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Light-induced reversible local fusions of thylakoid membranes in the presence of dibucaine or tetracaine

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Abstract

The dynamics of structural changes in pea chloroplasts in the presence of 25–50 μM dibucaine or tetracaine has been examined using electron microscopy. The light-induced uptake of anesthetic cations by thylakoids is attended by the appearance of local fusions of stroma-exposed thylakoid membranes. The first membrane protrusions and interthylakoid contacts are observed after 4 s illumination and they become numerous by 10 s. As a result, a network of anastomoses is formed which is maintained during at least 10 min. These effects are reversible in the dark and can be reproduced several times. The formation of membrane fusions is inhibited by the addition of protonophore. It is supposed that the energy-dependent uptake of protonated anesthetics by thylakoids leads to an increase in positive surface charge and thus a lateral pressure on the inner side of the thylakoid membrane. The appearance of membrane protrusions (crinkles) having the positive curvature of their inner surface may be considered as a way of compensating for lateral pressure. Presumably, anastomoses result from the fusion of crinkles to adjacent thylakoids.

Keywords: Thylakoid structure; Local anesthetic; Membrane fusion; Permeant amine; Dibucaine; Tetracaine

1. Introduction

Local anesthetics from the group of tertiary amines are amphiphilic weak bases, which penetrate membranes primarily in the neutral form. An essential feature of such substances is their ability to be distributed among cell compartments depending on pH of their bulks [1]. This property seems to be important in the effect of tertiary amines on coupling organelles, in particular, chloroplasts. Laasch with co-workers found that the uptake of dibucaine by chloroplasts increases under illumination [2]. The accumulation of charged anesthetic molecules inside energized chloroplasts results in various functional effects, which are observed at low (10–100 μM) anesthetic concentrations [2–7].

It is known that local anesthetics interact with the components of biological membranes. The studies on liposomes indicate that tertiary amine local anesthetics are able to incorporate into the lipid bilayer, thus perturbing its structure [8–11], and bind to proteins [12]. Taking into account these considerations, one can assume that the light-induced accumulation of tertiary amine cations inside the lumen and their binding to the inner side of the thylakoid membrane would affect the balance of forces that stabilize membrane structure. As a result, rearrangements of the thylakoid system could be expected. The preliminary

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data obtained in our experiments confirmed this suggestion [13]. In the present study, the dynamics of structural changes of pea chloroplasts energized in the presence of local anesthetics dibucaine (DC) and tetracaine (TC) has been examined.

2. Materials and methods

Chloroplasts were isolated from pea leaves (*Pisum sativum* L.). All the steps of chloroplast isolation were performed at weak light and at a temperature of 0–4°C. Washed leaves were incubated in a refrigerator for 2–3 h and then homogenized with a blender in a medium (1:10, w/v) containing 0.4 M sucrose, 20 mM NaCl, and 20 mM Tris/HCl buffer (pH 7.8). The homogenate was filtered through 4 layers of gauze and centrifuged at $7000 \times g$ for 3 min. The pellet obtained was resuspended in a medium which contained 0.2 M sucrose, 10 mM NaCl, 5 mM $MgCl_2$, and 10 mM Tricine/NaOH buffer (pH 7.8). After centrifugation for 10 min at $2500 \times g$, the sedimented chloroplasts were suspended in the same medium up to chlorophyll concentration 3.5–4.0 mg/ml and stored in the dark at 0°C. Chlorophyll concentration was checked using the Arnon method [14].

In the experiments, chloroplasts were illuminated by white light ($250\ W \cdot m^{-2}$) at 20°C in a reaction medium containing 0.1 M sucrose, 0.2 mM Tricine, 0.2 mM Hepes (pH 7.95), 10 mM NaCl, 5 mM $MgCl_2$, 50 μM methyl viologen or phenazine methosulfate ± 5 μM diuron, and a suspension of chloroplasts (50 μg chlorophyll per ml). Samples for electron microscopy were collected directly from the reaction medium and immediately fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) either in the light or in the dark, depending on the experimental requirements. 1% OsO_4 was used for postfixation of samples. Further treatment of preparations for electron microscope examination was carried out by conventional methods [15]. TC uptake by illuminated chloroplasts was calculated from the stimulation of H^+ uptake [16,17] using the following formula: $\Delta[TC^+] = \Delta\Delta[H^+]/(1-\alpha)$, where $\Delta[TC^+]$ is the uptake of protonated TC, $\Delta\Delta[H^+]$ the stimulation of H^+ uptake in the presence of TC, and α the degree of protonation of the amine. Light-induced H^+ uptake by thylakoids was measured at pH 8.5 using a pH-sensitive electrode.

3. Results

As was mentioned above, DC and TC are permeant amines, which penetrate membranes primarily in the neutral form. In this case the illumination of chloroplasts should result in the protonation of amine inside thylakoids, due to the acidification of the lumen. At pH of the medium close to pK of amine, a stimulation of light-dependent H^+ uptake by thylakoids should be observed, which is proportional to the amount of amine cations accumulated inside the lumen in the bound and free states [16,17].

Fig. 1a shows the curves of light-dependent H^+ uptake by thylakoids in the absence and presence of 50 μM TC upon basal electron transport from water to methyl viologen. The addition of TC stimulates H^+ uptake. Hence, the TC uptake by thylakoids can be estimated. The dynamics of this process is pre-

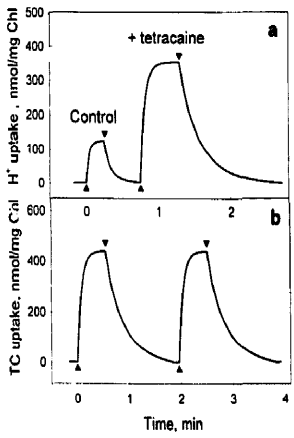

Fig. 1. Dynamics of light-induced H^+ (a) and TC (b) uptake by thylakoids. ▲, light on; ▼, light off. Concentration of TC was 50 μM. The reaction medium contained 0.1 M sucrose, 20 mM Tricine, 20 mM Hepes (pH 8.5), 10 mM NaCl, 5 mM $MgCl_2$, 50 μM methyl viologen, and the suspension of chloroplasts (50 μg chlorophyll per ml). TC uptake was calculated from the stimulation of H^+ influx in illuminated thylakoids (see Section 2). TC-dependent stimulation of H^+ influx was determined by subtraction of H^+ uptake curves in the presence and absence of TC.

sented in Fig. 1b. It can be seen that the light-induced TC uptake by thylakoids is completely reversible: its kinetics and amplitude are reproducible in several successive illumination cycles. In our previous study [17], we found a similar reversibility of structural changes in chloroplasts energized in the presence of TC or DC. So it would be interesting to conduct the time scanning of anesthetic-induced structural changes in chloroplast on the basis of TC uptake dynamics.

Pea chloroplasts used in the present study lose the envelope and stroma during isolation, but the architecture of the thylakoid system remains in the native state (Fig. 2a, b). The united fusiform membrane system consists of closed flattened membrane disks

Fig. 2. Thin sections of pea chloroplasts in the absence (a, b, c) and presence (d, e) of 25 μM DC. (a, b) Dark control; (c) after 10 min illumination; (d, e) formation of the network of anastomoses in the light after 60 s illumination; (e) membrane fusions at higher magnification. Bars equal 0.2 μm.

packed into stacks (granas), granal thylakoids, and single elongated thylakoids connecting granas (agranal thylakoids). As a rule, thylakoids in granas closely contact each other. Only one side of end grana thylakoids (end membranes) and small regions of inner grana thylakoids (margins) are in contact with the external medium. All agranal thylakoids contact directly the external medium (Fig. 2a, b). Both granal and agranal thylakoids in freshly isolated chloroplasts (dark control) are equal in thickness, which is close to 20 nm; the thickness of two thylakoid membranes in the grana is 15 nm, with the lumen being about 5 nm, which corresponds to their native state in a leaf. Normally, a preparation of isolated chloroplasts contains 5–10% of swollen thylakoids whose lumen is 5–10-times as great.

The illumination of chloroplasts (1–10 min) induces no marked structural alterations and does not increase the proportion of swollen chloroplasts (Fig. 2c).

Fig. 3. Thin sections of pea chloroplasts in the presence of 50 μM TC_in the dark (a), in the dark after illumination (1.5 min dark after 60 s illumination) (b), and in the light after 2 s illumination (c). Bars equal 0.2 μm.

The addition of 25–50 μM DC or TC to chloroplasts in the dark does not affect their structure (Fig. 3a). The illumination of the same preparations leads to considerable structural rearrangements. In the regions of thylakoids that contact the external medium, intermembrane anastomoses and local peak-shaped protrusions of membranes are seen (Fig. 2d, e; Fig. 4a; Fig. 5a). It can be assumed that the anastomoses result from the fusion of protrusions to nearby thylakoid membranes. The membranes fuse so that the intrathylakoid space of agranal and end granal thylakoids appears to be united. The lumen of anastomoses is the same wide that in thylakoids.

The number of interthylakoid anastomoses seen on the chloroplast sections vary from a few units to several tens. Sometimes, they are very abundant so

Fig. 4. Thin sections of pea chloroplasts in the presence of 50 μM TC in the light after 4 s (a) and 6 s (b) illumination. Peak-shaped protrusions are seen (arrow heads). Bars equal 0.2 μm.

that in the region of agranal thylakoids a network is formed, which appears as a foamy structure filling the entire space between granas that are not involved in the process (Fig. 2d, e; Fig. 5a). The meshes of the network appear as closed vesicles. In fact, however, they probably represent the external medium, which is as continuous as the membrane network itself.

The time scanning of intermembrane contact formation showed no structural changes during the first two seconds of illumination: the chloroplasts did not differ from the starting dark samples (Fig. 3c). After 4 s illumination, the first contacts between thylakoids were observed in 20% of chloroplasts. In addition, the protrusions of yet not fused membranes could be seen (Fig. 4a). After 6–8 s illumination, both the number of fusions in one chloroplast (Fig. 4b) and

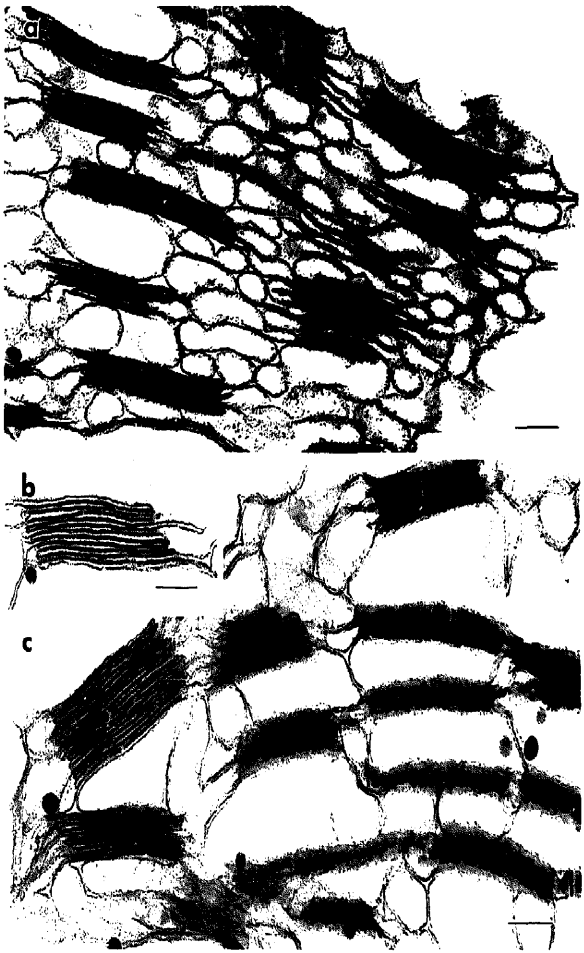

Fig. 5. Thin sections of pea chloroplasts in the presence of 25 μM DC in the light after 60 s (a) and 10 min (b, c) illumination. (a) Maximal development of the network of anastomoses; (b, c) degradation of membrane structures. Bars equal 0.2 μm.

the percentage of chloroplasts, in which membrane fusion took place, increased: intermembrane anastomoses were formed already in 50% of chloroplasts. A 10 s illumination produced anastomoses in 80% of chloroplasts, which was the maximum value for all preparations examined. The remaining 20% of chloroplasts did not contain anastomoses, but the volume of marginal and agranal thylakoids in them was considerably increased.

Thus, even a 10 s illumination is sufficient in order that chloroplasts become coated with a network of interthylakoid contacts. The maximum amount of anastomoses in the intergranal space of chloroplasts is observed after 60 s illumination (Fig. 5a).

The formed system of intermembrane contacts is maintained during 10 min. of continuous illumination, although some chloroplasts show signs of destruction. The destruction manifests itself in both a decreased number of interthylakoid contacts and a swelling of thylakoids themselves. The chloroplasts become more rounded and the granas somewhat crooked (Fig. 5b, c).

All these phenomena are reversible upon deenergization of organelles. Even 10 s after switching off the light, the chloroplasts return to the original state,

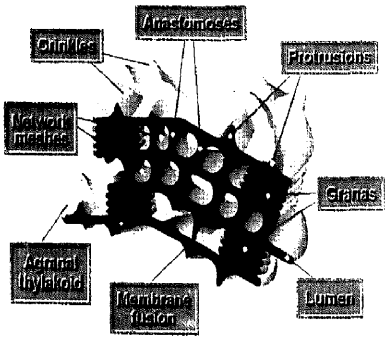

Fig. 6. Scheme for structural rearrangements of the thylakoid system in the presence of tertiary amine local anesthetics. The illumination of chloroplasts results in the appearance of membrane crinkles which are seen as peak-shaped protrusions on the sections. The inner membrane surface in the regions of crinkles has the positive curvature. The fusion of crinkles to nearby membranes leads to the formation of a network of anastomoses in the intergranal space. The meshes of the network are filled with external medium.

and only a few outgrowths can be seen on the membranes of some thylakoids (Fig. 3b). The ability of chloroplasts to form a system of anastomoses is retained after at least 10 cycles of illumination (60-s light/1.5-min dark regime), although a considerable destruction is observed.

Methyl viologen was mainly used as electron acceptor in the study; however, other acceptors (phenazine methosulfate ± diuron) did not affect the result. The addition of 2 μM chlorophenylhydrazone completely inhibited the light-induced structural rearrangements in the presence of DC and TC (data not shown).

4. Discussion

The fusion of biological membranes in the presence of local anesthetics has been considered previously in studies dealing with the effect of anesthetics on cell secretion and phagocytosis [18] as well as fusion of erythrocytes [19].

It is known that local anesthetics induce phase transitions in model systems consisting of simple lipids [20–22]. A pure glycolipid produces the hexagonal phase with the uncharged form and the cubic phase with the cationic form of TC. However, the addition of even one lipid, phosphatidylcholine, blocks phase transitions induced by TC and partially restores the molecular order of the bilayer [22]. High concentrations of uncharged TC molecules saturate the phosphatidylcholine membrane and induce phase transitions, whereas charged TC molecules incorporate into the bilayer to produce mixed TC-lipid micellae [11]. But there is no data on the lipid phase transitions when low concentrations of local anesthetics are added to the suspension of thylakoids.

Boulanger et al. [8] showed that the cations of TC interact with the polar headgroups of phospholipids and are localized at the interface of the bilayer, whereas uncharged molecules penetrate deeply into bilayer. In this process, the polar headgroups of phospholipids undergo stronger conformational changes than the hydrocarbon chains. It was proposed by Seelig et al. that the conformational alterations in the phospholipid headgroups are due mainly to changes in bilayer surface charge density under the action of the cationic forms of anesthetics [23].

It follows from the above studies that local anesthetics cause a disordering of the bilayer, which is highly expressed in the headgroup region rather than in the hydrocarbon one. The balance of electrostatic forces along the membrane surface seems to be important for anesthetic cations to be built up into the membrane. This statement was also confirmed by Shibata et al. [24,25] who found that the extent of TC incorporation into phosphatidylcholine membranes increased by the addition of negatively charged cardiolipin. The interaction of local anesthetics with membranes leads to the fluidization of the lipid matrix and expansion of membranes [18,26].

It is shown in the present study that the reversible binding of the cations of TC and DC inside thylakoids is attended by reversible structural rearrangements of the thylakoid membrane, the temporal characteristics of these processes being similar. Both of these effects depend on the generation of H^+ gradient across the photosynthetic membrane: they are inhibited by the addition of protonophore. The mechanism of this phenomenon is rather unclear. One possible explanation is the following.

In the dark the addition of a local anesthetic to the reaction medium leads to the distribution of charged and uncharged molecules between the inner volume of thylakoids, external medium, and membrane phase. The illumination of chloroplasts produces a concentration gradient of amine cations across the thylakoid membrane, which is equal to the H^+ gradient. The accumulation of the protonated amine in the lumen is responsible for the additional binding of amine cations to the inner side of the thylakoid membrane. It can be suggested that anesthetic cations interact with both the lipids of the inner membrane layer and the lumen-exposed proteins. In any case, the lateral pressure along the inner surface of the membrane rises, due to incorporation of anesthetic molecules and an increase in positive charge density. This may be a precondition for structural rearrangements compensating for increased pressure. One of the possible ways of such compensation is the appearance of membrane areas with the positive curvature of the inner surface. Presumably, this is just the cause of the formation of membrane crinkles, which have a peculiar form and appear as peak-shaped protrusions on the sections (Fig. 6).

The growth of a crinkle is supposed to be determined by two factors: the implication of the nearby membrane areas in the crinkle region and expansion of the membrane due to incorporation of anesthetic cations. If a crinkle comes in contact with an adjacent thylakoid, the two membranes fuse and form an anastomose. This process proceeds very rapidly, and we failed to register single steps. It seems likely that in the regions of crinkles, the membrane has features that make it predisposed to fusion. At physiological pH the outer surface of thylakoids is negatively charged [27]. With local anesthetics presented in the medium, the external surface of the thylakoid membrane may be neutralized to some degree, due to the binding of anesthetic cations. This may be important for the membranes to drift together and come into contact to each other [28].

In concluding, it should be mentioned that the lumen of anastomoses is similar to that of thylakoids. Therefore, even the maximum development of the network of membrane fusions should not lead to an increase in the total internal volume of thylakoids. This conclusion is consistent with the results of Laasch et al. [7], indicating that illumination in the presence of DC even decreases the osmotic space of chloroplasts. This observation is of interest if the structural effects of local anesthetics are compared with the effects of hydrophilic permeant amines, which cause thylakoid swelling [29–31]. Probably, the mechanism of action may differ in case of hydrophilic or lipophilic permeant amines.

References

[1] Rottenberg, H., Grunwald, T. and Avron, M. (1971) FEBS Lett. 13, 41–44.
[2] Günther, G. and Laasch, H. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 4, pp. 207–210, Kluwer, Dordrecht.
[3] Laasch, H. and Weis, E. (1988) Biochim. Biophys. Acta 936, 99–107.
[4] Laasch, H. and Weis, E. (1989) Photosynth. Res. 22, 137–146.
[5] Laasch, H. (1989) Planta 178, 553–560.
[6] Laasch, H. and Schumann, J. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 3, pp. 161–165, Kluwer, Dordrecht.
[7] Laasch, H., Schumann, J. and Günther, G. (1991) Planta 183, 567–574.
[8] Boulanger, Y., Schreier, S. and Smith, I.C.P. (1981) Biochemistry 20, 6824–6830.

[9] Kelusky, E.C. and Smith, I.C.P. (1984) Can. J. Biochem. Cell Biol. 62, 178–184.
[10] Kelusky, E.C., Boulanger, Y., Schreier, S. and Smith, I.C.P. (1986) Biochim. Biophys. Acta 856, 85–90.
[11] Frezzatti, W.A. Jr., Toselli, W.R. and Schreier, S. (1986) Biochim. Biophys. Acta 860, 531–538.
[12] Vanderkooi, G. and Adade, A.B. (1986) Biochemistry 25, 7118–7124.
[13] Opanasenko, V.K., Semenova, G.A., Agafonov, A.V. and Gubanova, O.N. (1995) Biochemistry (Moscow) 60, 2053–2057.
[14] Arnon, D.J. (1949) Plant Physiol. 24, 1–15.
[15] Semenova, G.A. (1995) Can. J. Bot. 73, 10, 1676–1682.
[16] Pick, U. and Avron, M. (1976) Eur. J. Biochem. 70, 569–576.
[17] Opanasenko, V.K., Gubanova, O.N. and Agafonov, A.V. (1995) Biochemistry (Moscow) 60, 687–691.
[18] Seeman, P. (1972) Pharmacol. Rev. 24, 583–655.
[19] Coakley, W.T., Nwafor, A. and Deeley, J.O.T. (1983) Biochim. Biophys. Acta 727, 303–312.
[20] Cullis, P.R. and Verkleij, A.J. (1979) Biochim. Biophys. Acta 552, 546–551.
[21] Menashe, M., Schmidt, C.F., Conley, T.G. and Bittonen, R.L. (1982) Biophys. J. 37, 199a.
[22] Auger, M., Smith, I.C.P. and Jarell, H. (1989) Biochim. Biophys. Acta 981, 351–357.
[23] Seelig, A., Allegrin, P.R. and Seelig, J. (1988) Biochim. Biophys. Acta 939, 267–276.
[24] Shibata, A. Ikawa, K. and Terada, H. (1995) Biophys. J. 69, 470–477.
[25] Shimooka, T., Shibata, A. and Terada, H. (1992) Biochim. Biophys. Acta 1104, 261–268.
[26] Johnson, R. (1982) Biochem. Pharmacol. 31, 1784–1787.
[27] Barber, J. (1982) Annu. Rev. Plant Physiol. 33, 261–295.
[28] Seddon, J.M. (1990) Biochim. Biophys. Acta 1031, 1–69.
[29] Crofts, A.R. (1967) J. Biol. Chem. 242, 3352.
[30] Izawa, S. and Good, N.E. (1966) Plant Physiol. 41, 544–552.
[31] Hind, G. and Jagendorf, A.T. (1965) J. Biol. Chem. 240, 3195–3201.