Study of the Effect of Ion Channel Modulators on Photosynthetic Oxygen Evolution

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Various ion channel activities can be recorded by electrophysiological methods in the outer and inner envelope membranes of chloroplasts as well as in the thylakoid membrane. However, most of these channels are poorly characterized from a pharmacological point of view. Furthermore, the molecular identity has been determined only for a few of them, preventing an understanding of their role in plant physiology. By allowing specific ion fluxes across plastidial membranes, these ion channels may either directly or indirectly regulate photosynthesis, as has been hypothesized earlier. We have determined the effect of various ion channel modulators [indole-3-acetic acid, 5-nitro-2-(3-phenylpropylamino)-benzoate, (−)-epigallocatechin-3-gallate, *p*-chlorophenoxyacetic acid, Konig's polyanion, Cs^+, Gd^{3+}, 4-aminopyridine, tetraethylammonium chloride, charybdotoxin, nimodipine, and cyclosporin A] on the efficiency of photosynthetic oxygen evolution in intact chloroplasts, broken chloroplasts, and isolated thylakoids. The data may improve our understanding of chloroplast ion channels and identifies inhibitors which may be exploited for electrophysiological studies.

INTRODUCTION

Chloroplasts are small organelles in plant cells, separated from the cytoplasm by double envelope membranes, that is, outer and inner envelopes. Chloroplast stroma contain a third membrane system, the thylakoid membrane, in which the light-driven reactions of photosynthesis take place. During photosynthesis, photons are absorbed by the antenna pigments, that is, protein-bound chlorophyll and carotenoids, and the excitation energy is transferred from the site of absorption, mainly the light-harvesting complexes, to the reaction centers. Here, excitation is converted into charge separation, which drives electron flow from photosystem II (PSII) to photosystem I (PSI) via the cytochrome b_6f complex. The net result of this process is the oxidation of water molecules, molecular oxygen evolution, the reduction of $NADP^+$, and generation of a proton gradient (ΔpH) across the membrane. The energy stored as ΔpH is exploited for ATP synthesis (for a recent review on photosynthesis, see, e.g., ref 1). Electron flow is strictly correlated with the evolution of molecular oxygen, the rate of which can be taken as a measure of photosynthetic efficiency.

Electrophysiological and pharmacological evidence strongly suggests that, as in other organisms, ion channels are also fundamental for the induction of specific cellular responses in plants. They are thought to participate in signaling in response to various endogenous and environmental stimuli, for example light, phytohormones, and pathogen attack.[2] In most cases, the channels involved in these processes have not yet been identified on a molecular level, and the investigation of the molecular identity and role of ion channels in the chloroplast (Chl) envelope and thylakoid membranes is still at its beginning stages.

As described above, during photosynthesis, a light-driven proton gradient is generated between the chloroplast stroma and the thylakoid lumen. Upon illumination, the flux of protons from the stromal to the lumenal side of the thylakoid membrane is expected to lead to the formation of a pH gradient, as well as the formation of a transmembrane potential, given that protons are positively charged. Instead, in sharp contrast to mitochondria [where $\Delta\mu_{H+}$ is composed of both ΔpH and $\Delta\Psi$ (ca. −180 mV)], transthylakoid $\Delta\mu_{H+}$ is mainly composed of ΔpH. This fact, along with the observation that the $\Delta\Psi$ difference dissipates relatively rapidly, was taken as evidence for the operation of ion channels in series with an electron-transfer chain (see, e.g., ref 3), which would electrically balance light-driven proton transport.

A role for cationic channels in counterbalancing and, in general, the existence of ion channels in the thylakoid membrane were hypothesized in the 1970s, thanks to the pioneer works of several groups.[4−11] It is, indeed, well-established by now that mainly K^+, Mg^{2+}, and small anions take part in counterbalancing and that changes in the concentrations of all these ions in the stroma are accompanied by passive H^+, K^+, Cl^-, and probably Na^+ transport across the envelope of intact chloroplasts. However, the use of specific channel inhibitors for the study of the ion-flux−photosynthesis relationship has been undertaken only in a few cases.[12−16]

Several chloride-, potassium-, and divalent cation-selective ion channels[17−29] and light-induced currents[30−33] have been recorded from all three chloroplast membranes. Patch-clamp experiments were performed on intact chloroplasts[18,19] and isolated thylakoid membranes.[17,20,23,25,28] A more frequently used approach consists of the isolation of purified membrane vesicles and their fusion into an artificial black lipid bilayer membrane. A detailed description of all observed activities is out of the scope of the present paper; therefore, only the channels whose pharmacology has been at least partially addressed are mentioned here.

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The outer envelope membrane (OE) contains several porins (for a review see ref 34) and Toc75, which is the channel-forming component of OE protein import machinery.[35] Direct patch clamping of intact chloroplasts revealed a 520 pS cationic channel[19] and a 159 pS anionic channel[18] in the OE. The former was sensitive to Konig's polyanion (PKA), a known inhibitor of mitochondrial VDAC (voltage-gated anion channel), while the latter was activated by KCl and has been proposed to play a role in osmoregulation. Other activities recorded from the OE have not been characterized pharmacologically. In the inner envelope membrane (IE), a 160 pS (in 150/25 mM KCl) potassium-selective channel has been found, which is inhibited by 10 mM tetraethylammonium (TEA^+), a general blocker of K^+ channels, and by 5 mM Mg^{2+} and Ba^{2+}.[21] A 100 pS (250/20 mM KCl) cation-selective channel has been shown to be activated upon the addition of 1 mM $MgCl_2$ and to be inhibited by 1 mM ATP.[24] Anionic channels have also been found in IE vesicles, namely, a 50 pS (250/50 mM KCl) channel, blocked by ferredoxin.[26] Finally, several channels have been revealed in thylakoid membranes,[17,20,23,25,28] but for none of them was the pharmacology investigated. For both a 100 pS chloride-selective channel and a 60 pS cationic channel, a "counter-balancing ion" role has been envisioned.

Besides ion channel activities, the chloroplast envelope transporters also have to be mentioned. Both the pharmacology and molecular identities of these transporters have intensively been investigated over the past decades, and the overall picture concerning transporters is much more complete than that of ion channels (for a recent review, see ref 36).

In the present work, we describe the effect of various channel modulators on photosynthetic oxygen evolution. To address a possible direct or indirect role of envelope channels in the regulation of light-driven oxygen evolution as well as of thylakoid channels and stromal proteins, measurements were performed on both intact and broken chloroplasts as well as on isolated thylakoid membranes. Preincubation with different ion channel modulators for 10 minutes was performed in order to avoid long-time effects and to record changes which are compatible with the action of fast-responding ion channels. A systematic study under these conditions has not been performed up to now to our knowledge.

MATERIALS AND METHODS

Isolation of Intact Chloroplasts for Oxygen Evolution Measurements. Intact chloroplasts were prepared as described.[37,38] Briefly, market spinach leaves were homogenized with 340 mM sorbitol, 0.4 mM KCl, 0.04 mM EDTA [(Ethylenedinitrilo)-tetraacetic acid disodium salt], and 2 mM Hepes at pH 7.8; filtered twice; and centrifuged for 10 s at 3500*g*. Pellets were resuspended in 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes at pH 7.0, and 0.2% BSA. Purity and intactness were checked by phase-contrast microscopy. The chlorophyll concentration was determined according to Arnon.[39]

Broken Chloroplasts. Broken chloroplasts were obtained by osmotic shock of intact chloroplasts as described.[37,40] Briefly, 60 μL of intact chloroplasts (corresponding to 1.3 mg/mL Chl concentration) were diluted in 620 μL of distilled water and left incubating for 5 min. The effect of osmotic shock was controlled using an inverted microscope (60 × 10 magnification). When added, inhibitors were included after the shock for 10 min. Prior to the measurements, 620 μL of twice-concentrated assay medium (see below) and $K_3Fe(CN)_6$ (2.7 mM final concentration) was added.

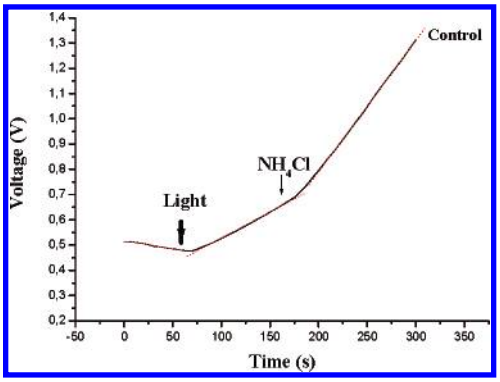

Figure 1. Representative Oxygen Evolution Measurement. Linear fittings of the zones corresponding to ox1 (between light and ammonium chloride application) and ox2 (following ammonium chloride addition) are shown. Evolution rates expressed in V/s are converted into μmol O_2/(mg Ch1 × h) by the F. I. P. program (see description in Materials and Methods).

Isolated Thylakoids. Thylakoids were obtained according to Polle and Junge.[41] Briefly, spinach leaves were homogenized with 400 mM sorbitol, 19 mM NaCl, and 100 mM tricine (pH 7.8) and centrifuged for 5 min at 1000*g*. The pellet was resuspended in 10 mM NaCl and 10 mM tricine (pH 7.8) and centrifuged for 10 min at 10 000*g*. The pellet was resuspended in a small volume of 100 mM sorbitol, 10 mM NaCl, and 10 mM tricine (pH 7.8) and diluted with the same medium that was used for intact and broken chloroplasts to a final chlorophyll concentration of 1.3 mg/mL. A total of 60 μL of this preparation was used for a single measurement.

Oxygen Evolution Measurements. Experiments were performed using a Clark electrode (Hansatech CBID) as described.[37,40] The assay medium for intact chloroplasts contained 330 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 50 mM Hepes−KOH (pH 7.6), 0.2% bovine serum albumin, and 10 mM D,L-glyceraldehyde. This latter substance was included to inhibit CO_2 fixation. The intactness of chloroplasts was assayed by the Hill reaction.[37] Chlorophyll concentration was equal to 60 μg/mL in all of the experiments, and oxygen evolution was measured at 25 °C in the dark, followed by the application of light with an intensity of 2000 $\mu Em^{-2}\,s^{-1}$. $K_3Fe(CN)_6$ (2.7 mM) was used as an electron acceptor, and 5.33 mM NH_4Cl was used as an uncoupler. A representative experiment is shown in Figure 1. Oxygen evolution under control conditions in intact chloroplasts without the uncoupler (ox1) was 15.11 ± 0.99 μmol O_2/(mg Chl × h) (n = 20), while in the presence of the uncoupler (ox2), it increased to 49.6 ± 28 μmol O_2/(mg Chl × h) (n = 20). In the case of osmotically shocked chloroplasts, the O_2 evolution was 48.6 ± 2.7 μmol O_2/(mg Chl × h) (n = 20) in ox1, while it was greater than 100 μmol O_2/(mg Chl × h) in ox2. With isolated thylakoids, O_2 evolution was 56 ± 10 μmol and 146 ± 33 O_2/(mg Chl × h) (n = 18) in ox1 and ox2, respectively. When applied, chloroplasts or thylakoids were incubated with the inhibitors for 10 min in the dark on the ice prior to measurement, to

allow the diffusion of inhibitors across the OE. In the case of inhibitors that are insoluble in water [e.g., CSA and 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid (nimodipine), controls were performed to exclude any effect of the solvent. The Fluorescence Induction Program (FIP) allowed the determination of the rate of oxygen evolution, expressed in V/s, by a linear fitting of the curves obtained under various conditions (see red dotted lines in Figure 1) and the expression of it as μmol O_2/(mg Chl $\times$ h) by taking into account the chlorophyll concentration, the volume of the experimental mixture, the oxygen content of air-saturated water at 25 °C, and calibration of the oxygen electrode. The absolute values measured under control conditions varied slightly among different preparations, and oxygen evolution values in the presence of the inhibitor were normalized accordingly. Experiments were repeated at least three times, and mean standard deviation ($\pm$SD) values are reported in the figures. Independent unpaired *t* tests were performed, and significant differences ($p < 0.05$) with respect to the control are marked with asterisks in the figures.

Materials. All chemicals were of the highest available purity and were purchased from Sigma [indole-3-acetic acid (IAA), 5-nitro-2-(3-phenylpropylamino)-benzoate (NPBB), (−)-epigallocatechin-3-gallate (EGC), *p*-chlorophenoxyacetic acid (CPA), CsCl, $GdCl_3$, 4-aminopyridine (4-AP), tetraethylammonium chloride, nimodipine, and cyclosporin A (CSA)] and Alomone Labs, Israel [charybdotoxin (CTX)]. Konig's polyanion was a kind gift from Prof. Konig.

RESULTS

To address the role of various envelope ion channels in the regulation of photosynthetic oxygen evolution, measurements were performed on freshly isolated intact spinach chloroplasts in the presence of various channel modulators. Intactness of the chloroplasts was estimated according to Hill[37] and is based on the fact that the electron acceptor K_3-$Fe(CN)_6$ does not cross the envelope membranes. Thus, in the case of intact chloroplasts, light-induced oxygen evolution (ox1) is expected to be small. In our case, intactness, calculated as described by Walker,[37] varied slightly among the various preparations and was 70−85%. Intactness was controlled using a phase-contrast microscope as well (600$\times$ magnification). The effect of the various inhibitors was assayed after 10 minutes of preincubation to allow their diffusion across the OE. Furthermore, to better evaluate their effect, in each set of experiments, measurements were repeated after the addition of NH_4Cl (ox2), which dissipates the transthylakoid proton gradient, thereby accelerating the photosynthetic rate and oxygen evolution.[42]

Stromal proteins, which may have a regulatory function on ion channels, are lost during the isolation procedure of thylakoid membranes. For this reason, inhibition of the thylakoid channels was tested on chloroplasts broken by osmotic shock. A 10-fold dilution of chloroplasts in distilled water invariably caused their rupture. Again, the effect of the inhibitors was assayed in the absence (ox1) and presence (ox2) of an uncoupler.

To understand whether stromal proteins eventually contributed to the modulation of the photosynthetic electron-transfer rate by channel inhibitors, experiments were performed in the same way and in the same assay medium using isolated thylakoids.

Attention is called to the fact that, especially when working with intact chloroplasts, since most substances are not freely permeable across biological membranes, a relatively high dose may be necessary to alter the photosynthetic process taking place in thylakoids. In any case, the doses used in this work are compatible with those described for affecting various ion channels in different systems.

Among chloride channel inhibitors, the effects of NPPB, IAA, EGC, CPA, and PKA were tested. This latter substance, a 1:2:3 copolymer of methacrylate, maleate, and styrene, is a known inhibitor of porins (see above) and did not induce any change in oxygen evolution with respect to the control at 100 μg/mL (not shown). This concentration was used because a few hundred micrograms per milliliter of PKA has been shown to partially inhibit mitochondrial porins in reconstituted systems.[43] NPPB has been shown to block Cl^- fluxes across the basolateral membrane of the thick ascending limb of the loop of Henle with a *K*i of 80 nM.[44] CFTR[45] and ClC-type Cl^- channels require higher concentrations (100 μM) for inhibition, while plant plasma membrane anion channels are blocked by NPPB with a *K*i of around 5−7 μM.[46] In our system, 200 μM NPPB caused a 93 $\pm$ 5% inhibition of both ox1 and ox2 in both intact and broken chloroplasts (not shown). These data are in agreement with the fact that NPPB is membrane-permeable and with the results of Bock and colleagues[16] on isolated thylakoids. These authors demonstrated that derivatives of arylaminobenzoates, widely used as chloride channel inhibitors, bind to the Q_B site on the D1 protein, acting as herbicides and blocking electron transport.

IAA has a different chemical structure and is used as an inhibitor of various chloride channels in animals, where it is efficient at around 100 μM (see, e.g., ref 47). In plants, however, IAA has been reported to behave as an activator of chloride channels of the plasma membrane by shifting their voltage dependence.[48] In plants, IAA plays an important role since it is an auxin growth hormone. Figure 2 shows that, in the presence of IAA, the oxygen evolution rate increases in the absence of the uncoupler but decreases in a concentration-dependent manner in the presence of NH_4Cl in both intact and broken chloroplasts. IAA is slightly membrane permeable, and its effects are comparable in intact and broken chloroplasts, suggesting such an action of IAA at the level of thylakoids. In accordance, a dose-dependent inhibition of oxygen evolution takes place in the presence of IAA in isolated thylakoids to a similar extent as that observed in broken chloroplasts. Instead, the increase of O_2 evolution in the absence of the uncoupler which was observed in both intact and broken chloroplasts is not revealed anymore when working with isolated thylakoids.

In the case of EGC (Figure 3), an inhibitor known to act on various channels in animal systems[49−51] with an IC_{50} of 100 μM for Kv1.5[51] and 166 μM for the anion-selective channel VacA,[49] a significant decrease was recorded only in the presence of the uncoupler in both intact and broken chloroplasts. The same effect was observed, in a dose-dependent manner, in isolated thylakoids, although inhibition was less pronounced in this system (55% inhibition versus 82% inhibition in intact and broken chloroplasts).

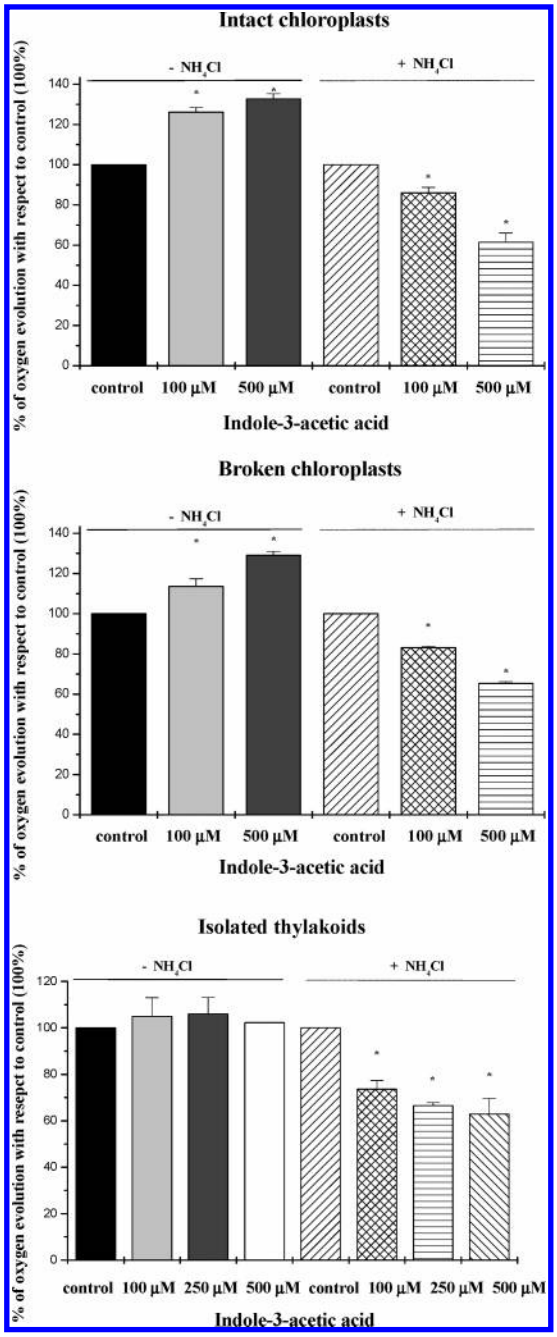

Figure 2. Effect of indole-3-acetic acid (IAA) auxin on oxygen evolution rates. Data were normalized with respect to oxygen evolution measured under control conditions (100%). Measurements were performed in the absence or in the presence of NH_4Cl as indicated. IAA concentrations used are indicated below the columns. Mean values ± SD are reported ($n \geq 3$). Asterisks mark significant differences with respect to the control. Oxygen evolution rates in the presence of the various inhibitors were normalized, throughout this work, to those measured under control conditions using the same chloroplast preparation.

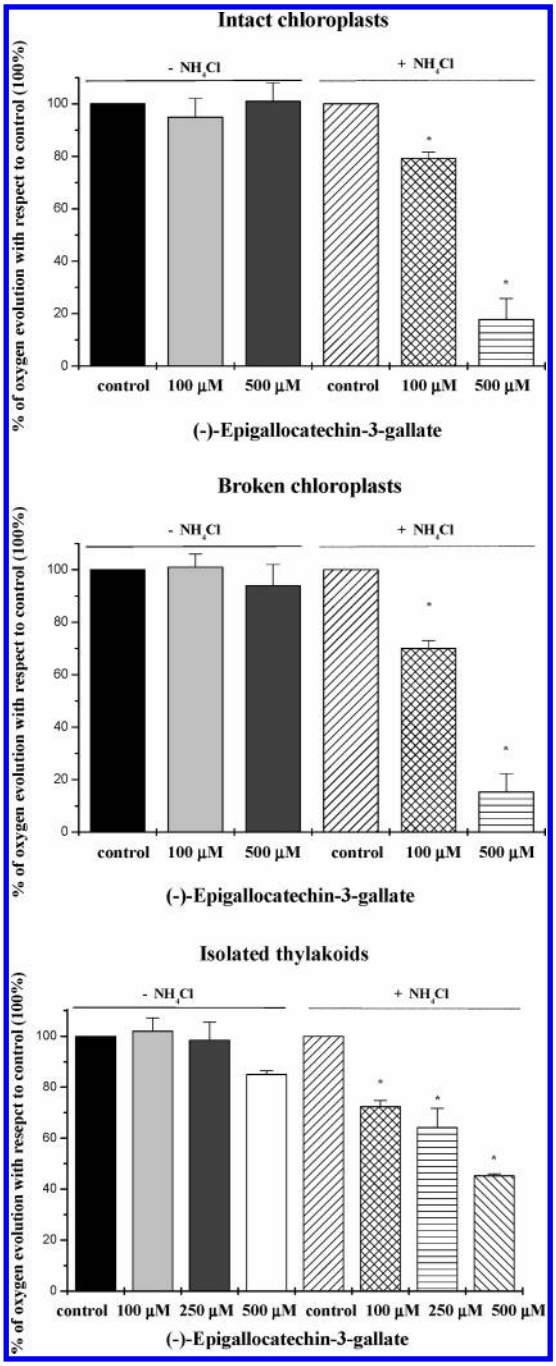

Figure 3. Effect of (−)-epigallocatechin-3-gallate (EGC) on oxygen evolution rates. Data were normalized with respect to oxygen evolution measured under control conditions (100%). Measurements performed in the presence of NH_4Cl are reported. The EGC concentrations used are indicated below the columns. Mean values ± SD are reported ($n \geq 3$). Asterisks mark significant differences with respect to the control.

Oxygen evolution was slightly but significantly diminished (by ≈30%) in the presence of 100 μM CPA, an inhibitor of chloride channels of the ClC family,[52] especially during ox1 (not shown). However, CPA diminished oxygen evolution only when added to intact chloroplasts, indicating that CPA-sensitive channels are not present in the thylakoid membrane. In accordance, a ClC family member has recently been shown to localize to the envelope membrane.[53]

Concerning cation channel inhibitors, the effects of Cs^+, Gd^{3+}, 4-AP, TEACl, CTX, and nimodipine were assayed. 4-AP, a classic inhibitor of potassium channels, increased oxygen evolution in ox1 at 1 mM. Given that this increased oxygen evolution level was the same as that obtained with control chloroplasts in ox2 and that 4-AP did not further increase the rate of oxygen evolution in the presence of an uncoupler (not shown), we concluded that 4-AP itself acts as an uncoupler, consistently with its amine character. Similarly, TEA^+ acts as an uncoupler at a 10 mM concentration in accordance with previous results.[54] Cesium, another blocker of potassium channels, significantly decreased the oxygen evolution rate in a concentration-dependent manner,

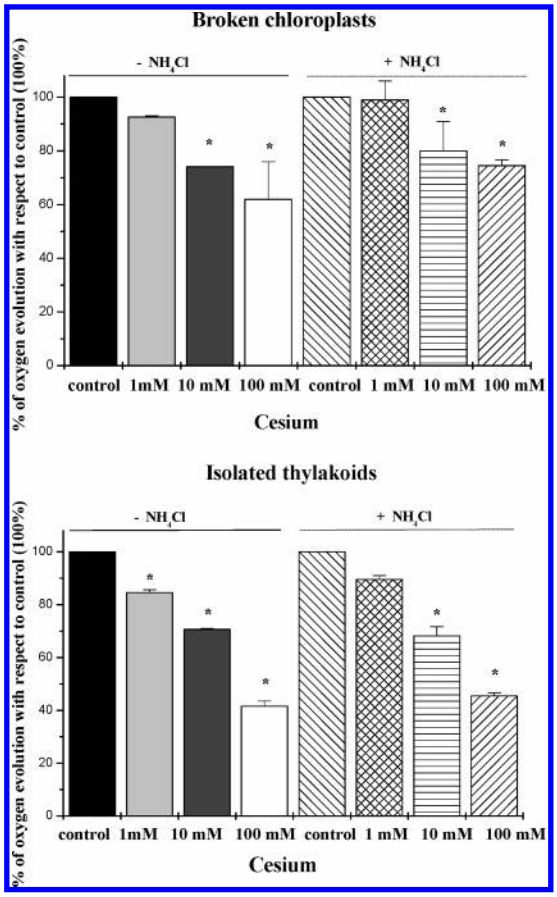

Figure 4. Cesium ion, a blocker of potassium channels, affects photosynthetic efficiency only when added to broken chloroplasts or to isolated thylakoids. Cs^+ had a significant inhibitory effect on oxygen evolution at 10 and 100 mM concentrations. Asterisks mark significant differences with respect to the control. Mean values ± SD are reported ($n \geq 3$).

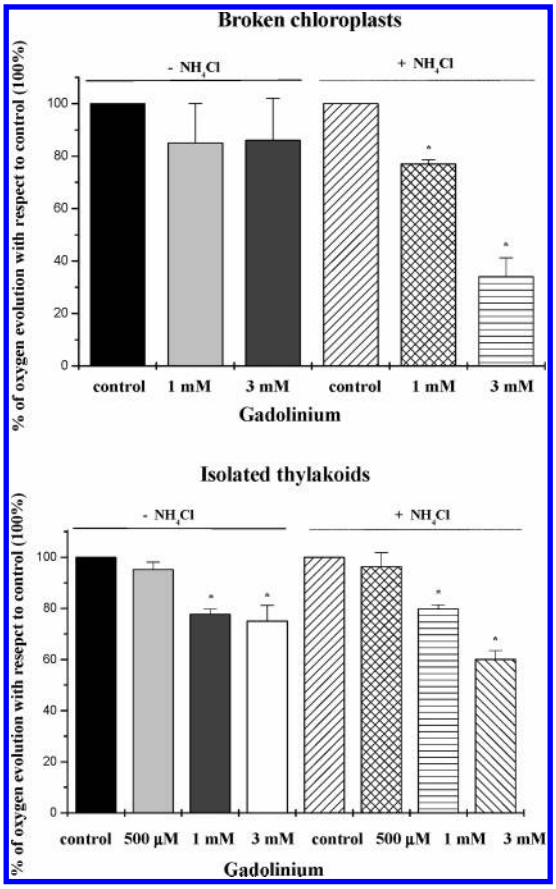

Figure 5. Effect of gadolinium ions on oxygen evolution rates. Data obtained with isolated thylakoids obtained by osmotic shock of intact chloroplasts are shown. Mean values ± SD are reported ($n \geq 3$).

as shown in Figure 4, when added to broken chloroplasts. Inhibition was to an even higher extent when cesium chloride was added to isolated thylakoids, while no effect was observed in intact chloroplasts even at the highest concentration (not shown). Different concentrations of Cs^+ were used up to 100 mM because various potassium channels have different sensitivities toward Cs^+,[55,56] complete inhibition being achieved by a few mM to 100 mM Cs^+.

CTX is a specific inhibitor of shaker-like voltage-gated and calcium-activated potassium channels, acting at nanomolar concentrations.[57] Since a voltage-dependent cation channel has been recorded in chloroplast membranes,[24] the effect of 10 nM CTX was tested on photosynthetic efficiency, but no significant differences were found.

The results with Gd^{3+}, an inhibitor of calcium as well as of stretch-activated ion channels, are reported in Figure 5. Gd^{3+} caused a marked dose-dependent decrease in oxygen evolution rate only in broken chloroplasts and in thylakoids, especially in the presence of an uncoupler. Gadolinium is not membrane-permeable and is known to block stretch-activated channels at micromolar concentrations in the tens; however, in some cases, higher concentrations are needed. For example, 0.5 mM gadolinium has been reported to inhibit Mid1 by 60%, a eukaryotic, nonselective, stretch-activated cation channel.[58] Furthermore, 1mM Gd^{3+} was necessary to block the hypo-osmotic shock-induced exit of lactose and ATP through large stretch-activated channels in bacteria.[59]

Nimodipine, a specific calcium channel inhibitor (especially of the L-type, at micromolar concentrations, see, e.g., ref 60), caused a drastic decrease in oxygen evolution rate at 50 μM to a similar extent in intact and broken chloroplasts as well as in isolated thylakoids (Figure 6), in accordance with its lipophilic nature.

Finally, the effect of CSA was tested, since a channel recorded directly in thylakoids resembled the mitochondrial megachannel (MMC),[28] known to be inhibited by CSA[61] via cyclophilins.[62] CSA is membrane-permeable and completely inhibits MMCs at low micromolar concentrations. Photosynthetic oxygen evolution was inhibited in a dose-dependent manner in intact chloroplasts to basically the same extent in the presence and absence of an uncoupler (see Figure 7). Interestingly, in broken chloroplasts, 10 μM CSA caused less inhibition than that at 1 μM, suggesting that, at higher concentrations, CSA may have additional effects. In sharp contrast, in isolated thylakoids incubated with CSA, oxygen evolution was not affected to a similar extent to that observed in broken chloroplasts, suggesting that the presence of a stromal factor (present in broken chloroplasts but largely lost during the isolation of thylakoids) is necessary for CSA action.

DISCUSSION

In the present work, we describe the effects of various channel modulators on photosynthetic oxygen evolution when added to intact or broken chloroplasts or to isolated

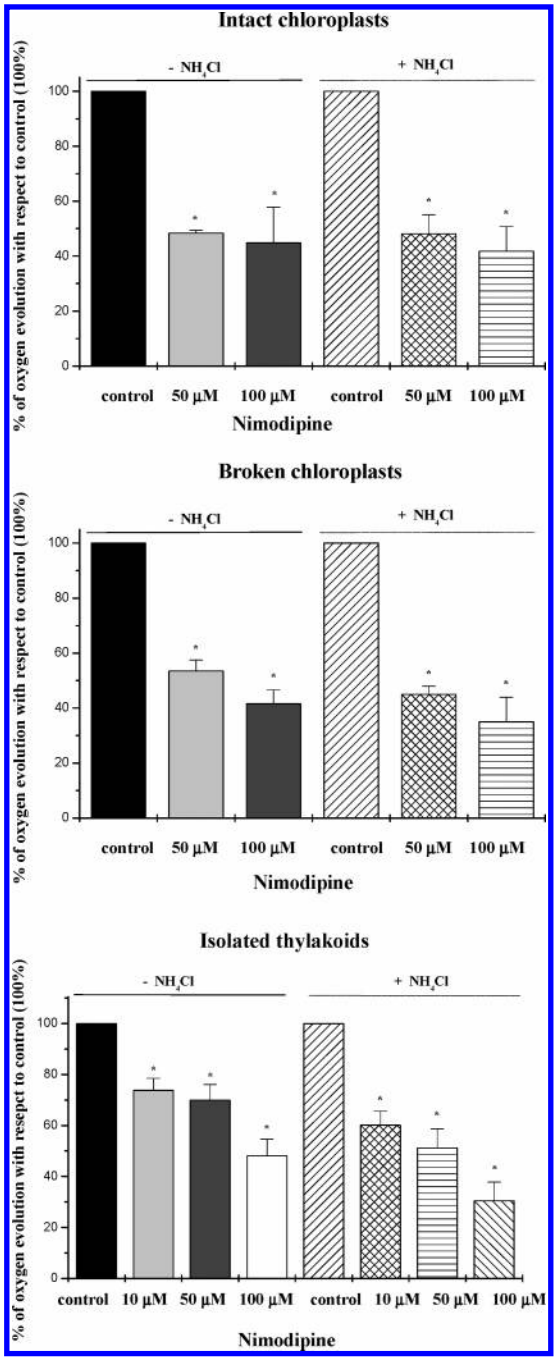

Figure 6. Nimodipine, a specific L-type calcium channel inhibitor, markedly decreases photosynthetic efficiency. Nimodipine is a membrane-permeable chemical. Mean values ± SD are reported ($n \geq 3$).

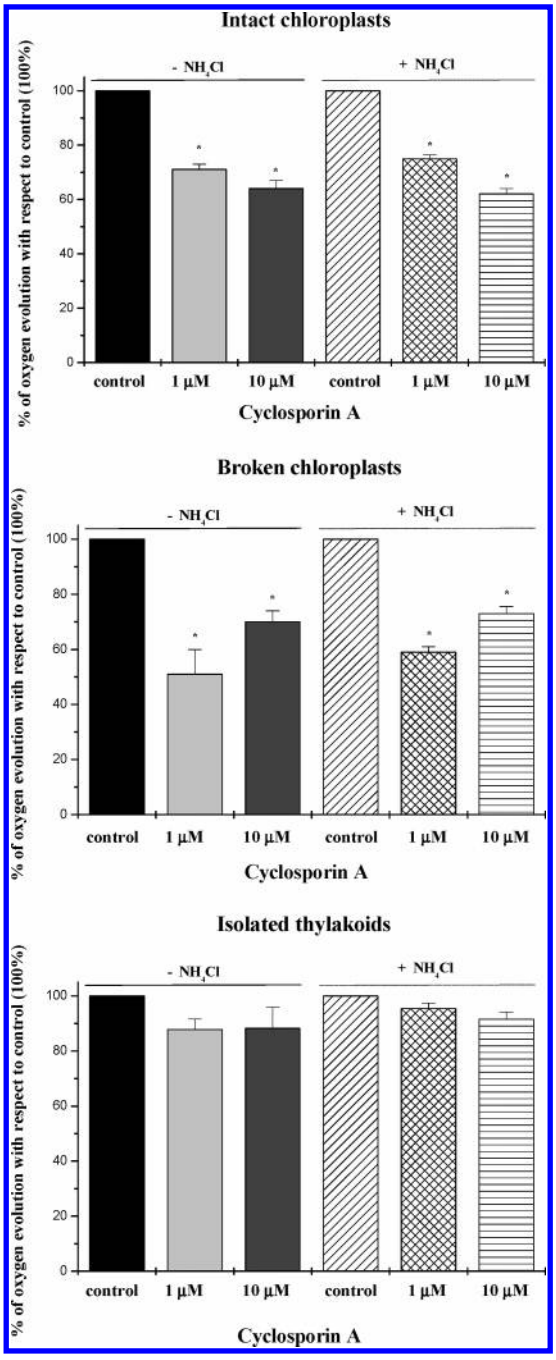

Figure 7. Significant inhibitory effect of cyclosporin A (CSA) on photosynthetic oxygen evolution in intact and broken chloroplasts but not in isolated thylakoids. CSA is slowly membrane-permeable. Mean values ± SD are reported ($n \geq 3$).

thylakoids. The rapid effect of some of the substances we used in an isolated system is compatible with the action of fast-responding ion channels rather than with the action of signaling components (e.g., kinases), even though this latter possibility cannot be excluded. The interpretation of the results we propose will need further confirmation by future experimental work (e.g., the biochemical isolation of inhibitor-binding channels). Furthermore, to address the role of envelope channels in the regulation of the dark reactions of photosynthesis, which also require specific optimal ionic conditions, additional assays (e.g., CO_2 fixation) will have to be performed. In any case, the results of the screening presented here may represent a valid starting point for further investigation into chloroplast ion channels, and they identify inhibitors which may be exploited for the pharmacological characterization of chloroplast membrane channels in reconstituted systems or in patch-clamp experiments. Furthermore, independently of the exact site of action of the used modulators, their effects on photosynthetic oxygen evolution efficiency described here can be taken into consideration when using them for plant physiology studies (for example, CSA is used for studies on apoptosis, a process in which a reduced photosynthetic efficiency may not be irrelevant).

As described in the Introduction, ion fluxes have been proposed to regulate photosynthesis. When considering the modulation of photosynthesis by ion channel inhibitors, it has to be taken into account that a complete inhibition of photosynthesis cannot be expected even by completely

inhibiting channel activity, since these channels are not part of the photosynthetic machinery itself and their function probably is not mandatory for photosynthesis to occur. Anion-selective channels are known to reside in various chloroplast membranes,[17,19,20,22,23,26,63] and a regulated chloride concentration seems to be ensured in chloroplasts by unknown mechanisms.[5] Chloride ion fluxes have also been proposed to regulate plastidial processes, including photosynthesis.[4−6,11] The effect of various chloride channel inhibitors was tested on photosynthetic oxygen evolution in a thorough study,[16] demonstrating that arylaminobenzoate derivatives, including NPPB, block electron transport. Our results obtained with NPPB are in agreement with previous observations.[16] Arylaminobenzoate derivatives were shown to block electron transport by directly binding to photosystem II rather than by chloride channel inhibition. Among the other chloride channel inhibitors tested were SITS and DIDS, neither of which inhibited oxygen evolution.

We report here, for the first time, the short-term effect of IAA-94, which is structurally different from the Cl^- channel blockers discussed above. IAA slightly, but significantly, stimulates oxygen evolution but shows the opposite effect in the presence of NH_4Cl, indicating a different mechanism with respect to arylaminobenzoates. The effect of this membrane-permeable molecule was quantitatively similar in intact and broken chloroplasts while somewhat different in isolated thylakoids. In this latter system, IAA did not cause a significant increase of oxygen evolution in the absence of an uncoupler, while inhibition during ox2 was confirmed. This observation suggests that, for IAA action during ox1, an unidentified stromal factor, which is largely lost during isolation of the thylakoids, is needed. IAA is an auxin and has been reported to have multiple effects on various channels and transporters in plants. Among them are anion[48] and potassium channels[64] as well as the proton ATPase of the plasma membrane (see, e.g., ref 65). The stimulatory effect of IAA on oxygen evolution may be explained by assuming that IAA stimulates not only the plasma membrane H^+-ATPase but also that present in thylakoids. In this case, an accelerated proton flux from lumen to stroma could explain an accelerated photosynthetic electron flux and oxygen evolution. Alternatively, if IAA had an activatory effect not only on plasma membrane chloride channels but also on those of chloroplasts, an increase of chloride influx into the lumen might result in an increased oxygen evolution either through improving the efficiency of the oxygen evolving complex (OEC)[66] or via the counterbalancing effect. Of course, we do not exclude that IAA acts by other mechanism(s) in isolated chloroplasts, and further experiments are required to understand its action during ox2. In any case, we would like to note that an action of IAA in isolated chloroplasts via regulation of gene expression (as occurs in the nucleus) is highly improbable. Although, IAA has been reported to increase Hill activity in chloroplasts isolated from IAA-treated plants, by 12−17% with respect to control plant chloroplasts, suggesting that activity of the photosynthetic apparatus is under phytohormone control also on a long-term scale.[67]

EGC has been recently shown to inhibit a chloride-selective channel formed by VacA toxin.[49] When an uncoupler is added to thylakoids, oxygen evolution increases several-fold. It is well-known that the presence of chloride ions is necessary for the oxygen evolving complex to be able to work at a maximal rate.[1,66] Thus, during ox2, an inhibition of Cl^- influx into the lumen by EGC acting on chloride-sensitive channels may explain the inhibition of oxygen evolution with respect to the control. EGC is a radical scavenger, and a chemical analogue of EGC, *n*-propyl gallate, has been recently shown to exert a long-term protective effect on protein degradation during light stress.[68] In our case, the effects of EGC are observable within 10 minutes, suggesting that long-term protection against photo-oxidation is not involved. Furthermore, if EGC acted as a scavenger to reduce oxygen evolution, an inhibitory effect would also be expected during ox1. We, however, revealed a significant effect of EGC only during ox2, even in isolated thylakoids. Whether EGC would act selectively on chloride channels in thylakoids is not known. EGC has recently been shown to increase cell membrane permeability to Ca^{2+} through the stimulation of calcium channels.[50] The stimulation of calcium flux into the lumen, however, would be expected to increase the photosynthetic rate (see below). EGC has a blocking effect on a shaker-like potassium channel, Kv1.5.[51] Since the molecular nature of the calcium channel(s) and the presence of shaker-like potassium channels in thylakoids are not known, this possibility cannot be tested. In conclusion, while the effect of EGC is clear and reproducible in all three examined systems (intact and broken chloroplasts and thylakoids), a determination of the exact method of its action requires further work.

Concerning cation channel inhibitors, a decrease of oxygen evolution was observed with Cs^+, Gd^{3+}, and nimodipine. Cs^+ has been recently reported to bind to the high-affinity Mn^{2+} site in the OEC Mn cluster, with a slightly greater affinity than Mn^{2+} itself.[69] The strong binding by Cs^+ to the apo-OEC is possible only after the release of the manganese cluster, since in intact native membranes 10 mM Cs^+ produced only a 6% decrease in the O_2 evolution rate relative to the control.[69] A comparable inhibition was observed in our experiments using 10 mM Cs^+ in both broken chloroplasts and isolated thylakoids. In light of these results, Cs^+ seems to affect photosynthesis independently from its action on potassium channels.

Gadolinium, a known blocker of calcium and stretch-activated channels, caused a significant decrease of oxygen evolution in the presence of an uncoupler, in thylakoids. Calcium ions are essential for oxygen evolution, since calcium depletion modifies the structure of the PSII oxygen evolving complex and results in an almost complete abolition of O_2 evolution.[70] The architecture of a cyanobacterial *Thermosynechococcus elongatus* OEC has been recently described and found to contain a cubane-like Mn_3CaO_4 cluster.[66] The inhibitory effect of gadolinium on oxygen evolution, observed both in broken chloroplasts and in isolated thylakoids and which is more pronounced during ox2, is probably due to a decreased influx of calcium ions into the lumen where the OEC is located. In agreement with our results, $LaCl_3$, although at submillimolar concentrations, has been shown to inhibit electron transport during photosynthesis.[12] Concerning the action of cesium and gadolinium when used at high concentrations, it has to be taken into account that these cations at high concentrations may act not only by directly binding to ion channel proteins but also by reducing surface potential and inducing a change in the

Table 1. Effect of Ion Channel Modulators on Photosynthetic Oxygen Evolution in the Absence of an Uncoupler[a]

ion channel modulator	concentration	effect in intact chloroplast	effect in broken chloroplast	effect in isolated thylakoid
Anion Channel Modulators				
5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB)	(200 μM)	----	----	----
indole-3-acetic acid (IAA)	(100 μM)	++	+	n.s.
	(500 μM)	++	++	n.s.
(−)-epigallocatechin-3-gallate (EGC)	(100 μM)	n.s.	n.s.	n.s.
	(500 μM)	n.s.	n.s.	n.s.
p-chlorophenoxyacetic acid (CPA)	(100 μM)	--	n.s.	n.d.
Konig's polyanion (PKA)	(100 μM)	n.s.	n.s.	n.d.
Cation Channel Inhibitors				
4-aminopyridine (4-AP)	(1 mM)	++++	++++	n.d.
tetraethylammonium chloride (TEACl)	(10 mM)	++++	++++	n.d.
cesium	(10 mM)	n.s.	--	--
	(100 mM)	n.s.	--	---
gadolinium	(1 mM)	n.s.	n.s.	-
	(3 mM)	n.s.	n.s.	-
nimodipine	(50 μM)	---	--	--
	(100 μM)	---	--	---
Other Inhibitor				
cyclosporin A (CSA)	(1 μM)	--	---	n.s.
	(10 μM)	--	--	n.s.

[a] n.s.: non-significant effects with respect to control. n.d.: not determined. +, ++, +++, and ++++: 0−25%, 26−50%, 51−75%, and 76−100% activatory effect with respect to control, respectively. -,- -,- - -, and - - - -: 0−25%, 26−50%, 51−75%, and 76−100% inhibitory effect with respect to control untreated sample, respectively.

voltage-dependence of voltage-gated channels.

Calcium channel inhibitors nifedipine, nicardipine, riodipine, and verapamil have been shown to inhibit electron transport and oxygen evolution quite strongly at submillimolar concentrations.[13,15,71] Furthermore, Ca^{2+} channel inhibitors can block the Ca^{2+}-dependent reactivation of oxygen evolution, and this blockage can be partially prevented by an activator of Ca^{2+} channels, CGP28392.[15] The drug used in the present study is nimodipine, which is a potent L-type calcium channel antagonist. The inhibitory effect of 50 μM nimodipine on oxygen evolution is in agreement with the effects of other calcium channel antagonists[13,15] and also with the requirement of calcium for OEC functionality.

As described above, direct patch clamping of the thylakoid membrane revealed the presence of a high-conductance channel[28] with electrophysiological properties similar to those of the mitochondrial megachannel.[72] MMC has been shown to correspond to the so-called permeability transition pore (PTP) and to be inhibited by CSA at micromolar concentrations.[61] Since CSA affects the electron transport rate in isolated mitochondria by inhibiting the PTP, the effect of this cyclic endecapeptide on oxygen evolution has been investigated in chloroplasts as well in the present study. Already, 1 μM CSA significantly decreases photosynthetic oxygen evolution efficiency both in intact chloroplasts and in broken chloroplasts during ox1 as well as during ox2, consistently with its ability to cross biological membranes. Importantly, no such effect is evident in isolated thylakoids (49% inhibition in broken chloroplasts versus 13% inhibition in isolated thylakoids), strongly suggesting that a stromal factor is necessary for the action of CSA to be exerted. CSA is an immunosuppressant which binds with high affinity to immunophilins called cyclophilins. In mitochondria, matrix cyclophilin D is necessary for CSA to inhibit the PTP.[62,73] A total of 15 isoforms of immunophilins have been localized in chloroplasts, 1 in the stroma and 14 in the lumen. Immunophilins display peptidyl-prolyl cis−trans isomerase (PPIase) activity and are thought to act as protein-folding catalysts. TLP40, an immunophilin identified in chloroplasts,[74] has been shown to regulate the activity of a photosystem II-specific protein phosphatase and the turnover of D1 protein[75] and could a priori be responsible for the observed effect of CSA in our system. However, TLP40 is a lumenal immunophilin, while a stromal factor seems to be required for the action of CSA. TLP40 is not sensitive up to 10 μM cyclosporin A, and we present data using 1 and 10 μM cyclosporin A. Furthermore, half-time of the dephosphorylation of LHCII, D1, and D2 in the presence of PPIase peptide substrate was more than 100 minutes.[74] Both TLP20, a spinach lumenal immunophilin,[76] and Roc4, a stromal cyclophilin,[77] are able to bind CSA and may a priori mediate the action of CSA on oxygen evolution. However, as mentioned above, our data indicate the involvement of stromal factor(s). It is tempting to speculate that Roc4 is required for CSA to be able to modulate photosynthesis. The possibility that Roc4, MMC-like channels, and CSA interact with each other cannot be excluded, but there needs to be experimental proof. The novel data presented here suggest that CSA may modulate photosynthetic oxygen evolution by the involvement of a stromal factor, and given the short time required for CSA to exert its effect, the participation of a MMC-like channel seems likely. A determination of the mechanism of action of CSA requires further experiments and may lead to the discovery of the existence of a CSA-sensitive ion channel in thylakoids.

In conclusion, the screening of the effects of ion channel modulators on light-driven photosynthetic activity led to stimulating results (for a summary, see Table 1) and, in some cases, confirmed previous data. It must be kept in mind that a lack of any effect does not exclude the presence of a channel in chloroplast membranes. Also, an effect of an ion channel inhibitor on oxygen evolution does not necessarily imply the presence of a channel, given that at least some of the molecules used may act in a channel-independent manner

(e.g., NPPB). Further work is required to exclude binding of the inhibitors (e.g., CSA) to regulatory proteins different from membrane channels, but there is no doubt that understanding the molecular details of ion flux regulation during photosynthesis will be of major importance for possible biotechnological applications and for a better elucidation of the complex mechanisms that modulate plant cell metabolism.

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