Effect of local anaesthetics on mitochondrial membrane potential in living cells

Michel GROUSELLE,* Olivier TUEUX,†† Philippe DABADIE,‡ Dinu GEORGESCAUD* and Jean-Pierre MAZAT†‡§

*Centre de Recherche Paul Pascal–CNRS, Château Brivazac, F-33600 Pessac, †Institut de Biochimie Cellulaire et Neurochimie–CNRS, 1 rue Camille Saint-Saëns, F-33077 Bordeaux Cedex, and ‡Department of Medical Biochemistry and Molecular Biology, Université de Bordeaux II, 146 rue Léo-Saignat, F-33076 Bordeaux Cedex, France

Using the laser dye rhodamine 123, we demonstrated that local anaesthetics can reach mitochondria in cell culture and reversibly decrease, or even collapse, their transmembrane potential. This effect is highly dependent on the lipid-solubility of the local anaesthetic and can be facilitated by the presence of a lipophilic anion.

INTRODUCTION

Pharmacoactive amines with local-anaesthetic properties have numerous and diverse actions on isolated mitochondria. Local anaesthetics have been found to inhibit mitochondrial electron transport at several points along the respiratory chain [1], and to inhibit the F_1 ATPase [2] and adenine-nucleotide transport [3]. Furthermore, they have been reported to uncouple oxidative phosphorylations in isolated mitochondria [4,5]. However, it is not known whether these results are relevant to the mitochondria inside a cell. In fact, it has not been demonstrated whether local anaesthetics have access to the mitochondria when applied on the outside of cells, nor whether they produce *in vivo* the same effects as those observed on isolated mitochondria. The aim of this work is to answer these questions. To this end, we have used cultured fibroblasts labelled with the laser dye rhodamine 123, which has been extensively employed as a fluorescent stain of mitochondria in living cells [6–8]. Since rhodamine 123 is an aromatic cation, it has been assumed that in whole cells it is electrophoretically distributed into the mitochondrial matrix in response to its high transmembrane potential, as has been demonstrated on isolated mitochondria [9]. Agents known to depolarize or de-energize mitochondria, such as uncouplers and respiratory inhibitors, decrease rhodamine-123 fluorescence of mitochondria in cultured cells [6]. Using this property, we tested four local anaesthetic molecules, which had different lipid-solubilities (see Table 1). We demonstrate that the local anaesthetics with the highest lipid-solubility, when applied outside a cell, can reach the mitochondria and collapse their transmembrane potential, as indicated by the fluorescence-intensity decrease of the rhodamine 123. A method is developed to analyse the experimental data and to quantify the effects of the local anaesthetics, allowing us to correlate their action with their lipid-solubility.

MATERIALS AND METHODS

Chemicals

Bupivacaine and lidocaine were gifts from Laboratoires Roger Bellon. Other chemicals were from Sigma.

Cell culture and microscopy

3T3 fibroblasts were obtained and grown by standard methods

in Eagle's minimum essential medium plus 1 mM-pyruvate, 1 mM-glutamine, NaHCO_3 (0.35 g/l) and 10% (v/v) foetal-calf serum. They were grown on 30 mm-diam. round cover-slips in an air/ CO_2 (19:1) incubator, at 37 °C. Experiments were carried out after at least one replicative step. Cells were then mounted in a live-cell observation chamber, rinsed once with 2 ml of 10 mM Hanks Hepes Buffer (pH 7.2) (10 mM-sucrose/118 mM-NaCl/4.6 mM-KCl/1 mM- MgCl_2 /1.8 mM- CaCl_2), and then incubated with 10 μg of rhodamine 123/ml for 30 min at 37 °C, in the same buffer. After a change of medium, the chamber was mounted on an Olympus IMT2 inverted microscope, with the temperature maintained at 34–36 °C. Stained cells were examined by epifluorescent illumination, with an Olympus Dapo 40 UV ($\times 40$) or Dapo 100 UV ($\times 100$) NA 1.3 oil-immersion objective. The microscope was equipped with a 100 W mercury lamp attenuated by a neutral-density filter (0.2% transmission), a fluorescein excitation filter (490 nm) and an emission filter (510 nm). Emitted light was focused on a SIT (Silicon Intensified Target) Camera (SOFRETEC CF 152 NV 25 equipped with a TH 9655 tube) and images were observed on a Thompson 6124 video monitor and recorded with a SONY U-matic tape recorder. Images were recorded at different times for period of less than 5 s, in order to avoid photobleaching effects. Local anaesthetics were added after the recording of a reference image. On achievement of the uncoupling process, the chamber was perfused with fresh buffer containing no anaesthetic and the recovery was recorded 8–15 min later.

We checked that none of the local anaesthetics used interfered with the rhodamine fluorescence itself at the wavelengths used, by direct measurements using a fluorimeter.

Statistical analysis

To obtain a statistical quantification of the effect of various local anaesthetics on cell culture, we monitored the release of rhodamine 123 from the mitochondria of at least 15 cells in the same field. Images were recorded at regular time intervals. On each image, we counted the number of cells presenting de-energized mitochondria as monitored by the release of rhodamine 123. The distribution of the number of cells with de-energized mitochondria appearing in each time interval was established. The mean time of this distribution is taken as a characteristic time of the local anaesthetic under study.

Abbreviation used: TPB[−], tetraphenylboron.

§ To whom reprint requests should be addressed

Fig. 1. Effect of 2.5 mM-bupivacaine on mitochondria stained with rhodamine 123 in fibroblast cell culture

(a) Reference without bupivacaine; (b) 6 min after addition of 2.5 mM-bupivacaine; (c) 8 min after the solution was washed with the buffer to eliminate the bupivacaine. Bar = 10 μ m.

Fig. 2. Number of cells de-energized by local anaesthetics as a function of time

Key: $\blacklozenge$, etidocaine; $\bullet$, bupivacaine; $\blacksquare$, lidocaine; $\blacktriangle$, dibucaine. All anaesthetics were added at 2.5 mM.

RESULTS AND DISCUSSION

Fig. 1 shows the action of bupivacaine on a fibroblast culture. The rhodamine-123-stained mitochondria are shown in Fig. 1(a); 6 min after the addition of 2.5 mM-bupivacaine, the fluorescence intensity in the mitochondria decreases and a diffuse low-level fluorescence appears in the cytoplasm (Fig. 1b). This result can be compared with the effects of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 2,4-dinitrophenol and valinomycin, which dissipate the transmembrane potential [7]. This change is reversible, and mitochondria recover their original fluorescence intensity after the removal of the local anaesthetic (Fig. 1c). Similar effects were obtained with the other local anaesthetics.

Fig. 2 shows the plot of the number of cells presenting de-energized mitochondria as a function of time for the various local anaesthetics studied at a concentration of 2.5 mM. This is a cumulative representation of the effect that allows the calculation of an average time and an s.d. for each local anaesthetic studied (see the Materials and methods section). These mean times ($\pm$ s.d.) are shown in Table 1.

Table 1. Mean time of mitochondria de-energization after addition of local anaesthetic

Cells labelled with rhodamine 123 were incubated with local anaesthetic at a fixed concentration (2.5 mM). Image samples were recorded every 30 s or 1 min (depending on the local anaesthetic under study) for less than 5 s in order to avoid photobleaching effects. On each image, the number of de-energized cells was counted and the mean and s.d. of this cumulative distribution was calculated. The partition coefficients are determined in octanol/water at pH 7.4, according to ref. [12], and the pK_a values are taken from ref. [13].

Local anaesthetic	Partition coefficient	pK_a	Time (s) (mean $\pm$ s.d.)
Lidocaine	107	7.9	
Bupivacaine	748	8.1	485 ± 65 ($n = 25$)
Etidocaine	3855	7.7	355 ± 27 ($n = 15$)
Dibucaine	4375	8.8	164 ± 17 ($n = 35$)

Table 1 shows that the mean time correlates with the lipid-solubility of the local anaesthetics studied in this work. This is emphasized by the comparison of two local anaesthetics with similar pK_a values, but with different lipid-solubilities, such as lidocaine ($pK_a = 7.9$) and bupivacaine ($pK_a = 8.1$). Thus, even if the pK_a values of the local anaesthetics are slightly different, the parameter that seems to explain their effect on mitochondria appears to be their lipid-solubility.

These results are in agreement with ours [5] and those of Garlid & Nakashima [4] obtained with isolated mitochondria. We have shown that some highly lipophilic local anaesthetics, such as etidocaine and bupivacaine, can uncouple isolated mitochondria. Similarly, it is found here that these local anaesthetics de-energize mitochondria in cell culture. On the other hand, in accordance with Garlid & Nakashima [4], we

showed that some local anaesthetics with low octanol/water partition coefficients, such as lidocaine, do not uncouple isolated mitochondria. This result holds also for mitochondria in cell culture (Table 1). The addition of $5 \mu\text{M}$ -TPB⁻ (tetraphenylboron), a lipophilic anion known to promote the diffusion of cations through biological membranes, was necessary to observe an uncoupling effect of 2.5 mM-lidocaine on mitochondria in cell culture (Fig. 3). It was also observed that the presence of $5 \mu\text{M}$ -TPB⁻ decreases the concentration of bupivacaine or etidocaine necessary to promote an uncoupling of mitochondria inside the cells (results not shown). These results substantiate the model proposed by Garlid & Nakashima [4] involving the formation of a neutral complex ($\text{R-NH}^+\text{-TPB}^-$) for transport across the cytosolic and mitochondrial membranes.

These results point out three salient features in the action of local anaesthetics on mitochondria *in vivo*.

(1) Local anaesthetics can reach mitochondria when applied to a cell culture medium.

(2) This process depends on their lipid-solubility and can be enhanced by the presence of a lipophilic anion such as TPB⁻.

(3) Local anaesthetics can have the same effects on mitochondria inside the cells as on isolated mitochondria, i.e. they can decrease, and probably even collapse, the mitochondrial transmembrane potential. As for isolated mitochondria, this result is probably due to two different effects of the local anaesthetics: first, a true uncoupling of mitochondria by either an electrophoretic mechanism (as by K^+ in the presence of valinomycin) or a classical uncoupling effect by a futile cycle of H^+ (analogous to that proposed for weak acid uncouplers [5]). Secondly, their accumulation inside the mitochondria can lead to the inhibition of some complexes of the respiratory chain, of the ATP synthase, or of adenine nucleotide transport. These two effects will lead synergistically to a strong decrease of the mitochondrial metabolism, and in particular of the ATP synthesis.

Thus, besides their classical effects on ionic channels [10], local anaesthetics could have other side-effects, particularly on mitochondrial energy metabolism. However, the physiological relevance of these observations is questionable. It is well known that local anaesthetics have toxic effects, particularly at the cardiac level [11], but these effects seem to occur at lower concentrations. Several explanations can be found. The mechanism of toxicity could be completely different, and might involve not the mitochondria but direct targets at the cytoplasmic membrane level of the cardiac cells, such as ionic channels. The cardiac fibres can also accumulate the local anaesthetics more easily than can fibroblasts, perhaps owing to the presence of natural lipophilic anions that play the role of TPB⁻ in our experiments. In these latter conditions, the toxicity mechanism of local anaesthetics could involve the perturbation of the mitochondrial energy metabolism for which we have found evidence in this paper.

We thank Professor J. Koenig and C. Aymard for culture of fibroblasts. We are indebted to Professor B. Guérin for his constant interest in this work, to Dr. L. Letellier, Dr. A. Ghazi and Dr. M. Rigoulet for their critical reading of the manuscript, and to Dr. A. Heape for correcting the English. This work has been supported by the CNRS, Bordeaux II University and The Région Aquitaine.

REFERENCES

1. Chazotte, B. & Vanderkooi, G. (1981) *Biochim. Biophys. Acta* **636**, 153–161
2. Chazotte, B., Vanderkooi, G. & Chignell, D. (1982) *Biochim. Biophys. Acta* **680**, 310–316
3. Fayle, D. R. H., Barritt, G. J. & Bygrave, F. L. (1975) *Biochem. J.* **148**, 527–531

Fig. 3. Effect of 2.5 mM-lidocaine in the presence of $5 \mu\text{M}$ -TPB⁻ on mitochondria stained with rhodamine 123 in 3T3 fibroblast cell culture

(a) Reference without 2.5 mM-lidocaine, but with TPB⁻ ($5 \mu\text{M}$); (b) 7 min after the addition of 2.5 mM-lidocaine. It was checked that TPB⁻ alone had no effect at this concentration. Bar = $10 \mu\text{m}$.

4. Garlid, K. D. & Nakashima, R. A. (1983) *J. Biol. Chem.* **258**, 7974–7980
5. Dabadie, P., Bendriss, P., Erny, P. & Mazat, J.-P. (1987) *FEBS Lett.* **226**, 77–82
6. Johnson, L. V., Walsh, M. L. & Chen, L. B. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 990–994
7. Chen, L. B., Summerhayes, J. C., Johnson, L. V., Walsh, M. L., Bern, S. D. & Lampidis, T. J. (1982) *Cold Spring Harbor Symp. Quant. Biol.* **46**, 141–155
8. Chen, L. B. (1988) *Annu. Rev. Cell Biol.* **4**, 155–181
9. Emaus, R. K., Grunwald, R. & Lemasters, J. J. (1986) *Biochim. Biophys. Acta* **850**, 436–448
10. Hille, B. (1977) *J. Gen. Physiol.* **69**, 497–515
11. Covino, B. G. & Vassalo, H. G. (1976) *Local Anaesthetics, Mechanism of Action and Clinical Use*, pp. 131–140, Grune and Stratton, New York
12. Nys, G. G. & Rekker, R. F. (1973) *Chim. Thér.* **5**, 521–535
13. Verderame, M. (1986) *Handbook of CNS Agents and Local Anaesthetics*, p. 348, CRC Press, Boca Raton, FL

Received 20 March 1990/3 July 1990; accepted 23 July 1990