Lidocaine and ATPase Inhibitor Interaction with the Chloroplast Envelope

Weihua Wu and Gerald A. Berkowitz*
Horticulture Department, Cook College, Rutgers-The State University of New Jersey,
New Brunswick, New Jersey 08903

ABSTRACT

Photosynthetic capacity of isolated intact chloroplasts is known to be sensitive to K⁺ fluxes across the chloroplast envelope. However, little is known about the system of chloroplast envelope proteins that regulate this K⁺ movement. The research described in this report focused on characterizing some of the components of this transport system by examining inhibitor effects on chloroplast metabolism. Digitoxin, an inhibitor of membrane-bound Na⁺/K⁺ ATPases, was found to reduce stromal K⁺ at a range of external K⁺ and inhibit photosynthesis. Scatchard plot analysis revealed a specific protein receptor site with a Kᵣ for digitoxin binding of 13 nanomolar. Studies suggested that the receptor site was on the interior of the envelope. The effect of a class of amine anesthetics that are known to be K⁺ channel blockers on chloroplast metabolism was also studied. Under conditions that facilitate low stromal pH and concomitant photosynthetic inhibition, the anesthetic, lidocaine, was found to stimulate photosynthesis. This stimulation was associated with the maintenance of higher stromal K⁺. Comparison of the effects on photosynthesis of lidocaine analogs which varied in lipophilicity suggested a lipophilic pathway for anesthetic action. The results of experiments with lidocaine and digitoxin were consistent with the hypothesis that a K⁺ channel and a K⁺-pumping envelope ATPase contribute to overall K⁺ flux across the chloroplast envelope. Under appropriate assay conditions, photosynthetic capacity of isolated chloroplasts was shown to be much affected by the activity of these putative envelope proteins.

The chloroplast inner envelope is an osmotic barrier, and its free permeability is limited to small uncharged molecules such as H₂O, NH₃, CO₂, and O₂ (4). During light-induced stromal alkalization (8), a pH gradient is maintained between the stromal compartment and the cytoplasm. However, H⁺ movement can occur across the envelope. Stroma to cytoplasm H⁺ flux is facilitated, and regulated, by a not well-characterized system of membrane proteins. The integrated action of these proteins results in the maintenance of a transenvelope gradient (in the light) of nearly 1 pH unit (25). Net efflux of H⁺ from the illuminated chloroplast may also occur apparently against this chemical gradient (6, 8).

Previous work has established that overall H⁺ flux across the chloroplast envelope is modulated by several systems. K⁺ fluxes, either directly (14) or indirectly (3), are somehow linked to H⁺ countermovement. The action of an envelope ATPase (again, either directly or indirectly) is required to maintain an alkaline stroma under certain conditions (14, 17). Chloroplast envelope-bound Mg²⁺ also contributes to the regulation of H⁺ flux, possibly at the site that links H⁺ and K⁺ counterfluxes (11, 14, 17, 20).

In a recent study from this laboratory, several inhibitors were identified as affecting the ability of the chloroplast to maintain an alkaline stromal pH in the light (17). The cardiac glycoside digitoxin was found to cause stromal acidification and photosynthetic inhibition. It was speculated that this compound acted by binding to, and inhibiting, an envelope ATPase in a fashion similar to the previously reported action of oligomycin (14). Under conditions that facilitated net H⁺ influx, the amine anesthetic lidocaine was found to increase stromal pH. Because this anesthetic’s action in animal systems is mediated by the blockage of monovalent cation channels (2), it was speculated that lidocaine’s effects on chloroplast metabolism were due to a restriction of K⁺ efflux through an ion channel, indirectly resulting in a reduction of H⁺ import.

Identification of these pharmacological agents as affecting chloroplast metabolism offers some new probes with which the envelope proteins regulating H⁺ fluxes can be further characterized. For example, digitoxin has been reported to only affect the Na⁺/K⁺ pump in animal systems (21); Na⁺/K⁺ ATPases have not been previously in plant membranes. Identification and characterization of the digitoxin-binding site on the chloroplast envelope could aid in characterizing the nature of this ATPase. Ion channels have not been previously reported to be present in the chloroplast envelope. Further work with lidocaine could aid in characterizing that component of the envelope transport system. It was the objective of the work reported in this study, then, to further characterize how these pharmacological agents interact with chloroplast metabolism.

MATERIALS AND METHODS

Plant Material

Spinach (Spinacia oleracea var ‘Melody’) seeds were planted in pots containing 2000 cm³ potting mix (1:1, v/v, peat:vermiculite) and thinned to three to five plants/pot after 2 weeks. Pots were watered twice/week with tap water and once/week with complete (Peter’s Geranium Special plus
Chamber fertilizer. Plants were grown in a growth chamber with a 10-h light (480 μmol/m²/s) period at 21°C (day)/16°C (night). Fully expanded, nonsenescent leaves taken from 6- to 8-week-old plants were used for chloroplast isolation.

**Chloroplast Isolation**

Intact (routinely >90%) chloroplasts were isolated from spinach using procedures described previously (17). Briefly, leaves were homogenized in 50 mL grind medium containing 0.33 mM sorbitol, 50 mM Hepes-NaOH (pH 6.8), 2 mM Na₂EDTA, 1 mM MgCl₂, and 1 mM MnCl₂. After centrifugation (750g for 50 s in a Sorvall RC5C centrifuge and SS-34 rotor), pelleted chloroplasts were resuspended in 5 mL grind medium and carefully layered on top of an 8-mL cushion (40% Percoll and 0.5% [w/v] BSA in grind medium) in 15-mL tubes. These step gradients were then centrifuged (2000g in an HS-4 rotor for 1 min). Intact chloroplasts were recovered in the pellet and resuspended in a small volume (0.5–1.0 mL) of grind medium. All steps in the isolation procedure were carried out at 0 to 2°C.

**Thylakoid Membrane Isolation**

After isolation, intact chloroplasts were suspended in 20 to 30 mL buffer (20 mM Hepes-KOH, pH 7.6, with 4 mM MgCl₂), vortexed, and kept on ice for 2 min. Thylakoids released from osmotically shocked chloroplasts were recovered by centrifugation at 1500g (HS-4 rotor) for 4 min. The green pellet was resuspended in 1 to 2 mL ATPase assay medium as described by Hargarter and Ort (7) which contained 0.1 mM sorbitol, 20 mM Hepes-KOH (pH 7.8), 5 mM DTT, 20 mM KC1, and 4 mM MgCl₂ and stored on ice until used.

**Photosynthesis**

CO₂-supported O₂ evolution of intact chloroplasts was measured with Hansatech (Decagon Instr., Seattle, WA) O₂ electrodes at 25°C and 1500 μmol/m²/s PAR supplied by the 500 W bulb of a slide projector focused through a water heat filter. Typically, 50 μL grind medium containing 20 to 30 μg Chl was added to 1 mL final volume photosynthesis reaction medium (0.33 mM sorbitol, 50 mM Hepes-NaOH [pH 7.6], 2 mM Na₂EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 0.25 mM NaH₂PO₄, 5 mM NaHCO₃, and 1000 units/mL catalase) with additions as noted in the text. In some experiments, the chloroplasts were exposed to a “high Mg²⁺” treatment. In this case, 5 mM extra Mg²⁺ was added (as the chloride salt) to the standard reaction medium, which already contained 2 mM divalent cations and 2 mM EDTA. In this case, the chloroplasts were exposed to high levels of free external Mg²⁺, which increases the amount of chloroplast envelope-bound Mg²⁺ (20).

**Stromal K⁺**

Stromal K⁺ at varying external K⁺ was ascertained using silicone oil microcentrifugation. Four 400-μL microcentrifugation tubes were used as replicates for each measurement. Each microfuge tube contained a bottom layer of 20 μL 14% (v/v) HClO₄, 100 μL silicone oil mixture (as described in ref. 17), and a top layer of 200 μL photosynthetic reaction medium with chloroplasts equivalent to approximately 20 μg Chl. The tubes were incubated at 25°C in a water bath with 1200 μmol/m²/s PAR supplied by banks of 500 W incandescent floodlights placed on either side of the waterbath. After 10 min, tubes were microcentrifuged for 15 s in a Beckman microfuge B. Frozen tubes were cut in the oil layer, and the bottom portion of the tube with the HClO₄ fraction was placed in a 1.5-mL microfuge tube with 480 μL water, vortexed, and centrifuged for 1 min. The supernatant was assayed for K⁺ using a Perkin-Elmer 2280 atomic absorption spectrophotometer (Norwalk, CT). All samples and standards were made up to contain 0.2% NaCl; absorbance was measured at a wavelength of 766.5 nm. K⁺ in the HClO₄ fraction of the microcentrifuge tubes that centrifuged with the chloroplasts, but was external to the stroma, was estimated and subtracted from the total in each tube to ascertain stromal K⁺ levels. This was done by measuring the [¹⁴C]sorbitol-permeable space, as described below, under the various treatments for the chloroplast preparation and assuming that the external K⁺ equilibrated into this volume. Stromal K⁺ concentration was derived from a measure of the stromal volume (ascertained as described below) under the various treatments.

**Chloroplast Volume**

The dual-label silicone oil microcentrifugation technique developed by Heldt et al. (8) as modified by Peters and Berkowitz (17) was used to ascertain stromal volume and sorbitol-permeable space associated with chloroplasts. Chloroplasts were incubated in 200 μL photosynthetic reaction medium which contained 12.5 μCi/mL [¹⁴C]sorbitol and 5 μCi/mL [¹¹H]H₂O above layers of 100 μL silicone oil and 20 μL HClO₄ in 400-μL tubes. After 5 min, chloroplasts were separated from the medium above the silicone oil layer by microcentrifugation for 15 s. H and ¹⁴C in the supernatant and the HClO₄ fraction of the tubes were ascertained using dual-label dpm programs with external standards ratio quench correction on a Beckman 3801 liquid scintillation spectrophotometer. The ¹¹H₂O volume associated with the chloroplasts represented the sum of the stromal and sorbitol-permeable space. The [¹⁴C]sorbitol space represented the sorbitol-permeable (i.e. extrstromal) volume of reaction medium associated with the chloroplasts in the HClO₄ fraction. The difference between the two was taken to be a measure of stromal volume.

**Digitoxin-Binding Studies**

Digitoxin binding to the chloroplast envelope was estimated using silicone oil microcentrifugation of chloroplasts incubated in solutions containing [¹¹H]digitoxin and [¹⁴C]sorbitol. Intact chloroplasts were incubated under the same conditions used for the stromal K⁺ determinations except labeled sorbitol (at 12.5 μCi/mL) and digitoxin (at 26.1 Ci/mmol) were added to the photosynthetic reaction medium. In addition to the labeled sorbitol and digitoxin, a second set of tubes was made up with unlabeled cardiac glycoside added above that supplied...
by the addition of labeled digitoxin. Chloroplasts in the 400-
µL microfuge tubes were incubated in the light at 25°C for 5
min prior to centrifugation for 15 s. The [14C]sorbitol pelleting
with the chloroplasts in each tube was used to estimate and
subtract out the [3H]digitoxin in the HClO4 fraction that was
not associated with the chloroplast. The amount of labeled
digitoxin bound to the chloroplast envelope in the presence
of both labeled and nonlabeled (10 µM) digitoxin was sub-
tracted from the amount of labeled digitoxin bound to the
chloroplast envelope in the presence of only labeled digitoxin.
The differences represent specific binding of a ligand to a
receptor molecule and were used in Scatchard Plot analyses
(1) to distinguish between specific binding of digitoxin to a
receptor molecule and nonspecific association of this inhibitor
with the chloroplast envelope.

Reagents

[14C]sorbitol and [3H]H2O were obtained from ICN (Irvine,
CA) and [3H]digitoxin was from New England Nuclear (Bos-
ton, MA). Silicone oils were from William F. Nye Co. (New
Bedford, MA), and Hapes was from Research Organics (Cleve-
land, OH). Bupivacaine was obtained as a gift from the
manufacturer (Sterling-Winthrop Research Institute, Renssel-
laer, NY). Lidocaine analogs QX-222 and QX-314 were ob-
tained as a gift from the manufacturer (Astra Pharmaceutical,
Westborough, MA). Lidocaine prepared as the HCl salt was
obtained from Research Biochemicals (Natick, MA). All other
reagents were from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

Envelope Protein Inhibitor Effects on Photosynthesis

Incubation of chloroplasts in solutions containing milli-
molar levels of unchelated Mg2+ leads to stromal acidification
in vitro, resulting in photosynthetic inhibition (11, 17). It can
be speculated that, under this condition, the activity of chlo-
roplast envelope proteins that facilitate H+ influx would be
necessary to maintain maximal rates of photosynthesis. Con-
versely, inhibition of an envelope protein whose activity re-
sults in H+ influx should increase photosynthesis when an H+
extrusion system has been “turned off.” These relationships
are demonstrated in the series of experiments shown in Table
I. In three separate experiments, addition of Mg2+ to the
reaction medium inhibited photosynthesis by about 30%. In
the presence of added Mg2+, exposure of chloroplasts to the
ATPase inhibitor digitoxin or oligomycin resulted in further
photosynthetic inhibition. These ATPase inhibitors decrease
the stromal pH of the illuminated chloroplast (11, 17). The
data in Table I are consistent with previous assertions in the
literature (14, 15) which postulate that ATPase-facilitated H+
efflux from the stroma is necessary for optimal photosyn-
thesis. In further studies, it was determined that, after iso-
lation, chloroplasts become increasingly sensitive to the Mg2+
and the ATPase inhibitor treatments. During a 2.5-h period
when chloroplasts were left in grind medium at O°C, photo-
synthetic capacity (measured at 25°C) was hardly diminished;
however, the photosynthetic inhibition caused by Mg2+ and
digitoxin greatly increased (data not shown). Possibly, the
increased sensitivity to these treatments with time may reflect
a general collapse in the ability of the illuminated chloroplast
to maintain high stromal pH, which may be related to a loss
in stromal K+ (Fig. 1).

It should be noted that not all previous studies support the
contention that the activity of an envelope ATPase contrib-
utes to the maintenance of an alkaline stromal pH in the
light. Reduction of the ATP level (using coupling factor
inhibitors) in the illuminated chloroplast was not found to
affect stromal pH in two different studies (5, 19). However,
stromal [ATP] in the presence of the inhibitors was apprecia-
ble, 30% in one study (19) and in the other (5), 70% of the
content found under control conditions. Possibly, envelope
ATPase activity was not much affected by this reduction in
ATP level. The finding (as shown in Table I) that several
chloroplast envelope ATPase inhibitors inhibit photosynthesis
and that this inhibition is reversed by treatments (lidocaine)
that increase stromal pH (17) suggests an important physio-
logical role for the oligomycin- and digitoxin-sensitive ATP-
ase. In the presence of Mg2+ and digitoxin, photosynthetic
stimulation due to lidocaine addition averaged 26% in the
three experiments shown in Table I. Lidocaine stimulated
photosynthesis by an average 46% in the presence of Mg2+

---

**Table I. Mg2+, ATPase Inhibitor, and Lidocaine Effects on Photosynthesis of Isolated Chloroplasts**

In this table, the D and O treatments refer to 6.5 µM digitoxin and 20 µg/mL oligomycin, respectively. Lidocaine was at 100 µM and Mg2+ was supplied to the reaction medium at 5 mM (above the level chelated by EDTA). Results of three separate experiments are presented; data are shown as means ± se (n = 4).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Photosynthesis</th>
<th>% Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
</tr>
<tr>
<td></td>
<td>µmol O2/(mg Chl/h)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>143 ± 2.7*</td>
<td>101 ± 3.2</td>
</tr>
<tr>
<td>Mg2+</td>
<td>103 ± 3.2</td>
<td>69 ± 2.5</td>
</tr>
<tr>
<td>+D,</td>
<td>68 ± 2.4</td>
<td>48 ± 3.0</td>
</tr>
<tr>
<td>+D, lidocaine</td>
<td>83 ± 2.4</td>
<td>58 ± 2.5</td>
</tr>
<tr>
<td>+O</td>
<td>46 ± 1.9</td>
<td>32 ± 2.0</td>
</tr>
<tr>
<td>+O, lidocaine</td>
<td>67 ± 7.0</td>
<td>54 ± 3.5</td>
</tr>
</tbody>
</table>

* For each experiment, the rate under the various treatments is compared with the control rate.
and oligomycin in these experiments. Because lidocaine is known to be a K⁺ channel blocker (2), the photosynthetic stimulation caused by this anesthetic (Table I) suggests that preventing K⁺ loss from the stroma by restricting flux through an ion channel may greatly affect photosynthesis. Further studies addressed this hypothesis.

Measurement of lidocaine effects on stromal K⁺ did suggest that at least one K⁺ transport site across the chloroplast envelope may be a K⁺ channel. Previous work has shown that 100 mM K⁺ or above is required in incubation media to prevent K⁺ loss from isolated chloroplasts and to maintain high rates of photosynthesis (3, 18). In the presence of Mg2⁺ and the ATPase inhibitor digitoxin, exposure of chloroplasts to lidocaine did result in higher stromal K⁺ concentrations at a wide range of external K⁺ (Fig. 1A). The effect of lidocaine on photosynthesis (Table I), then, is likely associated with a reduction in K⁺ loss from the stroma (Fig. 1A).

**Characterization of Amine Anesthetic Action**

Physiological effects of local amine anesthetics are related to their ability to reach the receptor site of the cation channel, which resides deep within the channel pore toward the interior surface of the membrane (9, 22). It is currently theorized that the receptor site is most readily reached by a compound such as lidocaine via a hydrophobic route involving diffusion across the lipid portion of the membrane (10, 22). Characterization of the interaction of anesthetics with ion channels, therefore, has been facilitated by comparison of the relative efficacy of different analogs that vary in lipophilicity (9, 10, 26). Analogues of lidocaine that are less lipophilic have been found not to penetrate as well through the membrane to the receptor site; their pharmacological action is less pronounced (10). The experimental compounds QX-222 and QX-314 have structures similar to the tertiary amine lidocaine, except they are quaternary amines and, therefore, have a fixed charge. The fixed positive charge at the amine nitrogen is surrounded by ethyl groups in the structure of QX-314, making this quaternary amine substantially more lipophilic than QX-222 despite its cationic property (10). In the structure of the local amine anesthetic bupivacaine, the 3° amine nitrogen resides in a piperidine ring and is substituted with a butyl group, making it substantially more lipophilic than lidocaine (23, 26). Lidocaine hydrochloride is a preparation of the anesthetic that, when delivered to an aqueous solution at approximately pH 8, delivers a slightly lower level of the pharmacologically active free base form. The relative lipophilicity of these anesthetic analogs, from lowest to greatest, is as follows: QX-222 < QX-314 < lidocaine HCl < lidocaine < bupivacaine. In a series of experiments, the relative ability of these anesthetic analogs to stimulate photosynthesis in the presence of the ATPase inhibitors was found to be correlated with their relative lipophilicities. A compilation of results from four such experiments is shown in Figure 2. Photosynthesis in the presence of Mg²⁺ and digitoxin or oligomycin averaged 57% of the control rate in these experiments. As compared with this inhibited rate, the anesthetics QX-222, QX-314, lidocaine HCl, lido-
caine, and bupivacaine stimulated photosynthesis by 0, 41, 42, 48, and 63%, respectively. In the absence of the acidifying treatments (Mg²⁺, digitoxin, oligomycin), the anesthetics had no significant effect on photosynthesis in these experiments (data not shown).

Direct effects of these anesthetic analogs on the putative chloroplast envelope ion channel were not monitored in these experiments. The effects these compounds have on photosynthesis as shown in Figure 2 likely result from their effects on stromal K⁺ currents as noted in Figure 1A. The differential effects these local amine anesthetics have on photosynthesis allow for some preliminary speculations concerning the molecular nature of the ion channel. In all likelihood, the channel structure is similar to amine anesthetic-sensitive channels in animal systems. The drug receptor site is readily accessible either from within the membrane through the channel walls or from the aqueous phase on the interior side of the membrane; in either case the pathway involves movement through a hydrophobic phase. The analog QX-222 had no effect on the chloroplast, supporting this notion. Bupivacaine was found to work as well or better than lidocaine. The 10-fold increase in the lipid solubility of bupivacaine over lidocaine (23) further supports the notion of a lipophilic pathway for access to the receptor site in the channel. All the anesthetics that did affect the chloroplast in these studies (QX-314, lidocaine, bupivacaine) have a common dimethyl-benzene ring in the lipophilic portion of the molecule away from the substituted amine. This ring structure is thought to afford tight binding to a hydrophobic component within the channel pore (9).

**Preliminary Characterization of the Digitoxin Receptor**

We speculate that digitoxin inhibits photosynthesis (Table I) because of inhibition of an envelope ATPase. However, the possibility remains that the site of digitoxin inhibition is the thylakoid ATPase. This issue was addressed by comparing the effects of digitoxin and oligomycin, along with a known inhibitor of coupling factor activity (N,N'-dicyclohexylcarbodi-imide; 13) on ATPase activity of purified thylakoid membranes using the ATPase assay described by LeBel et al. (12) and light-activating the thylakoid preparation as described by Hargarter and Ort (7). Whereas N,N'-dicyclohexylcarbodi-imide substantially inhibited ATPase activity of these preparations, digitoxin and oligomycin had no effect (data not shown), supporting the contention that the effects these inhibitors have on photosynthesis were mediated by an inhibition of the chloroplast envelope ATPase.

Digitoxin (along with other compounds such as ouabain and digoxin which make up a class of drugs known as cardiac glycosides) is known to specifically inhibit only the Na⁺/K⁺ ATPase, which is a ubiquitous component of all known animal cell plasma membranes (21). The effect this inhibitor has on photosynthesis (Table I) raises the intriguing possibility that its receptor site on the chloroplast envelope is an Na⁺/K⁺ ATPase. Therefore, a series of experiments were undertaken to further characterize the nature of the interaction of this inhibitor with the chloroplast.

It is clearly documented that the digitoxin-binding site on the Na⁺/K⁺ ATPase in animal membranes in completely accessible from the external solution (21). It is for this reason that, in studies of cardiac glycoside effects on this ATPase, ouabain is typically used in place of digitoxin (24). Ouabain is highly water soluble (lipid insoluble), and digitoxin is highly lipid soluble (24). This extreme difference in lipid solubility does not affect the relative efficacy of these cardiac glycosides; this finding has been used to support the contention that the inhibitor-binding site on this ATPase is on the outer surface of animal cell membranes (24).

Cardiac glycoside interaction with the chloroplast envelope ATPase may be qualitatively different. Whereas digitoxin routinely inhibits photosynthesis of isolated chloroplasts (Table I), ouabain at concentrations up to 10-fold the effective concentration of digitoxin had no effect on chloroplast photosynthesis (Table II). These data suggest that the receptor site on the chloroplast envelope ATPase is not accessible to ouabain but is reached by digitoxin, which can readily diffuse within the interior of the chloroplast envelope membrane.

Further studies were undertaken to examine digitoxin binding to a receptor site on the chloroplast envelope. Increasing concentrations of digitoxin resulted in an increasing extent of [³H]digitoxin associated with the chloroplast (Fig. 3A). Significantly, the presence of an excess of unlabeled ouabain had no effect on the extent of [³H]digitoxin bound to the chloroplast (Fig. 3A). These data are consistent with the hypothesis that the cardiac glycoside receptor site on the chloroplast envelope ATPase is not accessible to the nonlipophilic compound ouabain.

Despite the finding of substantial effects of digitoxin on chloroplast photosynthesis (e.g. Table I), no data have been presented to demonstrate that digitoxin association with the chloroplast is a function of binding of this ligand to a specific receptor site of a protein on the chloroplast envelope. Especially because of the lipophilic nature of this inhibitor, the possibility exists that digitoxin association with the chloroplast could be nonspecific; the compound could simply diffuse into the envelope interior and be associated with the chloroplast in a nonspecific manner.

Attempts were made to demonstrate specific ligand to receptor site binding of this ATPase inhibitor with the chloroplast using Scatchard Plot analyses which allow for identification of binding to a specific receptor despite the occurrence of a large excess of nonspecific binding (1, 16). These experiments involve the quantification of [³H]digitoxin binding to the chloroplast in the presence and absence of additions of

<table>
<thead>
<tr>
<th>Table II. Comparison of Cardiac Glycoside Effects on Photosynthesis in the Presence of 5 mm Mg²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cardiac glycoside concentrations are in parentheses.</strong></td>
</tr>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Mg²⁺</td>
</tr>
<tr>
<td>Mg²⁺, digitoxin (6.5 μM)</td>
</tr>
<tr>
<td>Mg²⁺, ouabain (6.5 μM)</td>
</tr>
<tr>
<td>Mg²⁺, ouabain (65 μM)</td>
</tr>
</tbody>
</table>

* Rate under the various treatments is compared with the control.
Assuming Chl. from this analysis (21). Scatchard average concentration of site for receptor Chl, nmol/mg calculated results analyses shown (120). Plot analysis means ± SE of bound binding Figure 2. Measurement of bound [3H]digitoxin in the presence and absence of an excess of unlabeled ouabain (A) or unlabeled digitoxin (B). Binding in the presence of only labeled digitoxin (O) is compared with binding in the presence of 1 mM ouabain (△) in A. In B, binding of labeled digitoxin in the absence (O) and presence (□) of 10 μM added unlabeled digitoxin was measured. The differences (\( V \)) is the calculated specific binding (1). It should be noted that, although the maximum ouabain concentration can be made relatively high (i.e. at 1 mM), digitoxin at 10 μM approaches the limit of solubility in aqueous solutions. The experiment shown in B was repeated a total of three times; results in each case were similar to those shown. All data are shown as means ± SE (n = 4).

unlabeled digitoxin; the specific binding component of total [3H]digitoxin association with the chloroplast can be deduced from this analysis as shown in Figure 3B. In the Scatchard Plot analyses shown in Figure 4, a high affinity specific receptor site for digitoxin was revealed. The average \( K_m \) for the two experiments shown of 12.68 nM is similar to the \( K_m \) for cardiac glycoside binding to the Na+/K+ ATPase in animal cell membranes, which ranges between 2 and 23 nM, depending on the particular mix of cations present during the assay (21). Scatchard Plot analysis also allows for estimation of the concentration of receptor sites for the ligand (Rt in Fig. 4). The average value of the two experiments shown; 1.30 × 10^{-3} nmol/mg Chl, is equivalent to 7.83 × 10^{11} receptor sites/mg Chl. Assuming a total chloroplast stromal volume of 20 μL/mg Chl and individual chloroplast shape as a sphere with a 4-μm diameter, these experiments yield a digitoxin receptor site (i.e. possible envelope ATPase) concentration of 5.2 × 10^9 binding sites/cm² of the chloroplast inner envelope.

The data presented in Figures 3 and 4 indicate that a chloroplast ATPase other than the coupling factor likely has a receptor site that binds specifically to digitoxin. Although the issue is still not resolved, at least some previous studies have shown that chloroplast envelope ATPase activity contributes to the maintenance of high stromal pH and optimum photosynthetic capacity (14, 17). The experiments in this study that suggest that this ATPase has a specific binding site for digitoxin lead to the intriguing speculation that the physiological effects of this envelope ATPase on chloroplast metabolism may be mediated by its action as a K+ pump. Data shown in Figure 1B support this contention. At a range of external K+ concentrations, exposure of chloroplasts to digitoxin resulted in a decrease in stromal K+. Maury et al. (14) previously noted a reduction in K+ uptake due to treatment of intact chloroplasts with oligomycin. This body of work, then, suggests that the activity of a K+-pump ATPase in the

Figure 3. Measurement of bound [3H]digitoxin in the presence and absence of an excess of unlabeled ouabain (A) or unlabeled digitoxin (B). Binding in the presence of only labeled digitoxin (O) is compared with binding in the presence of 1 mM ouabain (△) in A. In B, binding of labeled digitoxin in the absence (O) and presence (□) of 10 μM added unlabeled digitoxin was measured. The difference (\( V \)) is the calculated specific binding (1). It should be noted that, although the maximum ouabain concentration can be made relatively high (i.e. at 1 mM), digitoxin at 10 μM approaches the limit of solubility in aqueous solutions. The experiment shown in B was repeated a total of three times; results in each case were similar to those shown. All data are shown as means ± SE (n = 4).

Figure 4. Scatchard Plot analysis of specific binding of digitoxin to a receptor site on a protein of the envelope. Data shown in A are recalculated from the experiment shown in Figure 3B. Data in B are from a second experiment. The slope of the linear regression (i.e. the broken line) represents the negative reciprocal of the \( K_m \) for ligand to receptor interaction. Abscissa intercept of the regression (labeled Rt) yields the total receptor number/mg Chl. Each data value was calculated from binding data with four replications per concentration and are shown as means ± SE.
envelope may be important for the normal metabolic function of the chloroplast. No information is presented in this report, however, to delineate how ATPase-driven K+ fluxes are linked to H+ counterflow. Digitoxin-sensitive K+ uptake, as shown in Figure 1B, could only be indirectly linked to H+ counterflow. This possibility could explain why some researchers have concluded that envelope ATPase activity is not essential to maintain high stromal pH. With the isolated chloroplast in vitro, high stromal pH may be dependent on K+ uptake only under certain incubation conditions as discussed previously (14, 17, 20). It should also be pointed out that no data have been presented here to delineate whether or not the oligomycin- and digitoxin-sensitive chloroplast envelope ATPases are the same proteins. Because these inhibitors both cause photosynthetic inhibition that is linked to stromal acidification (17) and reduction of stromal K+ (Fig. 7, also see ref. 14), this possibility appears plausible.

CONCLUSION

Data presented in this report has focused on substantiating the assertion that local amine anesthetics, such as lidocaine, and the cardiac glycoside, digitoxin, affect chloroplast metabolism because of interaction with chloroplast envelope proteins. Documentation of these interactions is significant for several reasons. K+ movements across the chloroplast envelope greatly affect photosynthetic capacity due to concomitant H+ counterfluxes (3, 11, 14, 17). The use of these inhibitors as probes in this study has allowed the identification of a K+ channel and a K+ pump (which could be an Na+/K+ or a K+/H+ pump) ATPase as possibly contributing to the regulation of K+ exchange across the envelope. These proteins may play an important function in maintaining high stromal pH in the light and, hence, allowing for maximal photosynthetic activity.

Characterization of the interaction of these inhibitors with chloroplast envelope proteins is also significant in that an extensive body of medical literature exists regarding the nature of both lidocaine and digitoxin interaction with membrane proteins. This information should allow for further insights to be made regarding both the chloroplast envelope ATPase and the K+ channel.

LITERATURE CITED