EFFECTS OF LOCAL ANESTHETICS ON CELL

MORPHOLOGY AND MEMBRANE-ASSOCIATED

CYTOSKELETAL ORGANIZATION IN BALB/3T3 CELLS

GARTH L. NICOLSON, JAMES R. SMITH, and GEORGE POSTE. From the Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, California 92112, the Department of Developmental and Cell Biology, University of California, Irvine, California 92664, and the Department of Experimental Pathology, Roswell Park Memorial Institute, Buffalo, New York 14263

It is generally considered that local anesthetics act directly at the level of the plasma membrane (10, 23, 45). In addition to their anesthetic effects on excitable membranes (4, 45, 50), local anesthetics produce a variety of effects on membranes, including expansion (47), altered osmotic fragility (46), and inhibition of cell spreading (43), movement (13, 40), adhesion (31, 42), and fusion (36). Several studies have shown that local anesthetics interact with membrane lipids (4, 7, 11, 14, 18, 33, 35, 45), particularly acidic phospholipids (14, 33, 35, 45), and are also able to displace Ca^{2+} from membranes (7, 33, 34, 48). Local anesthetics are considered to interact with membranes by both hydrophobic and electrostatic interactions in close proximity with the anionic groups of acidic phospholipids (7, 14, 35).

Local anesthetics are known to produce molecular disordering in lipid bilayers and to enhance the fluidity of phospholipids in membranes (20, 35). These effects occur only at very high anesthetic concentrations and are probably not related to anesthesia. At much lower concentrations, tertiary amine local anesthetics cause modifications in cell agglutinability (37-39) and mobilities of certain surface receptors (37-39, 47). Specifically, these receptors appear to be "uncoupled" from cytoplasmic controls (37-39), which appear to correspond to cytoskeletal elements associated with the plasma membrane (38, 39, 43, 47). We have

examined the effects of tertiary amine local anesthetics on the organization of the cytoskeletal system (microfilaments and microtubules) in BALB/3T3 cells and have found that low physiologically active concentrations of dibucaine, tetracaine, and procaine induce dramatic disruption of submembrane cytoskeletal organization.

MATERIALS AND METHODS

Dibucaine HCl, tetracaine HCl, and procaine HCl were obtained from Mann Research Laboratories (Orangeburg, N.Y.). Dimethylsulfoxide and glutaraldehyde were products of Sigma Chemical Co. (St. Louis Mo.) and Fisher Scientific Co., (Pittsburgh, Pa.).

Mouse BALB/c 3T3 (A·31) were obtained from Dr. S. Aaronson (National Institutes of Health, Bethesda, Md.) and grown in Dulbecco-modified Eagle's medium (DMEM) with 10% calf serum as described previously (37–39). Cells were grown on 60-mm tissue culture dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) until confluent and the media replaced with fresh media (37°C) containing various concentrations of the local anesthetics. Cells were incubated at 37°C, and each dish was monitored for gross morphological changes using an inverted microscope. After the incubation, the cells were fixed at 20°C with 1.5% glutaraldehyde, washed in phosphate-buffered saline, and postfixed for 1 h in 2% osmium tetroxide at 20°C. The fixed cells were either removed at this point or embedded directly on the dishes. For cell removal, the fixed cells were carefully lifted off the substrate with the use of a Teflon policeman and were then oriented by centrifugation (100 g) onto soft agar pellets. The cell pellets were dehydrated with ethanol-propylene oxide, stained in block in the 30% ethanol step with 4% uranyl acetate for 5 min, and embedded in Spurr's resin (Electron Microscopy Sciences, Warren, Pa.). For direct embedding, the cell monolayers were dehydrated in ethanol, stained in block, and embedded in Epon 812 (Electron Microscopy Sciences). The Epon-cell layers were then stripped from the plastic plates and

sectioned parallel to the growth plane using a Reichert (Vienna, Austria) Model OMU-9 ultramicrotome. Sections were stained with 0.4% lead citrate and observed in a Hitachi model HU-12 at 75 kV.

RESULTS

Local anesthetics produced dramatic morphological changes in confluent 3T3 cells. BALB/3T3 are normally flat, polygonal, endothelioid cells (Fig. 1). The addition of tetracaine (≥ 0.5 mM), dibucaine (≥ 0.1 mM), or procaine (≥ 1 mM) for 15–30 min at 37°C caused alterations in cell shape. At the lower anesthetic concentrations some cell rounding occurred (Fig. 2). At higher concentrations more cells rounded up, particularly those cells at the edges of cell groups, until all the cells in the culture were rounded (Fig. 3). These effects were reversible within 1–2 h upon washing cells in fresh media (37°C) without anesthetics. At the highest anesthetic concentrations used, the cells floated off the culture plate as found by Rabinovitch and De Stefano (44). The most dramatic changes in morphology were usually complete within 15 min, though some cells in each population appeared to be more resistant to anesthetic action than others, and some remained attached to the substrate. Generally, less confluent cultures were more susceptible to local anesthetic effects. The dose-response relationship of tetracaine-induced changes in 3T3 cell morphology is shown in Fig. 10. Dibucaine ($> 2 \times 10^{-4}$ M) and procaine ($> 10^{-3}$ M) produced similar changes in cell shape; the differing potencies of the tertiary amine local anesthetics used here corresponded well with their octanol-water partition coefficients (16, 43).

When confluent 3T3 cells were examined by electron microscopy, they appeared as flat, elongated cells (Fig. 4) with an extensive subplasma

FIGURE 1 Control BALB/3T3 cells. Fig. 1–3 by phase microscopy. Bars equal 20 μm. × 328.

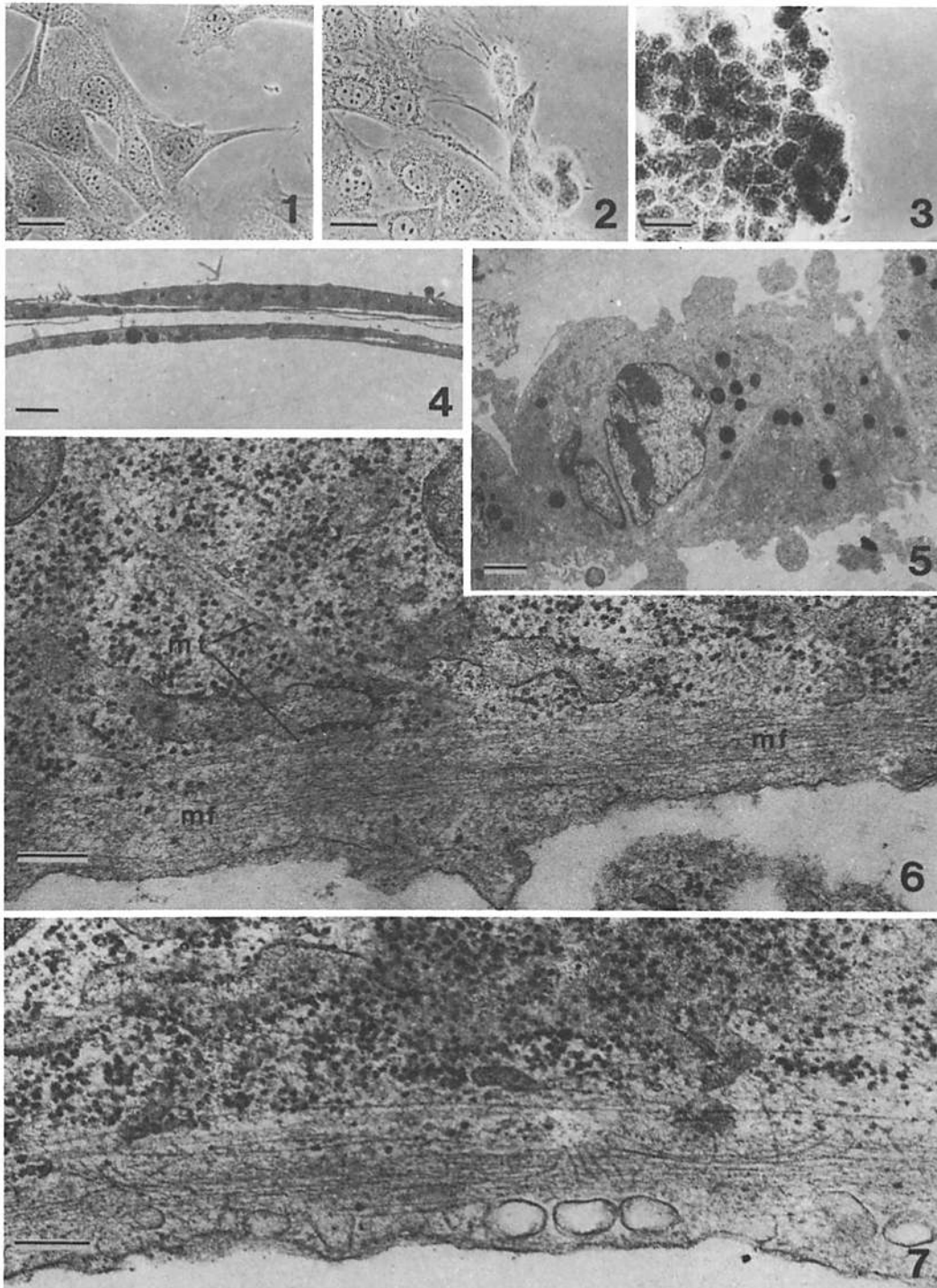FIGURE 2 3T3 cells treated with 0.5 mM tetracaine for 15 min at 37°C.

FIGURE 3 3T3 cells treated with 1.0 mM tetracaine for 30 min at 37°C.

FIGURE 4 Control 3T3 cells fixed *in situ*, removed from substrate, and oriented by centrifugation in an agar pellet. Bar equals 2 μm. × 3,000.

FIGURE 5 3T3 cells treated with 1.0 mM tetracaine for 30 min at 37°C; otherwise legend is identical to Fig. 4.

FIGURE 6 Control 3T3 cell embedded *in situ*. Microtubule, *mt;* microfilaments, *mf.* Bar equals 0.2 μm. × 52,500.

FIGURE 7 Legend is the same as in Fig. 4. Bar equals 0.2 μm. × 52,500.
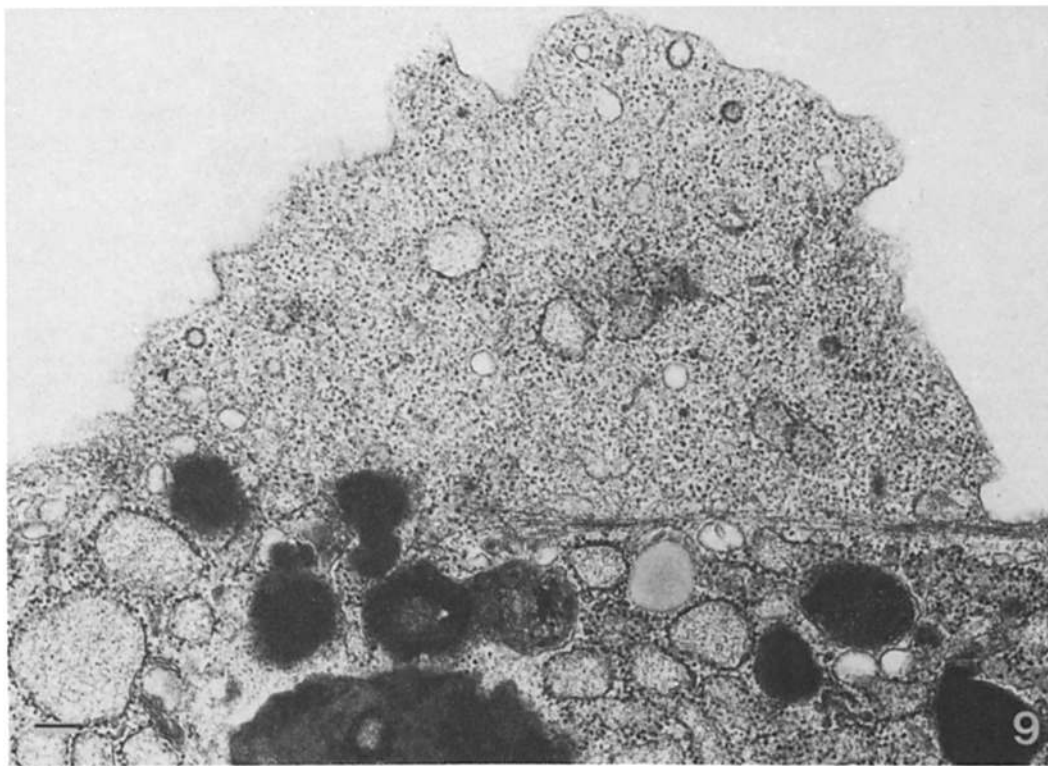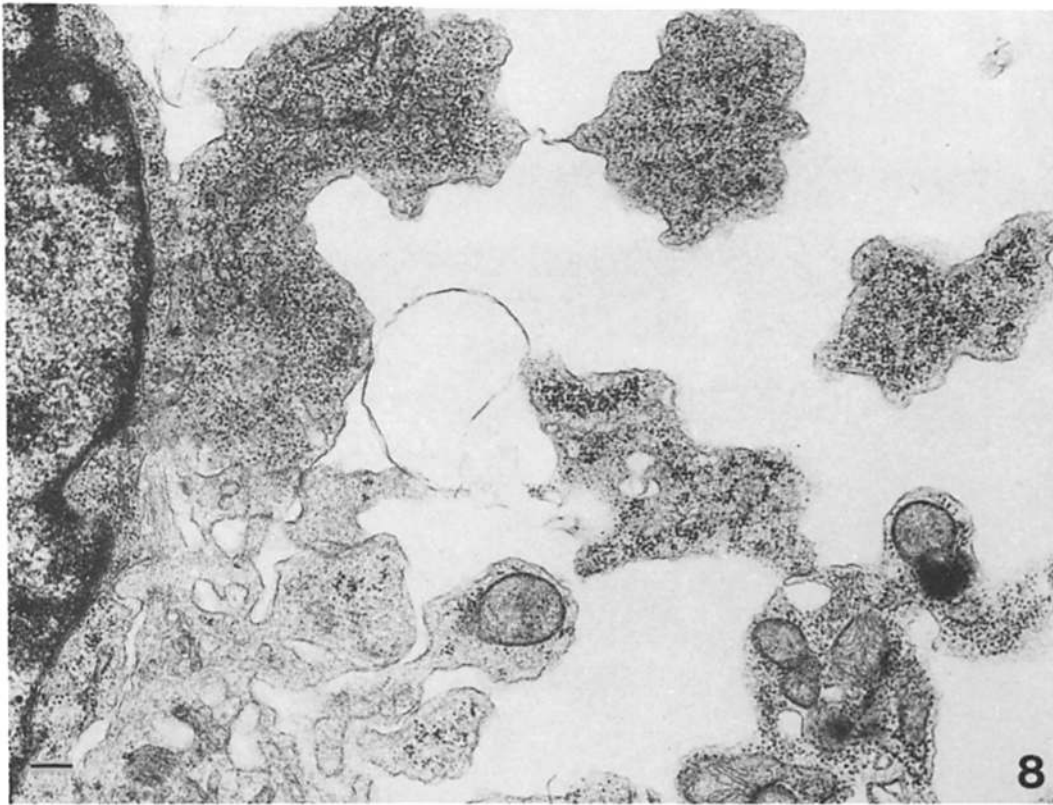
FIGURE 8   3T3 cell treated with 1.0 mM tetracaine for 30 min at 37°C; otherwise legend is the same as in Fig. 4. Bar equals 0.2 μm. × 27,000.

FIGURE 9   3T3 cell treated with 1.0 mM tetracaine for 5 min at 37°C and embedded *in situ*. Bar equals 0.2 μm. × 25,000.
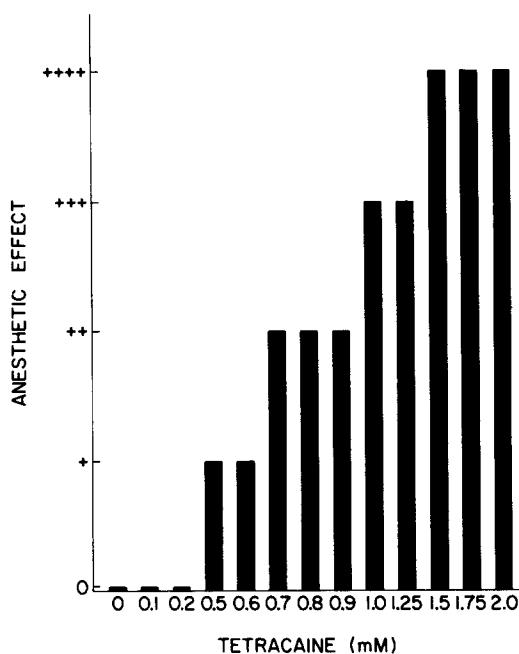
FIGURE 10 Dose-response of 3T3 cell morphology to tetracaine. Cells were incubated for 30 min at 37°C in various concentrations of tetracaine and scored for morphological alterations as follows: 0, normal morphology (see Fig. 1); +, some cell rounding (see Fig. 2); ++, ~50% of cells rounded; +++, ~100% of cells rounded (see Fig. 3); ++++, all cells detach from substrate.

DISCUSSION

The present experiments have shown that tertiary amine local anesthetics induce structural changes in cellular cytoskeletal systems, particularly those associated with the plasma membrane. These findings provide direct evidence to support recent proposals that the action of local anesthetics in modifying ligand-induced redistribution of surface receptors (37-39) results from the action of these drugs on membrane-associated cytoskeletal systems involved in transmembrane control of receptor mobility and topography (3, 9, 28-30). The evidence that local anesthetics can affect cellular cytoskeletal systems obtained in these earlier studies was indirect and was based on the observation that the action of local anesthetics in modifying cellular responses to lectins and antibodies could be mimicked by treating cells with drugs which act on microtubules, such as colchicine and the *Vinca* alkaloids, and by treatment with the microfilament disruptive drug, cytochalasin B (38, 39). Importantly, these two classes of drugs were only able to duplicate the action of local anesthetics when used together (38, 39), suggesting that local anesthetics were acting on both colchicine-sensitive microtubules and cytochalasin B-sensitive microfilaments. The present demonstration of anesthetic-induced changes in both microtubules and microfilaments provides experimental support for this proposal.

Ultrastructural alterations in microtubule structure in intact tissues after treatment with both local (17) and general (2) anesthetics have been described previously, and the local anesthetic lidocaine has been shown to impair microtubule assembly in vitro by inhibiting polymerization of tubulin subunits (17). To the best of our knowledge, however, changes in cellular microfilament systems induced by local anesthetics have not been described before, though a very recent report by Hinkley and Telser (19) indicates that halothane, a gaseous inhalational general anesthetic, causes microfilament breakdown in cultured mouse neuroblastoma cells.

The mechanism by which local anesthetics affect cellular cytoskeletal elements is unknown. The microfilament networks in several cell types have been shown to contain actomyosin components (reviewed in reference 1), and the action of local anesthetics in causing microfilament breakdown in 3T3 cells might be similar to the documented action of anesthetics in inhibiting actomyosin

membrane network of microfilaments and microtubules (Figs. 6-7). The addition of local anesthetics such as tetracaine (0.5-1 mM) caused dramatic changes in cell morphology and cytoskeletal organization within 15-30 min at 37°C. The normally flat 3T3 cells rounded up and appeared to contract, leading to the formation of numerous surface "blebs" (Fig. 5) which resembled the zeiotic protrusions produced by cytochalasin B (6) or cytochalasin D (15). These "blebs" lacked cytoskeletal elements and were irregular in shape (Figs. 5, 8, and 9). Within a few minutes after tetracaine treatment, in some cells the plasma membrane appeared to pull away from cytoskeletal elements, particularly at the edges of blebs (Fig. 9), while in other cells cytoskeletal structures could not be found (Fig. 8). Reduction in cytoskeletal organization was also seen in the interior cytoplasmic regions of the anesthetic-treated cells (Figs. 8 and 9). Again, some cells appeared to be more sensitive to the drugs than others, but all cells were affected at higher anesthetic concentrations.

contractile proteins from heart muscle (27, 41). Another feature of the pharmacological activity of local anesthetics, which might be relevant to their effects on microtubules, concerns the high affinity of these drugs for calcium ions (Ca^{2+}) and their capacity to displace Ca^{2+} from cellular membranes (reveiwed in reference 34). This property suggests that anesthetic-induced changes in the binding of Ca^{2+} to membranes (and membrane-associated structures) could perturb the linkage of integral membrane receptors within the membrane to cytoskeletal elements associated with the inner face of the plasma membrane. For example, the erythrocyte peripheral plasma membrane protein, spectrin, which exerts transmembrane control over the mobility of certain plasma membrane receptors (28, 30), is displaced from membranes by Ca^{2+}-chelating agents (24) and will only reassociate with lipid membranes in the presence of Ca^{2+} (21). In addition, the structural integrity of microtubules is particularly sensitive to Ca^{2+} concentration, and Ca^{2+} has been suggested to play a functional role in linking tubulin subunits (reviewed in 22, 32, 53). This raises the further possibility that local anesthetics could produce competitive inhibition of the Ca^{2+}-sensitive functions necessary for microtubule integrity. In this respect, the action of local anesthetics might be comparable to that of vinblastine, which has been shown to displace Ca^{2+} from tubulin subunits in vitro (54). Finally, anesthetic-induced release of Ca^{2+} from membranes might raise intracellular Ca^{2+} concentrations to levels ($> 10^{-5}$ M) sufficient to induce microtubule depolymerization (22, 32). None of the above mechanisms need be exclusive, and all three, together with others not identified here, could contribute to the observed alterations in cytoskeletal elements.

Previous interpretations of the effects of local anesthetics on cellular membrane have focused largely on the interactions of these drugs with membrane lipids (34, 35, 48, 49). The present results indicate, however, that these agents can also affect cellular cytoskeletal systems. In the light of these findings, the previously reported inhibitory effects of local anesthetics on such processes as cell spreading and motility (13, 43, 44), cell aggregation (52), endocytosis (43), axonal transport (5), exocytosis (8, 36), and effects on cell morphology (44, 49) might now be reasonably re-evaluated as possibly resulting (at least in part) from the action of these drugs on cellular cyto-skeletal systems rather than on membrane lipids alone, particularly since similar inhibition of these processes can be produced by treatment with drugs acting on microtubules and/or microfilaments (1, 8, 12, 25, 26, 36, 51).

SUMMARY

Tertiary amine local anesthetics (dibucaine, tetracaine, procaine) reversibly affect the morphology of untransformed BALB/3T3 cells and the organization of membrane-associated cytoskeletal elements. In the presence of these drugs cells contract and become rounded in shape with the appearance of numerous surface "blebs." Electron microscope examination of anesthetic-treated cells revealed significant reductions in plasma membrane-associated microtubules and microfilaments and/or their plasma membrane attachment. The relationship of the findings on local anesthetic-induced changes in cellular cytoskeletal systems is discussed in relation to previous proposals on plasma membrane organization and control of cell surface receptor topography and mobility.

REFERENCES

1. ALLISON, A. C., and P. DAVIES. 1974. Mechanisms of endocytosis and exocytosis. In Transport at the Cellular Level. M. A. Sleigh and D. H. Jennings, editors, Cambridge University Press, Cambridge. 419–446.
2. ALLISON, A. C., and J. F. NUNN. 1968. Effects of general anaesthetics on microtubules: a possible mechanism of anaesthesia. Lancet. 2:1326–1329.
3. BERLIN, R. D., J. M. OLIVER, T. E. UKENA, and H. H. YIN. 1974. Control of cell surface topography. Nature (Lond.). 247:45–46.
4. BLAUSTEIN, M. P., and D. E. GOLDMAN. 1966. Action of anionic and cationic nerve-blocking agents: experiment and interpretation. Science (Wash. D. C.). 153:429–432.

5. BYERS, M. R., B. R. FINK, S. J. KISH, and G. M. AASHEIM. 1975. Arrest of rapid axoplasmic transport in a sensory nerve *in vivo* by ligature or lidocaine treatment. *In* Progress in Anesthesiology. B. R. Fink, editor. Raven Press, New York. Vol. 1. 25-28.

6. CARTER, S. B. 1970. Cell movement and cell spreading: a passive or an active process? *Nature (Lond.).* 225:858-859.

7. CHEN, S. S. 1974. Studies of radiocalcium efflux in single barnacle muscle fibres. Effects of procaine and external divalent cations. *J. Physiol.* 238:313-328.

8. DOUGLAS, W. W. 1975. Exocytosis and the exocytosis-vesiculation sequence with special reference to neurohypophysis, chromaffin and mast cells, calcium and calcium ionophores. *In* Secretory Mechanisms of Exocrine Glands. N. A. Thorn and O. H. Petersen, editors, Academic Press, Inc., New York. 116-136.

9. EDELMAN, G. M. 1974. Origins and mechanisms of specificity in clonal selection. *In* Cellular Selection and Regulation in the Immune Response. G. M. Edelman, editor, Raven Press, New York. 1-38.

10. EYRING, H., J. W. WOODBURY, and J. S. D'ARRIGO. 1973. A molecular mechanism of general anesthesia. *Anesthesiology.* 38:415-424.

11. FEINSTEIN, M. B. 1964. Reaction of local anesthetics with phospholipids. A possible chemical basis for anesthesia. *J. Gen. Physiol.* 48:357-374.

12. FINK, B. R., M. R. BYERS, and M. E. MIDDAUGH. 1973. Dynamics of colchicine effects on rapid axonal transport and axonal morphology. *Brain Res.* 56:299-311.

13. GAIL, M. H., and C. W. BOONE. 1972. Procaine inhibition of fibroblast motility and proliferation. *Exp. Cell Res.* 63:252-255.

14. GIOTTA, G. J., D. S. CHAN, and H. H. WANG. 1974. Binding of local anesthetics to phosphatidylcholine and phosphatidylserine liposomes. *Arch. Biochem. Biophys.* 163:453-458.

15. GODMAN, G. C., A. F. MIRANDA, A. D. DEITCH, and S. W. TANENBAUM. 1975. Action of cytochalasin D on cells of established lines. III. Zeiosis and movements at the cell surface. *J. Cell Biol.* 64:644-667.

16. HANSCH, C., and S. M. ANDERSON. 1967. The structure-activity relationship in barbiturates and its similarity to that in other narcotics. *J. Med. Chem.* 10:745-753.

17. HASCHKE, R. H., M. R. BYERS, and B. R. FINK. 1974. Effects of lidocaine on rabbit brain microtubular protein. *J. Neurochem.* 22:837-843.

18. HAUSER, H., S. A. PENKETT, and D. CHAPMAN. 1969. Nuclear magnetic resonance spectroscopic studies of procaine hydrochloride and tetracaine hydrochloride at lipid-water interfaces. *Biochim. Biophys. Acta.* 183:466-475.

19. HINKLEY, R. E., JR., and A. G. TELSER. 1974. The effects of halothane on cultured mouse neuroblas-

20. HUBBELL, W. L., J. C. METCALFE, S. M. METCALFE, and H. M. MCCONNELL. 1970. The interaction of small molecules with spin-labelled erythrocyte membranes. *Biochim. Biophys. Acta.* 219:415-427.

21. JULIANO, R. L., H. K. KIMELBERG, and D. PAPHADJOPOULOS. 1971. Synergistic effects of a membrane protein (spectrin) and Ca^{2+} on the Na^+ permeability of phospholipid vesicles. *Biochim. Biophys. Acta.* 241:894-905.

22. KIRSCHNER, M. W., and R. C. WILLIAMS. 1974. The mechanism of microtubule assembly *in vitro. J. Supramol. Struct.* 2:412-428.

23. LEVER, M. J., K. W. MILLER, W. D. M. PATON, and E. B. SMITH. 1971. Pressure reversal of anaesthesia. *Nature (Lond.).* 231:368-371.

24. MARCHESI, V. T., and E. STEERS, JR. 1968. Selective solublization of a protein component of the red cell membrane. *Science (Wash. D.C.).* 159:203-204.

25. MASLOW, D., and E. MAYHEW. 1974. Histotypic cell aggregation in the presence of cytochalasin B. *J. Cell Sci.* 16:651-666.

26. MCCLURE, W. O. 1972. Effects of drugs upon axoplasmic transport. *Adv. Pharmacol. Chemother.* 10:185-220.

27. MERIN, R. G., T. KUMAZAWA, and C. R. HONIG. 1974. Reversible interaction between halothane and Ca^{2+} on cardiac actomyosin ATPase: mechanism and significance. *J. Pharmacol. Exp. Ther.* 190:1-14.

28. NICOLSON, G. L. 1974. The interactions of lectins with animal cell surfaces. *Int. Rev. Cytol.* 39:89-190.

29. NICOLSON, G. L. 1975. Concanavalin A as a quantitative and ultrastructural probe for normal and neoplastic cell surfaces. *In* Concanavalin A. T. K. Chowdhury and A. K. Weiss, editors, Plenum Publishing Corp., New York. 153-172.

30. NICOLSON, G. L. 1975. Restrictions on the lateral mobility of cell membrane components. *In* Functional Linkage in Biomolecular Systems. F. O. Schmitt, D. M. Schneider, and D. M. Crothers, editors, Raven Press, New York. 137-147.

31. O'BRIEN, J. R. 1962. Platelet aggregation. I. Some effects of the adenosine phosphates, thrombin, and cocaine upon platelet adhesiveness. *J. Clin. Pathol.* 15:446-455.

32. OLMSTED, J. B., J. M. MARCUM, K. A. JOHNSON, C. ALLEN, and G. G. BORISY. 1974. Microtubule assembly: some possible regulatory mechanisms. *J. Supramol. Struct.* 2:429-450.

33. PAPAHADJOPOULOS, D. 1970. Phospholipid model membranes. III. Antagonistic effects of Ca^{2+} and local anesthetics on the permeability of phosphatidylserine vesicles. *Biochim. Biophys. Acta.* 211:467-477.

34. PAPAHADJOPOULOS, D. 1972. Studies on the mechanism of action of local anesthetics with phospholipid

model membranes. *Biochim. Biophys. Acta.* **265**:169-186.

35. PAPAHADJOPOULOS, D., K. JACOBSON, G. POSTE, and G. SHEPERD. 1975. Effects of local anesthetics on membrane properties. I. Changes in the fluidity of phospholipid bilayers. *Biochim. Biophys. Acta.* **394**:504-519.

36. POSTE, G., and A. C. ALLISON. 1973. Membrane fusion. *Biochim. Biophys. Acta.* **300**:421-465.

37. POSTE, G., D. PAPAHADJOPOULOS, K. JACOBSON, and W. J. VAIL. 1975. Local anaesthetics increase susceptibility of untransformed cells to agglutination by concanavalin A. *Nature (Lond.).* **253**:552-554.

38. POSTE, G., D. PAPAHADJOPOULOS, K. JACOBSON, and W. J. VAIL. 1975. The effect of local anesthetics on membrane properties. II. Enhancement of the susceptibility of mammalian cells to agglutination by plant lectins. *Biochim. Biophys. Acta.* **394**:520-539.

39. POSTE, G., D. PAPAHADJOPOULOS, and G. L. NICOLSON. 1975. The effect of local anesthetics on transmembrane cytoskeletal control of cell surface receptor mobility and distribution. *Proc. Natl. Acad. Sci. U.S.A.* **72**: in press.

40. POSTE, G., and P. REEVE. 1972. Agglutination of normal cells by plant lectins following infection with non-oncogenic viruses. *Nat. New Biol.* **237**:113-114.

41. PRICE, H. L., T. OHNISHI, and G. S. PRESSMAN. 1975. Interaction of anesthetic agents with heart muscle proteins-mechanism of Ca^{2+}-halothane antagonism. *In* Progress in Anesthesiology. B. R. FINK, editor. Raven Press, New York, 617-622.

42. RABINOVITCH, M., and M. J. DE STEFANO. 1973. Manganese stimulates adhesion and spreading of mouse sarcoma I ascites cells. *J. Cell Biol.* **59**:165-176.

43. RABINOVITCH, M., and M. J. DE STEFANO. 1974. Macrophage spreading *in vitro*. III. The effect of metabolic inhibitors, anesthetics and other drugs on spreading induced by subtilisin. *Exp. Cell Res.*

88:153-162.

44. RABINOVITCH, M., and M. J. DE STEFANO. 1975. Cell to substrate adhesion and spreading: inhibition by cationic anesthetics. *J. Cell. Physiol.* **85**:189-194.

45. RITCHIE, J. M., and P. GREENGARD. 1966. On the mode of action of local anesthetics. *Ann. Rev. Pharmacol.* **6**:405-430.

46. ROTH, S., and P. SEEMAN. 1971. All lipid-soluble anaesthetics protect red cells. *Nat. New Biol.* **231**:284-285.

47. RYAN, G. B., E. R. UNANUE, and M. J. KARNOVSKY. 1974. Inhibition of surface capping of macromolecules by local anaesthetics and tranquillisers. *Nature (Lond.).* **250**:56-57.

48. SEEMAN, P. 1972. The membrane actions of anesthetics and tranquilizers. *Pharmacol. Rev.* **24**:583-655.

49. SHEETZ, M. P., and S. J. SINGER. 1974. Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. U.S.A.* **71**:4457-4461.

50. SKOU, J. C. 1954. Local anaesthetics. VI. Relation between blocking potency and penetration of a monomolecular layer of lipoids from nerves. *Acta Pharmacol. Toxicol.* **10**:325-337.

51. SPOONER, B. S., K. M. YAMADA, and N. K. WESSELLS. 1971. Microfilaments and cell locomotion. *J. Cell Biol.* **49**:595-613.

52. UNGAR, G. 1975. Effect of anesthetics on embryonic cell aggregation. *In* Progress in Anesthesiology. B. R. Fink, editor. Raven Press, New York. Vol. 1. 569-574.

53. WILSON, L., and J. BRYAN. 1974. Biochemical and pharmacological properties of microtubules. *Adv. Cell Mol. Biol.* **3**:21-72.

54. WILSON, L., J. BRYAN, A. RUBY, and D. MAZIA. 1970. Precipitation of proteins by vinblastine and calcium ions. *Proc. Natl. Acad. Sci. U.S.A.* **66**:807-814.