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INHIBITION OF PHOSPHOLIPASE A-INDUCED SWELLING OF MITOCHONDRIA BY LOCAL ANESTHETICS AND RELATED AGENTS

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Abstract—Phospholipase A was used to evaluate effects of local anesthetics and related agents on mitochondrial membrane conformation by recording the onset and rate of swelling following addition of the enzyme. Promethazine, chlorpromazine, cincaine, propranolol and butacaine in 3×10^{-5} to 2×10^{-4} M concentrations inhibited the swelling, their potency decreasing in the above-mentioned order. Laurylamine in 5×10^{-5}M concentration also increased the lag before onset of swelling in contrast to lauric acid, which like other anionic uncoupling agents shortened the lag. Spermine and spermidine in 10^{-5} M concentrations strongly inhibited swelling, the former being more potent, while polybrene, 1,4-diaminobutane and 1,5-diaminopentane were inactive. The agents which inhibit swelling also inhibited formation of lysolecithin and free fatty acids from mitochondria under influence of phospholipase A. No inhibition was found when detergent-dispersed mitochondria served as substrate for the enzyme. These results are discussed in relation to membrane conformation and charge distribution.

MITOCHONDRIA are composed of membranes whose functional and conformational states are intimately related. Conformational changes in membrane proteins would affect protein–protein and protein–phospholipid interactions. It is evident that such interactions, which may be allosteric,[1] could profoundly affect membrane permeability characteristics and the activities of membrane-bound enzymes.

Weinbach *et al.*[2–5] have indeed produced enzymatic and electron-microscopic evidence that various uncoupling agents change the membrane conformation. Using rat liver mitochondria as substrate for snake venom phospholipase A and proteolytic enzymes, they found lysis to be accelerated by uncoupling agents.

Many aromatic and aliphatic amines affect membrane-bound activities and may have strong pharmacological actions. Thus local anesthetics, including phenthiazines,[6] and also polyamines,[7,8] enhance the energy-dependent accumulation of divalent cations in mitochondria. Laurylamine and chlorpromazine are reported also to uncouple oxidative phosphorylation from respiration.[9] All these substances are known to be bound by phospholipids[10–13] and may exert their action on biological membranes by complexing with phospholipids[8,14] and proteins.[15] It was therefore of interest to study the subtle changes induced by the above-mentioned substances in the localization and state of membrane phospholipids by subjecting the membranes to enzymatic digestion by added phospholipase A.

MATERIALS AND METHODS

Rat liver mitochondria were isolated as described previously.[16] Their protein content was estimated by the Lowry method.[17] *Crotalus adamanteus* venom was used as a

source of phospholipase A (EC 3.1.1.4). In control experiments a purified preparation from *Vipera russelli* gave identical results. These preparations as well as cardiolipin, phosphatidylcholine and phosphatidylethanolamine were obtained from Koch & Light Co Ltd, Colnbrook, England. Butacaine sulphate was obtained from L & K Laboratories, Plainview, N.Y., Polybrene from Abbott Laboratories, North Chicago, Ill. and rotenone from Sigma Chemical Co, St Louis, Miss. Propranolol was kindly supplied by Dr V. Manninen, FCCP by Dr P. G. Heutler and the various phenthiazines by Orion Oy, Helsinki. Laurylamine, lauric acid and the hydrochlorides of 1,4-diaminobutane, 1,5-diaminopentane, spermidine and spermine were obtained from Fluka AG, Buchs, Switzerland.

Mitochondria were preincubated for 5 min at 25° in 225 mM mannitol, 75 mM sucrose, 10 mM tris, 5 μM rotenone, pH 7·9 before the addition of phospholipase. The swelling of mitochondria was followed by recording changes in light transmission using the Aminco-Chance dual wavelength spectrophotometer (American Instruments Co, Silver Springs, Md) set at 520 nm with the other wavelength excluded, slit 0·5 mm, light path 1·00 cm downward deflection indicating swelling.

Thin-layer chromatography of phospholipids was carried out on Silica-Gel plates F254, Merck AG, Darmstadt, Germany, in one dimension using a solvent mixture of chloroform–methanol–water (70:30:5) or in two dimensions.[18] For this the reaction was stopped by addition of 13 ml of a methanol–chloroform (8:5) mixture to 3 ml of the mitochondrial suspension with vigorous mixing. Then 11 ml chloroform and 3 ml water were added and after shaking the chloroform layer was filtered, 1–1·5ml methanol was added and the solvents evaporated under a stream of nitrogen. The residue was dissolved in chloroform–methanol (2:1) and a suitable aliquot applied to a plate which had been activated by heating 30 min at 120°. Phospholipids were visualized by immersing the plate in a tank with iodine vapours.

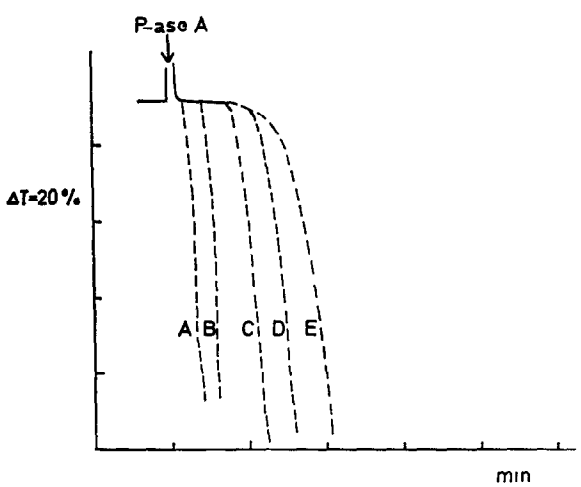

FIG. 1. Effect of varying the amount of mitochondria on the swelling induced by phospholipase A. 20 μl of a 0·2% solution of *Crotalus adamanteus* venom (P-ase A) was added to a final volume of 3·0 ml. Mitochondrial protein in A 0·43 ml, in B 0·72 mg/ml, in C 1·43 mg/ml, in D 2·15 mg/ml and in E 2·86 mg/ml.

RESULTS

Under suitable conditions, addition of phospholipase A to a mitochondrial suspension caused an extensive swelling, which set in after a short lag, Fig. 1, as previously shown by Weinbach and Garbus.[5] The lag can be prolonged by using more concentrated mitochondrial suspensions, i.e. by increasing the substrate concentration. The length of the lag is thus inversely proportional to the enzyme activity when the mitochondrial concentration is kept constant.

When the mitochondria had been preincubated with butacaine, propranolol, cincaine, chlorpromazine or promethazine, the lag was prolonged, the potency of the

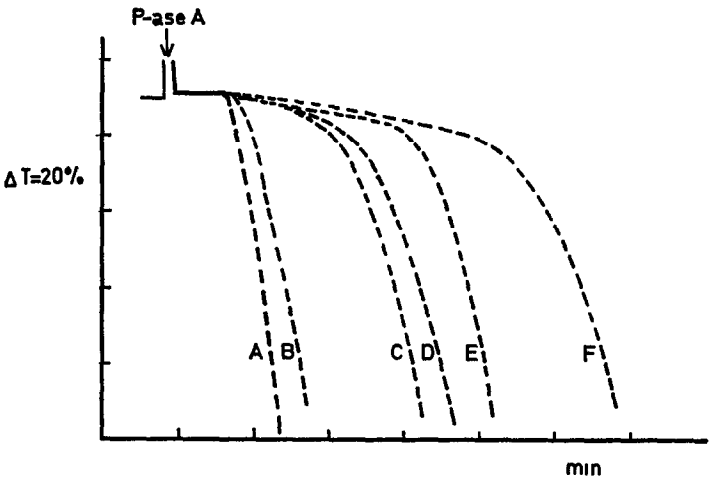

FIG. 2. Effect of propranolol and phenthiazines on the swelling of mitochondria induced by phospholipase A. A is the control, in B 67 μM butacaine, in C 33 μM propranolol, in D 33 μM cincaine, in E 33 μM chlorpromazine and in F 33 μM promethazine were present. Mitochondrial protein 1·4 mg/ml.

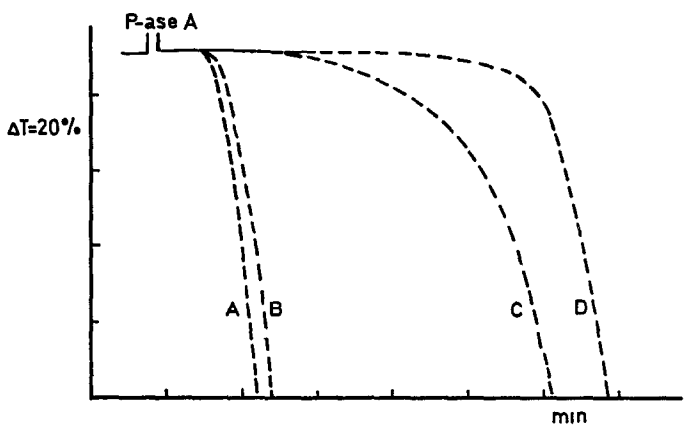

FIG. 3. Effect of various amines on phospholipase A-induced swelling of mitochondria. A is the control, 17 μM diaminobutane and 10 μg/ml polybrene gave an identical response. In B 17 μM diaminopentane, in C 17 μM spermidine and in D 17 μM spermine were present. Experimental conditions as in Fig. 2.

substances increasing in the above-mentioned order, Fig. 2. The rate of swelling was only slightly inhibited. However, with high enough concentrations of the more potent agents, swelling was fully abolished, Figs. 8 and 10. In control experiments it was found that, when these substances were added after the swelling had already set in, the rate of swelling was largely unaffected (not shown). The polyamines spermidine and especially spermine had similar effects at low concentrations, while diaminobutane and diaminopentane were almost inert, Fig. 3.

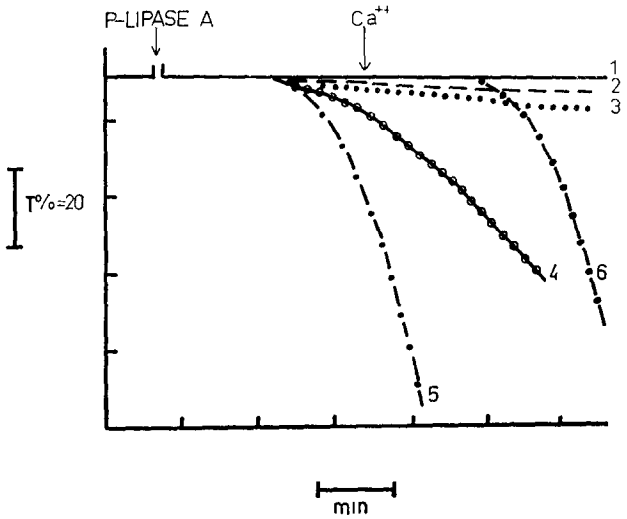

FIG. 4. Effects of EDTA, calcium ions and chlorpromazine on the phospholipase A-induced swelling of mitochondria. (1) 250 μM EDTA present. (2) 167 μM chlorpromazine present. (3) 167 μM chlorpromazine and 500 μM calcium chloride present. (4) control. (5) 500 μM calcium chloride present. (6) 250 μM EDTA present, 500 μM calcium chloride added were indicated. Mitochondrial protein 0·57 mg/ml.

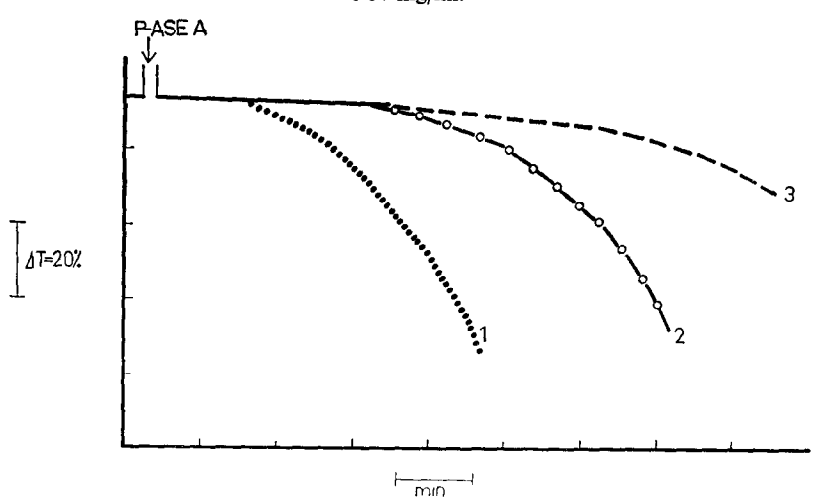

FIG. 5. Effect of the order of addition of calcium ions and propranolol on the phospholipase A-induced swelling of mitochondria. (1) Control. (2) 50 μM calcium chloride added 30 sec before the addition of 100 μM propranolol. (3) 100 μM propranolol added 30 sec before the addition of 50 μM calcium chloride. Mitochondrial protein 0·57 mg/ml.

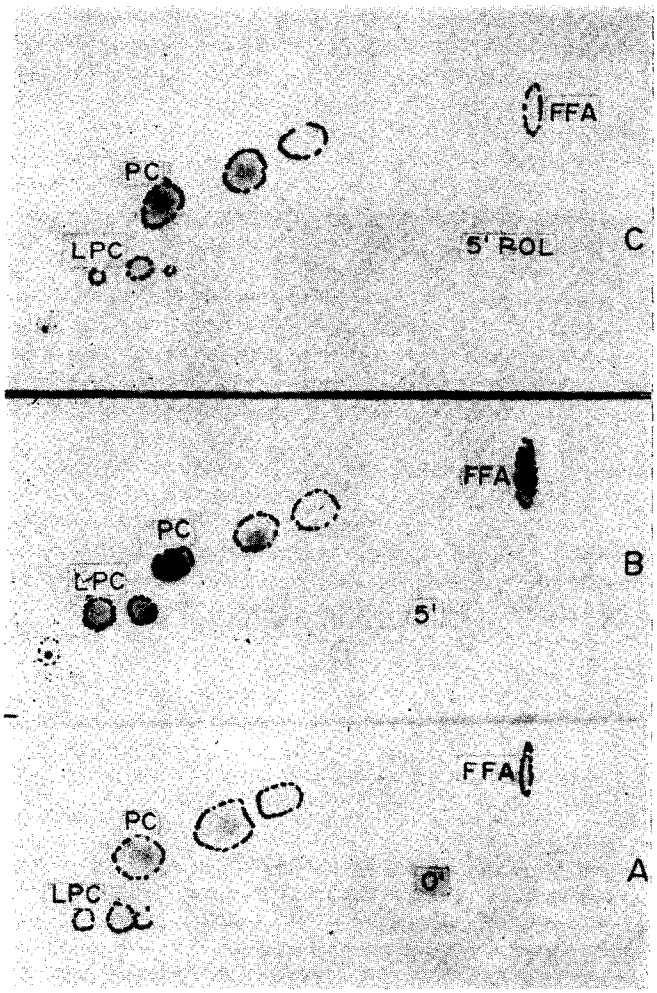

FIG. 6. Inhibition of propranolol of the formation of lysolecithin and free fatty acids during incubation of mitochondria with phospholipase A. P-OL = propranolol, PC = lecithin, LPC = lysolecithin, FFA = free fatty acids. Experimental conditions as in Fig. 1, mitochondrial protein 1·1 mg/ml. An amount phospholipids corresponding to half the extract from one incubation mixture was applied to the chromatographic plate. A. Control without incubation. B. Incubated for 5 min with phospholipase A. C. Incubated for 5 min with phospholipase A in the presence of 150 μM propranolol.

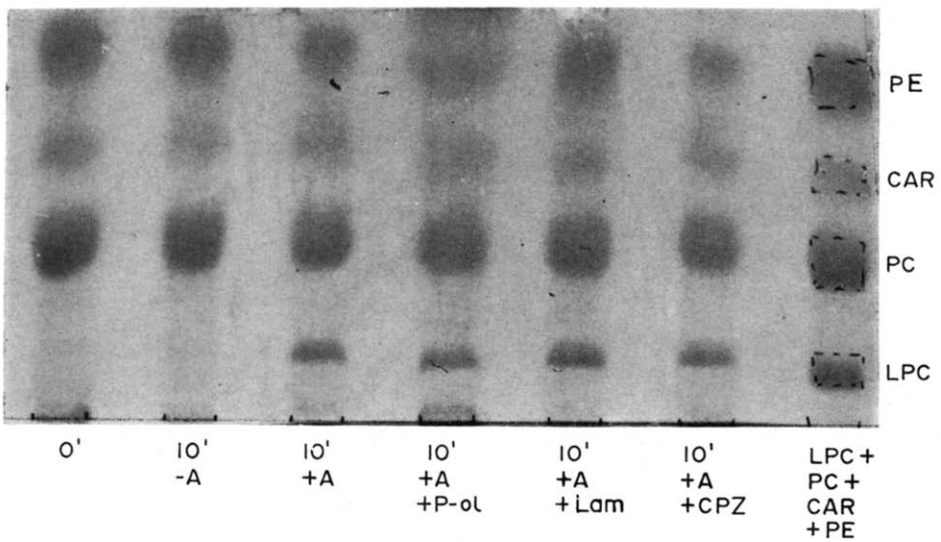

FIG. 7. Formation of lysolecithin from Triton X-100 treated mitochondria by the action of added phospholipase A. 100 μl of the mitochondrial suspension was treated with 50 μl 0·5 per cent solution of Triton X-100 before addition to the incubation mixture. The incubation time was 10 min, in other respects experimental conditions were as in Fig. 6. A = phospholipase A, P-ol = 150 μM propranolol, CPZ = 150 μM chlorpromazine, Lam = 50 μM laurylamine PC = lecithin, LPC = lysolecithin, CAR = cardiolipin, PE = phosphatidylethanolamine.

Since calcium ions are known to stimulate phospholipase A[19] activity, it was not surprising to obtain inhibition of swelling by addition of EDTA and release of inhibition by subsequent addition of calcium ions, Fig. 4. If, however, inhibition is produced by a substance such as chlorpromazine at the rather high concentration of 167 μM swelling remains inhibited after addition of calcium ions. The interaction of propranolol or phenthiazines and calcium ions is rather complex, calcium ions present in excess of 50 μM prevent the inhibition in a non-competitive manner. The order of addition of calcium and the inhibiting substance has a great effect, calcium being more potent when added before the agents, Fig. 5.

Figure 6 shows that lysolecithin and free fatty acids are produced by the action of phospholipase A on intact mitochondria. These have been separated by thin-layer chromatography. Propranolol in a concentration sufficient to prevent swelling greatly inhibited the hydrolysis. The same was found with the other compounds tested. Nor was there any demonstrable increase in lysolecithin and free fatty acids during incubation in the absence of added snake venom. This indicates that mitochondrial endogenous phospholipase A activity is absent in the incubation conditions.

In another series of experiments mitochondria were solubilized by Triton X-100 before addition of phospholipase A. In these conditions the substances used in this study did not inhibit the formation of lysolecithin and free fatty acids, Fig. 7. This clearly shows that the enzyme activity *per se* is not inhibited. Since there is a clearcut inhibition when intact mitochondria serve as substrate of the enzyme, it is likely that the inhibition is due to changes in membrane properties.

As previously shown by Weinbach and Garbus,[5] the so-called classical uncouplers,[20] like FCCP and DNP, accelerate the swelling of mitochondria induced by phospholipase A. It is seen in Fig. 8 that the anionic uncoupler lauric acid has an analogous effect, while an inhibition of swelling is found with the same amount of laurylamine. In this respect laurylamine behaves like chlorpromazine and the other agents studied by us. Laurylamine and chlorpromazine have been regarded as uncouplers of oxidative

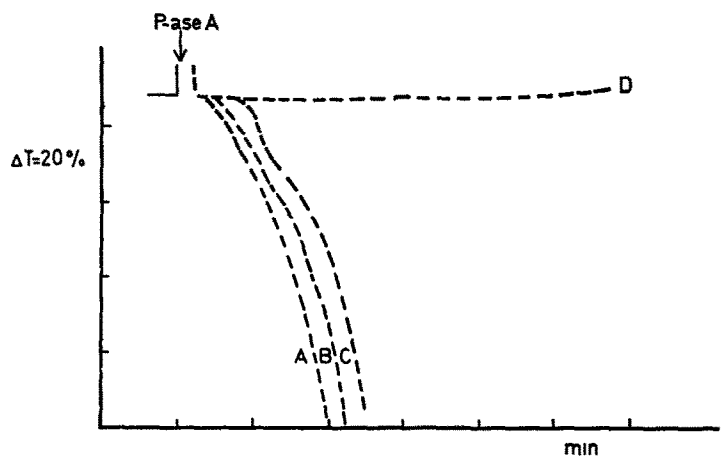

FIG. 8. Effects of laurylamine and lauric acid on the phospholipase A-induced swelling of mitochondria. A. 1 μM FCCP present, B. 100 μM lauric acid present, C. Control, D. 100 μM laurylamine or 167 μM chlorpromazine present. Experimental conditions as in Fig. 1, protein 2·0 mg/ml.

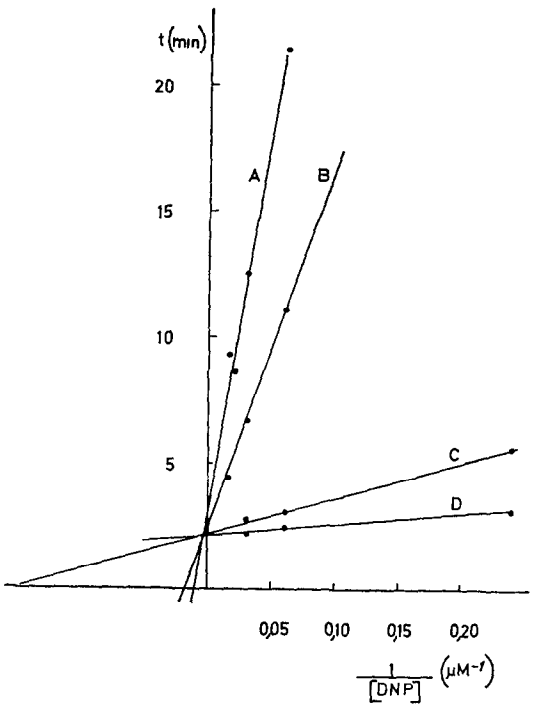

FIG. 9. Lineweaver–Burk plot of the interaction between chlorpromazine and DNP. The time needed to reach 50 per cent increase in optical transmission is plotted against the inverse concentration of DNP. Experimental conditions as in Fig. 1, protein 1·4 mg/ml. Chlorpromazine concentration in A 67 μM, B 50 μM, C 33 μM and D 17 μM.

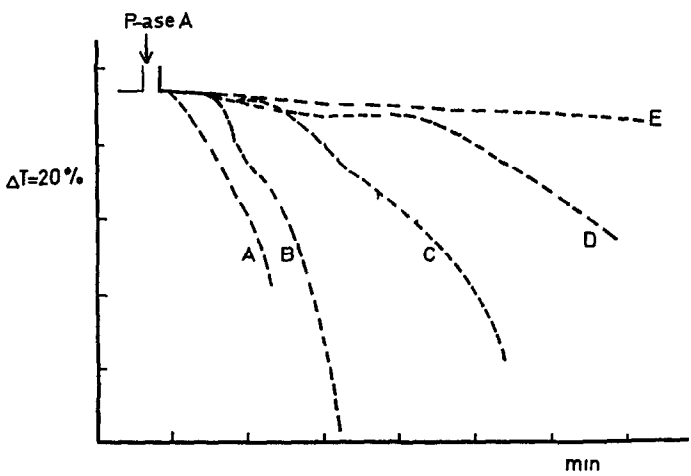

FIG. 10. Effect of the order of addition of FCCP and propranolol on the phospholipase A-induced swelling of mitochondria. Experimental conditions as in Fig. 1, protein concentration 2·0 mg/ml, 1 μM FCCP, 167 μM propranolol. A. FCCP alone, B. control, C. FCCP added prior to propranolol, D. propranolol added prior to FCCP, E. propranolol alone.

phosphorylation[9] even though their mode of action is obscure. It was thus of considerable interest to study the interaction of the lag-prolonging and lag-shortening agents. It is probable that the interaction is of a competitive nature, as judged from a Lineweaver–Burk plot, Fig. 9. The order of additions was again found to be of great importance. When propranolol was added before FCCP, the lag was much longer than when FCCP had been added first, Fig. 10. Similar results were obtained when chlorpromazine, butacaine, cincaine, promethazine or laurylamine was used instead of propranolol, or when FCCP was replaced by DNP.

DISCUSSION

In this study we have found that a number of lipophilic substances capable of becoming positively charged make mitochondria resistant towards lysis caused by added phospholipase A. The simplest explanation for this would be inhibition of the enzyme activity *per se*, however, no inhibition was found when phospholipase A was permitted to hydrolyze phospholipids in mitochondria disrupted by a non-ionic detergent, Fig. 7. This also makes it very unlikely, that these substances would act by displacing the activating calcium ions from the enzyme. Any effects of these agents are thus most likely the result from their interaction with mitochondrial membranes.

Many of the substances studied stabilize membranes, including mitochondria, against various swelling-inducing agents.[11,21] It may be argued that it is the swelling induced by the reaction products of phospholipase A, e.g. lysolecithin and free fatty acids,[22] that is inhibited, rather than the enzyme activity. However, we have clearly shown, Fig. 6, that the production of lysolecithin and free fatty acids by the action of phospholipase A on mitochondria is inhibited by propranolol and the other substances studied. Thus it seems evident that the effects are due to changes in the mitochondrial membranes resulting in resistance towards the action of phospholipase A.

Local anesthetics displace calcium from erythrocyte membranes[23,24] and may do so also in mitochondrial membranes. This would increase the calcium concentration in the medium, at least slightly, resulting in a stimulation of phospholipase A activity.[25] However, the opposite was found.

Jacobus and Brierley[15] found calcium ions to be bound mainly by phospholipids, the binding being non-competitively inhibited by zinc ions, which are preferentially bound to protein binding sites. They also showed that the binding of zinc ions is competitively inhibited by promethazine and chlorpromazine. We have found that calcium ions and local anesthetics interact in a noncompetitive manner. The great effect of the order of addition of calcium ions and the agents tested upon the susceptibility of mitochondria towards lysis induced by phospholipase A, may be due to changes in membrane binding sites induced by calcium ions and the various drugs, respectively.

The distribution and density of fixed negative charges in the membrane are probably of paramount importance in determining its conformational and permeability properties.[26] The agents used in this study are more or less positively charged and in addition contain hydrophobic regions. It is therefore conceivable that they accumulate in and at the membrane and associate with negatively charged groups of phospholipids and proteins. Hauser *et al.*[27] have indeed produced evidence that the interaction

of the chemically related local anesthetics procaine and tetracaine with phospholipid micelles is mainly electrostatic with some contribution from Van der Waals dispersion forces. By this mechanism the molecular organization and conformation of the membrane would necessarily be altered, which would be manifested as an altered response to the action of phospholipase A.[28] The phospholipase activities are susceptible to changes in the surface charge of their substrates.[29,30] We have also found a decrease in the net surface charge density of mitochondria in the presence of propranolol and related agents.[31] The changed properties of the membranes are also demonstrable as an expanded membrane volume.[23, 32–34]

A change in the density and distribution of fixed negative charges and in the conformation of the membrane readily accounts for the observed stimulation by local anesthetics[6] and by polyamines[7,8] of the energized uptake of divalent and monovalent[14] cations by mitochondria. It is interesting that divalent cations, which by binding to the membranes also would neutralize fixed negative charges, have similar stimulating effects on cation transport.[35,36] The same mechanism might lead to diminished uptake of anions, which might explain the competition between anionic uncouplers and the agents tested, Fig. 9. This mechanism is also in accordance with the explanation put forward by Weinbach and Garbus[5] for the stimulation of swelling by uncoupling agents.

It is noteworthy that chlorpromazine and laurylamine changed the response of the mitochondria towards phospholipase A in the opposite direction than classical uncouplers, including lauric acid, Fig. 8. In mitochondria which have been induced to undergo volume oscillations, addition of classical uncouplers induces an instant shrinkage[37] while the above-mentioned agents cause damping in the expanded, energized state.[14] It seems likely that the alleged uncoupling action of laurylamine and chlorpromazine is due not to energy dissipation but mainly to inability of the energy transfer enzymes to use the available energy for the synthesis of ATP in a membrane whose conformation and physical state have been altered.

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REFERENCES

1. J. P. CHANGEUX and J. THIÉRY, in *Regulatory Functions of Biological Membranes* (Ed. J. JÄRNEFELT), BBA Library Vol. 11, p. 116, Elsevier, Amsterdam (1968).
2. E. C. WEINBACH and J. GARBUS, *J. biol. Chem.* **240**, 1811 (1965).
3. E. C. WEINBACH, J. GARBUS and H. G. SHEFFIELD, *Expl cell Res.* **46**, 129 (1967).
4. E. C. WEINBACH and J. GARBUS, *Biochem. J.* **106**, 711 (1968).
5. E. C. WEINBACH and J. GARBUS, *Biochim. biophys. Acta* **162**, 500 (1968).
6. L. MELA, *Archs Biochem. Biophys.* **123**, 286 (1968).
7. L. SALGANICOFF, *Fedn Proc.* **27**, 527 (1968).
8. N.-E. L. SARIS, M. K. F. WILKSTRÖM and A. J. SEPPÄLÄ, 5th FEBS Symposium, **17**, 363 (1969).
9. H. LEES, *Biochim. biophys. Acta* **131**, 310 (1967).
10. M. B. FEINSTEIN, *J. gen. Physiol.* **48**, 357 (1961).
11. P. M. SEEMAN, *Int. Rev. Neurobiol.* **9**, 145 (1966).
12. R. A. DEMEL and L. L. M. VAN DEENEN, *Chem. Phys. Lipids* **1**, 68 (1966).
13. S. RAZIN and R. ROZANSKY, *Archs Biochem. Biophys.* **81**, 36 (1959).
14. A. J. SEPPÄLÄ, M. K. F. WIKSTRÖM and N.-E. L. SARIS, in *Biochemical Oscillations* (Eds. B. CHANCE, E. K. PYE and B. HESS), Academic Press, New York, in press.
15. W. E. JACOBUS and G. P. BRIERLEY, *J. biol. Chem.* **244**, 4995 (1969).

16. M. K. F. WIKSTRÖM and N.-E. L. SARIS, *Europ. J. Biochem.* **9**, 160 (1969).
17. O. H. LOWRY, O. H. ROSENBROUGH, N. J. FARR and R. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
18. G. ROUSER, G. GALLI and G. KRITCHEVSKY, *J. Am. Oil Chem. Soc.* **42**, 404 (1965).
19. O. HAYAISHI, in *Methods in Enzymology* (Eds. S. P. COLOWICK and N. O. KAPLAN), Vol. 1, p. 660, Academic Press, New York (1955).
20. L. ERNSTER and C.-P. LEE, *Ann. Rev. Biochem.* **33**, 729 (1964).
21. H. TABOR, C. W. TABOR and S. M. ROSENTHAL, *Ann. Rev. Biochem.* **30**, 579 (1961).
22. I. HONJO and K. OZAWA, *Biochim. biophys. Acta* **162**, 624 (1968).
23. W. O. KWANT and P. SEEMAN, *Biochim. biophys. Acta* **183**, 530 (1969).
24. W. O. KWANT and P. SEEMAN, *Biochim. biophys. Acta* **193**, 338 (1969).
25. N.-E. L. SARIS and A. J. SEPPÄLÄ, *Acta Chem. Scand.*, in press.
26. T. TEORELL, *Ann. N.Y. Acad. Sci.* **137**, 950 (1966).
27. H. HAUSER, S. A. PENKETT and D. CHAPMAN, *Biochim. biophys. Acta* **183**, 466 (1969).
28. J. A. RUPLEY, in *Methods in Enzymology* (Ed. C. H. W. HIRS) Vol. 11, p. 905, Academic Press, New York (1967).
29. A. D. BANGHAM and R. M. C. DAWSON, *Biochem. J.* **75**, 133 (1960).
30. A. D. BANGHAM and R. M. C. DAWSON, *Biochim. biophys. Acta* **59**, 103 (1962).
31. A. J. SEPPÄLÄ, S. NORDLING and N.-E. L. SARIS, *Scand. J. clin. Lab. Invest.* **25**, Suppl. **113**, 79 (1970).
32. P. SEEMAN, W. O. KWANT, T. SANKS and W. ARGENT, *Biochim. biophys. Acta* **183**, 490 (1969).
33. P. SEEMAN, W. O. KWANT and T. SANKS, *Biochim. biophys. Acta* **183**, 499 (1969).
34. P. SEEMAN and W. O. KWANT, *Biochim. biophys. Acta* **183**, 512 (1969).
35. L. ERNSTER and K. NORDENBRAND, Abstr. 4th FEBS Meeting, p. 108, Oslo 1967.
36. B. CHANCE and L. MELA, *Biochemistry* **5**, 3220 (1966).
37. L. PACKER and J. M. WRIGGLESWORTH, in *Energy Level and Metabolic Control in Mitochondria* (Eds. S. PAPA, J. M. TAGER, E. QUAGLIARELLO and E. C. SLATER), p. 125, Adriatica Editrice, Bari (1969).