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Depolymerization of Microtubules Alters Membrane Potential and Affects the Motional Freedom of Membrane Proteins

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ABSTRACT: Two independent lines of evidence were obtained indicating that microtubule depolymerization affects the functions and the physical state of membranes in intact Chinese hamster ovary cells. The first type of evidence was obtained by using the dye dihexyloxacarbocyanine iodide to measure membrane potential before and after treatment with several microtubule active agents. Microtubule depolymerization resulted in a decrease in cell fluorescence, whereas stabilization of microtubules with taxol resulted in an increase in cell fluorescence. These effects of the drugs were due to their interactions with microtubules and not to direct effects of the drugs on the plasma membranes for the following reasons: (1) effects were time dependent and required entry into the cells as indicated by the lack of fluorescence change in a multidrug-resistant mutant that does not accumulate antimicrotubule drugs and (2) a colcemid-resistant tubulin mutant did not show these effects on cell fluorescence. Evidence for altered motional freedom of membrane proteins in the plasma membrane was obtained by using electron spin resonance analysis of maleimide spin probe labeled cells. This study showed that depolymerization of microtubules results in increased motional freedom of maleimide-labeled sulfhydryl group containing proteins. Taken together, these data argue that microtubules function in mammalian cells to regulate the physical state of membranes and modulate membrane potential generated across cell membranes.

Several lines of evidence suggest that the microtubule network may interact with membrane components, modulating membrane potential (Vassilev et al., 1985), hormone responsiveness (Insel & Kennedy, 1978; Hagmann & Fishman, 1980), capping of receptors (Albertini & Clark, 1975; Malawista et al., 1978), and exocytosis (Wehland et al., 1982). Direct association of microtubules or tubulins with cell membrane components has been observed (Bhattacharya & Wolff, 1975; Bernier-Valentin et al., 1983; Pfeffer et al., 1983; Collot et al., 1984), and earlier studies have demonstrated that the microtubule system can directly influence plasma membrane dynamics such as microviscosity and fluidity (Berlin & Fera, 1977; Aszalos et al., 1985); however, the mechanism by which microtubules modulate different membrane functions is not yet fully understood.

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In this study we have extended our earlier findings indicating that microtubules influence the motional freedom of lipid components in plasma membranes (Aszalos et al., 1985). We present evidence here that the motional freedom of protein components as well as the membrane potential is influence by the microtubule system. For this study Chinese hamster ovary (CHO)[1] cells were chosen because these cells can be grown in suspension (Gottesman, 1985), because our previous studies were done with CHO cells (Aszalos et al., 1985), and because of the availability of specific mutants affecting uptake of antimicrotubule drugs (Ling & Thompson, 1973) and mutants affecting tubulin itself (Cabral et al., 1980) that could be used as biological specificity controls. Our results indicate that microtubules influence directly or indirectly the motional freedom of membrane proteins and alter the membrane potential of intact viable cells.

Materials and Methods

Growth of CHO Cells and Drug Treatment. CHO cells (10001) were grown in a gyrorotatory shaker bath at 160 rpm in suspension in α-modified minimal essential medium containing 10% fetal bovine serum (M. A. Bioproducts), 2 mM L-glutamine (Flow), 50 units/mL penicillin, and 50 μg/mL streptomycin (Flow) as previously described (Gottesman, 1985). Cells grown in monolayer culture were trypsinized the day prior to measurement of membrane potential or electron spin resonance (ESR) spectrometry, and 5×10^6 cells were inoculated into 20 mL of complete medium in a 125-mL glass bottle (2.5×10^5 cells/mL) for suspension growth. Cells were at a density of approximately 5×10^5 cells/mL at the time of the experiments (late log phase). Microtubule active drugs were added to cells 1 h prior to measurements from stock solutions as follows: colchicine (Sigma), 1 mg/mL in Me_2SO; vincristine (Sigma), 1 mg/mL in Me_2SO; colcemid (GIBCO), 10 μg/mL in H_2O; taxol (Developmental Therapeutics Branch, National Cancer Institute), 4 mg/mL in Me_2SO. The CHO cell line 11801 is a transformant of our wild-type cell line that carries three to five copies of a mutant β-tubulin gene (Cabral et al., 1980; Whitfield et al., 1985) and is temperature sensitive for growth; therefore, it was grown at 34 °C. Wild-type control cells were also grown at 34 °C when this cell line was studied. The CHO multidrug-resistant cell line C5 (Ling & Thompson, 1973) was from V. Ling (University of Toronto).

Membrane Potential Measurements. For membrane potential measurements, untreated and drug-treated aliquots of a CHO cell suspension (3×10^6 cells/mL) were stained with 0.5 μM dihexyloxacarbocyanine iodide [$DIOC_6(3)$; Molecular Probes, Inc., Junction City, OR) (stock solution, 50 μM dissolved in Me_2SO) in growth medium. This concentration of dye was nontoxic to cells as determined by a steady fluorescence intensity for the duration of the measurements, which was up to 1 h. The fluorescence intensity of this dye changes with the viability of cells and is a very sensitive indicator of cell viability (Shapiro, 1981). That the fluorescence intensity was indicative of the membrane potential was ascertained by the addition of agents known to change membrane potential according to Waggoner (1979). Elevated concentrations of extracellular K^+ (up to 120 mM), and 2 μg/mL gramicidin S, decreased the fluorescence intensity, while 2 μg/mL valinomycin increased the fluorescence intensity as expected for viable cells. No fluorescence quenching due to dye aggregation occurred under our experimental conditions, since no peak in the fluorescence intensity could be observed before the equilibrium fluorescence intensity was reached in the cells. This fact assured us that the observed fluorescence intensity change is indicative of membrane potential changes.

A Coulter EPICS V flow cytometer and its software were used for data acquisition and analysis. The laser was tuned to 488 nm, and the output was 400 mW. The flow rate of the cells was generally kept low, about 10^3 cells/s, to get good resolution. The forward-angle light scatter and green fluorescence signals were collected. The fluorescence usually was gated on the scatter signal to minimize artifacts. The coefficient of variation and the mean were calculated for each histogram. To obtain one histogram, 3×10^4 cells were analyzed.

ESR Spectrometry: Instrumentation. ESR spectra were recorded at X-band with a Varian E-9 Century series spectrometer (Varian Associates, Inc., Palo Alto, CA) operated at 9.5 kHz, 100-kHz field modulation, 4-G modulation amplitude, 100-G sweep range, and 20-mW microwave power. The scan time and receiver gain were changed according to requirements for obtaining a good spectrum. The temperature of the probe was set at 20 °C (±0.5 °C) by a Varian variable-temperature accessory using N_2 gas flow.

Labeling of Membrane Proteins for ESR Spectrometry. Spin-labeling of the membrane proteins was done basically as described by Grof and Belagyi (1983). Essentially, 20 mL of ethanolic solution of maleimide nitroxide (1 mg/mL) was taken to dryness in a conical centrifuge tube. The spin-label was then dissolved in 0.1 mL of phosphate-buffered saline (PBS), and a 50-μL cell suspension of $(2–4) \times 10^7$ cell/mL was added. After 15-min reaction time at ambient temperature, the suspension was pelleted in 30 s. The pellet was resuspended in 0.5 mL of PBS solution and the resultant suspension repelleted. This procedure was repeated twice again in order to wash out the unreacted maleimide spin-labels. The last supernatant contained no measurable amount of spin-label. The ESR signals of the final pellets were measured in a 50-μL micropipet capillary (Clay Adams Co., Persippany, NJ). The capillaries were sealed with Critoseal (Syva Co., Palo Alto, CA).

Evaluation of ESR Spectra. The motional freedom of membrane proteins was assessed from ESR parameters of maleimide spin probe reacted with sulfhydryl groups of proteins. Two types of sulfhydryl groups bind this spin label; namely, easily accessible mobile sulfhydryl groups and slow-moving sulfhydryl groups seeded deeper in the hydrophobic regions of the membranes (Grof & Belagyi, 1983). Therefore, the ESR spectra indicate the motional freedom of both types of proteins from which the slow-moving proteins can be characterized by the outer hyperfine coupling constant $2T_{\parallel}'$. The parameters usually measured in ESR experiments are $2T_{\parallel}$ and $2T_{\parallel}'$, which are components of the motionally averaged nitrogen hyperfine tensor from which the order parameter (S) and the polarity factor (a_n) are calculated (Hubbell & McConnell, 1971). Calculation of these parameters could not be done in our experiments because of the two superimposed spectra of the two types of proteins. The slow-moving, deeper proteins could be characterized to some extent by the outer hyperfine couplings, $2T_{\parallel}'$. In this case, since we could not calculate S or a_N, the meaning of a larger $2T_{\parallel}'$ could be either less motional freedom or less polar vicinity of the ESR probe (Freed, 1972).

Results

Membrane Potential Measurements. The membrane potential of wild-type and mutant CHO cells, untreated and

[1] Abbreviations: CHO, Chinese hamster ovary; $DIOC_6(3)$, dihexyloxacarbocyanine iodide; ESR, electron spin resonance; PBS, phosphate-buffered saline.

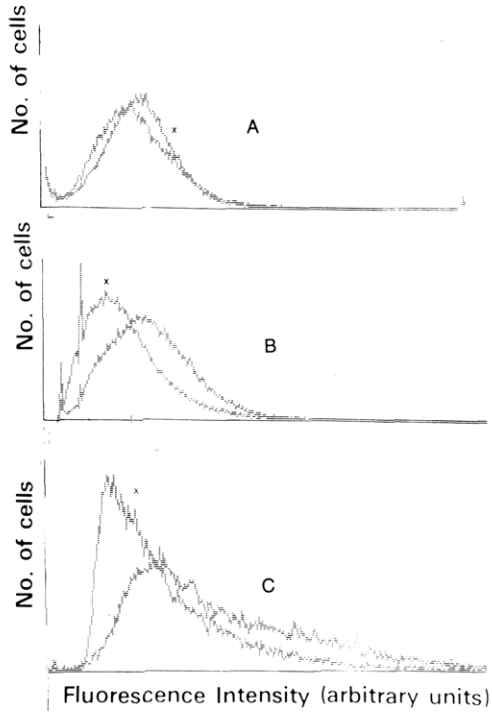

FIGURE 1: Cell fluorescence intensity change in wild-type CHO cells: (A) before and after (×) addition of 2 μg/mL valinomycin; (B) before and after (×) addition of 2 μg/mL gramicidin S; (C) before and after (×) increasing the extracellular [K^+] to 80 from 5 mM.

treated with microtubule depolymerizing agents, was measured by the fluorescent translocating dye method as described under Materials and Methods. The use of the flow cytometric technique allowed us to obtain information on a cell-by-cell basis. Usually, fluorescence of 3×10^4 cells was measured to obtain one histogram. That the fluorescence intensity was indicative of the membrane potential was determined by observing appropriate fluorescence intensity shifts upon the addition of known membrane potential changing agents as detailed under Materials and Methods and shown in Figure 1. Light scatter histograms of treated and untreated CHO cells were superimposable, indicating equal cell shapes of treated and untreated cells. The membrane potential of wild-type CHO cells decreases after treatment with 2.5 μg/mL vincristine or 2.5 μg/mL colchicine as shown in Figure 2. Addition of 2.5 μg/mL vincristine to untreated wild-type CHO cells followed by fluorescence intensity measurement within 3 min indicated very little fluorescence intensity changes as compared to that after 1-h treatment. From this fact, an ionophoric character of the applied microtubule depolymerizing agent can be ruled out. We observed a linear relationship between the membrane potential shift and the colcemid concentration upon addition of 0.05, 0.075, and 0.1 μg/mL colcemid (data not shown). Previously, we have shown that colcemid induces microtubule depolymerization in CHO cells with a similar dose–response (Aszalos et al., 1985).

Histograms showing changes of fluorescence intensity upon addition of 2.5 μg/mL colcemid, 10 μg/mL taxol, and 10 μg/mL taxol followed by 2.5 μg/mL colcemid to wild-type CHO cells are shown in Figure 3. Colcemid alone caused a decrease of fluorescence, while taxol increased the fluorescence of CHO cells. However, addition of colcemid after taxol treatment resulted in no significant change in the fluorescence intensity.

Two mutant CHO cell lines, a transformant carrying and expressing several copies of a mutant β-tubulin gene (11801) (Cabral et al., 1980; Whitfield et al., 1985) and a multi-

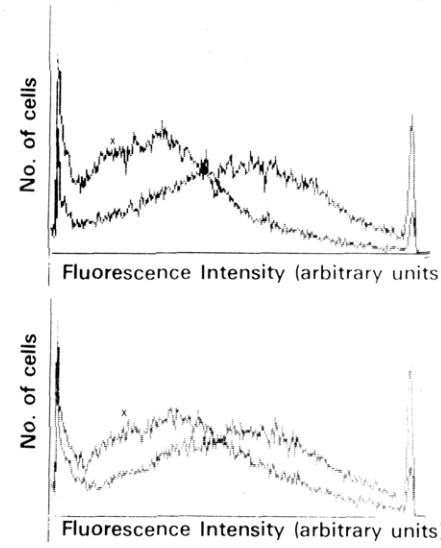

FIGURE 2: Histograms of 2.5 μg/mL vincristine-treated (top histogram), 2.5 μg/mL colchicine-treated (bottom histogram), and untreated wild-type CHO cells. In both cases treated cells yielded the left histograms (marked with an ×). Histograms were obtained as described in Materials and Methods and were superimposed electronically.

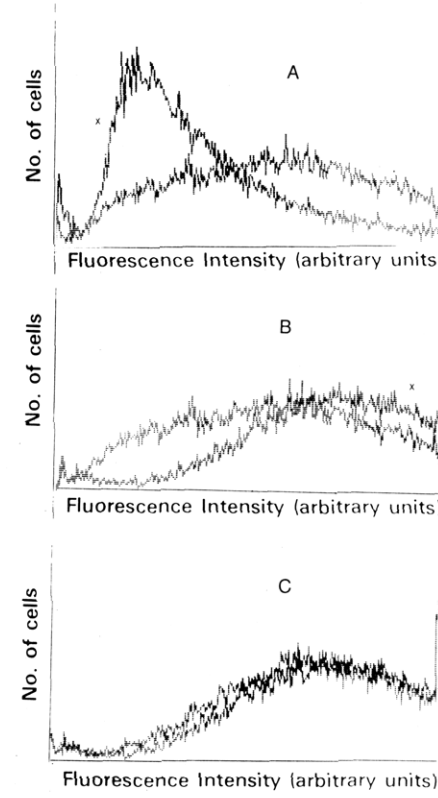

FIGURE 3: Histograms of 2.5 μg/mL colcemid (A), 10 μg/mL taxol (B), and 10 μg/mL taxol followed by 2.5 μg/mL colcemid-treated CHO cells (C). Histograms of treated cells are marked by an × in each case. Histograms were obtained as described in Materials and Methods and were superimposed electronically.

drug-resistant plasma membrane mutant (C5) (Ling & Thompson, 1973), were tested for membrane potential change after addition of 0.1 or 0.075 μg/mL colcemid. While the wild-type and revertant CHO cells responded to these treatments (Figure 4A,C), no fluorescence intensity change was observed with either of the mutant cells (Figure 4B and data not shown). The corresponding light scatter histograms for the treated and untreated wild-type cells are also shown in Figure 4D. Superimposing the light scatter histograms of 2.5

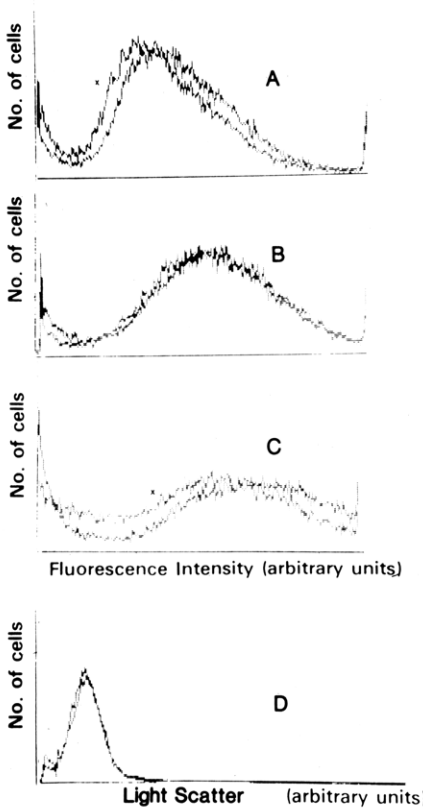

FIGURE 4: Cell fluorescence intensity change in wild-type, β-tubulin mutant (11801), and revertant CHO cells: (A) histograms of 0.075 μg/mL colcemid-treated (×) and untreated wild-type CHO cells; (B) histograms of 0.075 μg/mL colcemid-treated and untreated 11801 CHO cells; (C) histograms of 0.075 μg/mL colcemid-treated (×) and untreated revertant CHO cells; (D) light scatter histograms of 0.075 μg/mL colcemid-treated and untreated wild-type CHO cells.

μg/mL colchicine and vincristine-treated and untreated cells also indicated similar cell volumes and shapes for the treated and untreated cells. It is worth noting that while light scatter histograms of the wild-type and β-tubulin mutant cells were very similar, their membrane potentials were slightly different, as indicated by different positions of the mean fluorescence channel for each of these CHO cell lines.

Motional Freedom of Membrane Proteins of CHO Cells. The motional freedom of membrane proteins was assessed from the ESR parameters of a maleimide spin probe reacted with sulfhydryl groups of proteins. Sulfhydryl groups of proteins with easily accessible and highly mobile sulfhydryl groups, and also from proteins with slow-moving sulfhydryl groups buried deeper in the hydrophobic region of membranes (Grof & Belagyi, 1983), will bind this spin-label. Therefore, the ESR spectra indicate the motional freedom of both types of proteins. The slower moving proteins can be characterized by the outer hyperfine couplings, $2T_{\parallel}$. A typical spectrum obtained with the maleimide probe reacted with proteins of the CHO cells is shown in Figure 5. Results obtained with the different microtuble depolymerizing agents and taxol, a drug that stabilizes microtubules and inhibits their depolymerization, are shown in Table I.

Each depolymerizing drug used—colchicine, vincristine, colcemid—decreased the value of $2T_{\parallel}'$. Taxol, 10 μg/mL, had no effect alone. Also, no significant change in the $2T_{\parallel}'$ value could be seen when cells were pretreated with taxol (10 μg/mL) prior to colcemid (0.2 μg/mL) treatment. Changes in the $2T_{\parallel}'$ values caused by the microtubule depolymerizing drugs are significant according to standard deviation calculations ($p < 0.05$, calculated by Student's t test between treated and untreated cells). These results suggest that microtubule depolymerization results in increased motional freedom of the spin probe.

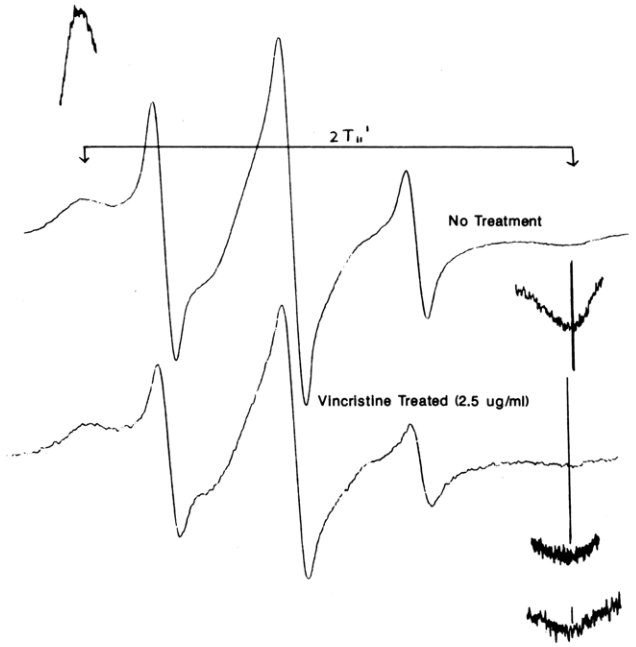

FIGURE 5: ESR spectra of maleimide spin probe bound to sulfhydryl groups of proteins of CHO cells: upper spectrum, no treatment; lower spectrum, vincristine- (2.5 μg/mL) treated cells. Labeling of the cells and other experimental conditions are detailed under Materials and Methods. For better precision, parts of these spectra were obtained with higher receiver gain, otherwise using the same experimental conditions.

Table I: ESR Parameters of Maleimide Spin Probe in CHO Cells

treatment (μg/mL)	$2T_{\parallel}' \pm$ SD[a] (n)
no treatment	58.4 ± 0.2 (8)
colchicine (2.5)	57.2 ± 0.3 (8)
vincristine (2.5)	57.4 ± 0.2 (8)
colcemid (0.2)	57.3 ± 0.1 (4)
taxol (10)	58.1 ± 0.2 (4)
taxol (10) followed by colcemid (0.2)	58.3 ± 0.2 (4)

[a] The numbers after the $2T_{\parallel}'$ values are standard deviations. Student's t test indicates $p < 0.05$ between treated and untreated samples.

DISCUSSION

It was reported previously that the motional freedom of ESR membrane probes increased if the microtubule system was depolymerized in CHO cells (Aszalos et al., 1985). In the current study, further evidence has been obtained to indicate that the microtubule system does affect, directly or indirectly, plasma membrane functions. We have shown that the motional freedom (or the polarity of the immediate environment) of proteins seeded in the lipid domain of membranes increases with depolarization of the microtubule system. We have also shown that the plasma membrane potential decreases when the microtubule system is depolymerized.

Penetration of the drug into the cells is a prerequisite for the above effects since insignificant membrane potential changes can be observed if drugs are added immediately before measurements. CHO mutant C5, which is resistant to many drugs because of decreased ability to accumulate these drugs, presumably owing to an alteration in membrane function (Ling & Thompson, 1973), fails to show membrane changes in response to colcemid treatment. This result serves as an additional control, indicating that the antimicrotubule agents must accumulate within the CHO cells to have an effect on

membrane potential.

It is also evident that the microtubule depolymerizing agents must interact with the microtubules after entering the cells to cause membrane potential changes for the following reasons: (1) the dose–response of the membrane depolarizing effect of colcemid is similar to the dose–response for depolymerization of microtubules for this drug (Aszalos et al., 1985; unpublished data); (2) taxol, a drug known to stabilize microtubules in cultured cells (Manfredi et al., 1982), blocks the membrane potential changing effect of colcemid; (3) a CHO mutant resistant to colcemid because of alterations in β-tubulin (Cabral et al., 1980; Whitfield et al., 1985) shows no membrane potential change upon colcemid treatment. However, the revertant cell line of the β-tubulin transformant does react to colcemid treatment as shown in Figure 4C. It seems reasonable to conclude from these findings that the observed membrane potential changes are mediated by microtubule depolymerization.

The dye $DIOC_6(3)$, used for the membrane potential measurements, also measures mitochondrial membrane potential changes (Rottenberg, 1979; Johnson et al., 1981; Wilson et al., 1985). It appears that under our experimental conditions the observed membrane potential changes are at least in great part due to changes at the plasma membrane level. This statement is supported by the following experimental facts: (1) We have done our experiments at 20 °C where the mitochondrial functions are suppressed. (2) According to the experimental results of Wilson et al. (1985), at a cell density of 10^6 cells/mL, the density used in our experiments, the plasma membrane contributes about 80% and the mitochondria about 20% to the measured membrane potential, i.e., fluorescence intensity. Microtubule depolymerization with 2.5 μg/mL vincristine or colchicine results in a fluorescence intensity decrease that is approximately 50% or more than double the 20% contribution of the mitochondria. (3) Under our experimental conditions, valinomycin, 2 μg/mL, increased the fluorescence intensity of the cells (Figure 1A), indicating plasma membrane and not mitochondrial involvement in the observed fluorescence intensity changes. If only the mitochondrial membrane potential was being measured under our experimental conditions, where no fluorescence quenching occurs due to dye aggregation, valinomycin treatment would yield a decrease in fluorescence intensity (Johnson et al., 1980). That the observed fluorescence intensities reflect membrane potentials in general is indicated by the appropriate fluorescence intensity changes after addition of elevated concentrations of extracellular K^+, gramicidin S, and valinomycin as shown in Figure 1.

The exact reason why the membrane potential changes with depolymerization of the microtubules is not known. However, it is conceivable that, with altered physical status of the membrane, as deduced from our membrane spin probe studies (Aszalos et al., 1985; and this work), the functional dynamics of different ion channels, such as K^+ channels, also change. It is interesting to note that the microtubule stabilizing drug taxol increases the cell fluorescence signal and that microtubule depolymerizing agents decrease it (Figure 3). It should be pointed out that addition of 80 mM extracellular K^+ yields about a 45% fluorescence intensity decrease, comparable (50%) to that observed with 2.5 μg/mL colchicine or vincristine treatment. Approximately 80% of the fluorescence intensity decrease caused by the addition of 80 mM extracellular K^+ is contributed by the plasma membrane, according to Wilson et al. (1985) as pointed out above. Another speculation can be proposed on the basis of the work of Vassilev et al. (1985) who have suggested that polymerized microtubules associated with membranes increase ionic conductance through the microtubule lumen. Both hypotheses presuppose that microtubules, directly or indirectly, are associated with the plasma membrane as has been proposed by others on the basis of direct biochemical evidence (Bernier-Valentin et al., 1983).

Spin-label studies of the motionally restricted protein sulfhydryl groups reflect results obtained with ESR probes of the lipid domain (Aszalos et al., 1985) and that of the above-discussed membrane potential studies. The motional freedom of the protein-bound spin probes increases if the microtubule system is depolymerized, and these changes are also hindered by taxol (Table I). These ESR results suggest that microtubule depolymerization, which we have previously shown results in increased motional freedom of the lipid domain in cell membranes, also affects mobility of proteins in the membrane. This finding raises the possibility that the more deeply located, slow-moving proteins may belong to ion channels or ion pumps or might interact with such pumps. Thus, changes in membrane potential could be a direct reflection of altered protein mobility in the membrane.

These studies demonstrate the physical and functional effects of microtubule depolymerization on the cell membrane. Taken together with previous published results, they argue for an important role of microtubules in modulating the physical state of, and consequently the function of, cell membranes.

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Binding of Local Anesthetics to Reconstituted Acetylcholine Receptors: Effect of Protein Surface Potential†

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ABSTRACT: Nicotinic acetylcholine receptor isolated from *Torpedo californica* electric organ is reconstituted into lipid bilayers of zwitterionic dioleoylphosphatidylcholine. These membranes are labeled with a spin-labeled quaternary amine local anesthetic (C6SLMeI), which has been shown previously to be a noncompetitive blocker of acetylcholine receptor–ion channel function in the micromolar concentration range. The electron spin resonance spectral component corresponding to protein-immobilized anesthetic spin-label can be resolved from the composite data spectrum by using spectral subtraction of lipid components. This protein-immobilized component is shown to represent C6SLMeI bound to a finite number of sites on the receptor. We demonstrate that C6SLMeI binds to the receptor as a function of the surface potential on the protein and suggest that the acetylcholine receptor reconstituted into zwitterionic phospholipid, which has no surface potential of its own, provides an excellent model system with which to study effects of protein surface charge. We hypothesize that the primary pathway of interaction of C6SLMeI with the acetylcholine receptor is via the aqueous medium.

Amphiphilic tertiary amine local anesthetics such as procaine, lidocaine, and tetracaine are known to interact hydrophobically with membrane lipids (Skou, 1954; Lee, 1977). On the other hand, there is much evidence that local anesthetics interact directly with a variety of membrane proteins. Yeh demonstrated a difference in sodium channel blocking potency between two isomeric forms of a local anesthetic in squid giant axon (Yeh, 1980). Hille (1977) demonstrated that there are both hydrophilic and hydrophobic pathways of interaction of local anesthetics with the sodium channel, suggesting that lateral diffusion to the protein from the lipid phase may not be required for pharmacological effect. The interactions of local anesthetics with sodium channels and acetylcholine receptors (AChR)[1] are similar in that the positively charged form is pharmacologically active at micromolar concentrations (Aracava et al., 1984; Blickenstaff and Wang, 1985) and are different in that the location of the pharmacologically relevant binding site(s) is (are) intracellular for the sodium channel and extracellular for the AChR (Aracava et al., 1984; Aracava & Albuquerque, 1984). Several electrophysiological studies on the acetylcholine receptor suggest a direct blockade of the ion channel by local anesthetics (Neher and Steinbach, 1978; Oswald et al., 1983; Ikeda et al., 1984), although there have been reports that some local anesthetics may block acetylcholine receptor–ion channel activity via the lipid bilayer (Koblin and Lester, 1979; Ribera et al., 1985).

Electrophysiological studies of local anesthetic binding are limited because only channel-associated activity is monitored. Biochemical approaches to determining binding sites on acetylcholine receptor membranes are difficult because of the noncovalent interactions of the local anesthetic with multiple sites on the receptor membrane. An accurate study of local anesthetic–membrane interactions in vivo must include careful characterization of the lipid domain, regardless of the location of the pharmacological local anesthetic binding site. The use of a spin-labeled local anesthetic and electron spin resonance spectroscopy (ESR) allows us to observe more directly the drug's interactions with membrane components.

Local anesthetic binding to membrane components is examined in a reconstituted membrane containing acetylcholine receptor (RAChR membrane). The defined composition of the RAChR membrane allows unambiguous identification of membrane components with which spin-labeled local anesthetic populations are associated. The use of the zwitterionic phospholipid dioleoylphosphatidylcholine permits examination

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[1] Abbreviations: AChR, acetylcholine receptor; carb, carbamylcholine; C6SL and C6SLMeI, tertiary and quaternary amine local anesthetic spin-labels, respectively (see Figure 1); C6MeI, quaternary amine local anesthetic without spin-label; DOPC, dioleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; ESR, electron spin resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NCB, noncompetitive blocker; PCP, phencyclidine; PMSF, phenylmethanesulfonyl fluoride; RAChR, reconstituted acetylcholine receptor; SDS, sodium dodecyl sulfate; TC, *d*-tubocurarine; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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