CELL SHAPE CHANGES INDUCED BY CATIONIC ANESTHETICS*

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Cell functions such as adhesion, movement, and phagocytosis involve changes in both cell shape and cell surface motility. These changes are the result of membrane perturbations caused by interactions of cells with substrates, particles, or other cells. Surface perturbations generate signals that are presumably transduced into changes in the state of aggregation and distribution of submembranous cytoskeletal constituents (1–3). Except for the role of Fc and C3 receptors in the interaction of particles or substrates with phagocytes (3), other cell surface-"sensing" components, as well as the nature of the membrane perturbations and the transducing mechanisms are unknown. Induced cell shape changes permit the analysis of factors involved in maintenance of cell shape and in cell motility. A few diverse agents promote rapid and reversible changes in cell shape. Examples are the spreading of cells induced by Mn^{2+} (4) and the cell rounding induced by high hydrostatic pressure or exposure to trypsin, strychnine, EDTA, or cytochalasins (5–9). Cationic anesthetics inhibit a variety of cell surface phenomena, including cell to substrate adhesion, spreading, phagocytosis, locomotion, cell fusion, capping of surface Ig, or cell mediated-cytotoxicity (10–16). We show here that exposure of cultivated macrophages to cationic anesthetics results in extensive and fully reversible contraction and rounding. Single cells were followed before, during, and after exposure to the drugs and the observations recorded in still pictures and time-lapse movies. We examined several features of anesthetic-induced rounding, investigated structure-activity relationships of the drugs, and compared anesthetic effects to those of EDTA.

Materials and Methods

Animals. Female Swiss Webster mice of 20–25 g body weight from either Carworth Div., Becton, Dickinson & Co., New City, N. Y. (CFW) or Taconic Farms, Germantown, N. Y. (TSW) were used as a source of macrophages.

Drugs. Lidocaine HCl, prilocaine HCl, QX222, and tetrodotoxin were kindly provided by Dr. Bertil Takman of Astra Pharmaceutical Products, Inc., Worcester, Mass. Chlorpromazine HCl and methochlorpromazine iodide were a gift from Dr. Glenn Ullyot of Smith, Kline & French Laboratories, Philadelphia, Pa. Procaine HCl and tetracaine HCl were purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. Dibucaine HCl was from Ciba Pharmaceutical Co., Summit, N. J. EDTA was from Fisher Scientific Co., Pittsburgh, Pa. Colchicine, benzocaine, tris(hydroxymethyl)aminomethane (Tris),[1] 4(2-hydroxyethyl)1-piperazine

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[1] *Abbreviations used in this paper:* D, Dulbecco's modified Eagle's tissue culture medium; DH, Dulbecco's medium with 10 mM HEPES; EGTA, ethylene-glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HEPES, 4(2-hydroxyethyl)1-piperazine ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

ethanesulfonic acid (HEPES), and ethylene-glycol-bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co., St. Louis, Mo.

Media. Tissue culture media and sera were purchased from Grand Island Biological Co., Grand Island, N. Y. Dulbecco's modified Eagle's tissue culture medium (D) plus 20% heat-inactivated fetal bovine or calf serum were used for macrophage cultivation. For the perfusion studies, bicarbonate-free D was buffered with 10 mM HEPES to pH 7.2 (DH). In some experiments Puck's saline A was similarly buffered with HEPES. The low Na medium contained 95% (vol/vol) 0.15 M Tris-HCl at pH 7.2, 5% (vol/vol) Dulbecco's medium, 2 mM Mg, and 10 mM HEPES.

Cultivation of Macrophages. Macrophages were collected and maintained in culture for 3–18 days on 25 mm round glass cover slips as previously described (17). There was considerable variability in cellular activity, as determined by extent of macrophage spreading and ruffling, between cultures prepared with different lots of serum.

Perfusion Studies. Macrophage cultures were mounted into a Sykes-Moore chamber (Bellco Glass, Inc., Vineland, N. J.) and perfused by gravity with DH at a rate of 0.3 ml/min. The total vol of the chamber and dead space in the tubing was 1.0 ml. The microscope stage was heated to 37°C by a Zeiss air stream incubator (Nicholson Precision Instruments Inc., Bethesda, Md.) and the temperature monitored by a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). After 10–15 min control perfusion in DH, cells were exposed to DH-containing anesthetics or other drugs for periods of 10–30 min, and then returned to DH perfusion to examine reversibility. Results were similar when 10% fetal bovine serum was added to DH. Still pictures were photographed on 35 mm Panatomic X film (Eastman Kodak Co., Rochester, N. Y.) using a long-distance condenser of 0.63 numerical aperture and a ×40 phase oil of numerical aperture 1.0 immersion objective. Time-lapse photomicrographs[2] at 40 or 60 frames per minute were taken with similar optics on 16mm Plus-X negative film (Eastman Kodak Co.) and a Sage cinemicrographic apparatus (Orion Research Inc., Cambridge, Mass.).

Results

Macrophage Cultures during Control Perfusion. Macrophages in any given culture varied widely in size, extent of spreading, numbers of microspikes, retraction fibrils, pinocytic activity and pseudopod formation, and withdrawal. Active and less active cells could be found side by side, and individual cells were seen to alternate between periods of higher and lower activity.

Macrophage Cultures Perfused with Anesthetics. Almost as soon as DH was exchanged with the anesthetic-containing medium, macrohages stopped putting out large pseudopods, reduced their ruffling activity, and recoiled the cell margins leaving retraction fibers along their circumference (Fig. 1 B–D). Larger cell processes withdrew (Fig. 1 E), and soon after, the macrophages, richly endowed with retraction fibrils, appeared quite refractile under phase contrast (Fig. 1 F). In the preparation illustrated, drug-free medium was perfused at 8 min, so that the medium in the chamber was fully exchanged at 11 min. At 12 min (Fig. 1 G) the cell showed a small petal-like process. Other processes formed as retraction fibers thickened (Fig. 1 H). These pseudopods developed ruffles at the tips (Fig. 1 I–K), and enlarged as the cell body flattened. Membrane activity was clearly increased during respreading when compared to control periods. This description was verified in over 40 perfusion experiments with still pictures. Cells rounded similarly when perfused with 1.5 mM tetracaine. Perfusion with lower concentrations of anesthetic (e.g., 1 mM tetracaine for 1 h) resulted in sustained contracture but did not progress to full rounding.

[2] The time-lapse movies were made at The Rockefeller University in collaboration with Dr. J. G. Hirsch. Copies of time-lapse sequences of rounding and respreading after lidocaine or EDTA can be obtained at cost from the authors.

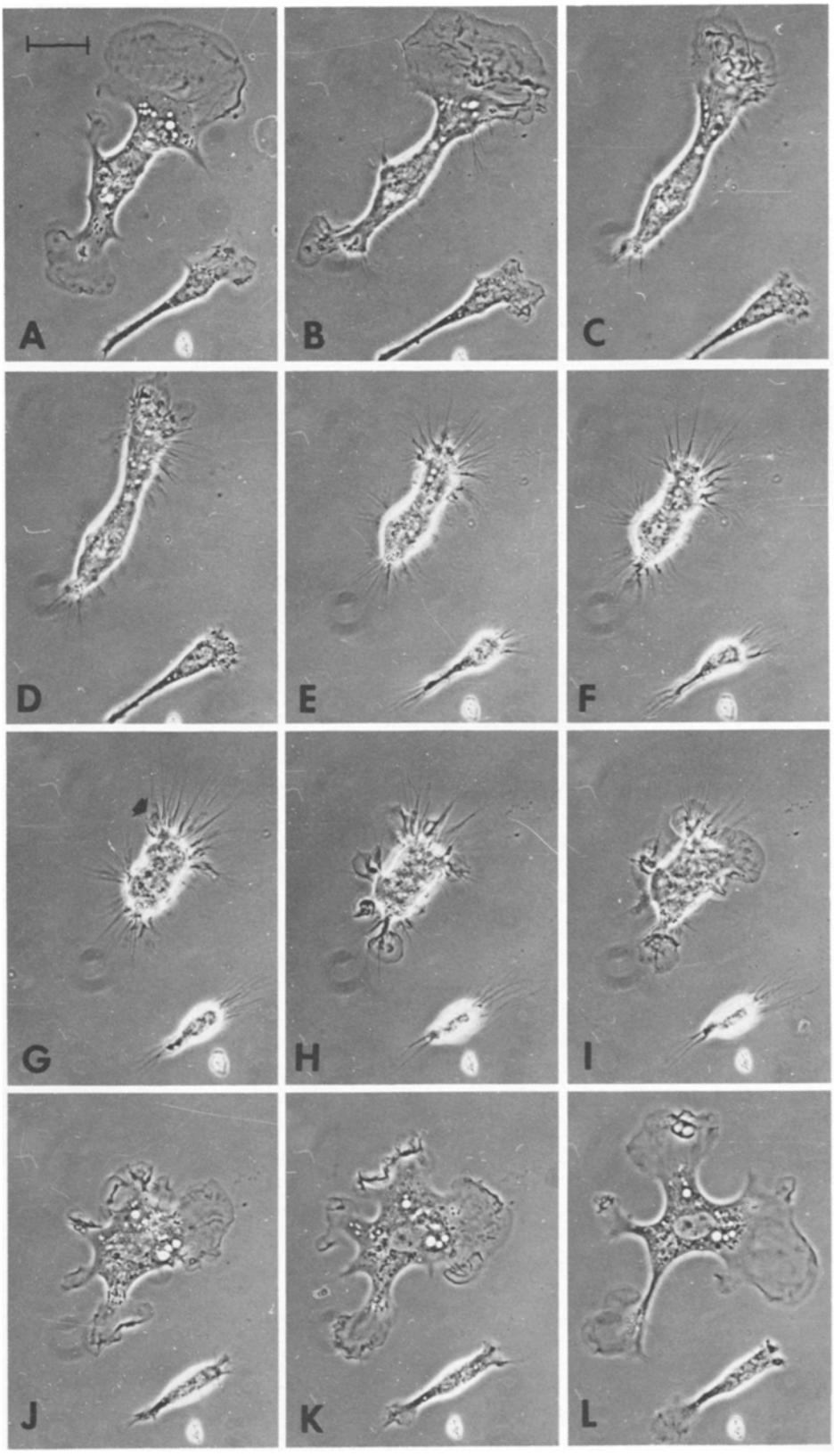

FIG. 1

Time-Lapse Studies. Films of eight separate preparations were available for analysis, all of them containing cycles of rounding and respreading with 12 mM lidocaine. The time-lapse studies revealed features which were not clearly perceived in visual observations and still pictures. (*a*) In 8 out of 10 cycles, membranes ruffled while cells rounded and petal-like processes were extended and withdrawn. In two other preparations, cells rounded with only minor membrane activity. More membrane activity was present in cells that rounded more slowly (about 15 min) than in cells that rounded over a short period of time (5–10 min). This relationship was supported by time-lapse studies of rounding with 1.5 mM tetracaine (three preparations with 12 cycles). Here complete rounding followed 4–8 min. of perfusion with the drug, and both ruffling and petal formation were less evident than in cells that rounded with lidocaine. Fig. 2 shows frames of the rounding of a cell perfused with 12 mM lidocaine. This rounding cycle required 17 min of exposure to the drug and, in contrast to the cell shown in Fig. 1, which rounded in 8 min, a variety of ruffles and petals were extended (arrows) even at late stages of rounding. Fig. 2 B–E illustrates the "backwards" ruffling or rolling motion as the cell contracted. In a few cells exposed to the anesthetics, processes or cell bodies retracted within a few seconds, as if suddenly released from their areas of adhesion to the substrate (18). (*b*) In well-rounded cells, a pulsation or throbbing-like motion was observed. This pulsation was also noticed in a few rounded cells present in control cultures. (*c*) Extensively rounded cells showed zeiotic activity which continued into the initial respreading period. Zeiosis was more prominent in cells rounded with tetracaine than with lidocaine, and has been reported in cells rounded by other agents (5–9). (*d*) Time-lapse studies vividly confirm the visual observations of the burst of membrane activity with respreading after removal of the drug. Petal-like extensions and small ruffles appeared at the tips of retraction fibrils and these fibrils thickened as if filled with cytoplasm. Simultaneously, pseudopodial and pinocytic activities increased. Respreading after anesthetic rounding is similar to, but faster than, the respreading of trypsinized tissue culture cells replated on glass or plastic (19).

Repeated Cycles of Rounding and Respreading. Macrophages underwent successive cycles of rounding with lidocaine followed by respreading, without showing signs of damage. Fig. 3 illustrates the maximum rounding and respreading stages of a single cell followed through eight such cycles over a total period of 228 min. After the last cycle, the preparation was further perfused with DH and the last picture, taken 6 h after the beginning of the experiment, shows that the cell did not appear damaged. Fig. 3 also demonstrates that the cell assumed different configurations in the different respreading cycles. Similar results were obtained with tetracaine.

FIG. 1. Effect of 15 mM lidocaine on cultivated macrophages in perfusion chambers. Perfusion with lidocaine started at 0 min and ended at 8 min, when it was followed by DH alone. Pictures were taken at: (A), 1 min; (B), 2.7 min; (C), 4 min; (D), 4.6 min; (E), 6.7 min; (F), 9 min; (G), 12 min; (H), 14.5 min; (I), 15.5 min; (J), 17.5 min; (K), 20.5 min; and (L), 23.5 min. Arrow in (*g*) points to small petal-like process, indicating the onset of respreading. 0 min was 65 min real time; this rounding-respreading cycle was the third of a series of eight. The large cell is also shown in Fig. 3. Note that the smaller cell rounded and respread slower. Bar equals 20 μm.

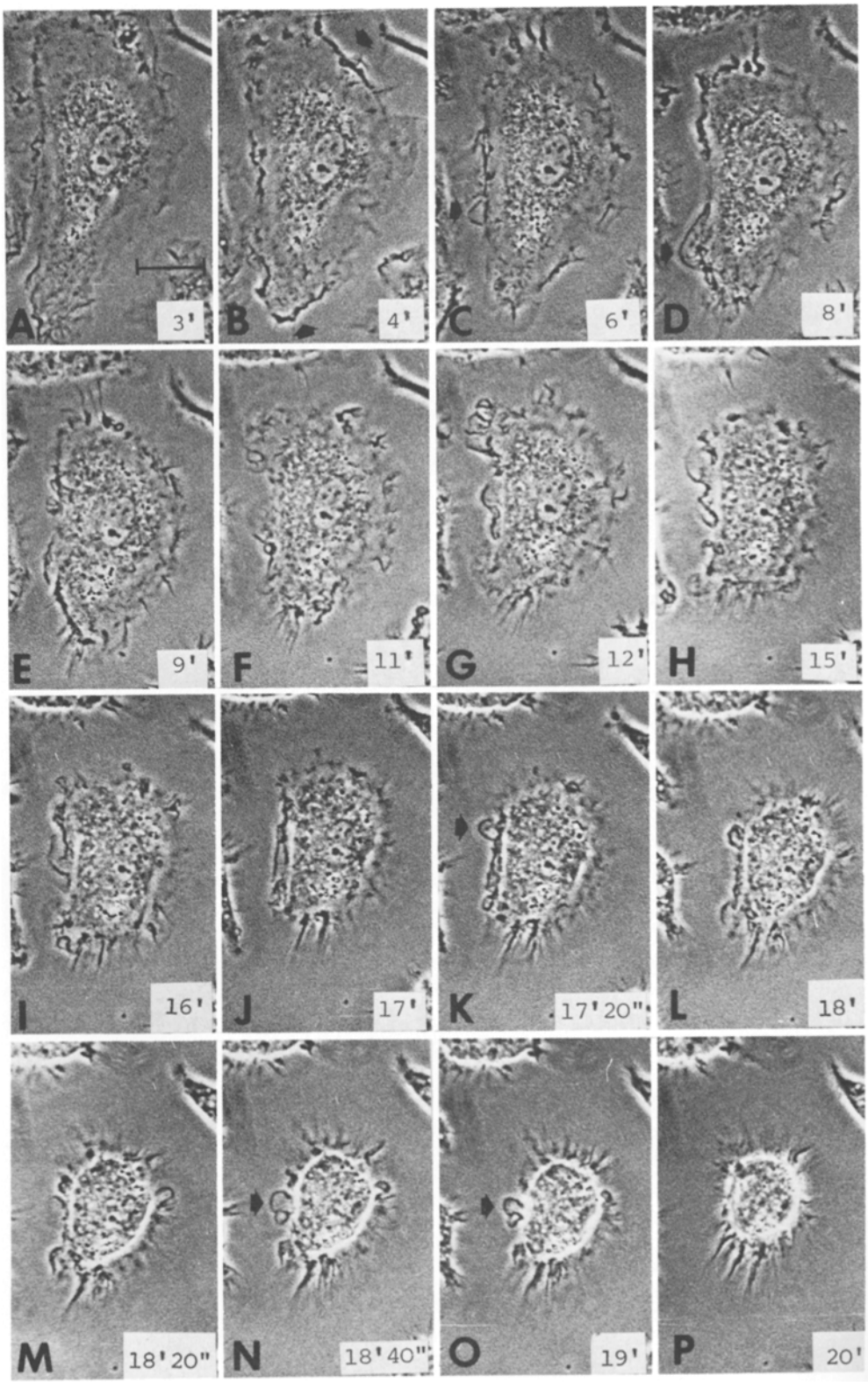

FIG. 2

Rounding Induced by EDTA. Rounding by 3 or 5 mM EDTA in DH as well as respreading in DH alone, were similar to those described above for lidocaine. The conclusion was drawn from 10 perfusion experiments with still pictures, and confirmed by viewing of time-lapse films of three separate preparations. In the three films, macrophages rounded with appreciable membrane activity. Confirming a previous report (8), repeated cycles with EDTA were also possible. Perfusion with the chelator in the presence of equimolar $CaCl_2$ did not induce rounding. Macrophages were also perfused with EGTA or EGTA-Mg in order to detemine whether rounding was related to the chelation of Ca or Mg. It is known that the affinity of EGTA for Ca is about 10^5 times that for Mg (20). Macrophages perfused with 3 mM EGTA or with 10 mM EGTA-10 mM Mg did not contract or round as they did during perfusion with EDTA. These results suggest that EDTA rounded the macrophages by chelating Mg, and not by chelating Ca. We have previously shown that induced macrophage spreading is also dependent upon medium Mg (21). However, macrophages did not round but showed only modest contraction when perfused for 20 min with Puck's saline A, a medium devoid of Ca or Mg. It is possible that cell depletion of divalent cations is better accomplished by chelation than by perfusion with divalent-free medium.

Structure-Activity Relationships of Anesthetics. Most local anesthetics at physiological pH occur in both the cationic (charged) and the base (uncharged) forms. In contrast to the charged form, the anesthetic base penetrates easily through cell membranes. Anesthetics block the action potential of nerve by interfering with the influx of sodium ions necessary for the generation of the action potential (22). Studies with peripheral nerves indicate that anesthetic cations affect the sodium channels from the inside. Thus, nondischargeable amines are relatively inactive when applied from the outside but are effective when they gain access to the axoplasmic face of the membrane (23). Molecules that are nearly uncharged at pH 7.0 such as benzocaine (24) or alcohols are also anesthetic, possibly due to their membrane-disturbing activity (25). Indeed, anesthetic action may result from a combination of the specific effect of the cations on the Na channels and the nonspecific membrane-disturbing activity of the hydrophobic moiety of the anesthetic molecules (26). We examined the effect of several anesthetics and their congeners in order to determine whether similar structure-activity relationships apply to macrophages. Our findings were as follows: (*a*) Concentrations of local anesthetics needed for cell rounding, correlated with their lipophilia as estimated by their octanol-water partition coefficients (25). Thus, rounding was obtained with 0.1 mM chlorpromazine, 0.4 mM dibucaine, 1.5 mM tetracaine, 12 mM lidocaine, and was minor with 20 mM procaine. Subtilisin spreading of macrophages was also inhibited in proportion to the octanol-water partition coefficients of the drugs (11). (*b*) Benzocaine at 5 mM (maximal concentration attained) rounded the macrophages similar to lidocaine, although to a lesser extent, perhaps because comparable doses could not be reached. (*c*) Prilocaine, a secondary amine anesthetic and a lidocaine

FIG. 2. Selected frames from time-lapse film of rounding induced by 12 mM lidocaine. Perfusion with the drug started at 0 min but effective concentration was only reached at about 3 min. Absolute times are indicated on the figures. Arrows point to ruffles and petal-like processes formed during rounding. Bar equals 30 μm.

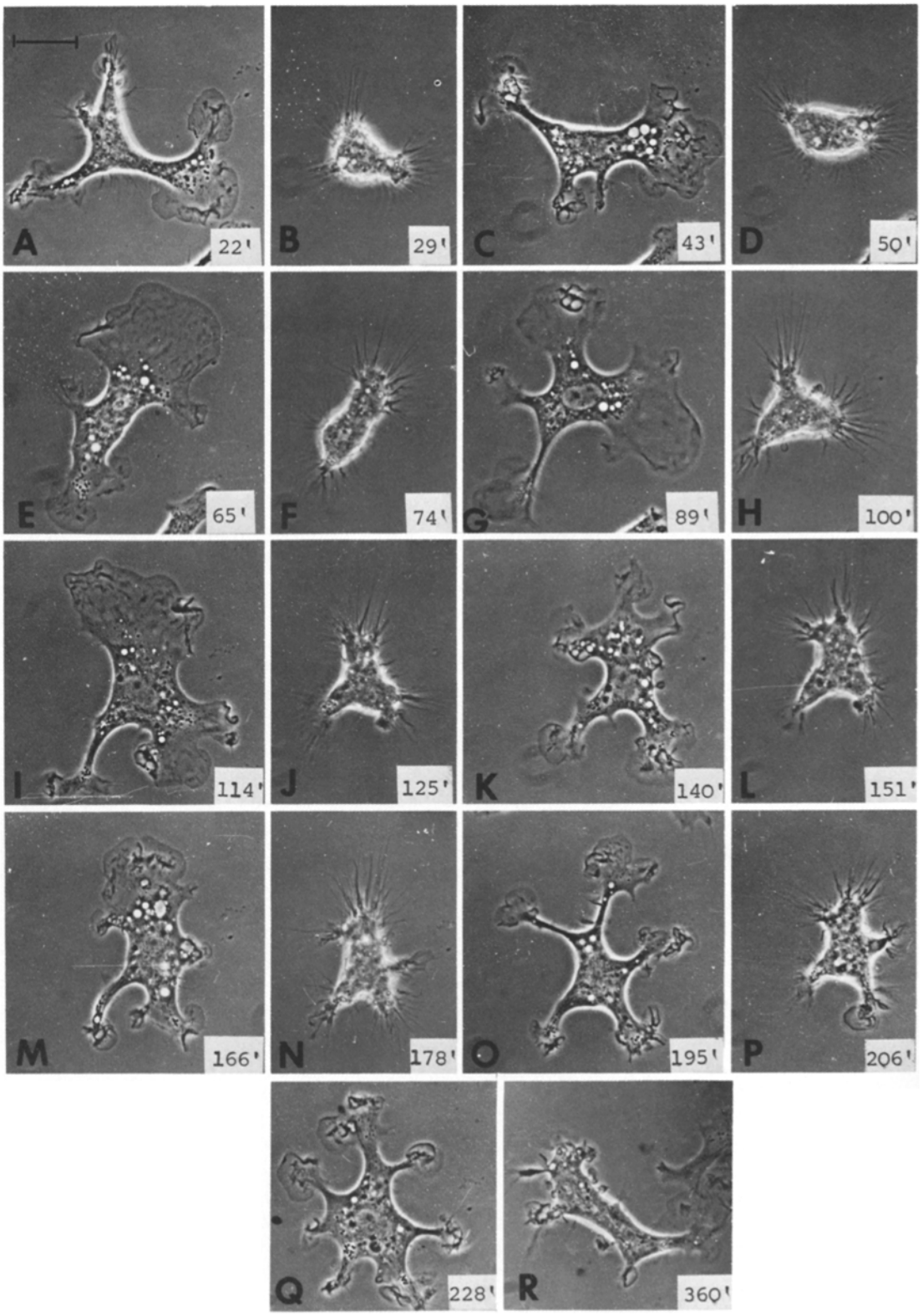
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FIG. 3

congener was active at 10 mM. (*d*) The quaternary congeners QX222 of lidocaine and methochlorpromazine were far less active than the parent compounds. Thus, only a minor response was obtained with 24 mM/30 min of the former (Fig. 4 B) or 0.4 mM/30 min of the latter compound. However, the same cells were able to respond to lidocaine (Fig. 4 D). (*e*) Tetrodotoxin, a drug that specifically blocks the sodium channels of nerve or muscle (24) was inactive at 10 μg/ml.

These results show that primary, secondary, or tertiary amine anesthetics are able to induce cell rounding. Effective agents were either uncharged or were present in both the charged and uncharged forms at physiological pH. Quaternary amine anesthetics, however, were of low activity, presumably because they are slowly permeant molecules. We have no evidence as to whether intracellularly present charged anesthetics can induce cell rounding. While nerve blocking and cell-rounding activities appear to be correlated, the ineffectiveness of tetrodotoxin suggests that cell rounding by anesthetics is not mediated by changes in sodium conductance.

Temperature Dependence of Rounding. In these experiments perfusion chambers could not be used and separate control and lidocaine-treated populations were studied. Macrophages were incubated at different temperatures for 10 or 20 min in DH alone or with 12 mM lidocaine. At 10, 15, or 22°C control cells showed some contraction (Fig. 5 C, E, and G) but in the presence of the drug the cells rounded extensively (Fig. 5 D, F, H, and J). At 5°C the effect of lidocaine was clearly reduced (not shown in Fig. 5). However, at 0°C, there was little or no rounding in both DH or DH-lidocaine-treated macrophages (Fig. 5 A and B).

Ionic Composition of the Medium. Local anesthetic blockade of nerve is potentiated by a lowered Na content in the medium and in some systems addition of Ca reduces the blocking effect (22, 27). The following experiments determined the effect of changes in the concentrations of Na or Ca in the medium on lidocaine rounding. Macrophage cultures perfused with Tris-HEPES Mg, a medium containing only 5% of the sodium present in DH, did not round over short periods of 10 min. Response to lidocaine when given in Tris-HEPES was not potentiated but similar to that in DH. In other experiments, 10 mM Ca added to DH or to DH-lidocaine did not modify the rounding response and permitted respreading comparable to that of cells perfused with DH alone. Furthermore, anesthetic rounding did not require Ca in the medium, as cells rounded in Ca, Mg-free phosphate-buffered saline, Puck's saline A, or in DH with 5 mM EGTA-Mg. To test whether lidocaine would induce rounding by interfering with the uptake of Mg, 10 mM Mg was added to DH or to DH-lidocaine. Neither rounding by lidocaine nor respreading in DH were affected by the additional Mg in the medium.

Test for Involvement of Microtubules. Local anesthetics have been reported to disrupt microtubules in axons and to inhibit polymerization of brain tubulin

FIG. 3. Successive cycles of rounding and respreading with 15 mM lidocaine in DH. Reading across the rows from left to right, pictures show maximum stages of respreading in DH and of rounding with lidocaine. Numbers represent absolute times. Eight cycles are shown; 1 (A, B); 2 (C, D), 3 (E, F), 4 (G, H), 5 (I, J), 6 (K, L), 7 (M, N), and 8 (O, P). Q and R show the same cell respread shortly after (Q) or 160 min (R) after the eighth rounding. Bar equals 20 μm.

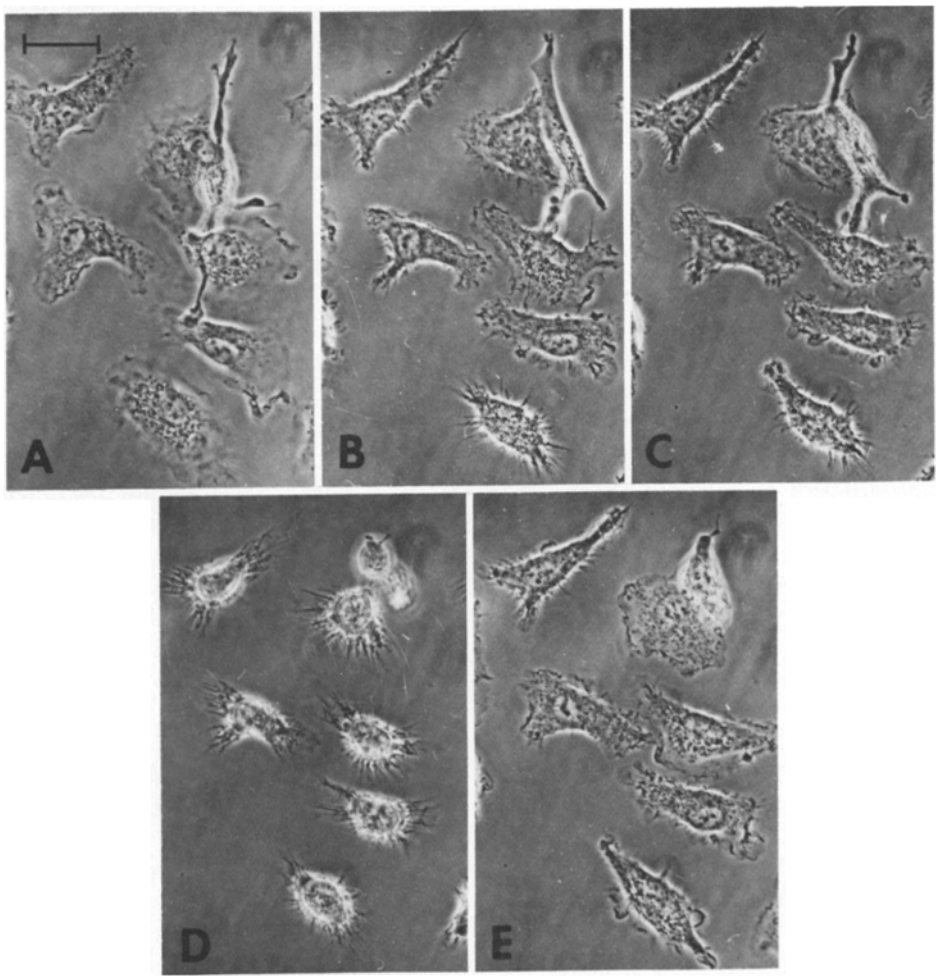

FIG. 4. Perfusion with QX222 followed by lidocaine. (A) control, DH; (B) 24 mM QX222 for 30 min; (C) DH for 30 min; (D) 12 mM lidocaine for 15 min; and (E) DH for 20 min. Bar equals 20 μm.

(28). It was of interest to determine whether macrophages pretreated with colchicine would respond to lidocaine. Cultures were exposed to 5 μM colchicine for 4, 12, or 24 h, perfused with lidocaine in DH-colchicine and then with DH-colchicine. Macrophages treated with colchicine assumed bizarre shapes (29) and were extremely ameboid as confirmed by time-lapse studies. Exposure to lidocaine in the presence of colchicine resulted in extensive rounding, and the cells respread when subsequently perfused with DH-colchicine. Therefore, polymerized tubulin may not be needed for anesthetic-induced rounding or for the respreading that follows removal of the anesthetic.

Discussion

We have shown that extensive cell contraction results from perfusion of cells with cationic anesthetics. Cells often exhibited ruffling, and petal extension and

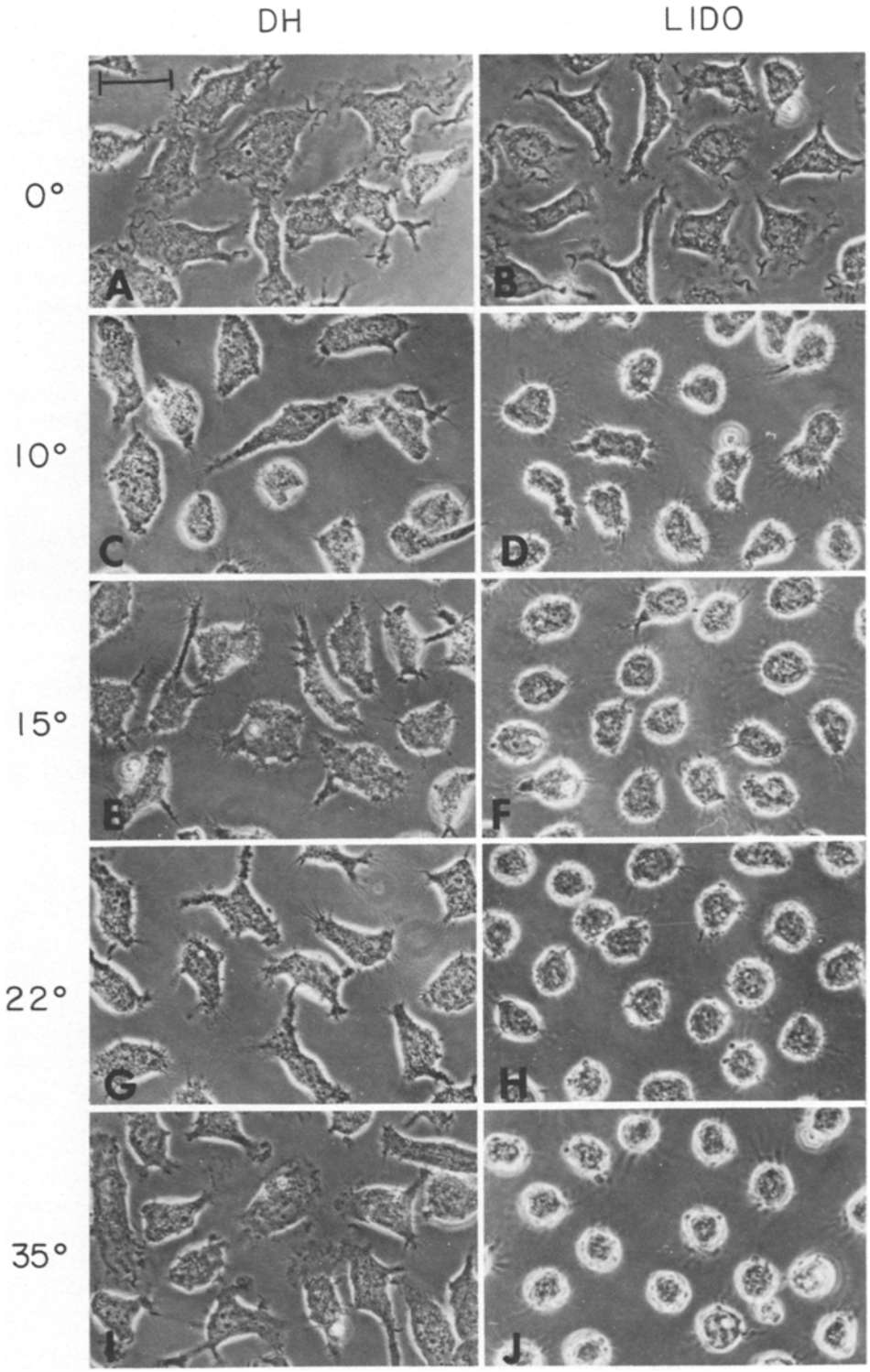

FIG. 5. Separate macrophage populations incubated for 20 min at different temperatures in DH (column on the left) or in DH with 12 mM lidocaine (column on the right). Effect of lidocaine is not detectable at 0 but is pronounced at 10°C and above. At 10, 15, and 22°C there is cellular contraction in DH alone. Bar equals 20 μm.

retraction as they rounded; this membrane activity correlated inversely with the rate of rounding. Similar rounding was obtained with EDTA. Anesthetic or EDTA effects were fully and rapidly reversible upon washing in drug-free medium.

Nerve blockade by local anesthetics is mediated by an effect of anesthetic cations on sodium channels accessible from the inner side of the plasma membrane (22–24). In addition to this "specific" effect, anesthetics have a nonspecific membrane-perturbing activity related to their lipid solubility (25). Our findings that the rounding effect of lidocaine was not influenced by the levels of Na in the medium and that the specific Na channel agent tetrodotoxin did not induce rounding indicate that the rounding effect is unrelated to changes in sodium conductance. In addition, ineffectiveness of externally applied nondischargeable and little permeant quaternary congeners most probably implies that the anesthetic target is intracellular.

A question to be resolved is whether anesthetic-induced rounding is metabolically dependent. We have shown that cells rounded extensively when exposed to lidocaine at temperatures between 10°C and 35°C (Fig. 5). This relative temperature independence contrasts with the temperature requirement of other membrane phenomena, such as cap formation or particle ingestion (30, 31). Recently, cell contraction and zeiosis induced by cytochalasin D were reported to be temperature dependent and reduced by high concentrations of metabolic inhibitors such as 2,4-dinitrophenol, antimycin A, or 2-deoxyglucose (9). Reversibility was not reported. Thus, metabolic dependence of cell rounding induced by different agents deserves additional evaluation.

Maintenance of the spread conformation probably requires the deposition of cytoskeletal elements in response to interactions of the plasma membrane with the substrate (32, 33). Such interactions, in turn may depend on the intrinsic membrane adhesiveness for the substrate (2, 3). Maintenance of the spread state can also be assumed to be metabolically dependent. In analogy with muscle contraction, ATP may be needed for divalent ion translocations or may be directly involved in the functioning of contractile proteins (34, 35). These considerations suggest that the anesthetics could affect: (*a*) the fluidity of the plasma membrane, (*b*) the putative mechanisms for transfer of information about the substrate, (*c*) the function of the contractile microfilaments, (*d*) divalent ion fluxes, or (*e*) intracellular levels of ATP.

Increased membrane fluidity may underlie the increased agglutinability by lectins of normal cells treated with cationic anesthetics (36). Transmembrane proteins postulated to convey information could be directly affected by the lipophilic anesthetic molecules or the function of these proteins could be interfered with by membrane disorganization. Anesthetics could affect the state of aggregation or the attachment of contractile microfilaments to the plasma membrane. Indeed, recent transmission electron microscopic studies of 3T3 fibroblasts exposed to cationic anesthetics revealed disorganization of subplasmalemmal microfilaments and microtubules (Dr. Garth L. Nicolson, personal communication). Disappearance of microfilaments was also reported to follow exposure of neuroblastoma cell cultures to halothane (37). Control of the function of microfilaments by free Ca^{2+} levels in the cell sap receives support from

recent experiments demonstrating ATP- and Ca-dependent contraction of isolated fibroblast cytoplasm (38). Anesthetics could increase the cell sap calcium by promoting release of the cation from mitochondrial stores or by inhibition of plasma membrane Ca and Mg ATPases (39). However, we observed only a modest contraction of macrophages perfused with 2 μM of the divalent ionophore-A23187 in either Ca-free or Ca-enriched medium (experiments in progress).

Experiments performed mainly with inhalation anesthetics and with barbiturates show that anesthetics can inhibit a variety of enzymes and affect intermediary metabolism (40, 41). Therefore cell rounding could be mediated by metabolic inhibition and lowered ATP levels. In experiments in progress, we found that inhibitors of electron transport or oxidative phosphorylation did not induce cell rounding in media containing glucose and thus did not mimic the effect of anesthetics on cells. Furthermore, even in glucose-free medium, the inhibitors induced less than maximal rounding when contrasted to the anesthetics. It is also of interest that levels of ATP were unchanged in fibroblasts that contracted by exposure to cytochalasin B (42).

Several cell lines in addition to macrophages respond by rounding when exposed to cationic anesthetics. This led to the development of a method to harvest cells from cultures after a brief exposure to lidocaine in serum containing tissue culture medium (43).

Finally, modulation of cell form by the anesthetics raises the possibility that locally released endogenous molecules of appropriate lipid solubility may be involved in the normal control of cell form, in formation and retraction of surface proceses, and in rounding preparatory to mitosis.

Summary

The effects of local anesthetics on cultivated macrophages were studied in living preparations and recorded in still pictures and time-lapse cine-micrographs. Exposure to 12 mM lidocaine or 1.5 mM tetracaine resulted in rounding in 10–15 min. Rounding was characterized by cell contraction, marked increase in retraction fibrils, withdrawal of cell processes, and, in late stages, pulsation-like activity and zeiosis. Cells showed appreciable membrane activity as they rounded. Respreading was complete within 15 min of perfusion in drug-free medium and entailed a marked increase in surface motility over control periods. As many as eight successive cycles of rounding and spreading were obtained with lidocaine without evidence of cell damage. The effects of anesthetics were similar to those observed with EDTA, but ethylene-glycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid-Mg was ineffective. Rounding was also induced by benzocaine, an anesthetic nearly uncharged at pH 7.0. Quaternary (nondischargeable) compounds were of low activity, presumably because they are slow permeants. Lidocaine induced rounding at 10°C and above but was less effective at 5°C and ineffective at 0°C. Rounding by the anesthetic was also obtained in media depleted or Na or enriched with 10 mM Ca or Mg. The latter finding, together with the failure of tetrodotoxin to induce rounding, suggests that the anesthetic effect is unrelated to inhibition of sodium conductance. It is possible that the drugs influence divalent ion fluxes or some component of the

contractile cells' machinery, but a metabolic target of action cannot yet be excluded.

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