THE INHIBITION OF STEROL SYNTHESIS BY ANESTHETICS

Susan J. FRIEDMAN and Philip SKEHAN
Department of Pharmacology, University of Colorado Medical Center, 4200 East 9th Avenue, Denver, CO 80262, USA

Received 23 February 1979
Revised version received 20 April 1979

1. Introduction

Local anesthetics are drugs which possess the capacity to block to conduction of nerve action potentials by inhibiting changes in membrane sodium conductance [1]. They have a number of additional effects upon membrane structure and function as well. Local anesthetics can associate with both the lipids and proteins of cellular membranes [2–5], stabilize erythrocytes to osmotic shock [6–8], increase membrane volume [7], displace adsorbed calcium [7], inhibit membrane transport [9,10], decrease cell adhesiveness [11], inhibit cell fusion [12], and alter surface receptor mobility [13,14].

A broad range of compounds with very different chemical structures possess local anesthetic activity. These include aromatic amines, butyrophenones, cyclohexanones, steroids, and phenothiazines [7].

Although the rapid biophysical effects of local anesthetics upon nerve sodium conductance and erythrocyte membrane stabilization have been investigated in detail, little is known about their longer term effects upon the cellular metabolism of membrane molecules. Here we report that a variety of drugs with local anesthetic activity share the ability to inhibit cholesterol synthesis by rat C_6 glioma cultures.

2. Materials and methods

Rat C_6 glioma cells (American Type Culture Collection) were cultured in Eagle's MEM plus 5% fetal calf serum and passaged at weekly intervals following dissociation with 0.25% trypsin containing 1 mM EDTA.

For [^{14}C]acetate incorporation studies, cells were plated at 7.5×10^4 cells/cm^2 (~50% of confluency) in T75 tissue culture flasks 20 h prior to labeling. To initiate labeling, medium was changed and cultures subsequently incubated at 37°C in fresh medium containing [2-^{14}C]acetate (55 mCi/mmol; 0.5 μCi/ml). At the end of the labeling period, which was usually 6 h, medium was removed and the cell layers washed 3 times with NKT (137 mM NaCl, 5.4 mM KCl, 1 mM Tris, pH 7.4) then scraped off with a rubber policeman and pelleted at low speed in a desk top centrifuge. Pellets were stored frozen at −20°C until use, then extracted with 2:1 (v/v) chloroform–methanol, dried, and dissolved in 0.1 M NaOH for protein determination by the Lowry method [15]. The chloroform–methanol extracts were dried under vacuum, redissolved in a small volume of chloroform–methanol, and cochromatographed with authentic neutral lipid standards on silica gel G thin-layer plates in a solvent system consisting of benzene–diethyl ether–ethanol–water (50:40:2:0.2, by vol.), followed by diethyl ether–hexane (6:94, by vol.) [16]. Approximately equal amounts of radioactivity were applied for each sample in a given experimental group. After chromatography, plates were exposed to iodine vapor to localize the lipid standards and then to Kodak Blue Brand X-ray film at −20°C to localize radioactivity spots. Each spot was scraped from the thin-layer plate, hydrated with water, and counted in Triton X–toluene/PPO/POPOP (1:4, by vol.) in a Beckman liquid scintillation counter. In control cultures, all of the [^{14}C]acetate-derived radioactivity in the sterol fraction was present in desmosterol and cholesterol as determined by argentation chromatography [17].

Adiphenine was the gift of Ciba-Geigy, and chlor-

promazine compounds were donated by Smith, Kline and French Labs. Ketamine was purchased from Bristol Labs., lidocaine from Elkins-Sinn, haloperidol from McNeil Lab., and tetracaine from Sigma. Lipid standards were purchased from Supelco and from Steraloids.

3. Results

Rat C_6 glioma cells incorporate [2-^{14}C]acetate into chloroform–methanol-extractable material at a constant rate for $\geqslant$ 7.5 h at 37°C (fig.1). After 6 h incubation with isotope, the sterol fraction (desmosterol plus cholesterol) of control cultures contains 4–13% of total extractable radioactivity. By contrast, when cultures are incubated with anesthetics during the labeling period, the entry of [^{14}C]acetate into the sterol fraction is inhibited by 60–98% (fig.2,3).

The reduction of [^{14}C]acetate incorporation into the sterol fraction is accompanied by a redistribution of the isotope into other lipid fractions. The nature of this redistribution varies with the anesthetic employed. For example, tetracaine, lidocaine, and pentobarbital divert the isotope flow primarily into the triglyceride and cholesterol ester fractions, while chlorpromazine diverts it into an unidentified lipid which migrates close to cholesterol on thin-layer plates (fig.3).

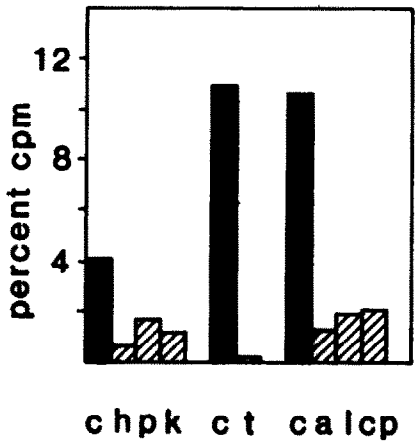

Fig.2. Inhibition of [2-^{14}C]acetate incorporation into sterols by anesthetic concentrations of drugs. Data is plotted as % of total lipid radioactivity present in sterols of control and drug-treated cultures. The results of 3 separate experiments are shown. Cultures in the first experiment (c,h,p,k) were drug-treated for 30 h prior to labeling. The other 2 groups were drug-treated and labeled for 6 h. *Abbreviations:* c, control; h, haloperidol (10^{-4} M); p, Na-pentobarbital (10^{-3} M); k, ketamine (10^{-3} M); t, tetracaine (5×10^{-4} M); a, adiphenine (3×10^{-4} M); l, lidocaine (2.5×10^{-3} M); cp, chlorpromazine (10^{-6} M).

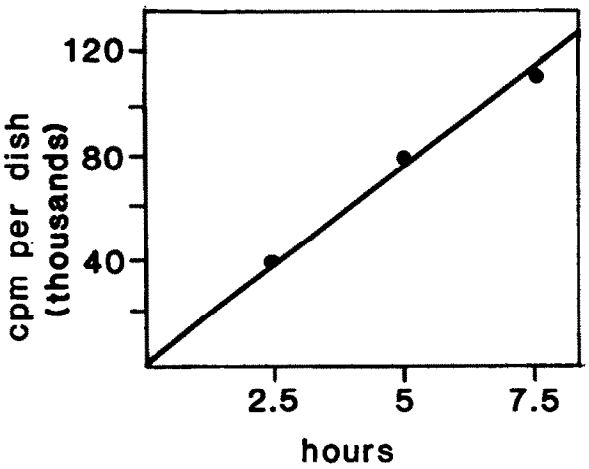

Fig.1. Time course of [2-^{14}C]acetate incorporation into cellular lipids. C_6 glioma cultures labeled with 0.5 μCi/ml [2-^{14}C]acetate (55 mCi/mmol) were analyzed for protein content and chloroform–methanol extractable radioactivity at the times indicated. Protein content/dish remained at ~0.1 mg during the labeling period. Values for duplicate samples were within 10% of the average shown.

The inhibition by anesthetics of [^{14}C]acetate incorporation into cholesterol and desmosterol appears to be a selective effect. Even after 24 h drug treatment the incorporation into total chloroform–methanol-extractable material is not inhibited (data not shown). Moreover, with the possible exceptions of tetracaine and pentobarbital, none of the anesthetics which we employed inhibited the synthesis of neutral lipids other than sterols.

The inhibition of sterol synthesis by the local anesthetics develops within 1–3 h after drug addition (fig.4). The effect is produced by anesthetic concentrations which are non-toxic even after 48 h continuous incubation. We have verified by electron microscopy that cells treated with anesthetics are ultrastructurally normal, and differ from controls only in containing an elevated number of osmiophilic droplets which we presume contain lipids.

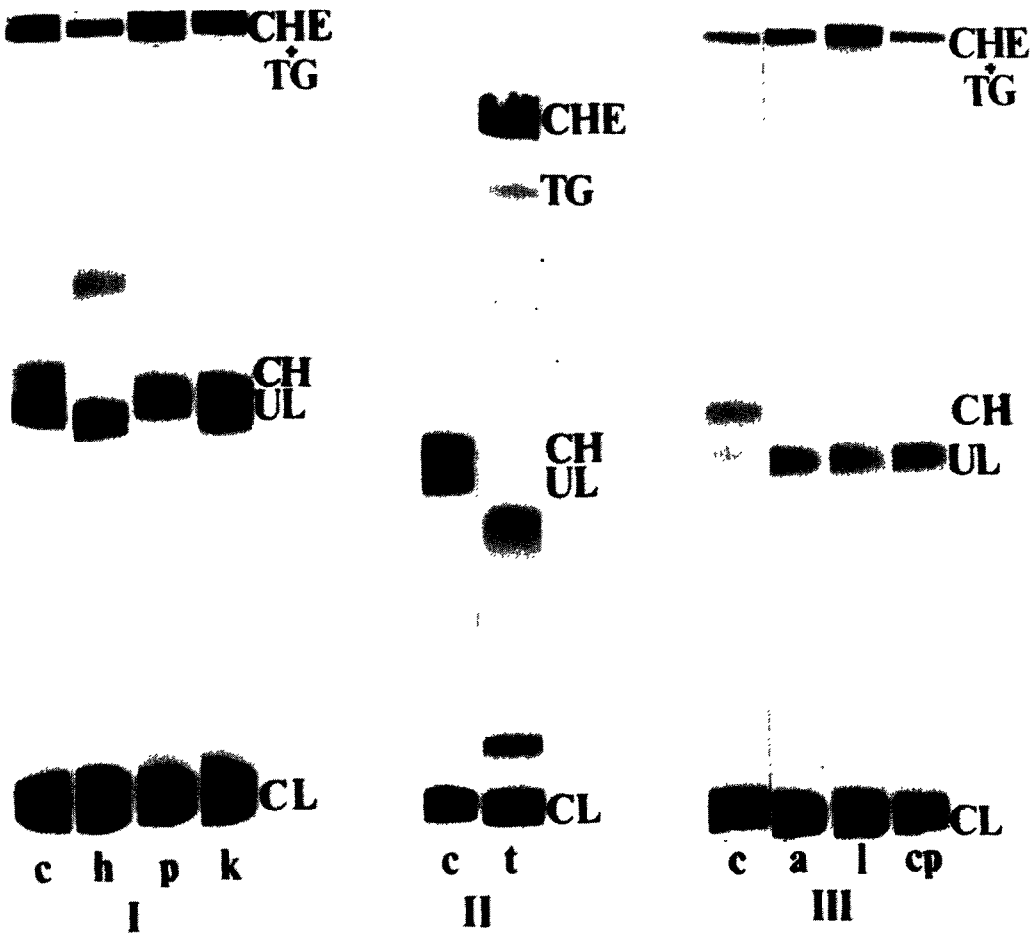

Fig.3. Autoradiographs of thin layers from experiments shown in fig.2. *Abbreviations:* CL, complex lipids; UL, unidentified lipid; CH, cholesterol plus desmosterol; TG, triglycerides; CHE, cholesteryl esters.

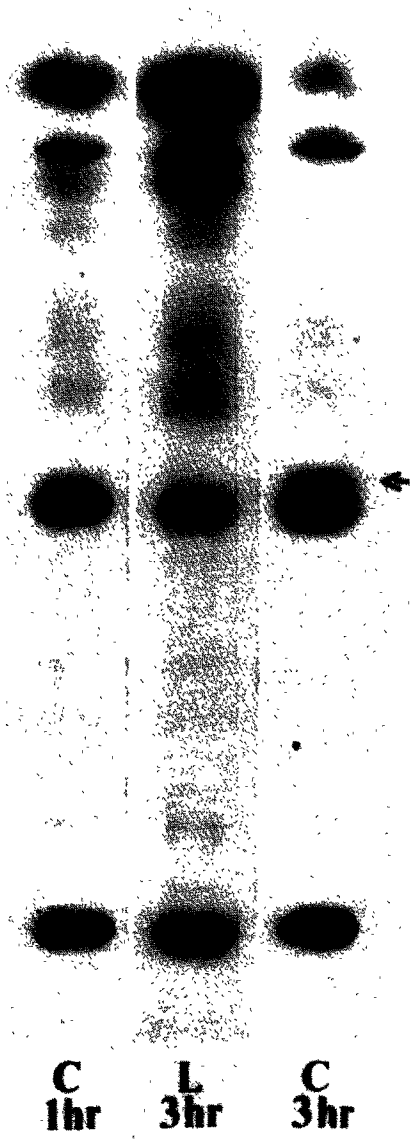

Fig.4. Time course of inhibition of [2-^{14}C]acetate incorporation into sterols by lidocaine. The incorporation of [2-^{14}C]-acetate into sterols was monitored at 1 h, 3 h and 5 h after addition of isotope (1 μCi/ml) to cultures which were simultaneously exposed to growth medium minus drug (C, control) or to growth medium + 2.5 × 10^{-3} M lidocaine (L, + lidocaine). The lipid extracts were chromatographed on thin layers and exposed to X-ray film. Results at 5 h were identical to those at 3 h. Desmosterol and cholesterol, the only sterols which are significantly labeled in gliomas, migrate as 1 band in this solvent system (indicated by the arrow).

4. Discussion

Current theories about the mechanisms of anesthesia have focussed primarily upon the biophysical consequences of anesthetic interaction with membrane lipids and proteins. One hypothesis postulates that anesthetics act by producing localized phase changes in membrane lipid domains [18–20]. A second hypothesis proposes that anesthetics act by direct association with amphipathic membrane proteins, altering their conformation and biological activity [21].

This investigation demonstrates that anesthetics are potentially capable of altering membrane properties by yet a third mechanism, one that is biochemical rather than biophysical and involves the inhibition of sterol synthesis.

A variety of drugs which chemically and pharmacologically are very different, but which all possess local anesthetic activity, were found to inhibit sterol synthesis by rat C_6 glioma cells. Effective agents which we have tested include aromatic amines (adiphenine, lidocaine, tetracaine), barbiturates (sodium pentobarbital), butyrophenones (haloperidol), cyclohexanones (ketamine) and phenothiazines (chlorpromazine) (fig.5).

It is unclear at present whether these several drugs all act by the same or by different mechanisms. Cholesterol biosynthesis can be interrupted at multiple sites [22,23] and even drugs which are close structural analogs of one another can inhibit the pathway at different sites [24]. However, the mechanism(s) by

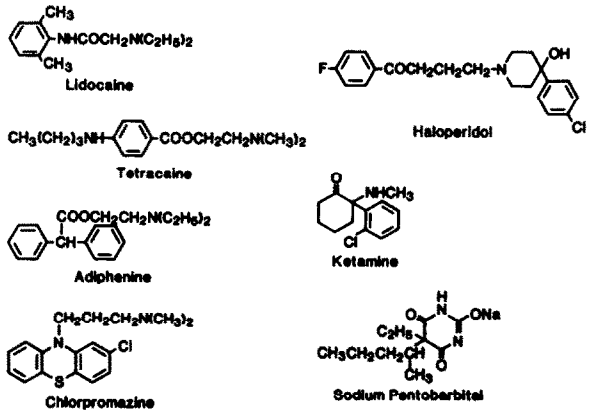

Fig.5. Structures of drugs used in this study.

which local anesthetics inhibit C_6 sterol synthesis is selective in that [^{14}C]acetate incorporation into total cellular lipids is unaltered. It is unlikely, therefore, that local anesthetics inhibit sterol synthesis by a general mechanism such as interference with acetate activation.

The inhibition of sterol synthesis by the anesthetics does not appear to involve exclusively hydrophobic or polar interactions, since drugs which interact with membranes in a predominantly hydrophobic manner (tetracaine and the phenothiazines) and drugs which interact in a predominantly polar manner (lidocaine) are both effective inhibitors [25–27]. Further, inhibitory activity is shown by drugs which are predominantly charged at the pH employed in our experiments (pH 7.2) (aromatic amines) and by drugs which are predominantly unionized (pentobarbital). Thus the mechanism of action would not appear to depend upon whether a drug is charged or neutral. Inhibition is produced by molecules in which the polar amine moiety forms part of an aliphatic ring structure (haloperidol), or is attached to an aliphatic ring (ketamine), or is contained in a flexible aliphatic chain of varying length and structure (lidocaine, tetracaine, adiphenine, chlorpromazine).

The relative order of potency of anesthetics employed here in inhibiting C_6 glioma sterol synthesis closely parallels that reported for their nerve conduction blockage [6] and their osmotic stabilization of erythrocyte membranes [6–8] (table 1).

The only obvious characteristic which all of these drugs have in common is their amphipathic structure. Not all amphipaths have anesthetic activity, however. Octanoic acid, a fatty acid which is reportedly devoid of anesthetic activity [28], also failed to inhibit cholesterol synthesis at 1.5×10^{-3} M, the lowest concen-

Table 1
Comparison of relative order of potency of anesthetics in nerve conduction blockage, protection of erythrocytes against hemolysis, and inhibition of C_6 glioma sterol synthesis

Anesthetic	Drug concentration (mol/l) required for		
	Nerve block[a]	Antihemolysis[b]	Sterol synthesis inhibition[c]
Chlorpromazine	1×10^{-5}	2×10^{-5}	0.5×10^{-5}
Haloperidol		2.2×10^{-5}	2×10^{-5}
Chlorpromazine methiodide		6×10^{-5}	5×10^{-5}
Tetracaine	6.8×10^{-5}	5×10^{-4}	3×10^{-5}
Adiphenine			5×10^{-5}
Na-pentobarbital	1.7×10^{-3}	3×10^{-4}	1×10^{-3}
Ketamine			1×10^{-3}
Lidocaine	3.8×10^{-3}	1.8×10^{-2}	2.5×10^{-3}

[a] Minimum concentration for conduction blockage in frog sciatic nerve at 22°C [7]
[b] Concentration for 50% antihemolysis of human red blood cells assayed for 10 min [6,8]
[c] The concentration for 50% inhibition of [2-^{14}C]acetate incorporation into glioma sterols was determined by treating cell cultures with 4–5 concentrations of each drug, separating sterols from other neutral lipids by thin-layer chromatography, and quantitating isotope incorporation into sterols either by liquid scintillation counting of the thin layers (for lidocaine and chlorpromazine samples) or by comparing density of film exposure for drug-treated and control samples (all other drugs). Densities were ranked as ± (little or no inhibition), ++ (50% inhibition), +++ (75% inhibition) or ++++ (100% inhibition). Only one concentration was tested for Na-pentobarbital (10^{-3} M gave 60% inhibition) and for ketamine (10^{-3} M gave 70% inhibition). The sensitivity of gliomas to drug inhibition of sterol synthesis varied with different batches of cells, but the relative potency order for drugs remained the same as shown in the table

tration which was non-toxic to gliomas. Thus, at the present time we suggest that whatever their molecular mechanism(s) of action might be, both a hydrophobic and polar moiety on a single molecule are required for the inhibition of cholesterol synthesis by anesthetic drugs.

Acknowledgements

We wish to thank Dr Albert Vatter for performing the electron microscopy, and Ms Judie Prasad for her excellent technical assistance. This work was supported by NIH grant CA-16154.

References

[1] Hille, B. (1966) Nature 210, 1220–1222.
[2] Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 265, 169–186.
[3] Feinstein, M. B. (1964) J. Gen Physiol. 48, 357–374.
[4] Denburg, J. L., Eldefrawe, M. E. and O'Brien, R. (1972) Proc. Natl. Acad. Sci. USA 69, 177–181.
[5] Sobel, A., Heidmann, T., Hofler, J. and Changeux, J. P. (1978) Proc. Natl. Acad. Sci. USA 75, 510–514.
[6] Roth, S. and Seeman, P. (1971) Nature New Biol. 231, 284–285.
[7] Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
[8] Elferink, J. G. R. (1977) Biochem. Pharmacol. 26, 2411–2416.
[9] Feinstein, M. B., Volpi, M., Perrie, S., Makriyannis, A. and Sha'ati, R. I. (1977) Mol. Pharmacol. 13, 840–851.
[10] Lacko, L., Wittke, B. and Lacko, I. (1977) J. Cell. Physiol. 92, 257–264.
[11] Giddon, D. B. and Lindhe, J. (1972) Am. J. Pathol. 68, 327–338.
[12] Poste, G. and Reeve, P. (1972) Exp. Cell Res. 72, 556–560.
[13] Ryan, G. B., Unanue, E. R. and Karnovsky, M. J. (1974) Nature 250, 56–57.
[14] Poste, G., Papahadjopoulos, D., Jacobson, K. and Vail, W. J. (1975) Nature 253, 552–554.
[15] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[16] Freeman, C. P. and West, D. (1966) J. Lipid Res. 7, 324–327.
[17] Copius-Peereboom, J. W. and Beekes, H. W. (1965) J. Chromatog. 17, 99–113.
[18] Lee, A. G. (1977) Mol. Pharmacol. 13, 474–487.
[19] Tsong, T. Y., Greenberg, M. and Kanehisa, M. I. (1977) Biochemistry 16, 3115–3121.
[20] Papahadjopoulos, D., Jacobson, K., Poste, G. and Shepherd, G. (1975) Biochim. Biophys. Acta 394, 504–519.
[21] Boggs, J. M., Roth, S. M., Young, T., Wong, E. and Hsia, J. C. (1976) Mol. Pharmacol. 12, 136–143.
[22] Steinberg, D. (1962) Adv. Pharmacol. 1, 59–159.
[23] Dempsey, M. E. (1968) Ann. NY Acad. Sci. 148, 631–646.
[24] Braun, G. A. (1969) Adv. Exp. Med. Biol. 4, 473–482.
[25] Fernández, M. S. and Cerbón, J. (1973) Biochim. Biophys. Acta 298, 8–14.
[26] Blaustein, M. P. and Goldman, D. E. (1966) Science 153, 429–432.
[27] Giotta, G. J., Chan, D. S. and Wang, H. H. (1974) Arch. Biochem. Biophys. 163, 453–458.
[28] Roth, S. and Seeman, P. (1972) Biochim. Biophys. Acta 255, 207–219.