Membrane Partition of Fatty Acids and Inhibition of T Cell Function†

Alberto Anel, Gary V. Richieri, and Alan M. Kleinfeld*

Division of Membrane Biology, Medical Biology Institute, 11077 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT: Short-term exposure to elevated levels of free fatty acids (FFA) perturbs a variety of cellular functions. It is frequently observed that cis-unsaturated fatty acids (FA) mediate these perturbations while trans-unsaturated or saturated FA are relatively inert. This dichotomy has generally been ascribed to the differential effects of these FA on membrane structure, specifically, that cis but not saturated FA alter lipid acyl chain order. Direct support for this view, however, is lacking because membrane partition of FA has not been determined for the relevant FA and for the conditions of the functional studies. Previous measurements of membrane partition of natural long-chain FA have relied on the determination of the amount bound to the membrane rather than the aqueous-phase concentration of FA. Because [FFA] is low, however, the partition coefficient (K_p) is relatively insensitive to the membrane-bound concentration and, therefore, accurate determinations of K_p require direct [FFA] measurements. In this study FA partition between the aqueous phase and either lipid bilayer vesicles or cytotoxic T lymphocytes (CTL) was measured using a recently developed fluorescent indicator of FFA. This indicator is composed of recombinant intestinal fatty acid binding protein derivatized with acrylodan and is termed ADIFAB. Using ADIFAB, partition coefficients were determined for seven saturated and cis-unsaturated FA under conditions that parallel those in which cis FA have been shown to inhibit CTL signaling. In general, K_p values were approximately an order of magnitude greater than previous determinations and were found to be *greater* for the noninhibitory saturated FA than the inhibitory cis FA. K_p values ranged from about 2×10^6 for stearic to 4×10^4 for linolenic acid, reflecting a strong dependence on FFA aqueous solubility and relatively little dependence on membrane composition. The K_p values together with measurements of the corresponding change in membrane lipid order, by fluorescence polarization, allowed the determination of the specific level of acyl chain perturbation by each FA as a function of its membrane concentration. Specific perturbation was generally found to increase with increasing degree of cis unsaturation. Parallel measurements of the cis FA inhibition of the concanavalin A-stimulated rise in CTL intracellular calcium demonstrates that lipid acyl chain perturbation and inhibition are linearly correlated. This correlation suggests that inhibition of CTL signaling results from perturbation of plasma membrane lipid order.

Exogenously added cis-unsaturated fatty acids (FA)[1,2] profoundly alter a variety of functions in a broad spectrum of cells, while trans-unsaturated and saturated FA often have little or no effect (Karnovsky et al., 1982; Badwey et al., 1984; MacDonald & MacDonald, 1988; Richieri & Kleinfeld, 1989, 1990; Richieri et al., 1990; Stephen et al., 1991). Under the conditions of many of these studies, relatively short-time (seconds to minutes) exposures to elevated levels of FA are sufficient to induce the observed perturbations. When measured, little or no FA esterification occurs during the short time needed to induce the perturbation observed in these studies and normal function can be restored by removing the added FA, indicating that the perturbation is due to a physical alteration of cell structure. Studies, primarily using fluorescent membrane probes, show that addition of cis FA to lipid bilayers disorders lipid acyl chains, but trans-unsaturated and saturated FA have little or no effect (Klausner et al., 1980; Kleinfeld et al., 1981; Richieri et al., 1990), suggesting that the lipid phase of the plasma membrane is a likely site of this perturbation.

The different effects of these two classes of FA on function and structure reflect either a differential ability of the specific FA to perturb lipid order or, more simply, the differential partition of the FA between the aqueous phase and membrane. To distinguish properly between these two possibilities it is essential to determine accurately the partition coefficients of the various FA under the same conditions as required to induce perturbation of cellular function. Although partition coefficients for the distribution of selected long-chain FA between membranes and the aqueous phase have been reported, such measurements have, in general, not been done under conditions which closely parallel the conditions that result in functional perturbation (Pjura et al., 1984; Meers et al., 1988). Moreover, accurate determination of the partition coefficients of the highly insoluble long-chain FA by conventional methods is quite difficult, primarily because the large partition coefficients of the FA result in very high levels of FA in the membrane

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* To whom correspondence should be addressed.

[1] Abbreviations: AA, arachidonic acid; ADIFAB, acrylodated intestinal fatty acid binding protein; BHT, butylated hydroxytoluene; chol, cholesterol; cmc, critical micelle concentration; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DHA, docosahexenoic acid; DMPC, dimyristoylphosphatidylcholine; DPH, 1-diphenyl-6-phenylhexatriene; EPC, egg phosphatidylcholine; FA, fatty acid (s); FFA, free fatty acid(s); I-FABP, intestinal fatty acid binding protein; LA, linoleic acid; LNA, linolenic acid; LUV, large unilamellar vesicle(s); OA, oleic acid; PA, palmitic acid; SA, stearic acid; SUV, small unilamellar vesicle(s); TMADPH, 1-[4-(trimethylamino)phenyl]-6-phenylhexatriene.

[2] The term free fatty acid (FFA) is used in this paper to designate the aqueous-phase monomer of either the acid, the sodium salt, or the ionized molecule. For the low concentrations used in this study it is likely that most of the molecules are ionized since short- and medium-chain fatty acids, under monomeric conditions, have an apparent pK_a of 4.8 (Small, 1986). The term fatty acid (FA) is used to refer to the other (non-free) states of this molecule, either protein or membrane bound or in an aggregate state, and FA_{total} is FA plus FFA.

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phase and consequently very low levels of aqueous-phase FFA.

We have recently developed a fluorescent probe (ADIFAB) that allows accurate determination of the concentration of FFA ([FFA]) under conditions appropriate for the studies of FA perturbation of cellular behavior (Richieri et al., 1992). In the present study we have used this method to determine the partition coefficients of seven long-chain saturated and cis-unsaturated FA between the aqueous phase and lipid bilayer vesicles or whole cells. These measurements were carried out under a variety of FA concentrations and FA:membrane ratios and demonstrate that under all conditions, including those which result in functional perturbation, the partition coefficients of saturated FA *exceed* those of the cis-unsaturated FA. Moreover, by using these partition coefficients together with measurements of the alteration of DPH fluorescence polarization, the specific membrane lipid disordering potency of each FA was determined. This allows the functional perturbation to be compared accurately with lipid acyl chain perturbation as a function of added FA concentration. The results demonstrate that the perturbation of cellular function is highly correlated with the degree of lipid acyl chain perturbation, strongly supporting the hypothesis that the initial event in FA perturbation of cellular function involves the alteration of plasma membrane lipid acyl chain order.

EXPERIMENTAL PROCEDURES

Materials. 1-Diphenyl-6-phenylhexatriene (DPH) and 1-[4-(trimethylamino)phenyl]-6-phenylhexatriene (TMADPH), were obtained from Molecular Probes, Eugene, OR; egg phosphatidylcholine (EPC) and dimyristoylphosphatidylcholine (DMPC) were from Avanti Polar Lipids, Birmingham, AL; and radiolabeled phospholipids and FA were from New England Nuclear, Boston, MA. Buffer in most studies was 140 mM NaCl, 5 mM KCl, 20 mM Hepes, and 1 mM NaH_2PO_4 at pH 7.4. In studies of the CTL calcium response, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 5.5 mM glucose were included in this buffer. Fatty acids and their sodium salts were purchased from Nucheck Prep (Illysian, MN). The FFA probe ADIFAB was prepared from acrylodan-derivatized recombinant rat intestinal fatty acid binding protein (I-FABP) as described (Richieri et al., 1992). ADIFAB is available from Molecular Probes Inc. (Eugene, OR).

Cell Lines. The alloantigen-specific murine CTL clone C35 (anti H-2K^b) was a gift from Dr. Linda Sherman, Scripps Institute and Research Clinic, La Jolla, CA. The CTL clone and the EL4 (H-2K^b) thymoma were cultured as described previously (Richieri & Kleinfeld, 1989, 1990; Richieri et al., 1990).

Lipid Vesicles. Small unilamellar vesicles (SUV) were prepared by probe sonication as described previously (Huang & Thompson, 1974; Storch & Kleinfeld, 1986), and large unilamellar vesicles (LUV) were prepared by the vesicle extrusion method essentially as described (Hope et al., 1985). For both types of vesicles, lipid was dissolved in chloroform, evaporated to dryness, and lyophilized overnight, and the dried lipid film was hydrated in buffer. With the exception of DMPC, which was sonicated and centrifuged at 30 °C, SUV were prepared at 4 °C. For LUV preparations the hydrated lipid was passed 10 times through two 0.1-μm polycarbonate filters (Nuclepore) at room temperature.

Cell Function. CTL signaling was monitored by the rise in intracellular calcium ($[Ca^{2+}]_i$) following stimulation with concanavalin A (Con A) at 10 μg/mL, as described previously (Richieri & Kleinfeld, 1989). To measure $[Ca^{2+}]_i$, suspensions of CTL were loaded with the fura-2 fluorescence indicator of free calcium (Grynkiewicz et al., 1985), at approximately 1 $\times 10^6$ cells/mL, and measured by ratio fluorescence at 34 °C, as described previously (Richieri & Kleinfeld, 1989).

Table I: Fatty Acid–ADIFAB Dissociation Constants[a]

fatty acid	K_d ($\times 10^6$ M)
stearic (18:0)[b]	0.08 ± 0.01
oleic (18:1)[c]	0.28 ± 0.01
palmitic (16:0)[c]	0.33 ± 0.01
docosahexenoic (22:6)[b]	0.43 ± 0.01
linoleic (18:2)[c]	0.97 ± 0.02
arachidonic (20:4)[c]	1.63 ± 0.03
linolenic (18:3)[c]	2.5 ± 0.1

[a] Equilibrium constants were determined at 37 °C and pH 7.4 using the methods described in Richieri et al. (1992). [b] Values determined in the present study. [c] Values determined in Richieri et al. (1992).

FA Handling and FFA Treatment. Sodium salts of the unsaturated, especially the polyunsaturated, FA were found to oxidize when maintained in the solid form, even at −20 °C and purged with argon. With increasing oxidation, the binding affinity to ADIFAB and, we assume, the membrane partition coefficient decreased. The increase in oxidation was also followed by monitoring the increase in absorption at 265 nm. Therefore, upon arrival, the sodium salt of each FA was dissolved at a FA concentration of 20–50 mM in deionized water plus 4 mM NaOH and 25 μM butylated hydroxytoluene (BHT). Appropriate dilutions of these stock solutions were made in water plus 4 mM NaOH but no additional BHT (BHT was found to bind to ADIFAB with a K_d of about 10 μM). All stocks were maintained under argon at −20 °C for long-term storage, but in no case for longer than 3 months. Even with these precautions substantial oxidation was often observed in DHA samples, the most unsaturated (22:6) of the FA examined in this study. Results reported here were obtained only with FA having negligible absorption at 265 nm. Fatty acids were added to the cells or vesicles from approximately 1 mM solutions of the sodium salts and immediately mixed by drawing the liquid in and out of a Pasteur pipette.

[FFA] Determination with ADIFAB. ADIFAB, which is the acrylodan-derivatized form of rat I-FABP, responds to FA binding by undergoing a shift in fluorescence from 432 nm, in the apo form, to 505 nm, in the holo form. As a consequence, [FFA] can be determined from the ratio of 505- to 432-nm fluorescence essentially by the same procedure as used to determine $[Ca^{2+}]$ with the fluorescent probes developed by Tsien and his collaborators (Grynkiewicz et al., 1985). Accordingly

$$[FFA] = K_d Q(R - R_0)/(R_{max} - R) \quad (1)$$

where R is the measured ratio of 505- to 432-nm intensities (with blank intensities subtracted), R_0 is this ratio with no FFA present, R_{max} is the value when ADIFAB is saturated, and $Q = I_F(432)/I_b(432)$, where $I_F(432)$ and $I_b(432)$ are the ADIFAB intensities with zero and saturating concentrations of FFA, respectively. Values of Q and R_{max} were found to be 19.5 and 11.5, respectively, in Richieri et al. (1992). Equilibrium constants (K_d) were obtained either from Richieri et al. (1992) or in the present study (SA and DHA) and the results are summarized in Table I.

Determination of Partition Coefficients. The coefficient describing partition of FA between membrane and aqueous phase is defined as $K_p = [FA]_m/[FFA]$ where $[FA]_m$ is the concentration of FA in the membrane phase and [FFA] is the

free fatty acid concentration.[3] K_p can be expressed in terms of quantities measured directly as

$$K_p = (([FA_{total}] - [FFA])/[FFA])V_a/V_m \quad (2)$$

where $[FA_{total}]$ is the added FA concentration, [FFA] is determined using ADIFAB fluorescence and eq 1, and V_a and V_m are the volumes of the aqueous and membrane phases, respectively. For lipid vesicles V_m/V_a was estimated as 10^{-3}/mM phospholipid, using an area per lipid molecule of 70 Å^2 and a bilayer width of 40 Å (Huang & Mason, 1978). Plasma membrane phospholipid was estimated as 10 μM for 1×10^6 cells/mL as described previously (Richieri & Kleinfeld, 1989). For studies employing low vesicle concentrations or cells, [FFA] was corrected for FA binding to the container walls as described (Richieri et al., 1992). The ADIFAB response is not significantly affected by cells or vesicles, nor does the probe bind to cells (data not shown).

In a typical K_p determination, LUV (100 μM) composed of EPC + chol (2:1) were equilibrated in a cuvette with 0.2 μM ADIFAB at 37 °C. The 505/432-nm intensity ratio (vesicle blank subtracted) was measured, yielding an R_0 value of 0.276. A measured R value of 0.75 was obtained after equilibration with 10 μM sodium oleate, yielding [free oleate] of 0.24 μM, according to eq 1. At this level of free oleate, 98% of the added FA is vesicle associated. Using the measured R values, V_a/V_m of 10^4, and eq 2, K_p is 4.1×10^5.

Fluorescence Polarization. Measurements of DPH and TMADPH fluorescence polarization were carried out using an SLM 8000C as described previously (Storch et al., 1989; Richieri et al., 1990). The fluorescence probes were added to vesicles (phospholipid concentration was 100 μM) from dimethylformamide (TMADPH) or tetrahydrafuran (DPH) at probe to phospholipid ratios of <0.005 while vortex mixing, and the mixtures were allowed to incubate for 1 h at 37 °C before measurement. Under these conditions, FA addition had no effect on fluorescence intensities and contributions from background fluorescence and scattering from vesicles were negligible.

Potential contributions to the total polarization (p_{ob}) from FA micelles was estimated according to (Weber, 1952)

$$1/p_{ob} = \alpha_m/p_m + \alpha_{mic}/p_{mic} \quad (3)$$

where α_m and α_{mic} are equal to the fractional fluorescence intensities from the membranes and micelles, respectively ($\alpha_m + \alpha_{mic} = 1$), and p_m and p_{mic} are their respective polarization values. To estimate these parameters, DPH (0.2 μM) intensities and polarization values were measured in DMPC SUV (100 μM) and separately in 30 μM FA dispersions, a concentration that exceeds the cmc for oleate and stearate by more than 5-fold (Richieri et al., 1992). Intensities and polarization values of DPH in FA dispersions were found to be respectively 0.09 ± 0.01 and 0.064 ± 0.004 for oleate and 0.03 ± 0.01 and 0.260 ± 0.005 for stearate, where α_{mic} was determined as the intensity in FA dispersions relative to that in DMPC SUV. Thus, even if micelles were to form and, as discussed in the Results, [FFA] levels in the present study were $\ll$30 μM, their relative intensity contributions would negligibly affect p_{ob}.

[3] At the pH used in these studies (7.4), partition involves two molecular species (HFA and FA$^-$), since a considerable fraction of the membrane-associated FA is protonated (Miyazaki et al., 1992). The K_p as used in here is, therefore, $[HFA + FA^-]_m/FFA^-]$, a mixture of the individual partition coefficients of the acid and ionized forms of the "fatty acid".

Table II: FA–Lipid Vesicle Partition Coefficients ($\times 10^{-4}$)[a]

fatty acid	EPC (L)	EPC + chol (L)	DMPC (L)	DMPC (S)
SA		220*		
PA	37 ± 2	55 ± 2	67 ± 2	70 ± 5
OA	36 ± 3	40 ± 2	48 ± 2	79 ± 2
DHA		14 ± 2		
LA	9 ± 1	8.8 ± 0.5	14 ± 2	15 ± 1
AA		8.4 ± 0.6	14 ± 2	16 ± 1
LNA			4.9 ± 0.5	5.5 ± 0.5

[a] Measurements were done at 37 °C using LUV (L) or SUV (S); for both types of vesicles, phospholipid concentrations were 100 μM. The composition of the EPC + chol vesicles was 2:1 egg phosphatidylcholine: cholesterol. All values, with the exception of those marked with an asterisk, are averages plus standard deviations of measurements made at several added (total) FA concentrations between 1 and 25 μM. Values marked with an asterisk were determined from a single FA concentration. For each FA concentration, 10 measurements were made and the averaged R value was used to determine [FFA]. The standard deviations in these values were approximately 1 nM.

Table III: FA–Lipid Vesicle Partition Coefficients ($\times 10^{-4}$)[a]

fatty acid	EPC + chol (L)	DMPC (L)
PA	38 ± 2	240 ± 30
OA	23 ± 2	32 ± 2
LA	4.2 ± 0.3	5.6 ± 0.4
AA		5.2 ± 0.5
LNA		1.5 ± 0.2

[a] Measurements were done at 15 °C using LUV (L) and phospholipid concentration of 100 μM. The values are averages plus standard deviations of measurements made at several added (total) FA concentrations between 1 and 25 μM. For each FA concentration, 10 measurements were made and the averaged R value was used to determine [FFA]. The standard deviations in these values were approximately 1 nM.

Table IV: Vesicle Concentration Dependence of FA Partition Coefficients ($\times 10^{-4}$)[a]

fatty acid	10 μM	100 μM
SA	150*	
PA	48 ● 9	56 ± 2
OA	52 ± 4	36 ± 4
DHA	13*	13*
LA	12 ± 4	9 ± 4
AA	9*	10*
LNA	3*	4*

[a] Measurements were done at 37 °C using LUV composed of EPC + chol. All values, with the exception of those marked with an asterisk, are averages and standard deviations of measurements made at several added (total) FA concentrations between 0.5 and 4 μM and 1 and 10 μM for the 10 and 100 μM samples, respectively. Values marked with an asterisk were determined from a single FA concentration. For each FA concentration, 10 measurements were made and the averaged R value was used to determine [FFA]. The standard deviation in these values were approximately 1 nM.

RESULTS

Partition of FA into Lipid Vesicles. FA partition coefficients were measured in a variety of lipid vesicles and the results of these studies are summarized in Tables II–IV. One of the most important features of these results, regarding the ability of FA to modulate cell function, is the large K_p values exhibited by all FA. As a consequence of these large K_p values, relatively small $[FA_m]$ variations (~20%) occur in the 100 μM vesicles used for these measurements, even though there is a 15-fold variation in K_p (Tables II–IV). The variation in K_p at these relatively high membrane concentrations primarily reflects differences in [FFA] rather than $[FA]_m$. The measured K_p are very likely equilibrium values since virtually identical results were obtained for FA vesicle incubation times

between 5 and 120 min, indicating that equilibrium occurred in less than 5 min (data not shown).

Measurements shown in Tables II and III were done using vesicles with a lipid concentration of 100 μM to ensure that the perturbation of the host vesicle by the added FA is minimized and therefore that the K_p values obtained represent an intrinsic vesicle property, rather than a vesicle–FA property. In order to duplicate more closely the conditions used to study FA perturbation of cell function, partition was also measured using vesicles at phospholipid concentrations of 10 μM. As mentioned in Experimental Procedures, this corresponds to the estimated plasma membrane phospholipd concentrations of the CTL at the levels typically used in these studies, $\sim 10^6$ cells/mL. Table IV lists the results of measurements comparing K_p values using 10 and 100 μM EPC + Cholesterol LUV. Surprisingly, although FA insertion does alter acyl chain order as discussed below, little variation in K_p was observed for added [FA] up to amounts that corresponds to as much as 34 mol % of the membrane lipid phase (about 85% of the added OA and PA partition into the 10 μM vesicle sample). Since K_p does not depend upon the fraction of FA in the membrane, most of the values listed in Tables II and III represent the average of K_p values for FA concentrations between 0.5 and 25 μM.

Consistent with the lack of sensitivity to FA concentration within the membrane, the partition coefficients are also relatively insensitive to vesicle lipid composition or structure (Tables II and III). Some increase in K_p, is exhibited, however, with increasing lipid order or degree of lipid saturation as seen by the increase in K_p values as the composition changes from EPC to EPC + chol to DMPC (Table II). A significant effect of vesicle state is observed, but only in gel-state DMPC (15 °C) and for saturated FA, where palmitic acid shows a more than 6-fold increase in K_p relative to the liquid crystalline phase (Table III). At the same time, this change in vesicle state and/or temperature has little effect on the K_p of the cis-unsaturated FA. These results are consistent with the previously observed partition preference of saturated FA for solid-phase lipid (Sklar et al., 1977; Klausner et al., 1980).

While FA partition into lipd vesicles shows relatively little dependence on composition and structure, very significant differences are observed as a function of FA molecular species. The results in Tables II–IV show that, for all vesicles, K_p increases with increasing saturation and chain length. The partition coefficient, for example, of stearic acid (18:0) is 40–50 times that of linolenic acid (18:3), and in the case of gel-state vesicles (Table III) the K_p of palmitic acid is 160-fold greater than linolenic acid. The variation of K_p with FA molecular species, at least for a liquid crystalline bilayer, parallels the aqueous-phase solubility of the FA: solubility increases and K_p decreases with increasing degree of unsaturation or decreasing chain length (Spector & Brenneman, 1972; Ashbrook et al., 1975; Richieri et al., 1992). Thus it is likely that FA aqueous-phase solubility plays a major role in determining K_p, and as a result the less soluble saturated FA have greater partition coefficients than the more soluble cis-unsaturated FA.

FA Partition into Cells. Measurements of FA partition into a murine CTL clone (C35) and a murine tumor (EL4) are shown in Table V. In general the magnitudes of K_p values are similar to those obtained with lipid vesicles and exhibit the same general trend; K_p decreases with aqueous solubility of the FA. The agreement between cell and vesicle K_p values is consistent with the lack of K_p dependence on membrane composition or structure and with the value of 10 μM for 10^6 cells/mL estimated for available cell lipid. Most importantly,

Table V: Partition Coefficients for FA in C35 and EL4 Cells ($\times 10^{-4}$)[a]

fatty acid	C35	EL4
SA	320 ± 120	
PA	110 ± 20	
OA	73 ± 8	83
DHA	19 ± 6	23
AA	14 ± 2	16
LA	10 ± 2	15
LNA	7 ± 1	6

[a] All K_p values were determined at 37 °C and the cell concentrations were 10^6/mL. C35 measurements represent averages of between 4 and 7 different FA concentrations between 1.5 and 10 μM. EL4 measurements were done using a single FA concentration for each FA.

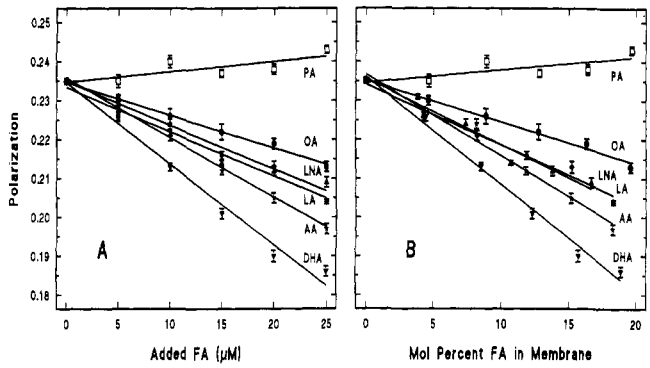

FIGURE 1: Effect of FFA on the fluorescence polarization in EPC + cholesterol LUV. In these studies the vesicle concentration (phospholipid content) was 100 μM, [DPH] was 0.2 μM, the indicated FA were added as their sodium salts, and the polarization measurements were done at 37 °C. Linear regressions for each FA data set are shown as solid lines. Data points are averages and standard deviations of 10 measurements. The results shown in this figure are representative of four separate studies. (A) Polarization results plotted as functions of added FA. (B) Results of (A) plotted as mole fraction of FA inserted into the bilayer according to the partition coefficients of Tables II–IV.

these results show that for cell concentrations typically used to study FA perturbation (see below), virtually all added saturated FA (>92%) and most of the OA (>88%), but lesser amounts of the polyunsaturated cis FA, partition into the cell membrane.

FA Perturbation of Lipid Acyl Chain Order. Previous studies have shown that changes in TMADPH and DPH polarization reflect changes in lipid order nearer to the surface and deeper in the bilayer, respectively (Prendergast et al., 1981; Storch et al., 1989). Steady-state polarization values of TMADPH and DPH were therefore monitored to determine the effect of added FA on lipid acyl chain order. Figure 1 illustrates the essential features of these results; addition of cis-unsaturated FA to LUV composed of EPC + cholesterol reduces DPH polarization, while saturated FA either have no effect or cause a slight increase in polarization. Figure 2 shows that addition of SA to DMPC vesicles above the phase-transition temperature (24 °C) increases the polarization values of both the surface-sensing (TMADPH) and the deeper-lying probe (DPH), while OA has no effect. At temperatures below the DMPC phase transition, addition of OA decreases polarization values and SA has little or no effect. Similar results were obtained using the other vesicles of different lipid composition and structure as described in Table I (data not shown). In general, the degree of cis FA induced perturbation in proportional to the degree of the lipid order of the bilayer. Less ordered vesicles such as pure EPC or DMPC above its phase-transition temperature exhibit proportionately smaller polarization value changes than with the EPC + cholesterol

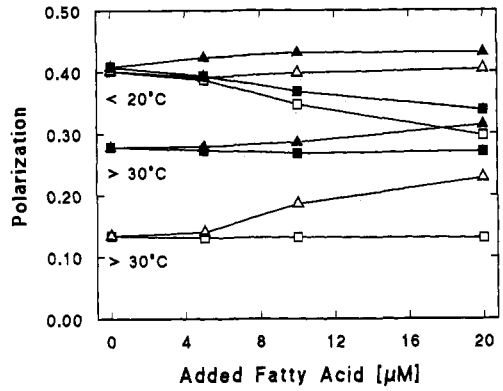

FIGURE 2: Effect of FFA on the fluorescence polarization in DMPC SUV. Stearic (△, ▲) or oleic (□, ■) acid was added to DMPC SUV either above (30 °C) or below (20 °C) the phase-transition temperature of DMPC (24 °C). DMPC concentration was 100 μM and DPH (△, □) or TMADPH (▲, ■) was <1 μM.

LUV of Figure 1, and the reverse is true for the saturated FA. Vesicles such as EPC + cholesterol are expected to provide a more accurate model of the bilayer phase of the plasma membrane, regarding lipid order, than pure phospholipid vesicles. Indeed, using TMADPH polarization, similar magnitude changes with cis-unsaturated FA treatment were observed previously in several CTL clones (Richieri et al., 1990) and with the C35 clone used in the present study (data not shown).

The degree of perturbation by added cis FA is proportional to its partition coefficient and to its *specific* perturbation potency, i.e., the change in polarization value per mole of lipid-phase FA. A relatively high concentration of lipid vesicles (100 μM) was chosen for these studies so that, with the exception of LNA (~80%), most (>90%) of the added FA would partition into the lipid bilayer and therefore evaluation of the specific potency would not be very sensitive to K_p. Using the measured partition coefficients, the results of Figure 1A have been replotted in Figure 1B as a function of the mole fraction of FA within the bilayer. When the data are replotted in this fashion, it is seen that the perturbation potency generally increases with the degree of cis unsaturation and, at the extrema, more than twice as much OA is needed to generate the same perturbation as DHA.

FA Perturbation of CTL Signaling. As with other murine CTL clones (Richieri & Kleinfeld, 1989; Richieri et al, 1990), C35 exhibits an increase of about 300 nM in intracellular Ca^{2+} levels within seconds of exposure to 10 μg/mL concanavalin A (Con A). We have previously shown that addition of cis-unsaturated but not saturated FA, a few seconds before Con A addition, inhibits the rise in $[Ca^{2+}]_i$ in a dose-dependent fashion, with complete inhibition occurring for added FA <10 μM. Figure 3 shows that the Con A-stimulated rise in $[Ca^{2+}]_i$ for C35 exhibits the same FA sensitivity as in these previous studies (DHA is new to this study). Thus cis-unsaturated but not saturated FA cause a dose-dependent inhibition of the rise in $[Ca^{2+}]_i$, with the degree of inhibition depending upon FA structure but in all cases occurring at added (total) FA less than 6 μM. These results are replotted in Figure 3, panels B and C as a function, respectively, of the mole fraction of FA in the membrane and the free concentration of each FA, using the measured partition coefficients. When the data are viewed this way (Figure 3B), one sees that the specific perturbational potencies vary considerably with FA structure, generally, the more unsaturated the FA, the more potent is its ability to inhibit CTL signaling. Moreover, as Figure 3C shows, perturbation of CTL signaling occurs at [FFA] < 0.5 μM. This is especially important regarding the physiological relevance of FFA perturbation, since it is the [FFA] levels that are regulated by the FA/albumin buffer system (Spector & Fletcher, 1978).

DISCUSSION

The results of this study demonstrate the following points: (1) The partition coefficients, for distribution between membranes and aqueous phase, of both saturated and cis-unsaturated FA are large. K_p values are so large, in fact, that virtually all saturated FA and most cis FA added to cells intercalate into the plasma membrane under conditions that are typical of FA perturbation studies. (2) All long-chain cis FA when added to membranes reduce the lipid acyl chain order, and in general the degree of perturbation increases with the number of cis bonds. (3) Added cis-unsaturated, but not saturated, FA inhibit CTL signaling and the degree of perturbation is directly proportional to the degree of lipid acyl chain perturbation.

Partition Coefficients. Measurements of natural long-chain fatty acid K_p values have been reported for a number of FAs in lipid vesicles, isolated plasma membranes, and whole cells using methods that physically separate free and membrane-bound radiolabeled FA (Pjura et al., 1984). These methods can suffer from several deficiencies that are especially critical when K_p is large, as it is for long-chain FA, and ultimately preclude the accurate determination of K_p values. First, complete separation of membranes and aqueous phase cannot be achieved, and therefore FA associated with the nonseparable membrane fraction must be subtracted from the observed supernatant radioactivity. For large K_p (small [FFA] and large $[FA_m]$), the FA associated with this fraction can easily exceed [FFA] by severalfold and therefore even small uncertainties in this quantity can preclude an accurate determination of [FFA]. For example, in the case of SA partition into 100 μM EPC + cholesterol LUV, >99.5% of the added SA is inserted into the bilayer and therefore a vesicle contribution to the supernatant of only 1% would represent more than twice the [FFA] level (0.5%). Second, since [FFA] is only a small fraction of $[FA_{total}]$, radioactive impurities with greater aqueous-phase solubilities could obscure true [FFA] levels (Vorum et al., 1992). Each of these factors results in increased apparent [FFA] levels and consequently smaller K_p values. The K_p values obtained in the previous studies (Pjura et al., 1984) are, in fact, about 10-fold lower than those obtained in the present study. Interestingly, the largest K_p value (8×10^5) in our previous study was obtained without physical separation using a fluorescent FA. The previous results, although different in magnitude, do exhibit the same trend, as found in the present study, with FA species SA > OA > LA [Table III of Pjura et al. (1984)], consistent with a significant aqueous-phase solubility dependence of K_p.

Studies of FA monolayers suggest that if FA in the aqueous phase were above the cmc, partition of saturated FA into the membrane might be negligible compared to cis FA, and this might account for the differential effects of FA perturbation on cellular function (MacDonald & MacDonald, 1988). As discussed above, however, CTL perturbation occurs at *added* FA concentrations <10 μM. Under these conditions virtually all the added SA (>95%) and most of the PA (>80%) partition into 10 μM vesicles or cells at 10^6/mL. After correction for wall binding, these results show that, for 10 μM added, SA and PA yield [FFA] of <0.3 and 0.8 μM, respectively, values that are below their respective cmcs (Richieri et al., 1992;

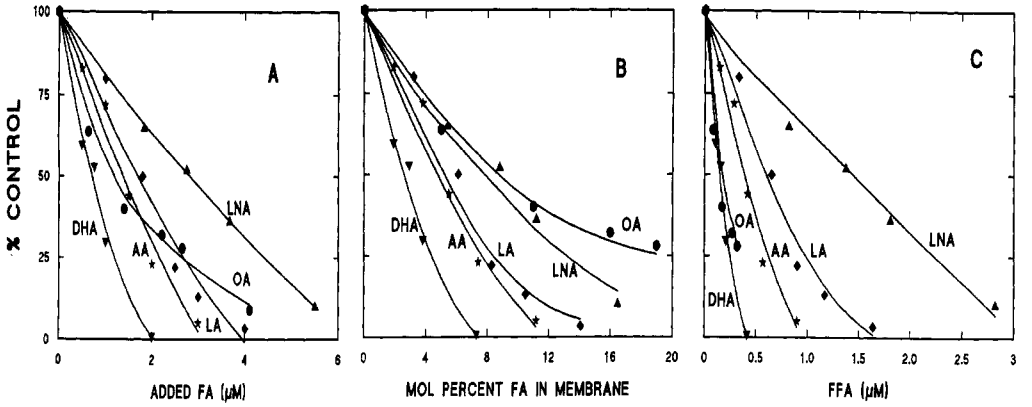

FIGURE 3: Cis FA inhibition of the rise in CTL $[Ca^{2+}]_i$. In these measurements fura-2-loaded CTL were stimulated with 10 μg/mL Con A and the peak $[Ca^{2+}]_i$ value was determined approximately 2 min after Con A addition. Values shown are relative to the CTL response in the absence of FA for which $[Ca^{2+}]_i$ values rose about 300 nM, as observed previously (Richieri & Kleinfeld, 1989). Saturated FA had no effect on the response [data not shown and Richieri and Kleinfeld (1989)]. Smoothed lines are shown through the data simply to guide the eye. (A) Inhibition of the response as a function of added FA. (B) Results of (A) plotted as the mole fraction of FA inserted into the membrane. (C) These same results plotted as a function of [FFA]. The abscissa values for (B) and (C) were obtained from the added FA concentration using the K_p values in Table IV.

data not shown). Thus under conditions of most in vitro studies of FA perturbation, [FFA] levels are below the cmc and more saturated than cis FA will partition into cell membranes.

Perturbation of Acyl Chain Order. The effect of FA on phospholipid acyl chains has been investigated by a variety of techniques, most of which were done using bilayers formed in the presence of FA [Pauls et al., 1983; Small, 1986; Ortiz & Gomez-Fernandez, 1987; Katsaras and Stinson (1990) and references therein]. All of these studies are consistent with an increase in order of the liquid crystalline phase of the bilayer in the presence of saturated FFA and a similar magnitude decrease in order of gel-phase bilayers with cis FFA. Similar results were obtained in the present studies, using preformed membranes, indicating that the association of both saturated and cis FA when added to preformed membranes reflects intercalation of FA into the membrane rather than some form of surface binding. Moreover, since the present results provide accurate K_p values for each of the FA, it is possible to determine the relative *specific* perturbation potency of each FA. As shown in Figure 1, with the possible exception of LNA, the greater the degree of cis-unsaturation, the greater the specific decrease in acyl chain order.

Mechanism of FA Perturbation of Cell Function. Studies of different functions in a variety of cells, as well as in isolated membranes, have demonstrated a sensitivity to perturbation by cis-unsaturated FA but not saturated FA (Karnovsky et al., 1982; Badwey et al., 1984; MacDonald & MacDonald, 1988; Richieri & Kleinfeld, 1989, 1990; Richieri et al., 1990; Stephen et al., 1991).[4] These effects appear, in at least some of these cases, to be due to a physical perturbation of cellular components since the effects are rapid, do not involve FA esterification, and can be reversed using albumin to extract the unesterified FA. Results from a number of these studies demonstrate that the cis FA effects are directed at specific targets; they are not simply due to a general toxic or lytic property of these FA. In the case of CTL, for example, cis

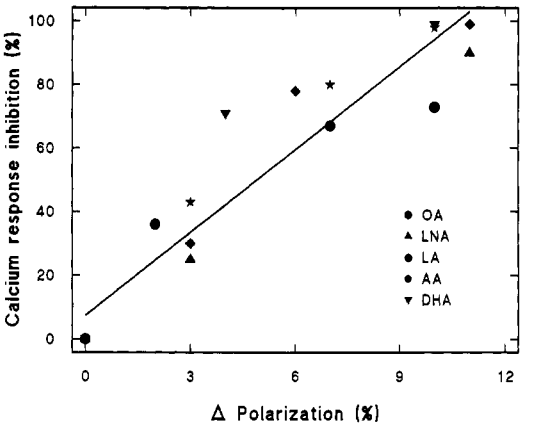

FIGURE 4: Correlation of the inhibition of the CTL $[Ca^{2+}]_i$ increase and acyl chain perturbation. This correlation is illustrated by plotting the percent inhibition along the ordinate and the percent decrease in polarization value (relative to the control value of 0.235) along the abscissa, for data points of Figures 1B and 3B corresponding to equal mole percent FA in the membrane. The solid line represents the linear regression through these data and has an r^2 value of 0.9.

FA inhibit the rise in $[Ca^{2+}]_i$, degranulation, and CTL-mediated killing of target cells but have no effect on antigen recognition, activation of phosphoinositol turnover, or CTL–target cell conjugation. The similarities of these kinds of perturbations suggest the possibility of a common origin. The results of the present study suggest that this mechanism probably involves the perturbation of lipid acyl chain order, rather than an allosteric and/or direct perturbation of specific proteins. More likely, this perturbation of acyl chain order alters the interaction between membrane lipids and specific proteins and this in turn alters the function of these proteins.

Evidence to support this view is that five different cis-unsaturated but no saturated FA inhibit the CTL response. This inhibition is dose-dependent and as shown in Figure 4 is linearly correlated with the degree of acyl chain perturbation. Figure 4 emphasizes that, after correction for the difference in partition, the dependence of the degree of inhibition can be represented solely as a function of the specific perturbation of acyl chain order, rather than on any specific structural feature of the FA. Thus, these results predict that determination of the degree of acyl chain perturbation for any other long-chain cis FA would uniquely predict its ability to perturb CTL function.

[4] Treatment of the human T cell line Jurkat with lauric (12:0) and myristic (14:0) acid has been reported to inhibit anti-CD3 activation of the rise in intracellular calcium levels at added FA levels <20 μM (Nordstrom et al., 1991). We find no inhibition of the Con A-stimulated calcium rise in CTL clones and no perturbation of lipid order in EPC–cholesterol vesicles at myristic acid concentrations up to 50 μM (data not shown). This is consistent not only with the lack of expected perturbation of membrane structure by saturated FA but also with the relatively high aqueous-phase solubility and therefore low K_p values of these short chain length FA.

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