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EFFECTS OF LOCAL ANESTHETICS ON MEMBRANE PROPERTIES

II. ENHANCEMENT OF THE SUSCEPTIBILITY OF MAMMALIAN CELLS TO AGGLUTINATION BY PLANT LECTINS

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SUMMARY

Treatment of untransformed mouse and hamster cells with the tertiary amine local anesthetics dibucaine, tetracaine and procaine increases their susceptibility to agglutination by low doses of the plant lectin concanavalin A. Agglutination of anesthetic-treated untransformed cells by low doses of concanavalin A is accompanied by redistribution of concanavalin A receptors on the cell surface to form patches, similar to that occurring in spontaneous agglutination of virus-transformed cells by concanavalin A. Immunofluorescence and freeze-fracture electronmicroscopic observations indicate that local anesthetics per se do not induce this redistribution of concanavalin A receptors but modify the plasma membrane so that receptor redistribution is facilitated on binding of concanavalin A to the cell surface. Fluorescence polarization measurements on the rotational freedom of the membrane-associated probe, diphenylhexatriene, indicate that local anesthetics produce a small increase in the fluidity of membrane lipids. Spontaneous agglutination of transformed cells by low doses of concanavalin A is inhibited by colchicine and vinblastine but these alkaloids have no effect on concanavalin A agglutination of anesthetic-treated cells. Evidence is presented which suggests that local anesthetics may impair membrane peripheral proteins sensitive to colchicine (microtubules) and cytochalasin-B (microfilaments). Combined treatment of untransformed 3T3 cells with colchicine and cytochalasin B mimics the effect of local anesthetics in enhancing susceptibility to agglutination by low doses of concanavalin A. A hypothesis is presented on the respective roles of colchicine-sensitive and cytochalasin B-sensitive peripheral membrane proteins in controlling the topographical distribution of lectin receptors on the cell surface.

INTRODUCTION

Concanavalin A and other plant lectins have been used extensively to study differences in the organization of the surface of untransformed and transformed cells

[1–3]. In general, cells transformed by oncogenic viruses or carcinogenic chemicals are agglutinated by doses of concanavalin A which do not affect untransformed cells, but the latter can be made susceptible to agglutination by similar low doses of concanavalin A by brief trypsinization [1–3]. The factors that determine these differences in agglutin ability are still poorly understood. Variation in cellular susceptibility to lectin agglutination does not result from simple differences in the number of lectin binding receptors exposed on the cell surface [4–11] and experimental investigation of the underlying mechanism(s) is now directed largely to identification of differences in the structural organization of the cell periphery in transformed cells and their untransformed counterparts.

Concanavalin A-induced agglutination of both untransformed and transformed cells is accompanied by redistribution of concanavalin A receptors on the cell surface from a random dispersed pattern to form "patches" [1, 8, 12–15]. This association between agglutination and redistribution of lectin receptors has prompted proposals that variations in cellular susceptibility to agglutination might reflect differences in the ability of receptors to move laterally within the plasma membrane to form patches. Specifically, it has been proposed [16–22] that the higher susceptibility of transformed cells to agglutination by concanavalin A results from a more "fluid" lipid matrix in the plasma membrane of these cells compared with the membranes of untransformed cells. Enhanced fluidity of the lipid membrane matrix could allow a greater rate of lateral movement of receptors within the membrane and favor the formation of patches of receptors after binding of concanavalin A to the cell surface.

In this communication we report that local anesthetics which increase the fluidity of phospholipids in model membranes, as described in detail in the preceding paper [23], also enhance the susceptibility of untransformed cells to agglutination by low doses of concanavalin A. Analysis of the cellular changes occurring in anesthetic-treated cells has revealed, however, that anesthetic-induced alterations in membrane fluidity might not be the primary mechanism responsible for the increased agglutination response, and that the major action of these drugs is on peripheral membrane proteins that regulate the distribution of lectin binding sites on the cell surface.

MATERIALS AND METHODS

Cells

BALB/c mouse 3T3 cells and BALB/c 3T3 cells transformed by simian virus 40 (SV3T3) were cultured in 60 mm plastic Petri dishes (Falcon Plastics, Oxnard, California) in Dulbecco's modification of Eagles medium supplemented with 10 % fetal bovine serum as described before [24]. Two variant SV3T3 cell lines, R-SV3T3-10 and R-SV3T3-14, showing reversion of their transformed properties, but which still retain rescuable SV40, were isolated from the SV3T3 cell population on the basis of their increased resistance to agglutination by concanavalin A using methods described elsewhere [25] and cultured under the same conditions as the parent SV3T3 cells. The baby hamster kidney (BHK) cell line, BHK21-C13, and BHK cells transformed by polyoma virus (PY-BHK) or Rous sarcoma virus (Bryan strain) (RSV-BHK) were also cultured in Dulbecco's modification of Eagles medium supplemented with 10 % fetal bovine serum as described before [26]. Culture media and sera were obtained from the Grand Island Biological Company (Grand Island, N.Y.).

Concanavalin A

Concanavalin A was purchased as a twice-crystallized preparation (Miles, Elkhart, Ind.) and purified further by affinity chromatography [8]. ^{3}H-labeled concanavalin A (specific activity $3.8 \cdot 10^6$ cpm per mg) was prepared from affinity chromatography purified concanavalin A using [^{3}H]acetic anhydride as described before [8, 27]. ^{3}H-labelled concanavalin A co-chromatographed with unlabelled concanavalin A on DEAE-cellulose and resembled native concanavalin A in its reactivity with rabbit anti-concanavalin A antibodies and cell agglutination properties [27]. Binding of ^{3}H-labeled concanavalin A to cells was measured at 0° C or room temperature (22 °C) as described previously [8]. The amount of ^{3}H-labeled concanavalin A bound to cells in the presence of α-methylmannoside (0.1 M) was subtracted from the amount bound in the absence of this compound. The results are expressed as specific binding of ^{3}H-labeled concanavalin A in cpm per $1 \cdot 10^7$ cells.

Cell agglutination by concanavalin A

Cellular susceptibility to agglutination by different concentrations of concanavalin A was measured as described previously [28]. Agglutination was assessed by microscopic examination of cell populations after incubation at room temperature for 20 min and scored as O, +, +−, +++ or ++++ for 0, 25, 50, 75 or > 90 % of cells agglutinated, respectively. The specificity of the agglutination reaction was confirmed by agglutination inhibition using 0.25 M α-methylmannoside, a specific haptenic inhibitor for concanavalin A.

Since Ca^{2+} exert a significant effect on the "fluidity" of phospholipids [23, 29], the agglutination assay for cells treated with local anesthetics was modified to accommodate the possibility that EDTA detachment of cells from petri dishes immediately before testing their agglutinability might alter the "fluidity" of membrane phospholipids and thus complicate interpretation of experimental observations on changes in agglutinability that might result from the ability of anesthetics to increase lipid fluidity [23]. Cells were therefore detached from the surface of petri dishes using 0.2 % EDTA in calcium and magnesium-free saline and then returned to complete medium supplemented with appropriate concentrations of local anesthetics for periods upto two hours at 37 °C. The susceptibility of cells to agglutination by different concentrations of concanavalin A was then determined, still in the presence of local anesthetics, and agglutination scored as described above.

Immunofluorescence assay of concanavalin A binding

The distribution of concanavalin A binding sites on the cell surface was determined by indirect immunofluorescence as described previously [8]. For staining, cells were exposed to concanavalin A for 30 min at 37 °C before final incubation with fluorescein-conjugated rabbit anti-concanavalin A antibodies for 1 h at 37 °C. Cells were finally fixed with 2.5 % glutaraldehyde for 15 min at room temperature. In some experiments cells were fixed with glutaraldehyde before incubation with concanavalin A and fluorescein-conjugated anti-concanavalin A antibodies. The distribution of specific immunofluorescence on individual cells was observed in a Leitz Ortholux microscope under ultraviolet light using UG-1 excitation and K-430 barrier filters. To determine the frequency of different immunofluorescence staining patterns on cells within any sample, at least 100 stained non-aggregated cells were observed under

1000× magnification. The term "uniform" fluorescence will be used to describe the staining pattern in which specific immunofluorescence was confined to the spherical outline of the cell. Work in this [8] and other laboratories [7, 30] has established that this pattern is characteristic of a randomly dispersed distribution of concanavalin A receptors on the cell surface. "Patchy" fluorescence describes staining patterns in which fluorescence occurs only in concentrated areas of the cell, including the central area, leaving other areas devoid of fluorescence including ring fluorescence at the cell margin. This pattern occurs only after redistribution and clustering of concanavalin A receptors to form "patches" [7, 8, 30].

Freeze fracture electron microscopy

Cells for freeze fracture observations of membrane ultrastructure were prepared and etched in a Balzers BA 360 freeze-etch apparatus and shadowed with platinum as described before [31]. Specimens were examined in a Phillips EM 300 electron microscope operating at 60 kV.

Fluorescence polarization

Fluorescence polarization measurements on intact cells were made in an Aminco Bowman spectrofluorimeter as described in the preceding paper [23] using 1,6-diphenylhexatriene (DPH) as a probe for membrane fluidity (vide infra). Cell suspensions ($1 \cdot 10^6$ cells per ml) were labelled with DPH in phosphate-buffered saline by blowing a 5 μl aliquot of DPH (10^{-3} M) dissolved in tetrahydrofuran into the cell suspension. The final concentrations of DPH and tetrahydrofuran were 1–8 μM and 0.5 % (v/v), respectively. The sample was then incubated for 1 h at room temperature in the dark, after which the cells were washed twice with phosphate-buffered saline and the final polarization measurements made using a 0.5 ml sample. The background signal from control cell suspensions without DPH was subtracted from all measurements.

Microscopy of DPH-stained 3T3 cells revealed that DPH fluorescence was present on the cell surface but significant cytoplasmic staining was also detectable within 5 min of exposure to the dye. In addition to diffuse low intensity staining over the general cytoplasm, numerous very small areas of intense punctate fluorescence were present, usually in the perinuclear cytoplasm. The size and distribution of the latter resemble closely the previously documented fluorescence staining pattern found in cells treated with acridine dyes in which fluorescence has been localized specifically to lysosomes [32]. No staining of the nuclear membrane was observed in DPH-treated cells. These observations with 3T3 cells conflict with those of Shinitzky and Inbar [33] using lymphocytes who reported that DPH fluorescence was confined strictly to the cell periphery. Our results suggest that DPH is distributed to both the plasma membrane and intracellular membranes.

Reagents

Glycerol, sucrose, dimethylsulfoxide and colchicine were obtained from the Sigma Chemical Company. Lumicolchicine was prepared by exposing colchicine to ultraviolet light in 95 % ethanol as described by Wilson and Friedkin [34]. [^{3}H]Colchicine (spec. act. 5 Ci/mol) was purchased from New England Nuclear (Boston, Mass.); dibucaine HCl, procaine HCl and tetracaine HCl from Mann Research

Laboratories (Orangeburg, N.Y.): cytochalasin B and diphenylhexatriene from the Aldrich Chemical Co. (Milwaukee, Wisc.) and vinblastine sulfate (VELBAN) from Eli Lilly and Co. (Indianapolis, Ind.).

RESULTS

The effect of local anesthetics on cellular susceptibility to agglutination by concanavalin A

Agglutination of untransformed 3T3 and BHK cells and the revertant SV3T3 lines, R-SV3T3-10 and R-SV3T3-14, requires significantly higher concentrations of concanavalin A than the corresponding cell lines transformed by oncogenic viruses (Table I). However, incubation of untransformed and revertant cell lines with the local anesthetic dibucaine for 30 min at 37 °C significantly increases their susceptibility to agglutination by low doses of concanavalin A (Table I).

The effect of different dibucaine concentrations and incubation times on the susceptibility of 3T3 cells to concanavalin A agglutination is shown in Fig. 1. Exposure of 3T3 cells to dibucaine ($1 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M) for as little as 10 min at 37 °C increased their susceptibility to agglutination by concanavalin A, though more marked changes in agglutinability required longer incubation times (Fig. 1). Dibucaine con-

TABLE I

THE EFFECT OF DIBUCAINE ON CELL AGGLUTINATION BY CONCANAVALIN A[a]

Treatment	Concentration of concanavalin A (μg/ml) for maximum cell agglutination[b]						
	Untransformed cells		Transformed cells			Revertant cells	
	3T3	BHK	SV3T3	PY-BHK	RSV-BHK	R-SV3T3-10	R-SV3T3-14
Untreated control	1400	850	50	80	30	1400	1200
Dibucaine ($1 \cdot 10^{-4}$ M)[c]	350	100	50	80	30	350	350
Dibucaine[c, d] ($1 \cdot 10^{-4}$ M) plus $CaCl_2$ (5 mM)	1350	850	50	N.D.	N.D.	N.D.	1200
Dibucaine[c, e] ($1 \cdot 10^{-4}$ M) plus $CaCl_2$ (10 mM)	1400	850	50	80	30	1200	1200

[a] Except where stated otherwise, the results represent mean values derived from six separate experiments.

[b] > 90 % cell agglutination by the stated concentration of concanavalin A after incubation at room temperature for 20 min. In any given experiment a minimum of three replicate cultures of the same cell type were tested to obtain a representative value of agglutinability.

[c] Cells were incubated in suspension in complete culture medium for 60 min at 37 °C and then assayed for agglutinability.

[d] Mean values derived from two separate experiments.

[e] Mean values derived from three separate experiments.

N.D. = not done.

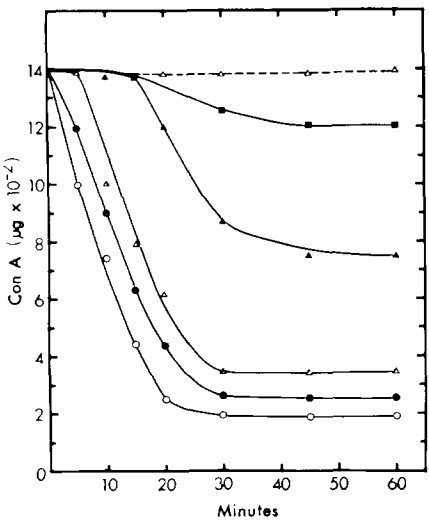

Fig. 1. The effect of dibucaine concentration and incubation time on the dose of concanavalin A required to induce maximum agglutination of 3T3 cells. Cells ($3 \cdot 10^6$ per ml) were incubated in suspension for the indicated times in serum-free medium supplemented with dibucaine HCl at the following concentrations: ■—■, $1 \cdot 10^{-5}$ M; ▲—▲, $5 \cdot 10^{-5}$ M; △—△, $1 \cdot 10^{-4}$ M; ●—●, $2 \cdot 10^{-4}$ M; and ○—○, $5 \cdot 10^{-5}$ M. Replicate control 3T3 cell cultures at the same density (△—△) were incubated in serum-free medium without dibucaine for the same periods.

centrations below $1 \cdot 10^{-5}$ M had little or no effect on cellular susceptibility to agglutination (Fig. 1). Incubation of 3T3 cells with dibucaine at concentrations greater than $5 \cdot 10^{-4}$ M for 15 min or longer produced progressive cell lysis.

The susceptibility of 3T3 cells to agglutination by concanavalin A was similarly enhanced by incubation with tetracaine ($5 \cdot 10^{-4}$ M) or procaine ($5 \cdot 10^{-3}$ M) (results not shown).

In contrast to the marked reduction in the dose of concanavalin A required to induce agglutination of dibucaine-treated untransformed cells, exposure of transformed cells to dibucaine did not alter their concanavalin A agglutination response (Table I).

Certain effects of local anesthetics on membranes are believed to result from their ability to displace membrane-bound Ca^{2+} associated with the anionic groups of acidic phospholipids [35, 36]. Moreover, the effects of local anesthetics on membranes can be reversed by increasing the Ca^{2+} concentration [35, 36]. Similarly, incubation of 3T3 cells in medium supplemented with dibucaine ($1 \cdot 10^{-4}$ M) plus additional Ca^{2+} (5 or 10 mM $CaCl_2$) effectively inhibited dibucaine-induced changes in cell agglutinability (Table I). The action of dibucaine in enhancing concanavalin A agglutination of untransformed cells was also rapidly reversed on returning cells to fresh medium without dibucaine.

The enhanced agglutination of dibucaine-treated cells by low doses of concanavalin A was accompanied by redistribution of concanavalin A receptors on the cell surface (Table II), similar to that described previously in concanavalin A-mediated agglutination of virus-transformed cells [12–15] and trypsinized untransformed cells [7, 8].

TABLE II

THE EFFECT OF DIBUCAINE ON CONCANAVALIN A BINDING, DISTRIBUTION OF SURFACE CONCANAVALIN A BINDING SITES AND AGGLUTINATION OF MOUSE 3T3 CELLS BY CONCANAVALIN A[a]

Treatment	Specific binding of [^{3}H]Con A (cpm per 10^7 cells)[b]	Cell agglutination by Con A[b]	Distribution of cell surface Con A binding sites[c]	
			uniform	patches
Untreated control	4635	0	−	−
Dibucaine ($1 \cdot 10^{-4}$ M)[d]	4387	+	+	+
Dibucaine ($1 \cdot 10^{-4}$ M) and glutaraldehyde prefixation[e]	4918	0	−	−
Dibucaine ($1 \cdot 10^{-4}$ M) plus $CaCl_2$ (10 mM)[d]	4717	0/+	+	−

[a] Mean values derived from four separate experiments.

[b] Binding of ^{3}H-labeled concanavalin A after 30 min at 0 °C (50 μg/ml) and cell agglutination by concanavalin A (500 μg/ml) at room temperature for 20 min measured as outlined in the methods.

[c] Determined by indirect immunofluorescence as described in the methods. + = > 75 % of cells examined show the specified pattern of specific immunofluorescence staining. The specificity of the staining reaction was confirmed by inhibition by 0.25 M α-methylmannoside and by omission of concanavalin A from the incubation mixture.

[d] Cells were incubated in suspension in serum-free medium for 30 min at 37 °C and then assayed for concanavalin A agglutinability, capacity to bind ^{3}H-labeled concanavalin A and distribution of concanavalin A binding sites.

[e] Cells incubated in dibucaine-supplemented culture medium for 30 min at 37 °C and then fixed with 2.5 % glutaraldehyde for 15 min at room temperature before testing for agglutinability, binding of ^{3}H-labeled concanavalin A and distribution of concanavalin A binding sites.

Fixation of dibucaine-treated cells with glutaraldehyde before addition of concanavalin A prevented the formation of patches of concanavalin A receptors (Table II) indicating that the receptors had been fixed in a dispersed distribution not involving large patches. Glutaraldehyde prefixation also inhibited the agglutination of dibucaine-treated cells (Table II). These results suggest that dibucaine per se does not induce redistribution of concanavalin A receptors to form patches but facilitates redistribution of the receptors once concanavalin A has bound to the cell surface.

Measurements of the binding of ^{3}H-labeled concanavalin A revealed that increased agglutinability of dibucaine-treated cells does not result from changes in the number of concanavalin A receptors exposed on the cell surface (Table II).

The effect of local anesthetics on membrane structure

Ultrastructural observations on the surface architecture of freeze-fractured 3T3 cells reveals a random distribution of intramembranous particles, the majority of which are single (Fig. 2A and Table III). However, in 3T3 cells incubated with

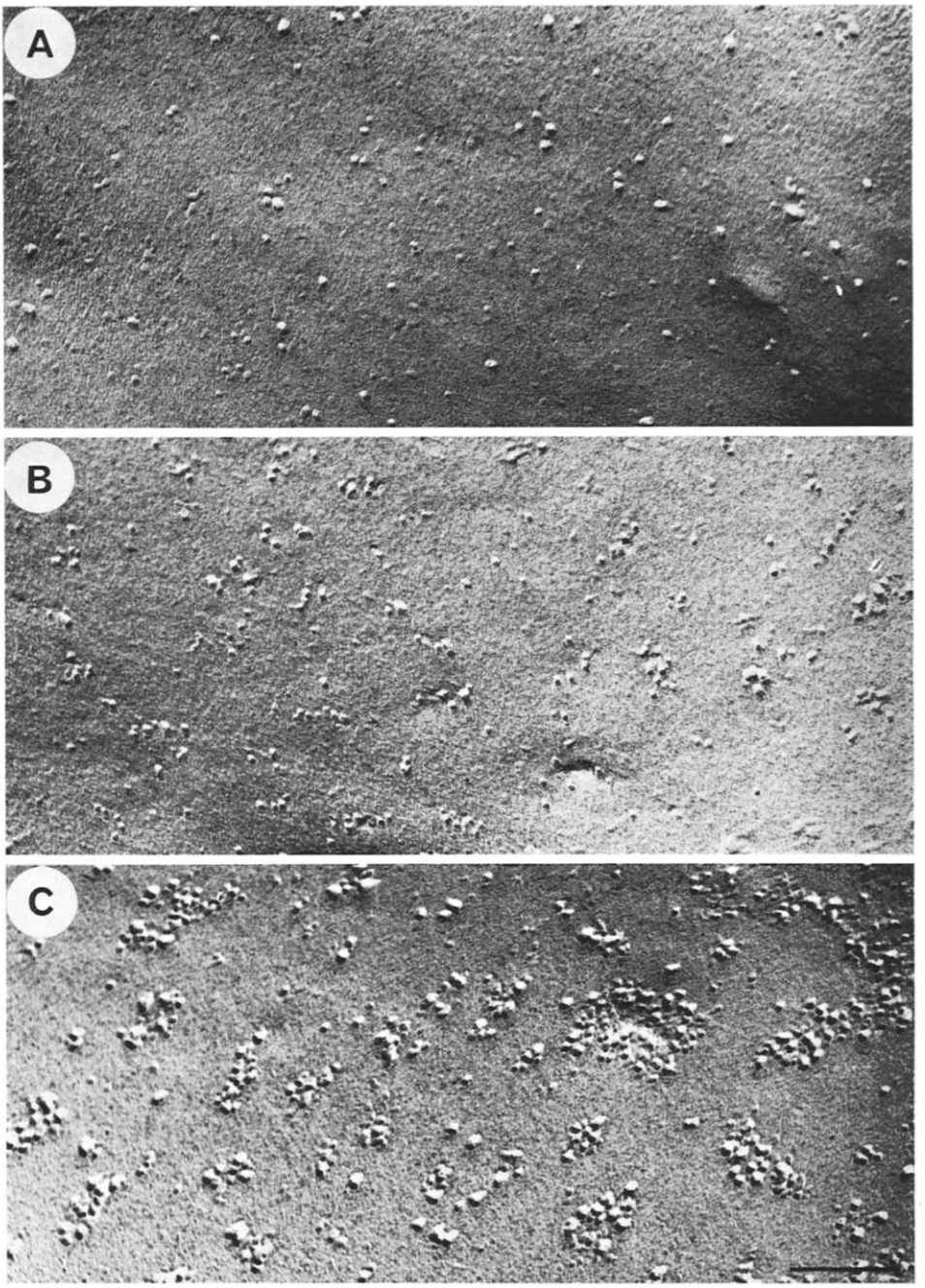

Fig. 2. Freeze-fracture electron micrographs of the surface of mouse 3T3 cells (A face). Bar = 0.1 μm. (A) Area of untreated control 3T3 cell surface showing predominant monogranular distribution of intramembranous particles. (B) Area of 3T3 cell surface after incubation for 1 h at 37 °C in serum-free medium supplemented with $1 \cdot 10^{-4}$ M dibucaine showing increased clustering of intramembranous particles. (C) Area of 3T3 cell surface after incubation for 1 h at 37 °C in serum-free medium supplemented with $1 \cdot 10^{-4}$ M dibucaine followed by incubation with concanavalin A (500 μg/ml) for 15 min at 37 °C showed marked clustering of intramembranous particles to form numerous large patches.

TABLE III

THE EFFECT OF DIBUCAINE AND CONCANAVALIN A ON THE DENSITY AND DISTRIBUTION OF INTRAMEMBRANOUS PARTICLES ON FREEZE-FRACTURED 3T3 CELL PLASMA MEMBRANES

Treatment	Number of intramembranous particles per μm^2 [a, b]	Single[c]	Clusters[c] < 5 particles	Clusters[c] > 5 particles
Untreated control	510 ± 53	47.2	38.9	13.9
Dibucaine[d]	491 ± 42	23.5	47.1	29.4
Dibucaine + Con A[e]	527 ± 65	22.6	32.2	45.2

[a] Mean values derived from counts of intramembranous particles in 0.1 μm^2 areas on electron-photomicrographs of the A fracture face of 3T3 cells at a magnification of 100 000 and counts on at least 15 individual cells.

[b] Particle dimensions were 85 ± 5Å (standard error of the mean) irrespective of whether the patches were distributed singly or within clusters.

[c] Percentage of the total number of intramembranous particles.

[d] $1 \cdot 10^{-4}$ M dibucaine for 1 h at 37 °C.

[e] $1 \cdot 10^{-4}$ M dibucaine for 1 h at 37 °C followed by addition of 500 μg/ml concanavalin A and further incubation at 37 °C for 15 min.

$1 \cdot 10^{-4}$ M dibucaine for 60 min at 37 °C the particle distribution is altered, and numerous small clusters of particles are present (Fig. 2B and Table III). In cells incubated for similar periods with dibucaine and then exposed to concanavalin A (500 μg/ml) for 10 min at 37 °C this redistribution of particles is even more marked (Fig. 2C) and more than 75 % of the particles are clustered (Table III).

These results indicate that dibucaine induces a change in the structural organization of the plasma membrane of 3T3 cells which permits spontaneous redistribution of intramembranous particles into small clusters (Fig. 2B). Extensive redistribution of intramembranous particles to form large clusters is seen only after binding of multivalent concanavalin A molecules to the cell surface (Fig. 2C). These results are in full agreement with the immunofluorescence data presented above.

Work in other laboratories [38-40] has demonstrated that the distribution of lectin receptors on the outer face of the plasma membrane corresponds closely to the distribution of intramembranous particles revealed within the hydrophobic core of the membrane by freeze-fracture. Thus, the marked clustering of intramembranous particles on dibucaine-treated cells induced by concanavalin A shown in Fig. 2C probably corresponds to the "patches" of specific concanavalin A immunofluorescence detected at the light microscopic level on dibucaine-treated cells exposed to concanavalin A (Table II).

Fluorescence polarization measurements on cells treated with local anesthetics: effect of anesthetics on membrane fluidity

Measurements on the behavior of the fluorescence probe diphenylhexatriene (DPH) in dibucaine-treated 3T3 cells and untreated control 3T3 cells are summarized in Table IV. The results indicate that incubation of cells with dibucaine for 1 h at 37 °C caused little change in the absolute values of DPH polarization compared with

TABLE IV

THE EFFECT OF DIBUCAINE ON DIPHENYLHEXATRIENE ROTATION IN 3T3 CELL MEMBRANES AT 25 °C[a]

Treatment	Concentration (M)	Polarization	Diphenylhexatriene	
			Lifetime[b] (ns)	Rotation rate[c] (MHz)
Untreated control	—	0.239	9.0	21
Dibucaine	$1 \cdot 10^{-4}$	0.238	8.0	23
Dibucaine	$2 \cdot 10^{-4}$	0.238	7.3	26

[a] Mean values derived from at least two separate experiments for each treatment. Maximum deviation from the mean did not exceed 4.5 % for any of the stated values within each experimental group.

[b] Apparent lifetime was calculated on the basis that all quenching shortens the lifetime by an amount proportional to the intensity loss. The lifetime for diphenylhexatriene embedded in a membrane at 25 °C was estimated as 9.0 ns using the data of Shinitzky and Barenholz [68].

[c] Calculated by methods described in the accompanying paper [23].

that in untreated control cells. However, a substantial quenching effect was observed in dibucaine-treated samples. If this quenching is assumed to be entirely dynamic, as documented for DPH in dibucaine-treated lipid vesicles [23], then the "apparent" lifetime of the DPH probe is actually decreased in dibucaine-treated cells (Table IV) and the probe rotation rate is increased (Table IV). These results are therefore consistent with the interpretation that dibucaine increases the fluidity of areas within the lipid bilayer monitored by the DPH probe.

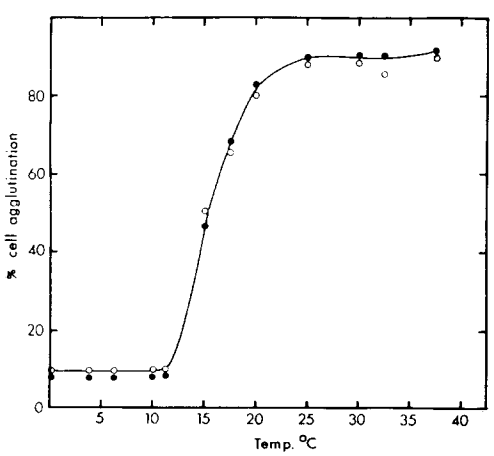

Fig. 3. The effect of temperature on agglutination of 3T3 cells by concanavalin A. 3T3 cells (○ – ○) and 3T3 cells pretreated with $2 \cdot 10^{-4}$ M dibucaine for 20 min at 37 °C (● – ●) were incubated at the indicated temperatures for 20 min with 1400 and 350 μg/ml concanavalin A, respectively. The proportion of the cell population agglutinated by concanavalin A was then determined at the same temperature.

The effect of local anesthetics on the temperature-dependence of cell agglutination by concanavalin A

Measurements of the temperature-dependence of concanavalin A-mediated agglutination of dibucaine-treated 3T3 cells indicate that despite a significant increase in their agglutinability, the temperature-dependence of the agglutination reaction is identical to that of untreated control cells (Fig. 3). The general features of the temperature-dependence of the concanavalin A agglutination response shown in Fig. 3 agree with previous observations on mouse LM [42] and 3T3 cells [43].

Effect of local anesthetics on the topographical control of concanavalin A binding sites on the cell surface

Reports from several laboratories indicate that the mobility of concanavalin A receptors during formation of "patches" or "caps" of receptors on the cell surface is regulated by peripheral membrane proteins associated with the inner face of the plasma membrane [44–52]. Treatment of cells with colchicine and other alkaloids such as vincristine and vinblastine has been shown to inhibit spontaneous agglutination of cells by concanavalin A [49, 53]. These alkaloids also influence the ability of multivalent ligands to induce redistribution of cell surface receptors to form caps [46–48, 54–56]. These observations have prompted the suggestion that the mobility of concanavalin A receptors is regulated by membrane-associated "colchicine-sensitive" proteins that probably correspond to microtubules.

In agreement with these previous findings, incubation of SV3T3 cells with colchicine for 60 min at 37 °C inhibited their subsequent agglutination by concanavalin A (Table V). Similar inhibition of spontaneous agglutination SV3T3 cells by concanavalin A was produced by preincubation with the *Vinca* alkaloid, vinblastine (Table V). However, lumicolchicine, a structural isomer of colchicine that does not bind to microtubule proteins [34], had no effect on concanavalin A agglutination of SV3T3 cells at concentrations comparable to those used with colchicine (Table V).

In contrast, the increased concanavalin A agglutination response displayed by

TABLE V

THE EFFECT OF COLCHICINE, VINBLASTINE AND LUMICOLCHICINE ON THE AGGLUTINATION OF SV3T3 CELLS BY CONCANAVALIN A[a]

Treatment	Concentration (M)	Cell agglutination by Con A[b]	Specific binding of [^{3}H]Con A (cpm per 10^7 cells)[b]
Untreated control	—	++++	4933
Colchicine[c]	10^{-6}	+	4495
Vinblastine[c]	10^{-5}	−	4860
Lumicolchicine[c]	10^{-6}	++++	4736

[a] Mean values from three separate experiments.

[b] Binding of ^{3}H-labeled concanavalin A (50 μg/ml) at 0 °C and cell agglutination by concanavalin A (150 μg/ml) at room temperature were measured as described in the methods.

[c] Cells were incubated in serum-free culture medium supplemented with the listed compounds for 1 h at 37 °C and then washed three times in phosphate-buffered saline before final incubation with concanavalin A to determine agglutinability.

TABLE VI

THE EFFECT OF COLCHICINE, VINBLASTINE AND LUMICOLCHICINE ON CONCANAVALIN A AGGLUTINATION OF 3T3 AND SV3T3 CELLS PRETREATED WITH DIBUCAINE[a]

Treatment	Cell agglutination by Con A		Specific binding of [^{3}H]Con A (cpm per 10^7 cells)[d]	
	3T3[b]	SV3T3[c]	3T3	SV3T3
Untreated control	0	++++	4664	4873
Dibucaine ($2 \cdot 10^{-4}$ M)[e]	++++	++++	4795	4581
Dibucaine ($2 \cdot 10^{-4}$ M) and colchicine ($1 \cdot 10^{-6}$ M)[f]	++++	+++±	4416	4825
Dibucaine ($2 \cdot 10^{-4}$ M) and vinblastine ($1 \cdot 10^{-5}$ M)[f]	++++	+++−	4907	4811
Dibucaine ($2 \cdot 10^{-4}$ M) and lumicolchicine ($1 \cdot 10^{-6}$ M)[f]	++++	+++±	4772	4938
Dibucaine ($2 \cdot 10^{-4}$ M) plus colchicine ($1 \cdot 10^{-6}$ M) and vinblastine ($1 \cdot 10^{-5}$ M)[f]	++++	+++−	4964	4588

[a] Mean values derived from three separate experiments.
[b] Agglutination by 350 μg/ml concanavalin A at room temperature after 20 min.
[c] Agglutination by 150 μg/ml concanavalin A at room temperature after 20 min.
[d] Binding of ^{3}H-labeled concanavalin A (50 μg/ml) after incubation for 30 min at 0 °C.
[e] Incubated with dibucaine for 30 min at 37 °C in serum-free medium.
[f] Incubated with dibucaine for 30 min at 37 °C in serum-free medium followed by incubation in fresh serum-free medium supplemented with the listed alkaloids and dibucaine for 1 h at 37 °C.

dibucaine-treated 3T3 cells was not inhibited by either colchicine or vinblastine (Table VI). Similarly, incubation of transformed SV3T3 cells with dibucaine before treatment with colchicine or vinblastine prevented the inhibitory effect of these alkaloids on subsequent cell agglutination by concanavalin A (Table VI). The failure of colchicine to inhibit concanavalin A agglutination of dibucaine-treated 3T3 and SV3T3 cells did not result, however, from anesthetic-induced alterations in the binding of [^{3}H]colchicine to cells (not shown). Combined treatment of cells with colchicine and vinblastine after initial incubation with dibucaine also failed to alter either cell agglutination or binding of ^{3}H-labeled concanavalin A (Table VI).

These results indicate that dibucaine alters the responsiveness of concanavalin A-mediated cell agglutination to microtubular disruptive drugs. The unresponsiveness of dibucaine-treated cells to these drugs suggests that dibucaine alters the functional state of the "colchicine-sensitive" peripheral proteins associated with the plasma membrane which have been proposed to act as "anchors" in restricting the movement of intrinsic membrane proteins carrying lectin receptors (see refs 46, 50, 51). Thus, colchicine and dibucaine may both be acting on a common target protein, though not necessarily via the same mechanism. If, however, both colchicine and dibucaine are considered as altering the activity of peripheral membrane proteins that normally restrict lectin receptor mobility, then it becomes necessary to explain why colchicine

inhibits cell agglutination by concanavalin A while dibucaine enhances this process.

Ultrastructural observations [51] have shown that treatment of SV3T3 cells with colchicine does not alter the random distribution of concanavalin A binding sites on the cell surface. However, exposure of colchicine-treated cells to concanavalin A results in the formation of a single massive cap-like aggregate of concanavalin A receptors in the center of the cell. As Ukena et al. [51] pointed out, this distribution would substantially reduce the probability of cell-to-cell contact in areas containing concanavalin A receptors, and thus limit the opportunity for linkage between clustered concanavalin A receptors on adjacent cells required for agglutination.

The formation of single large caps of concanavalin A receptors [56] and other cell surface receptors [54, 56] appears to involve microfilaments, since capping is inhibited by the drug cytochalasin B which specifically disrupts microfilaments. Since cells treated with colchicine and concanavalin A display a single cap of receptors, it is not unreasonable to suggest that the microfilament system is still functional in these cells. In contrast, the lack of capping of concanavalin A receptors on dibucaine-treated cells exposed to concanavalin A suggests that dibucaine may inhibit not only colchicine-sensitive peripheral membrane elements but also cytochalasin-sensitive microfilaments. Thus treatment of cells with dibucaine by impairing the colchicine-sensitive peripheral proteins that act as "anchors" for concanavalin A receptors would facilitate concanavalin A-induced redistribution of its receptors, while the simultaneous action of dibucaine to inhibit contractile microfilaments would prevent redistribution of the receptors into a single cap.

If dibucaine inhibits both colchicine-sensitive and cytochalasin-sensitive peripheral proteins then it should be possible to duplicate the action of dibucaine by exposing cells simultaneously to cytochalasin B and colchicine.

TABLE VII

THE EFFECT OF COLCHICINE AND CYTOCHALASIN B ON AGGLUTINATION OF 3T3 CELLS BY CONCANAVALIN A

Treatment	Concentration	Concentration of Con A (μg/ml) for maximum cell agglutination[a]
1. Untreated control		1400
2. Cytochalasin B[b]	5 μg/ml	1400
3. Cytochalasin B[b]	20 μg/ml	1400
4. Colchicine plus cytochalasin B[c]	$1 \cdot 10^{-6}$ M 5 μg/ml	475
5. Colchicine plus cytochalasin B plus dibucaine[d]	$1 \cdot 10^{-6}$ M 5 μg/ml $2 \cdot 10^{-4}$ M	350

[a] $>$ 90 % cell agglutination by the stated concentration of concanavalin A after incubation at room temperature for 20 min.

[b] 1 h at 37 °C.

[c] 1 h at 37 °C.

[d] Cells incubated for 1 h at 37 °C in medium containing colchicine and cytochalasin after which dibucaine containing serum-free medium was added and the sample incubated for a further 30 min at 37 °C.

As shown in Table VII, incubation of 3T3 cells with both colchicine and cytochalasin B enhances their susceptibility to agglutination by concanavalin A without altering the capacity of cells to bind ^{3}H-labeled concanavalin A.

Although the increase in concanavalin A agglutinability in cells treated with both colchicine and cytochalasin B, is not as great as that caused by dibucaine alone (Table I), further experiments in which 3T3 cells were incubated in medium containing colchicine and cytochalasin B and then with dibucaine before final exposure to concanavalin A revealed that dibucaine produced an additional small increase in cellular agglutinability (Table VII). The effect of dibucaine was not additive, however, suggesting that its primary action in enhancing cell agglutination probably results from its action on microtubules and microfilaments. The additional increase in cell agglutinability found in cells treated with colchicine, cytochalasin B and dibucaine compared with just colchicine and cytochalasin B might result from the additional action of dibucaine in increasing the fluidity of membrane lipids as discussed earlier.

Further support for the conclusion that dibucaine acts on cytochalasin-sensitive microfilaments, as well as on colchicine-sensitive proteins, was provided by the finding that treatment of SV3T3 cells with dibucaine reversed the inhibition of concanavalin A agglutination by colchicine (Table VIII). A similar reversal of colchicine-induced inhibition of agglutination was produced by cytochalasin B (Table VIII).

TABLE VIII

EFFECT OF DIBUCAINE AND CYTOCHALASIN B ON COLCHICINE-INDUCED INHIBITION OF CONCANAVALIN A AGGLUTINATION OF SV3T3 CELLS

Treatment	Concentration	Cell agglutination by Con A[a]
Untreated control	—	+ + + +
Colchicine[b]	$1 \cdot 10^{-6}$ M	−
Colchicine plus dibucaine[c]	$1 \cdot 10^{-6}$ M $2 \cdot 10^{-4}$ M	− + − +
Colchicine plus cytochalasin B[d]	$1 \cdot 10^{-6}$ M 5 μg/ml	+ − + −

[a] Agglutination by 150 μg/ml concanavalin A at room temperature after 20 min.

[b] 1 h at 37 °C.

[c] Cells incubated in colchicine-containing medium for 1 h at 37 °C followed by incubation in dibucaine-containing medium for 30 min at 37 °C.

[d] Cells incubated in colchicine-containing medium for 1 h at 37 °C followed by incubation for 1 h at 37 °C in medium supplemented with cytochalasin B.

DISCUSSION

Effect of dibucaine on membrane fluidity

The present experiments have shown that treatment of untransformed cells with tertiary amine local anesthetics enhances their susceptibility to agglutination by concanavalin A. This agglutination of anesthetic-treated cells is accompanied by redistribution of lectin receptors on the cell surface similar to that described previously in concanavalin A-mediated agglutination of cells infected with oncogenic [1, 2, 7, 14]

and nononcogenic viruses [8, 27] and in untransformed cells after brief trypsinization [1, 2, 7, 8]. Immunofluorescence and freeze-fracture data described here indicates that anesthetics per se do not induce extensive clustering of lectin receptors into large patches. Instead, these drugs modify the plasma membrane so that the formation of patches of receptors is facilitated following binding of multivalent concanavalin A molecules to the cell surface. Further evidence that anesthetics do not directly induce patches of concanavalin A receptors is provided by the finding that glutaraldehyde fixation of anesthetic-treated cells before exposure to concanavalin A inhibited subsequent agglutination and the clustering of concanavalin A receptors indicating that the receptors had been fixed in a distribution not involving large patches.

Dibucaine does, however, induce small clusters of intramembranous particles (Fig. 2B). This may reflect increased random movement of the particles as a result of dibucaine-induced alterations in general membrane fluidity (vide infra). This situation may be analogous to the formation of particle clusters of similar size in lymphocytes incubated with 25–50 % glycerol for 2 h [58].

As outlined in the introduction, several investigators have proposed [16–22] that the higher susceptibility of transformed cells to concanavalin A agglutination compared with untransformed cells results from a more "fluid" lipid matrix in the plasma membrane of these cells which facilitates lateral mobility of concanavalin A receptors and their redistribution to form patches after binding of concanavalin A to the cell surface. However, definitive evidence to support this proposal is still lacking.

The present fluorescence polarization measurements indicate that dibucaine causes a small but significant increase in the fluidity of the lipid environment in the vicinity of the diphenylhexatriene probe. It is not known, however, whether this consists of a small change in the "bulk" lipid matrix of membranes or reflects a much larger fluidity change occurring in specific regions of the membrane. At dibucaine concentrations ($1 \cdot 10^{-4}$–$5 \cdot 10^{-4}$ M) that induce alterations in cellular agglutination our studies on model membranes reported in the preceding paper [23] have shown that the "fluidizing" effect is confined to acidic phospholipids. This effect was even more marked in acidic phospholipid membranes that had been previously stabilized by Ca^{2+} [23], indicating that an increase in fluidity would also result from displacement of membrane-bound Ca^{2+} by dibucaine. It is perhaps reasonable to suggest therefore that in intact cells dibucaine ($< 5 \cdot 10^{-4}$ M) could induce changes in the fluidity of domains of acidic phospholipids within the plasma membrane without significant change in the fluidity of the bulk membrane lipids. Although such specific domains have yet to be formally demonstrated in natural membranes, experiments with mixed lipid bilayers indicate that Ca^{2+} induces segregation of acidic and neutral phospholipids into separate domains [29, 59].

Effect of dibucaine on peripheral membrane proteins

The change in membrane fluidity induced by dibucaine in intact cells is not sufficient, however, to alter the temperature-dependence of the concanavalin A agglutination reaction (Fig. 3). This observation assumes importance in view of recent work by Horwitz et al. [43] which has shown that incorporation of unsaturated fatty acids in the membranes of cultured cells, which would be expected to increase the fluidity of the lipid membrane matrix, produced a significant reduction in the temperature at which concanavalin A agglutination began but the dose of concanavalin A

required to produce cell agglutination was unchanged. This suggests that the striking enhancement of cell agglutination and reduction in the dose of concanavalin A required to achieve agglutination in dibucaine-treated cells may not result primarily from changes in fluidity of the bulk membrane lipid. Instead, the effect of dibucaine may reflect a combined action on both the fluidity of specific membrane lipid domains and on other membrane components that influence the function of concanavalin A receptors. For example, the present work indicates that dibucaine affects the functional properties of peripheral membrane proteins involved in regulating the topography of lectin receptors on the cell surface.

The present experiments have confirmed that colchicine and Vinca alkaloids inhibit concanavalin A agglutination of transformed cells. In contrast, concanavalin A-mediated agglutination of dibucaine-treated untransformed cells is not sensitive to these alkaloids. We interpret this as indicating that dibucaine causes functional dislocation of the colchicine-sensitive proteins associated with the plasma membrane. If, as suggested [46, 50], these colchicine sensitive elements act as "anchors" to restrict concanavalin A receptor movement, then functional dislocation of these elements by dibucaine should enhance receptor mobility and favor redistribution of concanavalin A receptors after binding of concanavalin A molecules to the cell surface. The present results are consistent with this interpretation.

Based on this scheme, inactivation of these peripheral proteins by colchicine or vinblastine, though not necessarily by the same mechanism as dibucaine, would also be expected to enhance concanavalin A receptor mobility and cellular susceptibility to concanavalin A agglutination. However, as reported previously [49, 51, 53], and confirmed here, colchicine inhibits concanavalin A agglutination. An explanation for this apparent paradox can be offered using the observation by Ukena et al. [51] that colchicine treatment does enhance concanavalin A receptor redistribution but, unlike dibucaine-treated cells in which concanavalin A produces several large patches of concanavalin A receptors, concanavalin A induces a single large "cap-like" aggregate of lectin receptors. This latter distribution would substantially reduce the possibility of contact between cells in areas containing concanavalin A receptors necessary to initiate the agglutination. Distribution of receptors into numerous large patches distributed over the entire cell surface, as in dibucaine-treated cells, would be much more efficient in promoting agglutination.

The formation of caps of lectin binding sites [56] and other cell surface receptors [54, 56] has been shown to involve structural elements sensitive to cytochalasin B, a drug that is believed to disrupt microfilaments [61]. Microfilaments are not affected by either colchicine or the *Vinca* alkaloids. Thus, colchicine treatment would increase the mobility of concanavalin A receptors by inhibiting the "anchor" function of colchicine-sensitive peripheral proteins but the resulting "unanchored" receptors may still be linked to a functional microfilament system. The latter, in the absence of the opposing anchor function, would be dominant and redistribute lectin receptors into a single large cap. Evidence that microfilaments possess acto-myosin components and can function as a contractile system has been reviewed by Allison [62].

We therefore envisage the colchicine-sensitive elements as playing a role as a skeletal element anchoring concanavalin A receptors, while cytochalasin B-sensitive microfilaments act as an opposing contractile system. The topography of concanavalin A receptors at any time would reflect the interplay between these two systems.

If this proposal is correct then the present finding that the concanavalin A receptors on dibucaine-treated cells are redistributed to form large patches, rather than a single cap, can be interpreted as indicating that dibucaine produces functional alteration in both the colchicine-sensitive and the cytochalasin B-sensitive systems of peripheral proteins. Strong support for this proposal is provided by the present observation that combined treatment of untransformed 3T3 cells with colchicine and cytochalasin B mimiced the effect of dibucaine by enhancing cellular susceptibility to

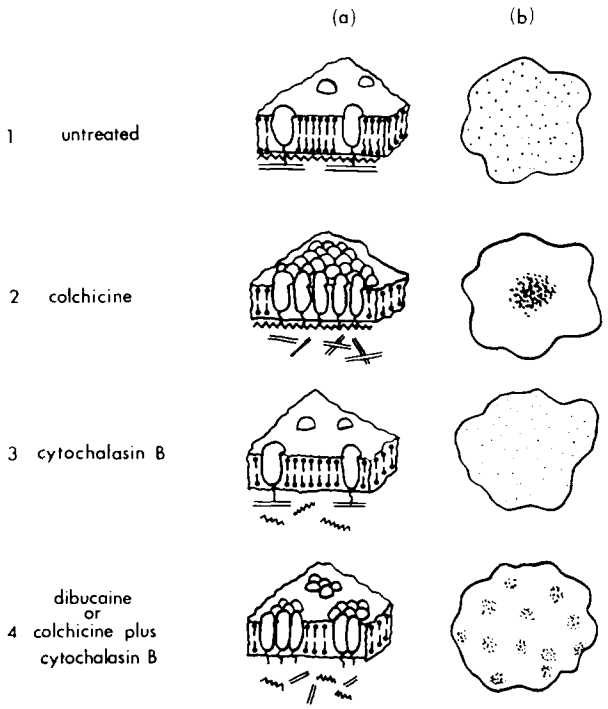

Fig. 4. Schematic representation of the effect of colchicine, cytochalasin B and dibucaine hydrochloride on the distribution of concanavalin A receptors on untransformed mouse 3T3 cells. (a) segment of plasma membrane showing intrinsic membrane proteins carrying concanavalin A receptors embedded within a lipid bilayer and linked on the inner membrane face to microtubules (=) and microfilaments (-WW-). (b) representation of the planar view of the distribution of concanavalin A receptors over the upper surface of the entire cell. (1) Untreated 3T3 cells: (a) the intrinsic proteins are linked to both microtubules and microfilaments; and (b) concanavalin A receptors remain randomly dispersed after binding of concanavalin A to the cell surface. (2) 3T3 cells treated with colchicine: (a) disruption of microtubules by colchicine allows concanavalin A induced aggregation of intrinsic proteins which are still linked to an intact microfilament system resulting in redistribution of concanavalin A receptors into a single large "cap" as shown in (b). (3) 3T3 cells treated with cytochalasin B. (a) despite disruption of microfilaments by cytochalasin B, intrinsic membrane proteins remain "anchored" by their connection to microtubules and binding of concanavalin A to the cell surface does not induce redistribution of concanavalin A receptors which remain randomly dispersed as shown in (b). (4) 3T3 cells after treatment with dibucaine or combined treatment with colchicine and cytochalasin B. (a) disruption of the linkage of intrinsic membrane proteins to both microtubules and microfilaments allows concanavalin A-mediated redistribution of concanavalin A receptors to form multiple "patches" distributed over the entire cell as shown in (b).

agglutination. The ability of dibucaine to reverse colchicine-induced inhibition of agglutination of transformed cells also lends support to the general hypothesis that local anesthetics impair both microtubule and microfilament activities. Finally, the recent observation that local anesthetics inhibit antibody-induced capping of surface IgG molecules on the surface of lymphocytes [63] further suggests that local anesthetics can impair microfilaments.

The essential features of the hypothesis formulated above are summarized in Fig. 4. The proposal that cytochalasin-sensitive and colchicine-sensitive peripheral membrane proteins act as opposing influences in regulating the distribution of cell surface receptors has not been advanced before but parts of the present hypothesis have been derived from the scheme proposed previously by Edelman [46] and Berlin [50].

Ca^{2+} and membrane organization

The nature of the linkage between membrane proteins carrying lectin receptors and peripheral membrane proteins is still unclear. The action of dibucaine in altering the linkage between these membrane components could result from several non-exclusive mechanisms.

Firstly, the ability of local anesthetics to displace membrane-bound Ca^{2+} [35, 36] could induce changes in the organization of peripheral proteins involved in regulating the distribution of cell surface receptors. For example, the peripheral membrane protein spectrin which has been shown to be involved in the trans-membrane control of lectin receptors in erythrocytes [44, 45] is released from membranes by Ca^{2+}-chelating agents [64], implicating a Ca^{2+}-bridge mechanism. In addition, spectrin interacts with acidic phospholipids at pH 7.4 only in the presence of Ca^{2+} [65]. By competing for such Ca^{2+}-binding sites, dibucaine and other local anesthetics could perturb the functional linkage between intrinsic membrane components and spectrin or analogous peripheral proteins.

Another aspect of the displacement of Ca^{2+} from membranes by anesthetics that might affect the mobility of lectin receptors concerns the overall effect of Ca^{2+} on the organization of the lipids in membranes. Studies with model membranes have shown that Ca^{2+} are important in influencing the overall packing and motional freedom of phospholipids. The binding of Ca^{2+} to phospholipids creates a more stable bilayer structure and may promote segregation of different phospholipid species into domains (vide supra). If similar domains exist in natural membranes they could provide an organizational framework for the distribution of specific membrane components, notably proteins. The displacement of Ca^{2+} from membranes could initiate dispersal of such rigid domains and introduce opportunities for more random intermixing of membrane components, a process that would no doubt be accelerated by specific binding of multivalent ligands such as lectins to the cell surface.

Local anesthetics may also induce direct breakdown of microfilaments and microtubules and/or interfere with the polymerization of subunits to inhibit assembly of these structures. Electronmicroscopic evidence that local anesthetics reduce the number of microtubules in tissues has been reported [66, 67] and lidocaine has been shown to produce dose-dependent inhibition of microtubule repolymerization in vitro [67].

A possible unifying mechanism, involving both of the actions of dibucaine

listed above, is that the peripheral proteins controlling lectin receptor mobility are linked by Ca^{2+} to rigid domains of acidic phospholipids which surround the receptor proteins. Displacement of Ca^{2+} from membranes by dibucaine would then lead to dislocation of the peripheral proteins, dispersal of rigid lipid domains and greater translational mobility for the receptors. The function of the peripheral proteins might be further impaired by direct effects of anesthetics on their structural integrity.

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