Biochimica et Biophysica Acta, 1019 (1990) 19–28
Elsevier

BBABIO 43243

A comparative study of the effects of procaine, lidocaine, tetracaine and dibucaine on the functions and ultrastructure of isolated rat liver mitochondria

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(Received 21 March 1990)

Key words: Procaine; Local anesthetic; Oxidative phosphorylation; Membrane potential; Ultrastructure; (Liver mitochondria)

The effects of procaine, lidocaine, tetracaine and dibucaine (10^{-5}–10^{-2} M) were tested on isolated rat liver mitochondria by measurements of the respiratory rates and of the membrane potential and by electron microscopy. A general concentration-dependent stimulation of the basal state (respiration before ADP addition) was observed for all local anesthetics studied. Up to the concentration of 10^{-3} M, the order of stimulation was: procaine < lidocaine < dibucaine < tetracaine. However, with the exception of dibucaine, which inhibited state-3 respiration (ADP present) in a strictly concentration-dependent manner, the other drugs had a biphasic effect: slight stimulation of state 3 at low and moderate concentrations ($\leqslant 10^{-3}$ M) and inhibition at higher concentrations. Nevertheless, due to a stronger stimulation of the basal state, the acceptor control ratio decreases progressively (uncoupling effect) as the concentration of the drugs increases. The only exception to this observation is procaine in the range of 10^{-5}–10^{-4} M, where the stimulation of the two respiration states (although small) is approximately equal and thus the uncoupling effect is absent or negligible. Membrane ptoential recordings suggested that membrane integrity and phosphorylation capacity were negatively affected at high drug concentrations ($> 10^{-3}$ M), especially in the case of tetracaine and dibucaine, when $5 \cdot 10^{-3}$ M even produced the collapse of the membrane potential and complete loss of the phosphorylation ability. Electron microscopy confirmed these effects, showing an abundance of either swollen or supercondensed mitochondria, with many membrane ruptures. The action mechanisms of the tertiary amines studied are discussed in terms of interaction of drug with the lipid bilayer and with the membrane proteins. It is concluded that both the inhibitory and the uncoupling effects are dependent, in the first place, on the degree of hydrophobicity of each local anesthetic.

Introduction

There is increasing evidence that local anesthetics of the tertiary amine type interfere with the mitochondrial metabolism in a complex way. Thus, both inhibition of the respiration [1,2] and uncoupling of oxidative phosphorylation [3,4] have been reported. However, the inhibition of the activity of the respiratory chain components is in contrast to the stimulation of the electron transport, as required by the uncoupling effect. Such a stimulation has been demonstrated for some local anesthetics by Garlid and Nakashima [3], although only in the presence of low concentrations of added lipophilic anions. Relatively recently [5,6], we have shown that concentrations of procaine and procaine-based preparations (Gerovital H_3 and Aslavital) in excess of 10^{-3} M produce concomitant inhibition of respiration (state 3) and uncoupling of oxidative phosphorylation, mainly associated with the stimulation of state 4, even though lipophilic anions were not used. A similar behavior was confirmed by Dabadie et al. [4] for the more hydrophobic amine bupivacaine, whereas lidocaine (less hydrophobic than bupivacaine but still more hydrophobic than procaine) was shown to require the presence of lipophilic anions for effective uncoupling.

In addition to these complex actions, a direct inhibition of the mitochondrial ATP synthase by local anesthetics was also demonstrated [7–10].

As regards the procaine and procaine-based preparations mentioned above, their action is also more complicated than presented so far. Thus, in the range of

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; diS-C_2-(5), 3,3′-diethylthiadicarbocyanine iodide; P_i, inorganic phosphate; ACR, acceptor control ratio.

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10^{-5}–10^{-4} M procaine, we were able to detect a slight but systematic stimulation of state-3 respiration, whereas higher concentrations were progresively inhibitory [5,6]. This biphasic relationship must not be confused with the one reported by Dabadie and co-workers [4] for bupivacaine action on state 4. It must be mentioned that our functional investigations were accompanied by electron microscopic studies which revealed that low concentrations of procaine (around 10^{-5} M) did not change the ultrastructural aspect of mitochondria (as compared to the control), whereas concentrations higher than 10^{-3} M produced structural alterations.

In an attempt to clarify the complex actions of the local anesthetics on oxidative phosphorylation and for a better understanding of the effects of procaine on mitochondrial functions and ultrastructure, we have extended recently this dual type of study to a series of teritary amines of increasing hydrophobicity. Thus, along with procaine, we have studied the effects of lidocaine, tetracaine and dibucaine on respiratory parameters, phosphorylation, membrane potential and ultrastructure of rat liver mitochondria, the present paper representing the full account of our data and interpretations, although some preliminary results have already been reported [11]. Unlike our previous work with procaine [5,6], when the respiration was stimulated by several successive additions of small amounts of ADP, this time we employed hexokinase and glucose to regenerate ADP and stimulate the respiration continuously. The selection of this variant was initially motivated by our efforts to reproduce as much as possible the conditions used previously by Aslan et al. [12], who had reported incredibly high stimulations of respiration (state 3), of over 300%, by concentrations close to 10^{-5} M procaine (added as Gerovital H_3), which could not be confirmed in the variant used in our previous work [5,6]. However, apart from this, the use of hexokinase and glucose seems to be more appropriate, since, under these conditions, the mitochondrion not only synthesizes ATP but also uses it for glucose phosphorylation, i.e., it performs more than one metabolic task, thus being closer to its in vivo requirements. Therefore, we have extended this variant to all tertiary amines employed in the present study.

Materials and Methods

Isolation of mitochondria

Mitochondria were isolated from the livers of freshly decapitated (unfasted) white female rats (180–200 g) of an in-bred Wistar line, essentially according to Johnson and Lardy [13]. The isolation medium was composed of 250 mM sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA and 0.05 mM EGTA, while the washing and suspending medium lacked the chelating agents.

Measurement of the respiratory parameters

Respiration rates were measured polarographically at 25 °C, in a 0.5 ml cell, with a Clark oxygen electrode (Yellow Springs, OH), in a medium consisting of 100 mM sucrose, 80 mM KCl, 5 mM Tris-Hepes (pH 7.3), 5 mM KP_i, 2 mM $MgCl_2$ and 0.5 mM EDTA. This composition will be referred to as the phosphorylation medium. 5 mM potassium succinate, 10 mM glucose, 0.6–1.0 units of hexokinase and 0.2–0.3 mg bovine serum albumin were added to this medium in the oxygraph cell. Mitochondrial suspension (usually 0.5 mg protein, as determined according to Petterson [14]) was injected through the stopper capillary and a basal respiration rate was recorded for about 1–2 min. This is referred to as the basal state, since it is not truly identical to state 4, which results after the exhaustion of the ADP. For a normal (control) trace, the respiration was then stimulated by addition of 0.1–0.2 mM ADP, leading to the so-called state 3. The ratio between the state 3 and the basal state, known as the acceptor control ratio (ACR), is one of the indices calculated by us and used as an indirect measure of mitochondrial integrity and phosphorylation ability. In the cases in which the effects of the tertiary amines were studied (tested), the drugs (previously buffered at pH 7) were added in concentrations varying from 10^{-6} to 10^{-2} M, usually on the basal state, 1 min after the addition of mitochondria. Occasionally (at high drug concentrations), they were added on state 3, in order to observe the direct effect on the stimulated respiration. When the drugs were added on the basal state, ADP was introduced 1 min after the drug addition. Letting the drug act for up to 10 min, before or after ADP addition, did not change the results significantly, except for high concentrations of dibucaine.

Respiratory rates and ACR values were calculated from the oxygraphic traces and the significance of the percent differences between pair recordings (with and without drug) were estimated by the paired *t*-test.

Estimation of membrane potential

Membrane potential generated by succinate respiration and the kinetic behavior of this potential following the addition of the tested drugs and ADP were monitored by spectrophotometric recording of the absorbance changes (at 660 nm) of the potential sensitive probe diS-C_2-(5), in a Specord-M40 spectrophotometer (Carl Zeiss, Jena), based on principles described previously [15–17]. Because this type of spectrophotometer does not allow a dual-wavelength recording, as customary for the kind of kinetic measurements performed by us, we took advantage of the programming capacity of the instrument, adding the potential probe only in one cuvette (both containing the same amount of mitochondria) and compensating the absorbance difference by a zero-adjusting program. Then all the ad-

ditions were made identically and concomitantly into both cuvettes. This way, any unspecific response (such as swelling) is cancelled out and only the specific response of the potential probe is recorded. In our experience, if the general precautions in working with this probe (see Refs. 15–17) are respected, this method gives results comparable to the dual-wavelength recordings.

Since relatively low concentrations of cyanine dyes may uncouple mitochondria and collapse the membrane potential [18,19], we have used very low concentrations of dye (usually 2.5 μM). Moreover, the occasional use of safranine (8 μM), as a control, gave results practically identical to those obtained with diS-C_2-(5), except that the kinetic changes were not followed so finely and promptly. However, it should be mentioned that we have not measured absolute values of the membrane potential but used the absorbance differences comparatively, in a qualitative manner. The kinetic behavior of the membrane potential gives, in fact, much more useful information than the amplitude. Specific details of this type of experiment can be found in the corresponding figure legends.

Electron microscopy

Mitochondrial ultrastructure was studied with a TESLA-BS-500 electron microscope. For electron-microscopic preparations, the 0.5 ml sample was taken directly from the oxygraph cell, 2 min after the drug addition (for the basal state) or 1 min after ADP addition (for state 3), the drug action lasting in both cases 2 min and the total incubation time of mitochondria being 3 min. Therefore, the control samples were also taken after 3 min of incubation (no drug added). The 0.5 ml aliquot was injected into a plastic microtube containing approximately 0.5 ml of a prefixing medium (1% glutaraldehyde solution in 150 mM phosphate buffer, pH 7.4). Mitochondria were then sedimented by centrifugation at 7000 × *g*, for 15 min. The pellet obtained was further processed according to current techniques for electron microscopy.

Chemicals

All the chemicals used were of analytical grade. Procaine (hydrochloride form) was from Hoechst (F.R.G.), lidocaine (free base), tetracaine, dibucaine (both, hydrochloride form), Hepes, ADP, rotenone, hexokinase and bovine serum albumin were from Sigma, Tris from Merck and diS-C_2-(5) from Eastman-Kodak.

Results

Respiratory parameters

The effects of different concentrations of procaine on the respiratory parameters recorded with succinate are presented in Fig. 1 as relative variations. The absolute values of the control parameters were given in our

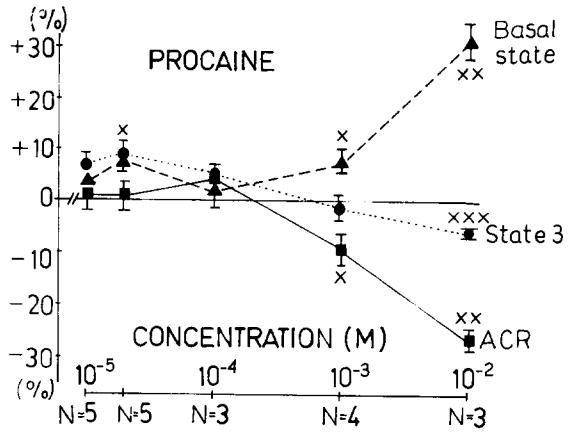


Fig. 1. Effects of various concentrations of procaine on the respiratory parameters (expressed as percent variation vs. control). Rat liver mitochondria are incubated with succinate and the indicated procaine concentration, as described in Materials and Methods. Acceptor control ratio (ACR) is calculated as the ratio between state 3 and basal state. ▲------▲, basal state; ●·····●, state 3; ■———■, ACR. Vertical bars illustrate the standard error of mean. Statistical significance is calculated according to the paired *t*-test. ×, $P < 0.05$; ××, $P < 0.01$; ×××, $P < 0.001$. *N* represents the number of pair recordings utilized at the corresponding drug concentration.

preliminary report [11], but for completion we present here the limits of those values. Thus, the basal respiration rate varied from 29.0 to 33.5 nanoatoms O/min per mg mitochondrial protein, whereas for the stimulated state this rate varied between 110.4 and 156.0, the acceptor control ratio varying mainly from 4 to 5. As can be seen in Fig. 1, low concentrations of procaine (10^{-5}–10^{-4} M) are not totally without effect. Although small, the systematic positive differences for the two respiration states and especially for state 3 could indicate a slight stimulation of respiration. In fact, at $2 \cdot 10^{-5}$ M procaine, the increase of state 3 is statistically significant ($P < 0.05$). Above 10^{-4} and especially above 10^{-3} M, there is a divergent effect: a very significant increase of the basal state, a small (but very significant) decrease of state 3 and also a very significant decrease of ACR. The moderate stimulation of the basal state suggests a partial uncoupling of oxidative phosphorylation, whereas the small decrease of state 3 indicates a slight but clear inhibitory effect on respiration. Similar results were obtained in our previous studies [5,6] not only with succinate but also with glutamate plus malate as substrates.

In a manner similar to that used for procaine, the effects of lidocaine are presented in Fig. 2. As can be seen, up to 10^{-4} M, the effects are not significant for any of the three parameters studied. For state 3, this is true even up to 10^{-3} M lidocaine. However, the basal state is strongly increased above 10^{-4} M, whereas ACR is equally decreased (uncoupling effect). State 3 is moderately inhibited above 10^{-3} M. Compared to procaine, the effects of lidocaine are partially similar, although somewhat greater.

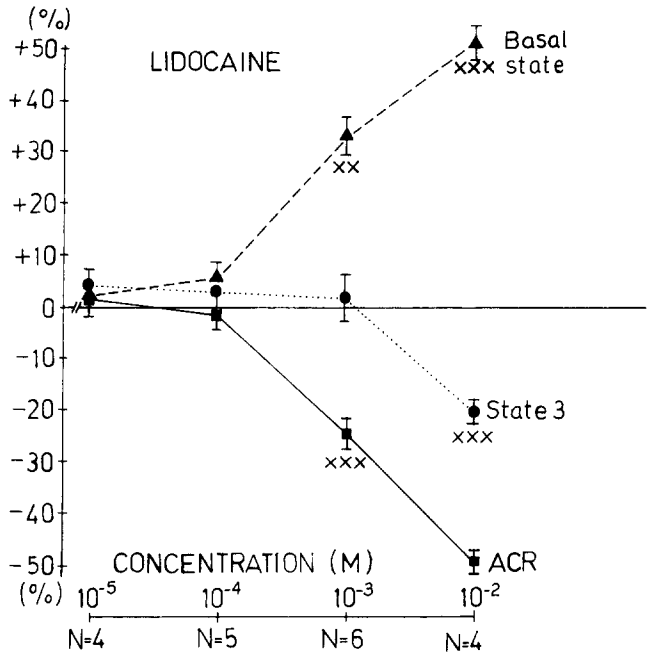


Fig. 2. Effects of lidocaine on the respiratory parameters of rat liver mitochondria. Explanations as in Fig. 1.

Tetracaine (Fig. 3) and dibucaine (Fig. 4) have much stronger effects than procaine or even lidocaine. Characteristic to tetracaine, one can identify a moderate stimulation of respiration around 10^{-4} M, followed by an abrupt inhibition above 10^{-3} M. The basal state is

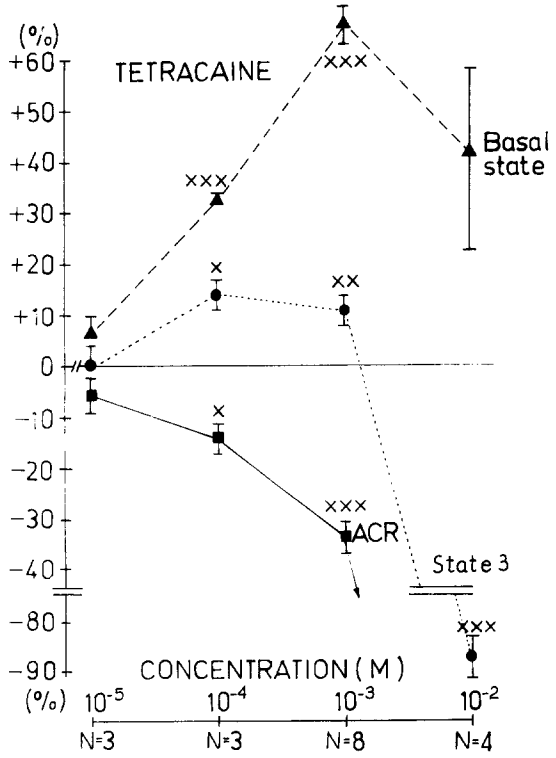


Fig. 3. Effects of tetracaine on mitochondrial respiratory parameters. Details as in Fig. 1.

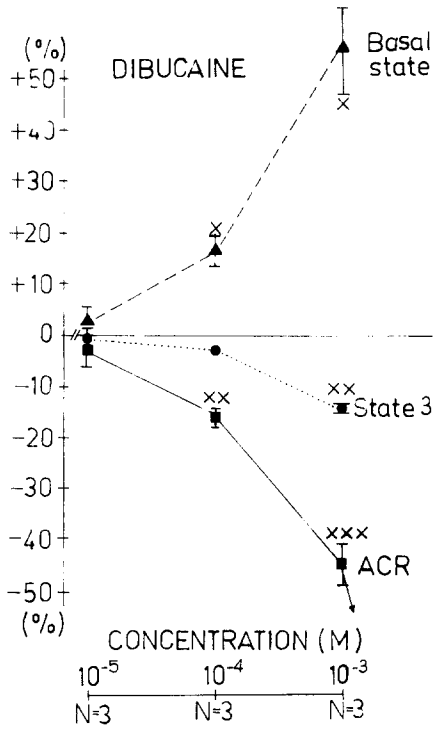


Fig. 4. Effects of dibucaine on the respiratory parameters of mitochondria. Explanations as in Fig. 1.

strongly stimulated up to the concentration of 10^{-3} M, after which this stimulation diminishes. ACR decreases progressively, more pronouncedly above 10^{-3} M. These phenomena indicate a strong uncoupling effect, followed by an equally strong inhibition of respiration, as the concentration increases. In the case of dibucaine, the inhibition of respiration (state 3) begins already at 10^{-4} M. Consequently, the stimulation of the basal state appears somewhat less strong than with tetracaine. At 10^{-2} M, any respiration and phosphorylation are completely abolished. Moreover, for longer incubation periods, this complete inhibition occurs already at 10^{-3} M dibucaine.

As can be seen from Figs. 1–4, with the exception of dibucaine, which inhibits state-3 respiration in a strictly concentration-dependent manner, the other drugs have a biphasic effect: slight stimulation of state 3 at low and moderate concentrations ($\leqslant 10^{-3}$ M) and inhibition at higher concentrations. Procaine deserves a special mention, since in its case the stimulation of state 3 is higher than that of the basal state, in a large concentration range. A similar biphasic effect has been described by Dabadie et al. [4], for bupivacaine action on state 4 (this state, however, is analogous to our basal state and not state 3). As pointed out by these authors [4], the maximum stimulation of respiration by ionophoric uncouplers (in the presence of bupivacaine) changes the biphasic aspect of the effect of this anesthetic (stimulation of state 4 at low concentrations, followed by inhibition at higher concentrations) into a monotonously increas-

ing inhibition. We could have probably demonstrated the same thing for tetracaine, fr example, where a similar biphasic effect on the basal state can be noted from our results. Nevertheless, even without the use of antibiotic uncouplers, it is clear from the results presented above that dibucaine has the strongest inhibitory effect, followed by tetracaine, lidocaine and, last, procaine. In fact, the relative inhibitory efficacy of the drugs, established by us, is in good agreement with the results obtained by Chazotte and Vanderkooi [2] on uncoupled submitochondrial particles, by the use of a more quantitative approach. As regards the uncoupling effect, the order of potency seems to be: tetracaine, dibucaine < lidocaine < procaine. As we shall see next, this sequence, although probably apparent (as discussed later), is reinforced by the membrane potential results.

Membrane potential

As the most important component of the proton electrochemical potential generated by respiration [20], the membrane potential should be affected by drugs which either inhibit electron transport or increase membrane permeability. So far, the oxygraphic results have indicated that the tertiary amines employed in this study are able to inhibit and at the same time uncouple respiration (the last process most likely by increasing membrane permeability). Our recordings of membrane potential confirm the oxygraphic results and even offer some additional information, due to the fact that they are performed in the absence of hexokinase and glucose and thus repeated alternations of state 3 and 4 can be recorded, as reported in our previous work [5,6].

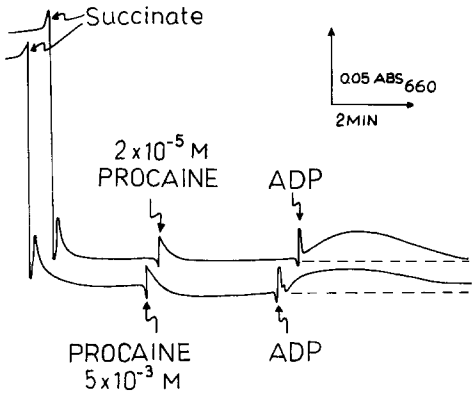


Fig. 5. Kinetics of the membrane potential in the presence of procaine and ADP. 1.1 mg mitochondrial protein is present in each of the two cuvettes. The suspending medium (1.5 ml) is composed of 100 mM sucrose, 80 mM KCl, 5 mM Tris-Hepes (pH 7.3), 5 mM KP_i, 2 mM $MgCl_2$, 0.5 mM EDTA and 8 μM rotenone. 2.5 μM diS-C_2-(5) is added to the sample cuvette and the absorbance difference electronically compensated by a zero adjustment program. The additions indicated on each trace are identical and made concomitantly into both cuvettes: 6.6 mM succinate, procaine (as specified) and 0.2 mM ADP.

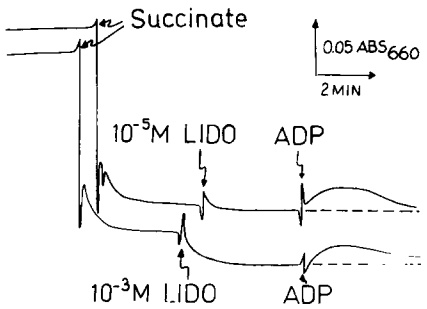


Fig. 6. Effects of lidocaine on the kinetics of membrane potential. Details as in Fig. 5, except that ADP is 0.15 mM.

Fig. 5 is an illustration of the effects of procaine on the kinetics of membrane potential, both under non-phosphorylating and phosphorylating conditions. The trace recorded with the addition of $2 \cdot 10^{-5}$ M procaine is indistinguishable from the control (not presented). Moreover, it can be seen that even at $5 \cdot 10^{-3}$ M procaine, the effect is not too strong, the mitochondria preserving their phosphorylation ability, as deduced from the particular aspects of the two curves, which are not much different. The small decrease of the membrane ptoential induced by ADP additions is due to the efficient energy utilization for ATP synthesis, and it occurs despite the fact that the respiration is stimulated several-fold by ADP addition, as known from the oxygraphic results. In fact, similarly to the situation in an usual oxygraphic record (i.e., without hexokinase and glucose), where, after ADP exhaustion, the respiration rate resumes approximately its initial level, the amplitude of the membrane potential also regains its initial level, as can be seen from our figure. Although not much exploited, this fact is known from the literature [21]. In our experience (Tarba, C., unpublished data), the depth of the decrease upon ADP addition and the degree of recovery after ADP exhaustion may be related to the functional state of the mitochondria (degree of coupling and phosphorylation efficiency). Following ADP addition, well-coupled mitochondria show clear indentations on the membrane potential recordings and full recovery after ADP exhaustion, whereas badly coupled mitochondria either do not show any indentation or only a shallow one, without a clear recovery. From this point of view, the trace in which a higher concentration of procaine is added (in Fig. 5) indicates a relatively low degree of uncoupling, which is in accord with the respiration measurements.

As can be seen from Fig. 6, the membrane potential kinetics are also only slightly affected by lidocaine, i.e., the phosphorylation ability of mitochondria is only partially decreased by 10^{-3} M drug, whereas 10^{-5} M has no effect. However, the apparent small increase of the membrane potential upon addition of 10^{-3} M lidocaine is in contradiction with its moderate uncoupling effect (as known from respiration measurements). This

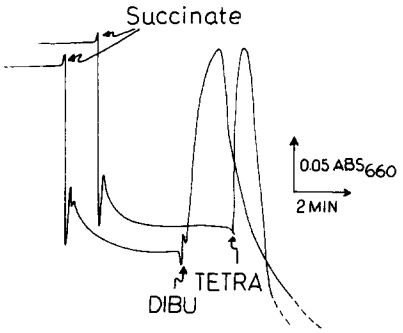


Fig. 7. Collapse of the membrane potential by tetracaine and dibucaine. Both anesthetics are added at a concentration of $5 \cdot 10^{-3}$ M. See Fig. 5 for other details.

discrepancy will be satisfactorily explained after presenting the effects of tetracaine and dibucaine on the membrane potential.

Fig. 7 illustrates the effects of tetracaine and dibucaine. At $5 \cdot 10^{-3}$ M, either of the two drugs collapses the membrane potential immediately after its addition. A concentration of 10^{-3} M or even lower also collapses the membrane potential, although more slowly (not presented). As can be seen from Fig. 7, the kinetics of dissipation are faster in the case of tetracaine. Knowing from the oxygraphic results that dibucaine has a stronger inhibitory effect, the only explanation of this observation is a faster and more potent uncoupling action of tetracaine, which is (again) in line with the conclusions resulted from respiration measurements. The fact that following the collapse of the membrane potential the absorbance returns towards its previous level, and even passes it, is an artifact generated by the expulsion of the potential-sensitive probe (cyanine dye) from the membrane by the drugs, which, at the high concentrations used, successfully compete with the dye for the binding sites of the membrane. This is in agreement with what is known about the mechanism of response of diS-C_2-(5) [15–17] and the degree of penetration of the anesthetic amines into the membrane [22,23]. Among other characteristics, for example, there is a difference of over 20 nm between the absorption maxima of the bound and unbound form of the dye (see Refs. 15–17]), which results in a large absorbance change around 660 nm, upon either binding or releasing of the dye. In this respect, the use of tetracaine and dibucaine in liposomes, in which membrane potentials were generated by ion gradients and proper ionophores, gave results similar to those obtained with mitochondria. Moreover, the recording of the changes produced by tetracaine and dibucaine in the absolute spectrum of the dye bound to liposomes or mitochondria indicated an unequivocal shift of the spectrum toward that of the unbound form (results to be presented in full elsewhere). On this basis, the apparent small increase of the membrane potential observed at 10^{-3} M lidocaine (Fig. 6) is actually an artifact, due to the partial expulsion of the dye from the membrane, this drug also competing with the dye for the binding sites of the membrane (albeit less successfully than the other two drugs).

Mitochondrial ultrastructure

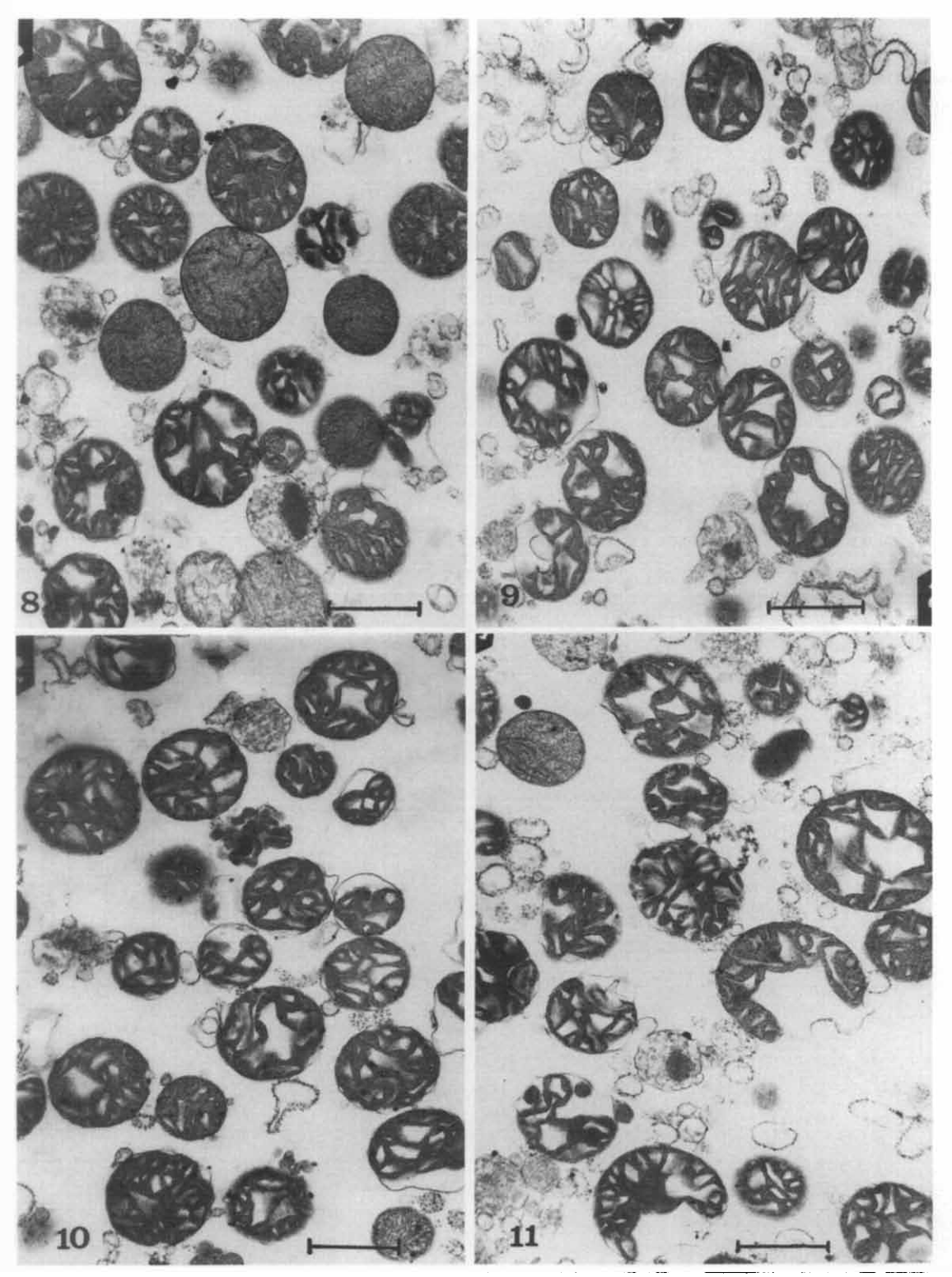
Despite the classical work of Hackenbrock [24], an unequivocal relationship between the mitochondrial ultrastructure and all its functional states has not been completely established. Nevertheless, Hertsens and Jacob [25] have recently extended this type of study by performing freeze-fracture electron microscopy of mitochondria forced into a well-defined metabolic state, and by the use of a computer-aided statistical methodology have shown that a clear discrimination could be made between different respiration states. Independently, with our more modest methodology we have reached similar conclusions and have used this principle of structure–function correlation (among others) in the study of effects of procaine [5,6].

To our knowledge, electron microscopy has not been used so far in the study of effects of anesthetics on mitochondria. However, as we shall see, this technique provides interesting information regarding the effect of drugs on mitochondrial ultrastructure, perfectly compatible with the functional results.

Figs. 8 and 9 show aspects of the control mitochondria in the basal state and state 3, respectively. In agreement with the literature description [24,25], the basal state is characterized by the presence of many mitochondria in the so-called orthodox configuration, i.e., organelles having a uniformly electrodense matrix, with poorly outlined cristae (Fig. 8). In state 3, the majority of the mitohcondria are in a condensed configuration, i.e., slightly contracted, with rather large intracrystal spaces (Fig. 9). In both figures, one or two swollen mitochondria with an overall rarefied matrix can also be observed.

We have obtained electron micrographs for both respiration states and for all anesthetics tested, at almost all concentrations employed in the oxygraphic studies. Nevertheless, only a few illustrative micrographs will be presented and discussed below. It should be mentioned, however, that despite the apparent inhomogeneity of our preparations, the effects of high concentrations of drugs (especially tetracaine and dibucaine) are clearly discernible by comparison with the corresponding controls and perfectly supported by a statistical study on larger populations (many micrographs of lower magnification).

The addition of 10^{-5} M procaine has no effect on the ultrastructure of state-3 mitochondria (Fig. 10, whereas 10^{-2} M procaine (Fig. 11) produces rather visiable changes, such as an increased number of supercondensed (falciform) mitochondria.



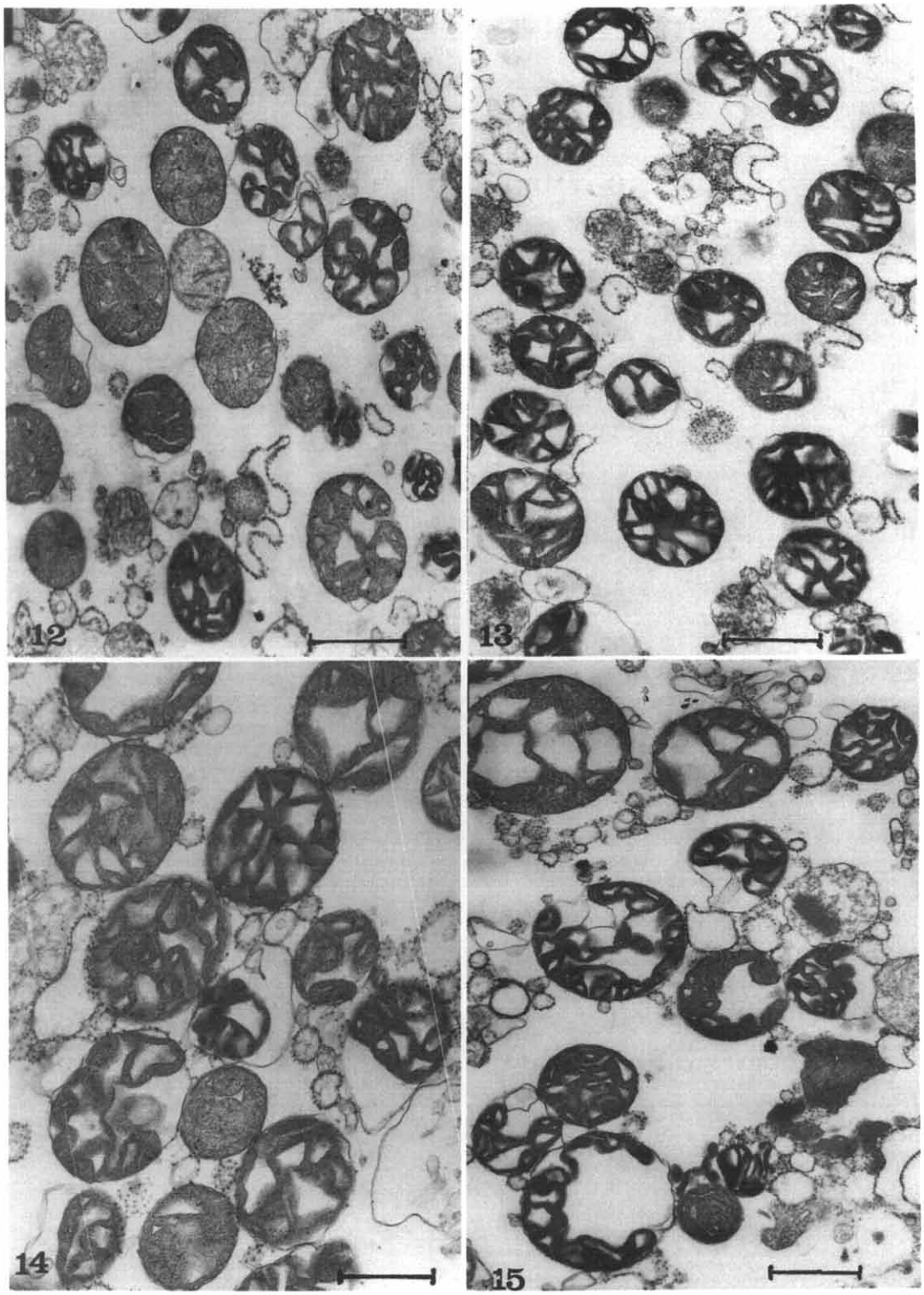
Figs. 8, 9. Ultrastructural aspects of control mitochondria in the basal state (8) and in state 3 (9). Bar is 0.5 μm.

Figs. 10, 11. State-3 mitochondrial ultrastructure in the presence of 10^{-5} M (10) and 10^{-2} M procaine (11). Bar, 0.5 μm.

In agreement with the functional results, 10^{-3} M lidocaine does not affect too much the mitochondrial ultrastructure. However, a moderate swelling can be observed in Fig. 12 (basal state), which is in line with the moderate uncoupling effect of this drug. Also, a slight supercondensation is apparent in Fig. 13 (state 3),

which may be attributed to the slight inhibitory effect of this drug.

Tetracaine and dibucaine, at a similar concentration, have much stronger effects. They both show almost identical effects on the basal state (swelling combined with supercondensation and membrane breaks; not presented here), whereas, on state 3, tetracaine (Fig. 14) does not have such strong effects as dibucaine (Fig. 15).



Figs. 12, 13. Effects of 10^{-3} M lidocaine on the basal state (12) and on state 3 (13). Bar, 0.5 μm.

Figs. 14, 15. Effects of tetracaine (14) and dibucaine (15) on state-3 mitochondrial ultrastructure. Both drugs are present at a concentration of 10^{-3} M. Bar, 0.5 μm.

whose action is characterized by extensive supercondensation and membrane ruptures, in line with its potent inhibitory effect. Thus, electron microscopy can be successfully used for estimating, at least qualitatively, the effects of anesthetic drugs on mitochondrial ultrastructure and, indirectly, function.

Discussion

In presenting our results, we have also discussed certain aspects bearing a close relation to the experimental data or indispensable for a clear understanding of the subject. In the present section, we will address the problem of the molecular mechanisms of the effects observed, their biological significance and practical importance.

As specified in the 'Introduction', there are certain literature reports which consider that the uncoupling mechanism of the tertiary amines is dependent mainly on the presence of the lipophilic anions, through ion pair formation with the cationic form of the anesthetic amine [3,4], which is predominant at a neutral pH. However, in talking about uncoupling, the authors of the above-mentioned reports have in mind a strong and specific action, comparable to that produced, for example, by special antibiotic uncouplers. It is indisputable that lipophilic anions can enhance the uncoupling process produced by tertiary amines, but the relevant question is whether the concentration of such anions is sufficiently high in vivo to influence decisively the uncoupling at relatively low concentrations of anesthetics. Since we are not aware of the existence of an intense energy dissipation in anesthetized tissues (which should be an inescapable consequence of a strong uncoupling) and because the uncoupling potencies of the drugs tested (in the absence of added lipophilic anions) are roughly proportional to their hydrophobicity, we consider that the principal mechanism of uncoupling, both in vitro and in vivo, takes place by drug dissolution into the membrane. As classically envisaged for the membrane effects of the local anesthetics [26,27], the interaction of these drugs with the membrane may lead to local disordering and increased local permeability. However, other mechanisms, such as electrophoretic entry of the cationic form and/or ion pair formation (as described in Refs. 3,4) may also play a secondary role in the process of uncoupling. Such secondary effects could expalin, for example, why tetracaine, even though less hydrophobic than dibucaine, seems to have a greater uncoupling potency, as judged by the degree of the basal state stimulation (at the concentration of 10^{-3} M). Nevertheless, if one considers the stronger inhibitory action of dibucaine, this inversion may be only apparent. In fact, if the uncoupling effect is appreciated in a more complex manner, through the decrease in ACR value, then there is a perfect correlation with the degree of drug hydrophobicity.

With regard to the inhibitory action of the local anesthetics, only the work of Chazotte and Vanderkooi [2], performed in uncoupled submitochondrial particles, deals with the whole series of tertiary amines studied by us. Their results (as well as ours) prove that the inhibition of respiration by these drugs is also proportional to their hydrophobicity. In agreement with what appears more or less explicitly in the literature, this relationship suggests two possible mechanisms of action, which are mutually compatible or even complementary. There is, first, the possibility of a direct interaction with the exposed parts (sites) of the enzymes, which could lead to partial protein denaturation and some loss of enzyme activity. However, since the hydrophobic sites of the proteins are usually masked or burried into the membrane, the most likely way of action is, again, by drug dissolution into the membrane lipid, from where it gets easy access to the binding sites of the enzymes.

The depression of enzyme activity by local anesthetic tertiary amines has also been clearly demonstrated for the mitochondrial ATP synthase [7–10], where a conformationally mediated mechanism has been proposed [8,10]. Other respiratory enzymes, especially cytochrome oxidase, may be affected through a similar mechanism. Indirectly, our data support such a proposal. However, we should observe that part of the inhibitory effects on oxidative phosphorylation in our study are due to the action of a drug on the mitochondrial ATP synthase. Moreover, due to the fact that the concentration dependence of enzyme inhibition is similar for the respiratory enzymes and for the ATP synthase (cf. Refs. 2 and 10), under the conditions employed in the present study, it is not possible to discriminate between the two effects, and our conclusions, based on the overall observations, may not be exactly applicable to the respiratory enzymes.

So far, in our discussion, we have not usually discriminated between the action of low (biologically relevant) and high (unphysiological) concentrations of drugs. Even though the study of the effects of high concentrations of local anesthetics and especially of the two potent uncouplers and inhibitors (tetracaine and dibucaine) may be useful for understanding the general mechanism of action of these drugs, we should not lose sight of the most important objective: the application of the knowledge gained from such studies to the understanding of the specific actions of biologically (pharmacologically) relevant concentrations of certain drugs. The problem with which we have been concerned lately is whether some of the effects of procaine observed in vivo, mainly the so-called 'energogenous effect' (stimulation of energy production and utilization by ATP-dependent systems) [28,29], may be explained on the basis of procaine actions demonstrated in vitro. What we

have found at physiological concentrations is a slight stimulation of respiration, without a significant change of the acceptor control ratio (present work, with succinate) or of the respiratory control ratio and of the phosphorylation efficiency, with either glutamate plus malate or succinate [5,6]. Even without invoking the rather singular observation tht low concentrations of local anesthetics (particularly, tetracaine) increase dramatically the membrane order of oriented lipid films [30,31], our results on effects of procaine strongly suggest the possibility of a mild stimulatory action of the low concentrations of procaine. However, other types of experiment are certainly needed before drawing a definite conclusion.

Acknowledgements

We are grateful to Professor N. Simionescu (Institute of Cellular Biology and Pathology, Bucharest) and to Dr. G.P. Serban (Clairol Research Laboratories, Stamford, CT) for the gift of certain important chemicals and for help provided in other ways.

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