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EFFECTS OF LOCAL ANAESTHETICS ON INTRACELLULAR FUSION PROCESSES

ENHANCEMENT OF CONCANAVALIN A-INDUCED MACROPHAGE VACUOLATION

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SUMMARY

The extensive vacuolation elecited in mouse peritoneal macrophages in response to interaction with concanavalin A is markedly enhanced by a simultaneous exposure to anaesthetics. The potency of enhancing vacuolation increases within the series of normal alcohols with chain length $C_{10} > C_8 > C_7 > C_6$. From the four tertiary amine local anaesthetics tested lidocaine and procaine are by far more effective than tetracaine and dibucaine. The latter two induce extensive cell shrinkage at concentrations at which the first two exhibit optimum enhancing capacity. Of the tested compounds chlorpromazine has the highest membrane/buffer partition coefficient and it exhibits its optimum enhancing effect on concanavalin A-induced macrophage vacuolation at the lowest drug concentration.

The binding of [^{3}H] concanavalin A as well as its internalization by macrophages incubated with the lectin for 15, 45 and 90 min are not affected significantly in the presence of decanol, procaine or chlorpromazine at concentrations of maximum enhancing effect on vacuolation. Thus enhancement of vacuolation does not stem from an increase in the rate or extent of concanavalin A interiorization. The rate at which vacuoles are generated is however markedly increased in the presence of chlorpromazine and the resulting vacuoles are of a larger diameter. At 2–5 fold the concentration required for inhibition of maximum enhancing effect, the drugs lead to extensive macrophage shrinkage and to depletion of intracellular ATP.

Phagocytosis of heat-killed yeast cells is reduced by tertiary amine anaesthetics at concentrations optimal for enhancement of concanavalin A-induced vacuolation.

Enhanced intracellular fusion of concanavalin A-bearing pinosomes to form vacuoles is discussed in terms of current ideas on factors affecting membrane fusion and the effects of anaesthetics on membrane organization of lipids, intramembraneous particles, glycoprotein receptors and the possible control by cytoskeletal elements. The results best fit the hypothesis that enhanced fusion correlates with membrane aggregation of both intramembraneous particles and concanavalin A receptor and the formation of areas relatively deplete of these structures and enriched in phospholipids.

INTRODUCTION

A wide variety of lectins with distinctly different structures and saccharide binding specificities have been shown to bind to randomly distributed membrane glycoproteins and bring about their redistribution to clusters and caps and to induce their interiorization via pinocytosis [1–4]. Macrophages respond to multivalent lectins ($\geqq 4$ sugar binding sites [5]) in a characteristic pattern hitherto unencountered in any other cell type. Upon interaction with concanavalin A the following sequence of events is being observed: (a) surface binding of the lectin and redistribution to form clusters of concanavalin A-glycoprotein conjugates; (b) an energy and temperature-dependent formation of numerous minute concanavalin A-bearing pinocytic vesicles; (c) intracellular fusion of vesicles to form vacuoles of medium (2–5 μm diameter) and large (> 5 μm diameter) size occupying most of macrophage cytoplasm and (d) vacuole disappearance in a 48 h period and regeneration of non-vacuolated morphology [6–8].

Involvent of the cytoskeleton in regulation of surface distribution of concanavalin A receptors and intracellular fusion processes is suggested from studies concerning the effect of cytochalasin B and colchicine [9] on concanavalin A-induced macrophage vacuolation cytochalasin B. which interferes with microfilament function (cytochalasin B-sensitive microfilaments) and stops cell mobility and ruffled membrane formation in macrophages inhibits concavalin A-induced vacuolation, and leads to formation of big clumps of fluorescent labelled concanavaline A on the surface. Colchicine exhibits the opposite effect of cytochalasin B, i.e. vacuolation is more pronounced and 4–6 fold the number of large size vacuoles dominate the cytoplasm.

Control of rates of concanavalin A-bearing pinosomes coalescence via surface membrane fluidity was suggested by the observation that hydrocortisone inhibits concanavalin A-induced vacuolation though neither binding of [^{3}H] concanavalin A nor its internalization are affected [10]. Previous studies in our laboratory thus seem to indicate a close correlation between the surface disposition and mobility of membrane components and subsequent intracellular membrane fusion events.

Local anaesthetics, tranquillizers and alcohols have diverse effects on membrane phenomena [11–20]. Those that were most extensively characterized seen to evolve from the fact that these compounds are lipid soluble and when inserted into the phospholipid domain of model or biological membranes they exert a "fluidizing" or disordering effect. Tertiary amine local anaesthetics and certain tranquillizers have also been shown to be able to displace membrane Ca^{2+} and occupy the vacant sites [11, 12]. Inhibition of virus-mediated cell fusion and inhibition of fusion of secretory granules with the plasma membrane in a wide range of cells [11, 13, 14] is consistent with the suggestion that displacement of Ca^{2+} from fixed sites within the plasma membrane is an essential step in the fusion reaction [15], and that the occupation of these sites by local anaesthetics inhibits the interaction of the sites, a prerequisite for fusion. Enchanced neurosecretion by anaesthetics has, however, also been observed [11, 16] and attributed to the fluidizing effect of the drugs on both the secretory granule membrane and the plasma membrane.

Local anaesthetics have been shown to affect the susceptibility of mammalian cells to agglutination by plant lectins [17–20]. The studies suggest that the effects

stem from a fluidizing effect in the lipid domains of the membrane, an induction of clustering of plasma membrane intramembraneous particles as well as from interference with the transmembrane cytoskeletal control of mobility and distribution of cell surface receptors.

Work from our laboratory has previously shown that chlorpromazine [21] markedly enhances the rate and extent of intracellular fusion of concanavalin A-bearing pinosomes. In the following we present evidence for enhanced concanavalin A-induced macrophage vacuolation and for reduced macrophage non-immune phagocytic activity due to interaction with various anaesthetic compounds: alcohols, local anaesthetics and a phenothiazine type tranquillizer.

The differential effect exhibited by the type of drugs studied suggests that independent sites of action of the drugs may be involved and contribute synergistically to the enhanced fusion capacity.

MATERIALS AND METHODS

Media. Dulbecco's phosphate-buffered saline, Dulbecco's modified Eagle medium (medium) and heat-inactivated newborn calf serum (serum) were obtained from Grand Island Biological Co. (New York). Medium supplemented with 100 units/ml of penicillin and 100 μg/ml of streptomycin and phosphate-buffered saline were sterilized by Millipore filtration.

Aneasthetics and miscellaneous compounds. Chlorpromazine · HCl : Taro, Pharmaceutical Ind. Haifa, Israel; dibucaine · HCl: I.C.N. Pharmaceutical Ind. Plaineview, N.Y.; Tetracaine · HCl: Kupat Holim, Israel: lidocaine · HCl; Boots Co. Ltd., Nottingham, England. Procaine · HCl, dibutyryl-3',5'-cyclic AMP, dibutyryl-3',5'-cyclic GMP and ATP: Sigma Chemical Co., St. Louis, Mo *n*-Hexanol and *n*-heptanol: B.D.H., Poole, England; *n*-octanol: Fluka, A. G., Buchs, Switzerland; *n*-decanol: Merck, Darmstadt, Germany; theophylline: Mann Research Labs. Inc., New York.

Concanavalin A. Concanavalin A twice crystallized was obtained from Miles-Yeda (Rehovot, Isreal). Acetylation of the lectin with [^{3}H] acetic anhydride [22] yielded [^{3}H] concanavalin A with a specific activity of $6.5 \cdot 10^6$ cpm/mg. Fluorescein isothiocyanate -labelled lectin was obtained from Miles-Yeda (Rehovot, Israel).

Collection and cultivation of macrophages. Peritoneal macrophages were aseptically collected from BALB/C strain male mice, weighing 20-25 g, following essentially the method of Cohn and Benson [23]. Peritoneal exudate cells suspended in Dulbecco's modified Eagle medium were allowed to attach (1 h, 37 °C) on either 25-mm diameter Corning cover glasses (for light microscope observations) or on Falcon plastic tissue culture dishes (35 × 10 mm, Falcon Plastics Div., Bioquest, Oxnard, Calif.) (for analysis of [^{3}H] concanavalin A binding and internalization). Exudate cells ($0.5 \cdot 10^6$) were applied in 0.15 ml medium to cover glasses which were placed in 35 × 10 mm Falcon tissue culture dishes and in 0.6 ml medium directly on the tissue culture dish. After the phase of cell attachment the plates were thoroughly rinsed with phosphate-buffered saline to remove non-adhering cells. Macrophages were then cultivated for 48 h at 37 °C in 2 ml of tissue culture medium consisting of 20 % serum in Dulbecco's modified Eagle medium in, a CO_2 incubator (5 % CO_2/air mixture). Culture medium was changed once after 24 h of incubation.

Induction of macrophage vacuolation by concanavalin A treatment. Macrophage cultures were incubated for 90 min at 37 °C with 5 or 20 μg/ml of concanavalin A in medium in the presence or absence of the specified drugs. The treated cultures were washed twice in phosphate-buffered saline, fixed (2 % glutaraldehyde in phosphate-buffered saline, 30 min, 4 °C) and stained (May-Grünwald-Giemsa). When fluorescent concanavalin A was used cell monolayers on cover glasses, fixed in glutaraldehyde (as above) were inverted on a drop of glycerol 0.5 M sodium-carbonate, pH 9 (1 : 1 v/v) and the circumference of the cover glasses was fixed to a microslide by nail varnish. Cells were examined and photographed with a Karl Zeiss large fluorescence microscope (Ultraphot).

Binding and internalization of [^3H] *Concanavalin A.* Macrophage cultures were treated with 20 μg/ml of [^{3}H] concanavalin A in medium for 15, 45 and 90 min at 37 °C. Monolayers were washed (four times in phosphate-buffered saline) and incubated (30 min, 24 °C) in the buffered saline containing 0.1 M of α-methyl-D-mannoside, a specific concanavalin A inhibitor. [^{3}H] concanavalin A, displaceable by the inhibitor from [^{3}H] concanavalin A-treated macrophage is defined as the fraction of cell-associated lectin that is attached to the outer surface of the cell membrane (surface-bound lectin). The fraction of internalized concanavalin A (the fraction remaining after α-methyl mannoside treatment) was dissolved with the cells by 0.1 % of sodium lauryl sulfate. Blank experiments (tissue culture dishes incubated with 20 % serum in medium for 48 h) were run in parrallel to account for lectin adsorption to the culture dish and were used for correcting counts from experimental cultures. Radioactivity was measured by scintillation counting with a Packard (type 3380) scintillation counter.

Ingestion of yeast cells. Yeast cells, *Saccharomyces oviformis*, were taken from a stationary culture grown on an agar slant. Heat-killed yeast cells were obtained by boiling yeast cell suspensions in phosphate-buffered saline (Ca^{2+}, Mg^{2+} free) for 60 min. The cells were then washed twice in phosphate-buffered saline and resuspended in medium at a concentration of $15 \cdot 10^6$ cells/ml. Macrophage monolayers were incubated at 37 °C with 1 ml of yeast cell suspensions for the period specified in the text, in the presence or absence of the specified drugs. Monolayers were washed thoroughly, fixed (2 % glutaraldehyde in phosphate-buffered saline, 30 min) and stained (May-Grünwald-Giemsa).

Determination of cellular ATP content. Cellular ATP content was assayed by the firefly luciferin-luciferase method [24]. Buffered extract of dessicated firefly lantern (Sigma) was reconstituted and incubated for 24 h at 4 °C. Cell monolayers were washed twice in phosphate-buffered saline and covered with 1 ml of 0.4 M $HClO_4$. After an incubation of 30 min at 4 °C the supernatants were collected and kept at −20 °C until assayed. A 10 μl aliquot was used for each assay. Calibration curves with ATP were carried out in the presence of 10 μl of $HClO_4$. Total cell protein determination was carried out according to Lowry et al. [25].

RESULTS

Recent studies from our laboratory have indicated that concanavalin A induces extensive macrophage vacuolation in a dose-, time-, energy- and temperature-dependent pattern [5–7]. The total number of vacuoles (> 2 μm diameter) reaches

a saturation value at low concanavalin A concentration (10 μg/ml) but the size distribution is markedly affected by membrane-perturbing agents [9, 10, 21]. The effect of various types of anaesthetics and of theophylline on concanavalin A-induced macrophage vacuolation at two concentrations of the lectin is summarized in Table I. A range of drug concentrations was tested and the condition yielding optimal vacuolation quantitatively analyzed.

The response elicited in macrophages as a result of exposure to 5 μg/ml of concanavalin A is not uniform (Table I), i.e. 34 % of the cells are devoid of vacuoles (>2 μm diameter) and 7 % of the cells develop more than 10 such vacuoles per cell. A moderate number of large size vacuoles (>5 μm diameter) (10 per 50 macrophages) is also observed. Exposure to 20 μg/ml of concanavalin A reduces the non-responding cell population to 4.6 % and increases the number of cells developing more than 10 vacuoles to 34 %.

Addition of a homologous series of alcohols, C_6–C_{10}, results in a significant enhancement of macrophage vacuolation. The order of potency of the alcohols, $C_{10} > C_8 > C_7 > C_6$, and the concentrations required for optimal effects are closely related to the membrane buffer partition coefficient of these compounds [11]. In the series of the tertiary amine anaesthetics tetracaine is almost without effect on concanavalin A-induced vacuolation, while dibucaine has some effect when added in the presence of 20 μg/ml of concanavalin A. Lidocaine and procaine at a concen-

TABLE I

THE EFFECT OF ANAESTHETICS ON CONCANAVALIN A-INDUCED MACROPHAGE VACUOLATION

Macrophages were incubated in medium containing the specified additives for 90 min at 37 °C. Results are mean values of three groups of 50 macrophages enumerated in each of triplicate cultures ± S.E.

	Compound concentration (M)	Concanavalin A concentration (μg/ml)	Per 50 macrophages		
			No. cells devoid of vacuoles	No. cells with > 10 vacuoles per cell (2–5 μm diameter)	No. vacuoles (> 5 μm diameter)
—	—	5	17.2±1.1	3.6±0.8	10.3±2.7
Hexanol	$1 \cdot 10^{-3}$	5	15.0±1.0	5.5±1.0	14.3±1.4
Heptanol	$7.5 \cdot 10^{-4}$	5	13.2±1.0	7.5±1.9	17.5±1.9
Octanol	$4 \cdot 10^{-4}$	5	12.0±1.6	8.0±1.0	22.8±2.9
Decanol	$5 \cdot 10^{-5}$	5	6.7±1.3	12.0±1.2	26.2±2.3
Tetracaine	$1 \cdot 10^{-4}$	5	14.3±1.3	2.0±1.0	11.7±1.3
Dibucaine	$1 \cdot 10^{-4}$	5	12.0±0.8	4.3±1.0	8.3±1.6
Lidocaine	$1 \cdot 10^{-3}$	5	1.7±0.6	39.8±0.8	111.0±5.7
Procaine	$1 \cdot 10^{-3}$	5	1.5±0.3	38.2±1.4	81.5±5.2
Chlorpromazine	$5 \cdot 10^{-6}$	5	3.9±0.4	17.0±1.0	67.4±1.8
Theophylline	$2 \cdot 10^{-3}$	5	1.7±0.6	42.0±1.4	99.2±8.3

tration of 1 mM exhibit a remarkable enhancement of concanavalin vacuolation. At both concanavalin A concentrations (5 and 20 μg/ml) the same degree of vacuolation was observed, apparently the limit of possible vacuolation at the incubation period applied. It is impossible to compare the potencies of tetracaine, dibucaine, lidocaine and procaine since the inducers of vacuolation exhibit their effect at al concentration of 1 mM, a concentration at which the first two lead to 100 % cell shrinkage in the culture. Macrophage cultures that have been exposed for 30 min at 37 °C to 0.5 mM tetracaine or to 5 mM procaine in medium (last column, Table I) undergo an extensive cell shrinkage. Reincubation (24 h) of the shrunken cultures in 20 % serum medium results in recovery of the highly spread morphology of the culture. Chlorpromazine is very potent in enhancing concanavalin A vacuolation at concentrations 200-fold lower than those required for a comparable effect using lidocaine or procaine. Macrophages shrink at chlorpromazine concentration of 20 μM, and of the shrunken cells (30 min, 37 °C, medium, 50 μM in chlorpromazine) only about 50 % recover their spreading capacity upon reincubation in fresh serum for 24 h. Macrophages also shrink in the presence of 2 mM of octanol, replacement of the compound with 20 % serum medium for 24 h leads to recovery of spreading capacity of about 30 % of the cells the rest being non-viable.

The extent of concanavalin A-induced vacuolation is both dose- and time-dependent. It was therefore of interest to assess whether the different compounds

Concanavalin A concentration (μg/ml)	Per 50 macrophages			
	No. cells devoid of vacuoles	No. cells with > 10 vacuoles per cell (2–5 μm diameter)	No. vacuoles (> 5 μm diameter)	Range of concentration of anaesthetics tested (M)
20	2.3±0.8	17.0±1.4	30.7±2.3	–
20	6.5±0.8	14.0±1.7	34.4±1.7	$5 \cdot 10^{-4}$–$2 \cdot 10^{-3}$
20	7.7±0.9	18.0±1.8	28.5±1.4	$3.2 \cdot 10^{-4}$–$1.5 \cdot 10^{-3}$
20	3.2±0.9	22.0±2.3	42.7±3.5	10^{-4}–$2 \cdot 10^{-3}$*
20	3.6±0.8	26.0±1.8	68.5±2.7	$5 \cdot 10^{-6}$–$2 \cdot 10^{-4}$
20	6.8±1.1	7.1±1.9	36.0±3.8	$5 \cdot 10^{-5}$–$5 \cdot 10^{-4}$*
20	2.3±0.7	25.8±1.7	54.5±5.1	$1 \cdot 10^{-5}$–$5 \cdot 10^{-4}$*
20	1.8±0.6	20.8±2.2	102.6±5.6	10^{-3}–$5 \cdot 10^{-3}$*
20	1.3±0.4	33.3±3.1	112.3±3.5	$5 \cdot 10^{-4}$–$5 \cdot 10^{-3}$*
20	0.5±0.5	27.0±1.6	119.7±5.0	10^{-6}–10^{-4}*
20	0.5±0.5	45.0±1.1	130.6±5.5	$1 \cdot 10^{-3}$–$2 \cdot 10^{-3}$

* Macrophages were highly shrunken at this anaesthetic concentration both in the presence or absence of concanavalin A.

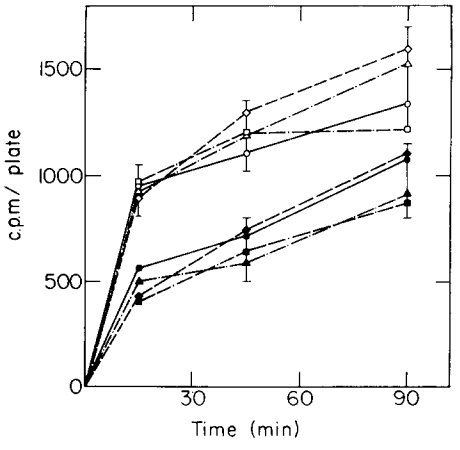

Fig. 1. The effect of local anaesthetics on binding (empty symbols) and internalization (full symbols) of [^{3}]concanavalin A (20 μg/ml). ○—○, control; ◇--◇, $5 \cdot 10^{-5}$ M decanol; □-.-□, 10^{-3} M procaine and △-.-△, $5 \cdot 10^{-6}$ M chlorpromazine. The experimental conditions are those given in Materials and Methods. The results are mean values with bars indicating standard errors of five culture plates. Differences between results obtained in the presence or absence of local anaesthetics were insignificant according to student's *t*-test.

used affect the kinetics of binding or interiorization of concanavalin A. Fig. 1 shows that decanol procaine and chlorpromazine do not exert any significant effect on either concanavalin A binding or internalization, at the three time points analyzed 15, 45 and 90 min. These findings seem to rule out the possibility that enhanced vacuolation stems from increased binding or uptake of concanavalin or from a difference in the rate of concanavalin A internalization. A kinetic difference in vacuole formation in macrophage cultures exposed to 40 μg/ml of concanavalin A in the presence and absence of 5 μM chlorpromazine was observed, however. A comparison of the time course of vacuole appearance under the above conditions is given in Fig. 2. Concanavalin A-induced vacuolation is normally observable at 60–90 min of incubation with the lectin at 37 °C. In the presence of chlorpromazine a marked vacuolation develops already at a 30 min incubation.

Trials to reverse chlorpromazine and lidocaine effects with Ca^{+2} were unsuccessful since macrophages undergo severe damage by Ca^{+2} at concentrations of 5–10 mM (in medium).

An incubation of macrophages (30 min, 37 °C) in medium in the presence of 5 and 10 mM of $CaCl_2$ results in about 50 % cell death in the culture as assessed both by trypan blue exclusion and morphological observation on stained preparations. A 90 min incubation period results in 100 % cell death in the culture. The cells show neither shrinkage nor swelling prior to loss of viability. It appears rather as membrane dissolution while the macrophages are still fully spread.

Respiratory inhibitors such as NaN_3 have been shown to inhibit concanavalin A-induced vacuolation [6]. In order to assess whether enhancement of vacuolation involves an increase in ATP pools within macrophages the effect of chlorpromazine on cellular ATP was studied. Fig. 3 indicates that at a concentration of 5 μM at which optimal enhancement of vacuolation was observed and up to 10 μM chlor-

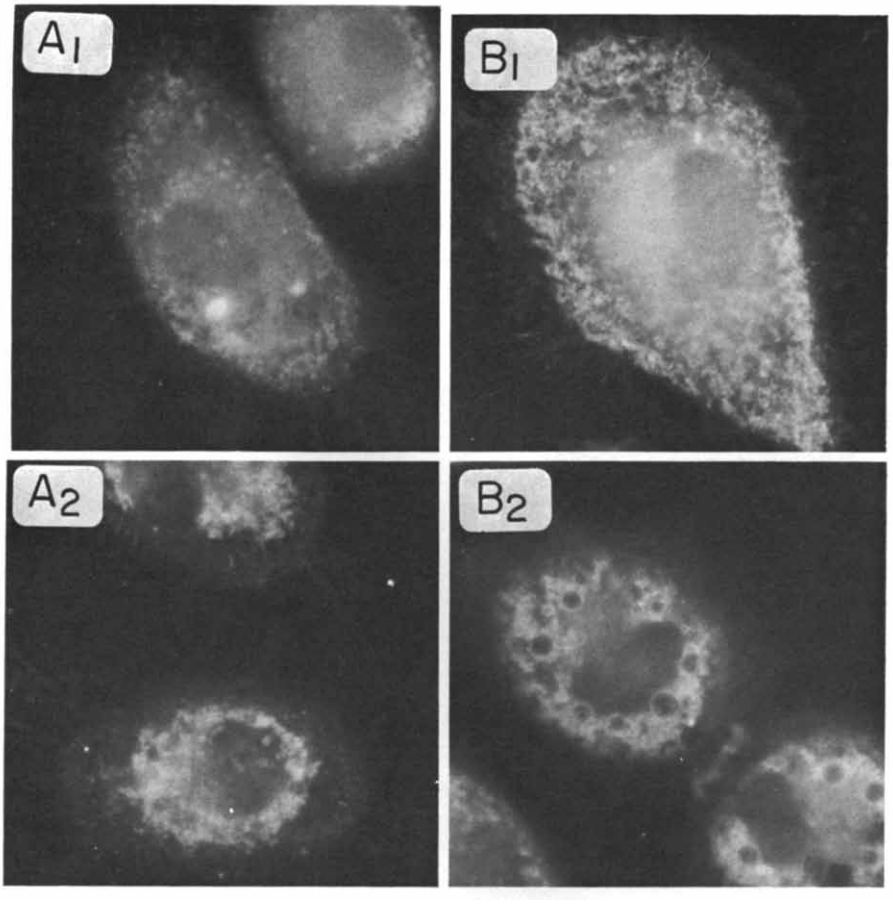

Fig. 2. See following page for legend.

promazine the level of cellular ATP does not exceed that observed in the control. It is interesting, however, that at concentrations of 20 μM of chlorpromazine where shrinking phenomena have been observed, ATP levels decrease steeply. A similar decrease has been observed with procaine at concentrations that lead to cellular shrinkage.

Theophylline has diverse effects on cells in tissue culture the most studied of which is the inhibition of phosphodiesterase activity. The result of this inhibition is a rise in intracellular cyclic AMP concentration. Since the cyclic nucleotides of AMP and GMP exert antagonistic effects on lysosomal enzyme release from leukocytes [26], a release that involves modulation of lysosome movement and fusion with the plasma membrane, we have looked at the effect of dibutyryl cyclic AMP and dibutyryl on concanavalin A (20 μg/ml)-induced vacuolation. Neither of the compounds (at a concentration of 1 mM) had any effect on concanavalin A vacuolation while theophylline at 1 mM concentration doubled the number of large size vacuoles. At 2 mM theophylline had the strongest enhancing capacity of all the compounds studied (Table I).

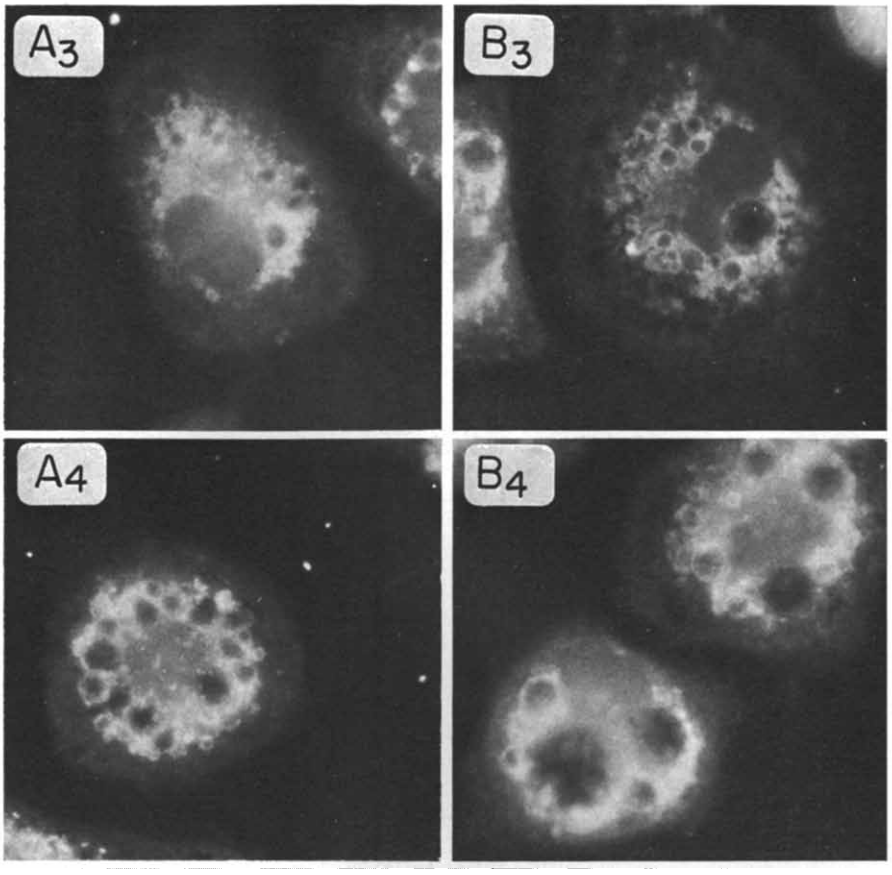

Fig. 2. Effect of chlorpromazine on the time course of concanavalin A-induced vacuole formation. Macrophages were exposed to fluorescein concanavalin A (40 μg/ml) in the presence (B) or absence (A) of $5 \cdot 10^{-6}$ M chlorpromazine, for (1) 10 min; (2) 30 min; (3) 60 min and (4) 90 min, at 37 °C. × 1500.

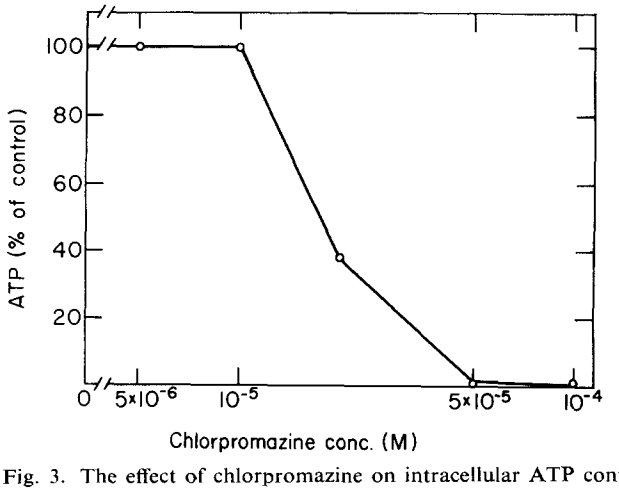

Fig. 3. The effect of chlorpromazine on intracellular ATP content. Macrophages were exposed to chlorpromazine in medium for 30 min at 37 °C. Control value of ATP in the absence of chlorpromazine (100 %) is 144 nmol/mg protein.

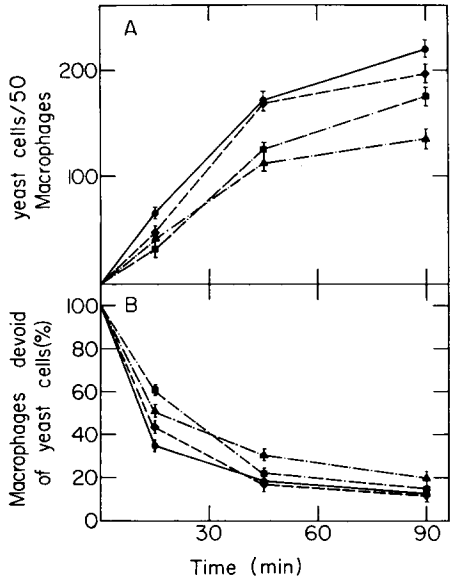

Fig. 4. (A) Effect of local anaesthetic on the ingestion of heat-killed yeast cells. (B) Percent of macrophages devoid of yeast cells. ● —●, control; ◆--◆, $5 \cdot 10^{-5}$ M decanol; ■-.-■, 10^{-3} M procaine and ▲-.-▲, $5 \cdot 10^{-6}$ M chlorpromazine. The results are mean values obtained from enumeration of ingested yeast cells in 20 groups of 50 macrophages in tetraplicate experiments. The bars represent standard errors.

The effect of anaesthetics on the phagocytic capacity of macrophages is given in Fig. 4. Decanol, procaine and chlorpromazine all exert a significant reduction in the phagocytic activity of macrophages at concentrations at which optimum enhancement of vacuolation was observed. The inhibitory effect is only slightly reduced upon approach to saturation in the phagocytic capacity, so that the effect is not merely a change rates of phagocytosis. Decanol has the least activity while chlorpromazine reduces the number of ingested heat-killed yeast cells by 37 %.

DISCUSSION

The importance of membrane fusion in a large number of cellular and subcellular activities has prompted many studies directed at the understanding of the molecular events preceding during and following the interdigitation of components of the fusing entities. There are also notable similarities between events in membrane fusion and the depolarization process in excitable cell membranes. In view of the diverse chemical agents that have been shown to induce in vitro the fusion reaction between cells [15] it is impossible to define the membrane components that are actually involved. Poste and Allison [15] in a comprehensive review devoted to membrane fusion suggest that one common feature to fusogenic agents would be their ability to introduce a "disorder" into a membrane so that the molecules in the membrane would gain sufficient motional freedom and possess free reactive sites to enable them to interact and establish stable linkages with molecules in adjacent membranes. In addition most of endocytotic and exocytotic processes that have been de-

scribed seem to depend on Ca^{2+} and metabolic energy for the triggering of the interaction between the two membranes but are inhibited by high Ca^{2+} or ATP concentrations [11, 14, 15, 27]. The exact location or the mechanism by which displacement of Ca^{2+} triggers diverse membrane phenomena is not known. Changes in the structural organization of membrane macromolecules are however, likely to ensure Ca^{2+} displacement [28–30].

Local anaesthetics and phenothiazine derivatives have been shown to inhibit membrane depolarization by increasing the dissociation of membrane Ca^{2+} and by competing with the Ca^{2+} for the same sites [11, 12]. Both types of compounds have been shown to prevent exocytosis and the release of secretory granules to inhibit cell fusion [11, 13, 14], and to prevent fusion of the lipid envelope of sendai virus with the host cell plasma membrane [31]. There are only rare cases where stimulation of fusion by chlorpromazine has been reported [11], amongst which is an increased quantal neurotransmitter release upon introduction of chlorpromazine into the medium in the presence or absence of Ca^{2+} [16].

The system described in the present communication is a system where rapid intracellular fusion processes take place. The pinosomes that undergo fusion are laden from within by a cross-linking agent i.e. concanavalin A. The fact that the pinosomes coalesce to big vacuoles is in itself a non-conventional phenomenon, since pinosomes that are non cross linked from within, fuse with each other to a limited extent and upon fusion with the lysosomal system they are degraded and undergo rapid shrinking, never exceeding a 0.5 μm diameter vesicle size. Introduction of the various compounds listed in Table 1 results in an enhancement of the fusion rates, an enhancement that does not stem from increased rate of concanavalin A interiorization (Fig. 1). The fact that no gross changes in concanavalin A interiorization have been observed does not exclude the possibility that a higher degree of clustering of concanavalin A-receptor conjugates has developed in a certain small fraction of the pinosomes. In line with our findings are the observations of Poste et al. [19] that local anaesthetics do not change the degree of lectin binding to fibroblasts.

Aliphatic alcohols have been shown to increase the membrane bound Ca^{2+} [11] and to increase the fluidity or disorder of phospholipid model membranes [11, 32, 33]. In general neutral anaesthetics increase membrane permeability to all ions including Ca^{2+}. The enhancement of concanavalin A pinosome fusion in the presence of aliphatic alcohols is thus observed under conditions that favour membrane binding of Ca^{2+} as well as its influx into the cytoplasm. The concentrations at which the different alcohols exhibit their optimum effect on concanavalin A-induced vacuolation are similar to those that are needed for a 50 % inhibition of osmotic hemolysis of erythrocytes. The potency correlates with the non-aqueous/aqueous partition coefficient of the members of the series [11].

Of the series of tertiary amine local anaesthetics tested (Fig. 5), lidocaine and procaine appear to produce the highest enhancing effect on intracellular fusion. Tetracaine and dibucanine affect the ability of macrophages to spread at lower concentrations than those leading to macrophage shrinkage when using lidocaine and procaine.

The four tertiary amine local anaesthetics are able to replace membrane-bound Ca^{2+} and to compete for its sites. Those anaesthetics have been shown to be

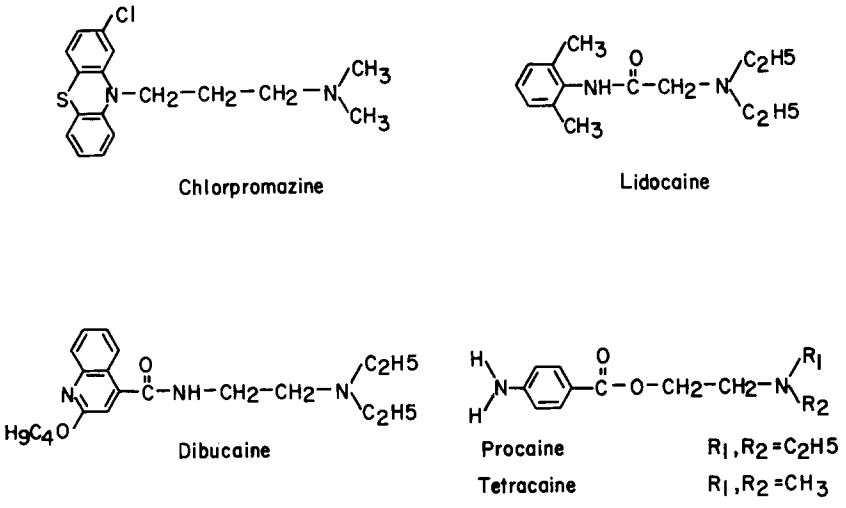

Fig. 5. Structure of anaesthetics used in the study.

inhibitory to virus-induced cell fusion with a reverse order or potency: dibucaine > tetracaine > procaine [13] and to inhibit fusion of secretory granules with the plasma membrane in a wide range of cells [11].

The effect of dibucaine on concanavalin A-induced fibroblast agglutination was totally reversed by 5 and 10 mM $CaCl_2$ [19]. It is worth noting at this point that fibroblasts and macrophages differ in their response to high Ca^{2+}. We have observed a striking cytotoxic effect on macrophages of 5 and 10 mM Ca^{2+} in experiments attempting to reverse the effect of local anaesthetics. Under comparable conditions mouse 3T3 fibroblasts spread on glass were totally resistant to Ca^{2+} confirming previous observations [19].

Apart from lipid solubility, Ca^{2+} displacement and a non-specific fluidizing effect in the phospholipid domain of both phospholipid model membranes and biological membranes, local anaesthetics and chlorpromazine have been shown to affect protein organization within the membrane [15, 17–20, 34]. Dibucaine has been shown to induce a change in the structural organization of the plasma membrane of 3T3 cells which permits spontaneous redistribution of intramembraneous particles into small clusters. Extensive redistribution of intramembraneous particles to form large clusters was seen only after concanavalin A binding to the cell surface. Immunofluorescence studies on the surface distribution of concanavalin A binding sites 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 cell surface. Poste et al. [18–20] suggest that the most important parameter affected by dibucaine involves the functional properties of peripheral membrane proteins (colchicine sensitive and cytochalasin B sensitive) regulating the topography of lectin receptors on cell surface.

The response of concanavalin A-treated macrophages to colchicine [9] and to local anaesthetics (Table I) is biphasic. At a certain dose both cause a dramatic enhancement in intracellular fusion of concanavalin A-bearing pinosomes whereas at a

higher concentration both lead to extensive cell shrinkage. Cell shrinkage may in fact be indicative of extensive dissolution of cytoskeletal supporting elements. Cytochalasin B in itself inhibits concanavalin A-induced vacuolation an inhibition that is not reversed by simultaneous treatment with cytochalasin B and colchicine [9]. The intactness of colchicine-sensitive elements is not indispensable for endocytotic processes, though they have been shown to regulate the composition of the interiorized plasma membrane vesicles [35].

Interpretation of effects of colchicine entirely on the basis of interaction with cytoskeletal elements has recently been challenged by Furcht and Scott [36]. They have shown that microtubule disruptive drugs, colchicine and vinblastine sulfate induce a dose- and time-dependent aggregation of intramembraneous particles at concentrations between 10^{-9} and 10^{-5} M. However, lumicolchicine, a derivative of colchicine that does not disrupt microtubules also promotes intramembraneous particle aggregation, suggesting that the drugs may have an effect on the plasma membrane in addition to disruption of microtubules.

It is tempting to suggest that the high enhancing capacity of theophylline on concanavalin A-induced macrophage vacuolation (Table I) could also evolve from membrane interactions and not necessarily from its inhibitory effect on a phosphodiesterase and concomitant changes in cellular cyclic AMP levels. It is of interest that an inhibitory effect of theophylline on 2-deoxyglucose transport in alveolar macrophages has been reported [37].

Our previous work and the results presented above are best integrated into the following working hypothesis. The site of membrane-membrane fusion involves a close apposition of membrane areas deplete or "poor" in protein moieties both those defined as concanavalin A-receptors and those identified as intramembraneous particles. Clustering of concanavalin A-receptors as a result of concanavalin A binding enhances the chance to find, in the interiorized vesicles, areas deplete of those receptors. Concanavalin A binding and clustering of receptors does not lead, however, to redistribution of intramembraneous particles. Local anaesthetics, chlorpromazine, alcohols and supposedly also theophylline affect membrane fluidity in a way inducing aggregation of intramembraneous particles. Treatment with both concanavalin A and anaesthetics exerts a synergistic effect on distribution of membrane components both concanavalin A-receptor conjugates and intramembraneous particles are highly aggregated and thus the probability of establishment of areas deplete of membrane proteins is enhanced hence force the observed enhancement of fusion processes. The proposed working hypothesis has strong support from recent findings of Lawson et al. [38]. Studying the process of mast cell degranulation in response to different specific stimuli they were able to show that areas of membrane contact prior to histamine release are deplete of both intramembraneous particles and of glycoprotein receptors. Redistribution of intramembraneous particles prior to fusion events was previously demonstrated [39, 40], but interpreted differently, i.e. it was suggested that membrane fusion is initiated by interdigitation of protein molecules on apposed membranes [15].

At high concentrations of the tested drugs cellular shrinking is observed (Table I) resulting concomitantly in impairment of microtubules and intracellular depletion of ATP (Fig. 3). It is noteworthy that treatments leading to ATP depletion have been shown to enhance concanavalin A-mediated cell agglutination [41] and that chlorpromazine has been shown to affect the energy metabolism of brain slices in

vitro, in particular leading to reduced ATP synthesis [42]. There is thus a delicate balance in the effect of the drugs being exhibited most dramatically at a concentration range at which the cells are just about to undergo shrinkage phenomena.

Phagocytosis involves several steps all of which could be affected by local anaesthetics: (a) the attachment stage and circumferential envelopment of the particles is normally coupled to reorganization of glycoprotein membrane components [35]; (b) fusion of the plasma membrane to form a phagosome; (c) involvement of the cytoskeleton in directed centripetal movement of the phagosome. Procaine and chlorpromazine do in fact affect the phagocytic capacity of macrophages reducing it by up to 40 % (Fig. 4). Decanol is less inhibitory than the tertiary amines. The inhibitory effects on phagocytosis are observed at drug concentrations that lead to maximal enhancement of concanavalin A-induced vacuolation.

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