration, and halothane and alcohols induce a transition of α-helix to β-sheet through modification of the protein–water interface [10,11]. Disruption of the protein structure is expected to result in a corresponding disruption of protein function, but secondary structural elements, such as α-helical domains, are not well accepted as a selective anesthetic and alcohol binding sites [6]. Higher order protein structural elements have been proposed to be more sensitive to disruption by anesthetics and alcohols [6,13].

Calmodulin-dependent events and processes are integral to synaptic transmission, and the inhibition of calmodulin-dependent processes has been implicated in the mechanism of alcohol and anesthetic action [14,15]. Because calmodulin (CaM)-modulated regulatory pathways are extremely complex biochemically and physiologically [16], it is difficult to determine if clinically relevant concentrations of alcohols and anesthetics affect them. The possibility that α-helical domains in proteins confer higher sensitivity of to alcohol and anesthetic action is pertinent to signaling through calmodulin because calmodulin itself is largely an

* Corresponding author. Wayne State University, Institute of Environmental Health Sciences, 2727 Second Ave. Suite 4000, Detroit, MI 48201 USA. Tel.: +1-313-961-7634; fax: +1-313-577-0082.
E-mail address: pmstemmer@wayne.edu (P.M. Stemmer).
[1] Current address: Southwest Regional Medical Center, Liberal, KS. 67901 USA.

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α-helical protein, and the calmodulin-binding domain of all calmodulin-binding proteins assumes an α-helix conformation when bound to calmodulin [17,18].

In the current study, we examine the effects of alcohols on one calmodulin-dependent enzyme system, the Ca^{2+}- and calmodulin-dependent phosphatase, calcineurin. Calcineurin provides a unique system to examine the effects of alcohols on calmodulin-dependent processes because it is an active phosphatase both in the absence and presence of calmodulin. The ability to differentiate between effects mediated through calmodulin and effects caused by disruption of other sites within the enzyme enables us to test the hypothesis that calmodulin-dependent activation is more sensitive to disruption by alcohols than the higher order tertiary and quaternary elements of protein structure that are important in enzyme activation. If the findings with peptides are pertinent to full proteins, then calmodulin-dependent enzymes may be sensitive targets for alcohol and anesthetic actions and the calmodulin-dependent activity of calcineurin is expected to be more sensitive to inhibition than the calmodulin-independent activity. Alternatively, if the higher order tertiary and quaternary elements of protein structure are more susceptible to disruption by alcohols and anesthetics, then alcohols are expected to have similar potency in the presence and absence of calmodulin.

2. Materials and methods

2.1. Calcineurin activity

Calcineurin phosphatase activity was assayed as previously described [19]. The peptide substrate, DLDVPIPGRFDRRVSVAAE, was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase (Promega). Calcineurin (10 nM), alone or with 0.1 μM calmodulin, was incubated in buffer containing 40 mM Tris–HCl pH 7.5, 100 mM KCl, 6 mM $MgCl_2$, 0.5 mM dithiothreitol and 0.1 mg/ml bovine serum albumin, 1 mM EGTA and enough $CaCl_2$ to produce the indicated concentrations of free Ca^{2+}. After warming the reactants to 30 °C, the ^{32}P phosphorylated peptide substrate was added to a final concentration of 1 μM and the dephosphorylation reaction allowed to proceed for 30 min. The reaction was stopped with 10% TCA, and free ^{32}P was separated from peptide by chromatography over AG-50W-X8 (Bio-Rad) resin. Free ^{32}P in the flow-through was quantitated by liquid scintillation counting. Calcineurin phosphatase activity is defined as the Ca^{2+} dependent activity and was calculated as the difference in activity between the samples containing Ca^{2+} and the samples containing only EGTA.

2.2. Ca^{2+} binding by flow dialysis

Ca^{2+} binding to calmodulin was measured by flow-dialysis as previously described [20]. The buffer used in these experiments consisted of 100 mM KCl, 1.0 mM $MgCl_2$ and 10 mM HEPES, pH 7.5. Calmodulin was present at 10 μM final concentration. A synthetic peptide, ARKEVIRNKIRAIGKMARVFSVLR, corresponding to the calmodulin-binding domain of calcineurin, was included in some experiments at 11 μM final concentration. Data were corrected for loss of Ca^{2+} and $^{45}Ca^{2+}$ from the flow well prior to calculating free and bound Ca^{2+} concentrations.

2.3. Fluorescence polarization

Fluorescence polarization measures changes in rotational freedom of a fluorescent probe [21,22]. This assay was used to estimate the affinity of calmodulin for Ca^{2+} and of calcineurin for calmodulin in the presence and absence of ethanol. Calmodulin, which had been engineered to contain a cysteine three residues away from the carboxyl terminus (CaM-T_{146}C), was a generous gift from Dr. Anthony Persechini [23]. The CaM-T_{146}C was labeled with fluorescein at a 1:1 ratio to create Fl-CaM by incubating the CaM-T_{146}C with fivefold excess of fluorescein-5-maleimide. The reaction was quenched with DTT and unincorporated reagents were separated from the Fl-CaM by gel filtration on sephadex G-25. Fl-CaM concentration was estimated using a fluorescein extinction coefficient of $\varepsilon_{495} = 78{,}000$. Polarization did not vary with Fl-CaM concentration between 0.1 and 2 nM whereas fluorescence intensity increased linearly in that range. All assays were run with 0.1 nM Fl-CaM in the binding mixture. Calcineurin was present at concentrations between 0.02 and 100 nM. Assay buffer contained 0.01 mg/ml BSA, 1 mM $MgSO_4$, 100 mM KCl and 10 mM HEPES (pH 7.5), 1 mM EGTA and $CaCl_2$ to produce the indicated concentration of free Ca^{2+}. Separate samples were prepared for each concentration of enzyme. Changes in fluorescence intensity were less than 10% and polarization values were not corrected for effects of intensity change.

2.4. Materials

$^{45}CaCl_2$ was purchased from Dupont (Boston, MA). Standard solutions of $CaCl_2$ were obtained from Gallard Schlesinger. The peptides were synthesized at the UNMC Protein Structure Core Facility. Calcineurin and calmodulin were expressed in *E. coli* and purified on calmodulin-sepharose and phenyl-sepharose columns, respectively. Isolated proteins were greater than 95% pure as judged by SDS-PAGE with Coomassie blue staining.

2.5. Statistical analysis

Phosphatase activity and fluorescence polarization data were analyzed using the equations built into the Prism software package. Ca^{2+}-dependent activation of calcineurin phosphatase activity and Ca^{2+} binding as detected by

fluorescence polarization measurement were analyzed according to the equation:

$$Y = Y_{min} + [(Y_{max} - Y_{min})/(1 + 10^{(\log EC_{50} - \log X)N_H})] \quad (1)$$

where Y is the measured value of activity or polarization, Y_{min} is the background value, Y_{max} is the maximum activity or polarization under the conditions of that run, X is the concentration of Ca^{2+} and N_H is the Hill coefficient. Binding of calcineurin to Fl-CaM was analyzed according to the equation:

$$Y = Y_{max} \times X/(K_d + X) \quad (2)$$

where Y is the measured change in polarization, Y_{max} is the maximum change in polarization under the conditions examined, X is the concentration of calcineurin and K_d is the dissociation constant. Non-overlapping 95% confidence limits are considered statistically significant.

3. Results

3.1. Inhibition of calcineurin phosphatase activity by n-alcohols

The concentration dependence for calcineurin inhibition by ethanol, butanol, and pentanol was examined in the presence and absence of calmodulin. Three alcohols were examined to establish that the longer chain alcohols have greater potency in enzyme inhibition, as is true for other enzymes [24–26]. Calcineurin inhibition in the absence of calmodulin reflects actions of these agents on the tertiary and quaternary elements of protein structure of the enzyme

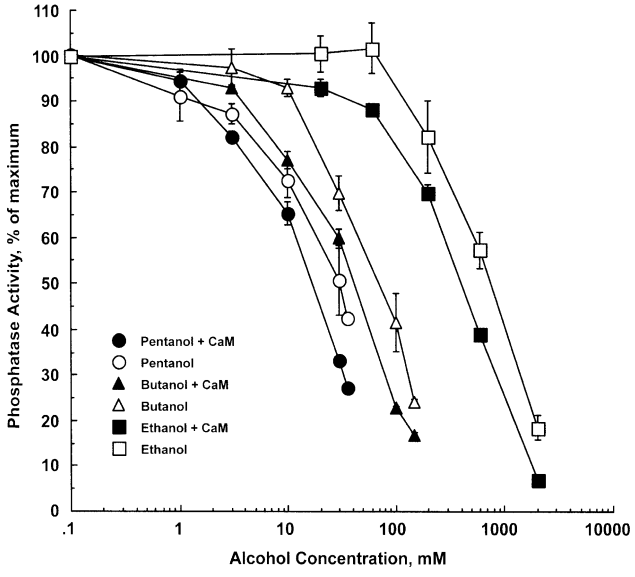

Fig. 1. Calcineurin inhibition by alcohols in the absence or presence of calmodulin. Calcineurin activity in the presence of increasing concentrations of ethanol, butanol, and pentanol was estimated by measuring release of ^{32}P from a phosphorylated peptide substrate. Data presented are the mean ± S.E., $n = 4$.

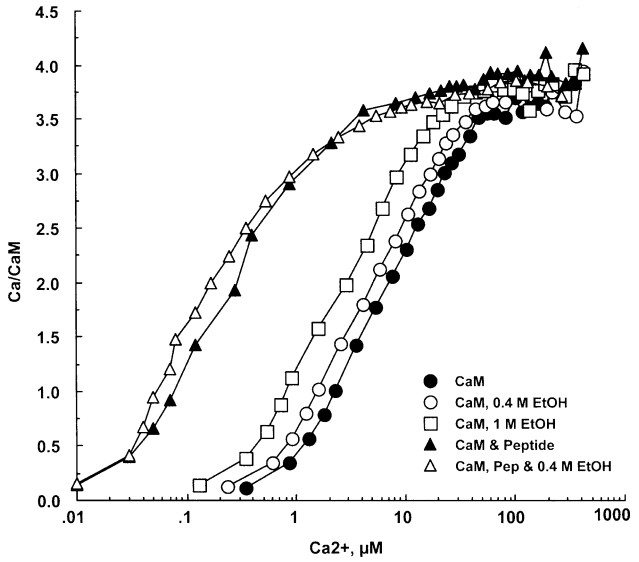

Fig. 2. $^{45}Ca^{2+}$ binding to calmodulin in the presence of various concentrations of ethanol and in the presence and absence of a target peptide. Calmodulin was incubated in the indicated concentrations of ethanol. $^{45}Ca^{2+}$ binding was quantified by flow dialysis. Data are pooled from two runs each condition.

or on the catalytic mechanism, whereas inhibition of calmodulin-dependent activity also can be caused by inhibition of the enzyme-calmodulin interaction. Fig. 1 shows that the enzyme is slightly more sensitive to the inhibitory effect of each alcohol tested in the presence of calmodulin. The maximum increase in potency of the alcohols was approximately twofold. Maximum activity of calcineurin in the presence and absence of calmodulin is 7.11 ± 0.18 and 0.38 ± 0.01 nmol/min/mg, respectively. The 95% confidence limits for inhibition of the phosphatase in the presence and absence of calmodulin are non-overlapping for each of the alcohols. These data demonstrate that the calmodulin-dependent enzyme activity is only slightly more sensitive to alcohols than calmodulin-independent activity.

3.2. Ca^{2+} dependence of the calcineurin/calmodulin/Ca^{2+} system

Calmodulin acts as a Ca^{2+} sensor protein. Therefore, any shift in the Ca^{2+} concentration dependence of a calmodulin-dependent enzyme activation would disrupt signaling through the system. The Ca^{2+} dependence of calmodulin-dependent calcineurin activity can be shifted by a direct effect on Ca^{2+} binding, at the step of calmodulin binding to the enzyme, or at an allosteric site necessary for enzyme activity. In order to determine which aspect of calmodulin-dependent activation is most sensitive to disruption by the alcohols, the Ca^{2+} dependence for each of these steps in activation was examined using ethanol as a representative alcohol. As shown by the leftward shift in the Ca^{2+} binding curves in Fig. 2, ethanol has a concentration-dependent effect to increase the affinity of calmodulin for Ca^{2+}. This

effect is seen in the presence and in the absence of a target peptide that increases the affinity of calmodulin for Ca^{2+}, indicating that the increased affinity of the calmodulin-target complex for Ca^{2+} does not eliminate the stimulatory effect of the alcohol. The concentrations of ethanol tested for effects on Ca^{2+}-binding approximate the IC_{50} and maximally inhibitory concentrations for enzyme inhibition. This demonstrates that enzyme inhibition by alcohols is concurrent with the alcohol-induced stimulation of Ca^{2+} binding to calmodulin.

3.3. Ca^{2+} dependence of calmodulin binding

Ca^{2+} binding to calmodulin and the Ca^{2+} dependence of calmodulin binding to calcineurin were monitored by changes in fluorescence polarization of fluorescein labeled calmodulin (Fl-CaM). In the absence of calcineurin, Fl-CaM undergoes a Ca^{2+}-dependent increase in polarization that is consistent with a conformation change in Fl-CaM as it binds to Ca^{2+} (Fig. 3). In the presence of 100 nM calcineurin, the measured change in polarization was 76.4 mPol units, as compared to 39.9 units in the absence of calcineurin. In the presence of calcineurin, the increase in polarization occurs at a lower Ca^{2+} concentration and exhibits cooperativity with respect to Ca^{2+} (Hill coefficient of 1.68 ± 0.23 in the presence of calcineurin and 0.69 ± 0.16 in the absence of the enzyme). Ethanol at 0.4 M caused a significant shift in the Ca^{2+} concentration dependence for changing polarization of Fl-CaM from (μM) 4.29 ± 0.57 to 1.92 ± 0.59. In the presence of calcineurin, 0.4 M ethanol induced a shift from (μM) 0.18 ± 0.04 to 0.14 ± 0.04. These data indicate that ethanol increases the affinity of Fl-CaM for Ca^{2+} to a

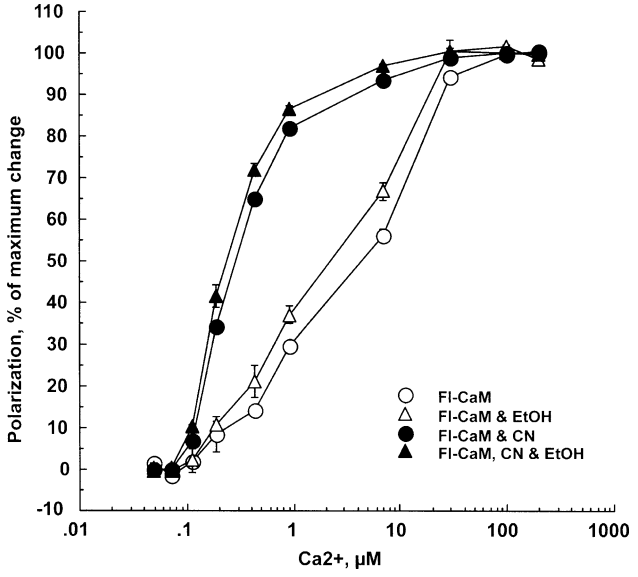

Fig. 3. Fluorescence polarization of Fl-CaM in the presence and absence of 400 mM ethanol and in the presence and absence of 100 nM calcineurin as a function of Ca^{2+} concentration. FL-CaM was incubated in the presence or absence of ethanol with increasing concentrations of $^{45}Ca^{2+}$. Values represent the mean ± S.E. for $n=4$.

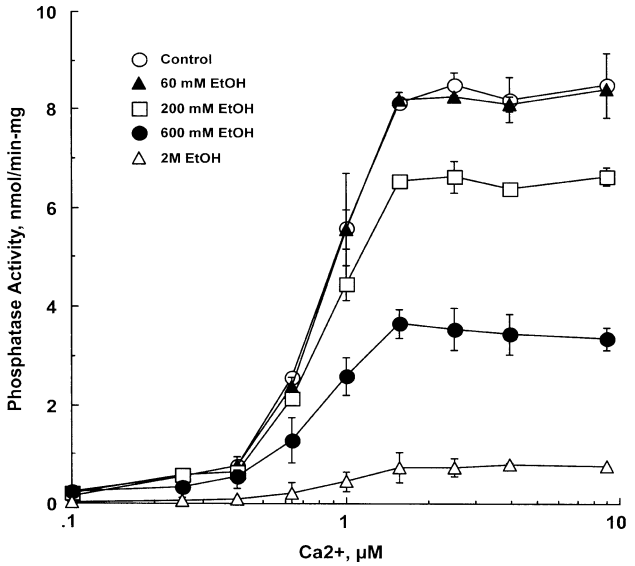

Fig. 4. Ca^{2+}-dependent activation of calcineurin in the presence of calmodulin and various concentrations of Ca^{2+}. Calcineurin activity was assessed in the presence of increasing concentrations of Ca^{2+} in the presence of the indicated concentrations of ethanol by quantifying release of ^{32}P from a phosphorylated peptide substrate. Values are the mean ± S.E. for $n=4$.

degree similar to that shown with flow dialysis in Fig. 2. In the presence of the target, however, the increase in Ca^{2+} affinity did not reach significance. These results confirm that the alcohol-induced increase in calmodulin affinity for Ca^{2+} that is observed for calmodulin in the absence of a target is reduced, but not eliminated, in the complete calcineurin/calmodulin/Ca^{2+} enzyme system.

3.4. Ca^{2+} dependence of calcineurin activation

The final aspect of the calcineurin/calmodulin/Ca^{2+} system that shows Ca^{2+} dependence is enzyme activity. Fig. 4 shows the Ca^{2+}-dependent stimulation of calcineurin in the presence of various concentrations of ethanol. There was no shift in the Ca^{2+} dependence of the enzyme for any concentration of ethanol tested with the range of k_{act} values for Ca^{2+} between 0.75 and 0.91 μM Ca^{2+}. Although the shifts in Ca^{2+} dependence caused by ethanol (Figs. 2 and 3) are only twofold, they are easily measured with the techniques available and are expected to be propagated through the system to cause a leftward shift in the Ca^{2+}-dependence of the enzyme activity. Therefore, the lack of an effect of ethanol on the Ca^{2+} dependence of the enzyme activation requires some explanation.

3.5. Ca^{2+} dependence of calmodulin binding to calcineurin

With the increase in calmodulin affinity for Ca^{2+} shown in Figs. 2 and 3, a corresponding increase in the affinity of calcineurin for calmodulin is expected. This should also be observed as an increase in sensitivity of the enzyme to

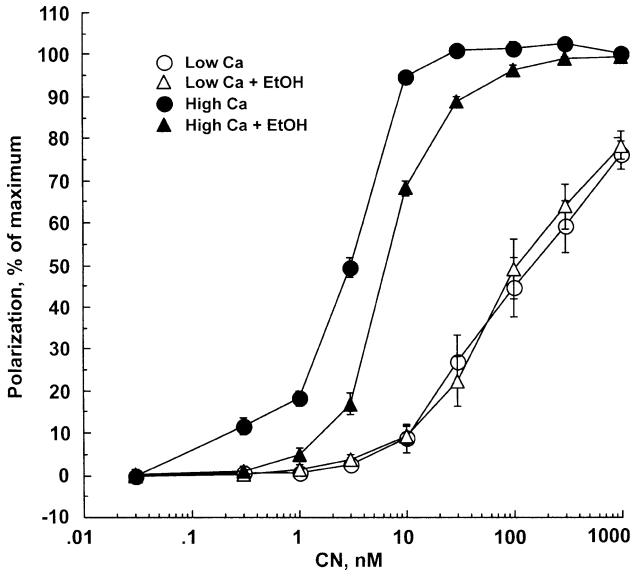

Fig. 5. Fluorescence polarization of Fl-CaM in the presence of 200 nM or 200 μM Ca^{2+} in the presence and absence of 400 mM ethanol as calcineurin concentration is varied. Fl-CaM binding to calcineurin is detected as an increase in fluorescence polarization. Values represent the mean ± S.E. for $n=4$.

activation by Ca^{2+}, which was tested and found not to occur. Therefore, calmodulin binding to calcineurin was measured directly to determine if the increased affinity of calmodulin for Ca^{2+} in the presence of ethanol is accompanied by a decrease in the affinity of calmodulin for the enzyme. The affinity of Fl-CaM for calcineurin in the presence and absence of 0.4 M ethanol was estimated by measuring changes in fluorescence polarization of solutions containing 0.1 nM Fl-CaM as a function of enzyme concentration. The Ca^{2+} concentration initially was set at 200 nM to reflect the expected cellular Ca^{2+} concentration for calcineurin activation. The Ca^{2+} concentration was increased to 200 μM to achieve complete saturation of Fl-CaM with Ca^{2+}. As can be seen in Fig. 5, the affinity of the enzyme for Fl-CaM increased as Ca^{2+} concentration was increased from 200 nM to 200 μM. Under low Ca^{2+} conditions, the affinity of Fl-CaM for calcineurin was not affected by ethanol. However, with Ca^{2+} at 200 μM, a concentration that is expected to saturate all Ca^{2+} binding sites on Fl-CaM, ethanol induced a rightward shift in the Fl-CaM binding curve, indicating that ethanol decreases the affinity of calmodulin for calcineurin.

4. Discussion

Two different questions are addressed in this study. The first is if calmodulin-dependent systems are sensitive targets for alcohols and anesthetics [14,15]. The second is if calmodulin, as an example of a predominantly α-helical protein, is sensitive to functional disruption by alcohols [10–12,27,28]. Presumably both results would be mediated by a destabilization of the α-helical secondary structure that is a prominent feature of calmodulin and calmodulin binding domains.

The three alcohols tested were each able to fully inhibit calcineurin phosphatase activity. However, because the IC_{50} for inhibition of calcineurin is well above the LD_{50} of the alcohols tested, it is clear that calcineurin is not a sensitive target of alcohols. With a complex phenomena such as anesthesia, however, even low potency effects may be significant [29]. In addition, the idea that calmodulin has a role in alcohol or anesthetic action is particularly difficult to dismiss because even modest effects on the Ca^{2+} dependence can have profound effects on synaptic processes and calmodulin is critical for the synaptic processes that are presumed to be the ultimate target of anesthetic action. With this in consideration and because calmodulin-dependent systems exhibit a high degree of positive cooperativity in respect to Ca^{2+}, (Hill coefficients between 3 and 4) [20,30], even a very modest shift in Ca^{2+} dependence will profoundly change signaling through calmodulin. Therefore, a full examination of the calcineurin/calmodulin enzyme system in regards to Ca^{2+} and disruption by alcohol was carried out.

Direct examination of the Ca^{2+} dependence for calmodulin activation of calcineurin indicates that the Ca^{2+} dependence is not changed by alcohols (Fig. 4). Previous reports that alcohol and anesthetics change calmodulin affinity for Ca^{2+} [15] are at odds with the absence of a shift in Ca^{2+} dependence for enzyme activation shown here. However, because the Ca^{2+} dependence of calmodulin-activated enzymes is dependent both on the affinity of calmodulin for Ca^{2+} and on the affinity of the target enzyme for calmodulin [30,31], multiple effects may be involved. The data from flow-dialysis and fluorescence polarization assays unequivocally demonstrate that ethanol increased the affinity of calmodulin for Ca^{2+} in a concentration-dependent manner over the concentration range in which it inhibits calcineurin (Figs. 2 and 3). The inhibition of calmodulin binding to calcineurin that was demonstrated in Fig. 5, provides a reasonable explanation for the apparent discrepancies within this study and between this and previous studies. The effect of alcohol to stimulate Ca^{2+} binding to calmodulin is approximately equal in magnitude and opposite in direction to the decrease in calmodulin affinity for calcineurin. At less than maximally stimulatory concentrations of Ca^{2+}, the effects cancel out and no shift in Ca^{2+} dependence is observed for either enzyme activation (Fig. 4) or for calmodulin binding (Fig. 5, low Ca^{2+} condition). When the Ca^{2+} binding sites are saturated, as in Fig. 5, high Ca^{2+} condition, the decrease in calmodulin affinity for calcineurin becomes evident.

These data are pertinent to both questions being discussed. In regard to the first, alcohols stimulate rather than inhibit the core function of calmodulin as a Ca^{2+} binding protein. This is also important to the question of the effects of alcohol on protein secondary structure, because calmod-

ulin assumes an α-helical structure when Ca^{2+} is bound. The alcohol-induced increase in calmodulin affinity for Ca^{2+} indicates that alcohol is likely to stabilize the α-helical structure of calmodulin. This has been shown for melittin [32,33], an α-helical peptide which binds to calmodulin. This result supports the work indicating that alcohols promote α-helix formation [34,35] in proteins and suggests that the evidence demonstrating alcohol destabilization of α-helixes in peptides [11,12,36] is not generalizable to proteins or to domains within proteins [6]. The inhibition of calcineurin by alcohols is complete and occurs without decreasing maximal calmodulin binding and presumably without disrupting the secondary structure of the α-helical elements important for calmodulin function.

These data indicate that a predominantly α-helical secondary structure for a protein or a domain within a protein is not a reliable indicator of alcohol or anesthetic potency toward that protein. Furthermore, the data are most consistent with the hypothesis that the tertiary elements of protein structure, which are critical for enzyme activity, are most sensitive to alcohols and anesthetics.

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