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Relationship between Respiration and Photosynthesis in Guard Cell and Mesophyll Cell Protoplasts of *Commelina communis* L.

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ABSTRACT

A mass spectrometric method combining $^{16}O/^{18}O$ and $^{12}C/^{13}C$ isotopes was used to quantify the unidirectional fluxes of O_2 and CO_2 during a dark to light transition for guard cell protoplasts and mesophyll cell protoplasts of *Commelina communis* L. In darkness, O_2 uptake and CO_2 evolution were similar on a protein basis. Under light, guard cell protoplasts evolved O_2 (61 micromoles of O_2 per milligram of chlorophyll per hour) almost at the same rate as mesophyll cell protoplasts (73 micromoles of O_2 per milligram of chlorophyll per hour). However, carbon assimilation was totally different. In contrast with mesophyll cell protoplasts, guard cell protoplasts were able to fix CO_2 in darkness at a rate of 27 micromoles of CO_2 per milligram of chlorophyll per hour, which was increased by 50% in light. At the onset of light, a delay observed for guard cell protoplasts between O_2 evolution and CO_2 fixation and a time lag before the rate of saturation suggested a carbon metabolism based on phospho*enol*pyruvate carboxylase activity. Under light, CO_2 evolution by guard cell protoplasts was sharply decreased (37%), while O_2 uptake was slowly inhibited (14%). A control of mitochondrial activity by guard cell chloroplasts under light via redox equivalents and ATP transfer in the cytosol is discussed. From this study on protoplasts, we conclude that the energy produced at the chloroplast level under light is not totally used for CO_2 assimilation and may be dissipated for other purposes such as ion uptake.

It is now established that guard cells present a high level of oxidative phosphorylations well in accordance with a high mitochondria/chloroplast ratio (24). The inhibition of stomatal opening under anoxia (34) or by respiratory inhibitors indicates a major role of respiration in the supply of energy (24). Generally, respiration rate was estimated from O_2 exchange rate in darkness (7, 31) or pH variations in the medium (32) and was unknown in light. Under illumination, the high alkalinization of the medium containing GCPs[1] was thought to be due to net CO_2 uptake; however, net O_2 evolution was only slightly positive or even negative, when the stoichiometry CO_2:O_2 should be 1 to be consistent with photosynthetic CO_2 fixation.

Presence of both photosystems in GCPs has already been ascertained by O_2 evolution under short actinic flashes (14) and by the fluorescence properties of chloroplasts (20, 36). In light, cyclic and noncyclic photophosphorylations may provide energy for stomatal opening (28). However, gross O_2 evolution has never been directly quantified; the assumption that the O_2 uptake rate is the same in darkness as in light should be proved. Moreover, CO_2 evolution and CO_2 uptake have never been continuously recorded in GCP suspensions, and results on CO_2 fixation with ^{14}C were ambiguous and dependent on contamination by MCPs (19).

GCPs have been shown to swell in light or low CO_2 concentration and shrink in darkness (8, 10), providing a valuable and simplified model for the study of guard cell physiology. Using a mass spectrometric method, which discriminates between the unidirectional fluxes of O_2 and CO_2, we report new data on respiration and photosynthesis during a dark to light transition. This experiment was conducted in parallel with MCPs to ascertain the method and to compare these two types of cell metabolism.

MATERIALS AND METHODS

Commelina communis L. seeds were germinated on moistened cellulose tissue for 2 weeks. The seedlings were then transferred to pots of coarse sand in a growth chamber with a 14 h photoperiod (25°C, RH 60%) followed by 10 h darkness (20°C, RH 80%). Light (150 μmol m^{-2} s^{-1} PPFD) was supplied by 400 W mercury lamps (HQI Osram, W Germany). The pots were watered three times a day with half-strength Hoagland solution.

Isolation of Guard Cell Protoplasts

Protoplasts were prepared from young fully expanded leaves from 5- to 7-week-old plants (7). The abaxial epidermis of 40 leaves was peeled and floated on medium A (10 mM KCl, 5 mM Mes/Tris, 20 μM $CaCl_2$, pH adjusted to 7 by addition of HCl), in Petri dishes. Osmotic pressure was adjusted to 340 mOsmol with mannitol. After 30 min, this medium was replaced by an incubation medium, *viz.* medium A + enzymic cocktail: 1% (w/v) cellulysin (Calbiochem Corporation, La Jolla, CA) + 0.2% (w/v) caylase 345L (Cayla, Toulouse, France) + 0.025% (w/v) pectolyase + 0.5% (w/v) BSA, pH adjusted to 6 with HCl. Petri dishes were gently stirred (40 rpm) on a water-bath at 30°C in room light. The incubation medium containing mainly MCPs and epidermal cell proto-

[1] Abbreviations: GCP, guard cell protoplast; MCP, mesophyll cell protoplast; PEPCase, phospho*enol*pyruvate carboxylase; RuBPCase, ribulose 1,5-biphosphate carboxylase.

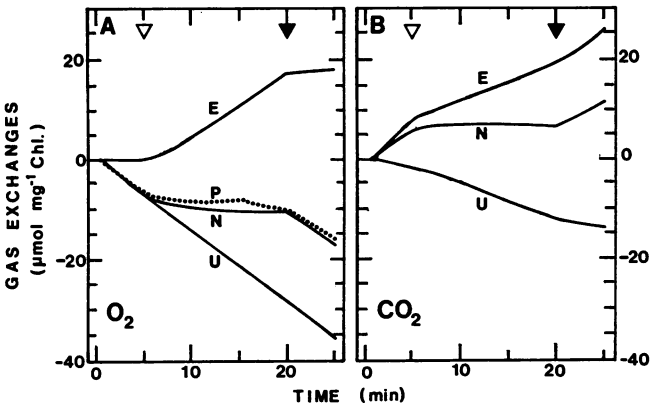

Figure 1. Typical effect of a dark to light transition on gas exchanges, in guard cell protoplasts of *C. communis* L., measured by mass spectrometry. A, O_2; B, CO_2. E, evolution; N, net evolution; U, uptake; open triangle, light on; closed triangle, light off. After 5 min, GCPs (1.5 10^6) were illuminated with red light at 800 μmol m^{-2} s^{-1}, for 15 min. Curve P corresponds to net O_2 evolution measured by polarography under the same conditions.

plasts was discarded 2 h later. Strips were washed with medium A and floated on fresh incubation medium for 2 or 3 h. Then incubation medium containing GCPs and remaining epidermal strips was filtered through a 60 μm nylon mesh. The filtrate was centrifuged (100*g*, 5 min). The pellet was washed twice, resuspended in medium A, and kept overnight at 4°C.

Isolation of Mesophyll Cell Protoplasts

MCPs were obtained from three fully expanded leaves. After removing the lower epidermis, the leaf pieces were floated for 30 min, adaxial side up, on 20 mL medium A (osmotic pressure adjusted to 540 mOsmol with mannitol) and then on incubation medium for 90 min. MCPs were filtered through a 100 μm nylon mesh, washed twice, centrifuged (100*g*, 5 min), and kept at 4°C.

Protoplast Viability and Contamination Level Determination

The protoplast viability was ascertained by observing the green fluorescence under blue light after fluorescein diacetate staining (35) and protoplast swelling under light. It was always over 95%. Aliquots of each GCP preparation (about 15 10^3 GCPs) were checked microscopically for mesophyll contamination. In all experiments, maximum contamination was below 2 or 3 10^{-3} mesophyll units per guard cell. A mesophyll unit represents either a MCP or an aggregate of mesophyll chloroplasts.

Chl content was determined according to the method of Lichtenthaler and Wellburn (16) after extraction in 80% acetone and protein content according to the Lowry method as modified by Peterson (23).

Measurement of O_2 Concentration by Polarography

After centrifugation (100*g*, 5 min), protoplasts (5 · 10^5) were resuspended in 1 mL of medium A buffered at pH 6.5 with 20 mM Mes/Tris and equilibrated with air. The suspension was placed in a Clark-type O_2 electrode (Hansatech, Kings Lynn, U.K.), thermoregulated at 25°C, and gently stirred by a magnetic flea (150 rpm). Before each experiment, protoplasts were illuminated for 5 min to give a rapid induction of photosynthesis. After 5 min in darkness, a red light, 800 μmol m^{-2} s^{-1} (LH7, Hansatech), was switched on for 15 min.

Mass Spectrometry Measurement of O_2 and CO_2 Fluxes

The principle of discrimination between O_2 evolution and O_2 uptake using MS and $^{18}O_2$ has already been described (6). In a similar way, discrimination between CO_2 evolution and CO_2 uptake was performed using $^{13}CO_2$.

The experimental chamber was identical to the polarographic electrode except that the teflon membrane was replaced by a polyethylene membrane. Dissolved gases passing through this membrane were collected on a three-collector mass-spectrometer (V.G. instrument MM 14-80, U.K.). Experimental procedure was the same as described above. After preillumination, 3 mL of $^{18}O_2$ and 0.5 mL of $^{13}CO_2$ (99% $^{18}O_2$ and 99% $^{13}CO_2$ purchased from CEA, Saclay, France) were injected into the suspension (1.5 10^6 GCPs) so that total O_2 and CO_2 concentrations were, respectively, 0.4 and 1 mM. Masses 32 ($^{16}O_2$), 36 ($^{18}O_2$), 44 ($^{12}CO_2$), 45 ($^{13}CO_2$) were recorded every 5 s for 30 min. Calculations of O_2 and CO_2 unidirectional fluxes were performed as already published (9, 22).

Table I. *Unidirectional Fluxes of O_2 and CO_2 in GCPs and MCPs from C. communis L. Determined by MS During a Dark to Light Transition*

After 5 min in darkness, light (800 μE m^{-2} s^{-1}) was switched on for 15 min. Rates were determined from the linear portion of curves. Values are means ± SE; number of experiments in parentheses.

Assay	O_2 Uptake	CO_2 Evolution	CO_2 Uptake	O_2 Evolution
		μmol mg^{-1} Chl h^{-1}		
GCP				
Dark	100 ± 14 (5)	93 ± 15 (6)	27 ± 13 (4)	0
Light	86 ± 13 (5)	59 ± 15 (6)	40 ± 15 (4)	61 ± 15 (4)
MCP				
Dark	8.1 ± 2.4 (11)	10.1 ± 2.4 (10)	−0.2 ± 2.5 (10)	0
Light	13.1 ± 2.5 (11)	7.0 ± 2.8 (10)	74 ± 15 (10)	73 ± 12 (11)

Chemicals

Unless specified, all chemicals were purchased from Sigma (Sigma Chemical Company, St Louis, MO).

RESULTS

During a typical experiment with GCPs (Fig. 1A), O_2 concentration measured by polarography decreased in darkness and was almost constant under light. At the end of illumination, initial O_2 consumption was recovered. MS was used to discriminate the unidirectional fluxes of O_2. Net O_2 variation under light was in fact due to a continuous O_2 influx only slightly inhibited under light and a high O_2 efflux which was inhibited by 10 μM DCMU. The results concerning net O_2 efflux during a dark to light transition, obtained either by polarography or by MS were concordant (Fig. 1A).

Simultaneous with O_2 measurements, MS was used to observe a small CO_2 influx in darkness stimulated by light (Fig. 1B). In contrast, CO_2 efflux decreased during illumination. Globally, net CO_2 variation in the medium, deduced from gross fluxes, led to a symmetric curve to net O_2 variation, as expected.

The means of several experiments with GCPs and MCPs are given in Table I. Respective protein and Chl contents were 190 and 3 pg per guard cell protoplast, 1400 and 120 pg per mesophyll cell protoplast. When working with GCPs, a difficulty arose due to mesophyll contamination. In the present study, mesophyll contamination was around 2 to 3 10^{-3}. Thus, chlorophyll contamination from mesophyll might reach 12%, which could lead to an overestimation of the photosynthetic properties of GCPs. However, when placed in the medium used for GCPs, MCPs burst and no O_2 evolution was observed under light. This may be due to osmotic stress and the inhibition by Tris of isolated chloroplasts.

Kinetics of CO_2 and O_2 uptake and evolution rates for GCPs and MCPs during a typical experiment are compared in Figure 2. In darkness, O_2 uptake matched well with CO_2 evolution (Fig. 2A and B). On a Chl basis, GCPs presented a very large respiration rate, 10 times as much as MCPs. However, on a protein basis this respiration ratio was only 2 (1.7 based on CO_2 evolution or 2.3 on O_2 uptake). Furthermore, as RuBPCase represents 35% MCPs proteins and is almost absent from guard cells (25), these two types of protoplasts seem identical as regards respiratory metabolism in darkness.

Kinetics of CO_2 uptake were very different (Fig. 2C and D); GCPs were able to fix CO_2 in darkness, in contrast to MCPs. When the light was switched on, CO_2 uptake increased slowly (Fig. 2C), reaching approximately half the rate of CO_2 fixation of MCPs.

The two types of protoplasts presented similar rates of O_2 evolution, but the relationship between O_2 evolution and CO_2 uptake was totally different (Fig. 2C and D). For MCPs, CO_2 uptake took place quickly and closely paralleled O_2 evolution; for GCPs, CO_2 uptake was delayed and remained lower than O_2 evolution (Table I).

During illumination, O_2 uptake was slightly decreased in GCPs, and considerably increased in MCPs (+ 62%). This increase was not due to photorespiration (CO_2 level was too high), but could be partly due to O_2 consumption in the chloroplasts, *e.g.* Mehler reactions.

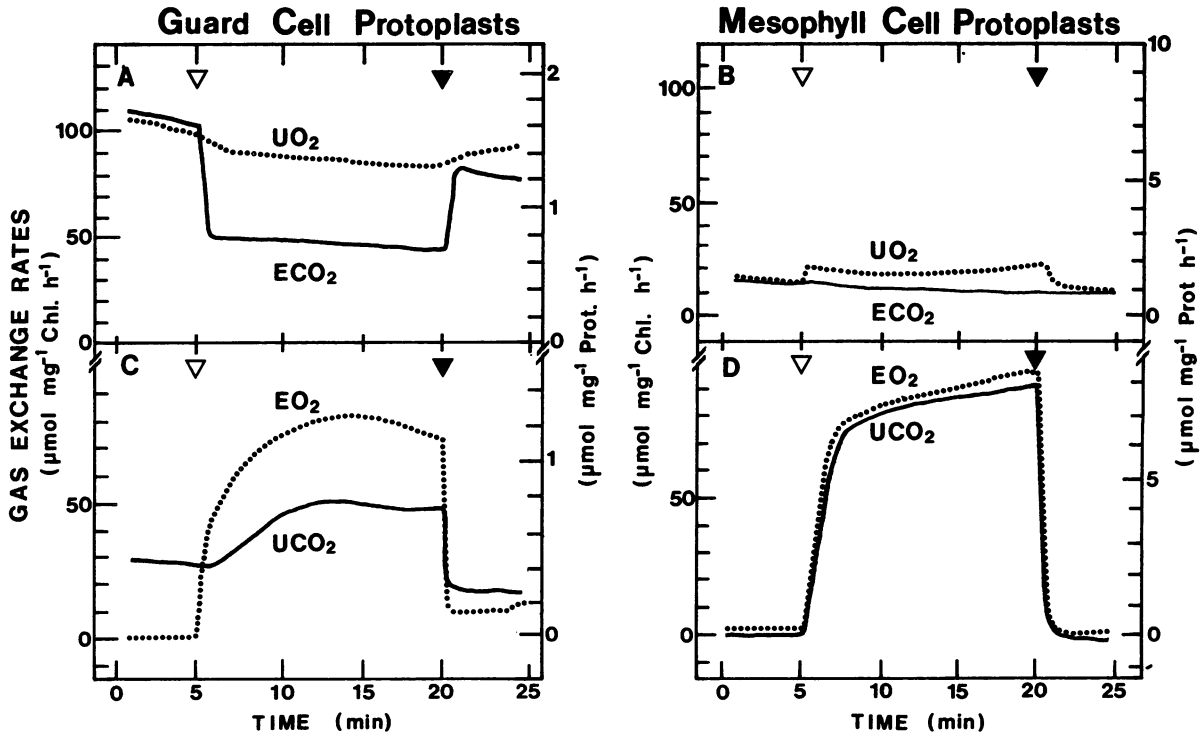

Figure 2. Comparison in the rates of gas exchanges between GCP and MCP, during a dark to light transition. A and C, GCP; B and D, MCP; E, evolution; U, uptake; open triangle, light on; dark triangle, light off. Experimental conditions were identical to Figure 1.

CO_2 evolution was decreased under light in both GCPs and MCPs. However, inhibition processes could be different according to the sudden slope change with GCPs, compared to the continuous decrease with MCPs.

DISCUSSION

Taken as a whole, the present results on O_2 and CO_2 exchanges obtained either by mass spectrometry or polarography are in accord with previous studies using other methods (Table II and Table III). However, O_2 evolution by GCPs as directly measured by MS led to lower values than those deduced from DCMU inhibition of PSII. Different reasons may be given to explain this discrepancy. It may be related to the growth conditions or to the experimental protocol (note that our polarographic data was also lower). However, determination of O_2 evolution by polarography is related to the inhibition of the PSII activity by DCMU and this supposes that O_2 uptake is not affected by photosynthetic activity. As shown by our results, such an assumption led to an overestimation of the O_2 evolution since O_2 uptake decreased under illumination (Table I). Interestingly, the present data indicates a comparable PSII activity in GCPs and MCPs on a Chl basis, confirming a normal functioning of the electron transport chain in the chloroplasts. Similarly, respiration rates in darkness, either based on O_2 uptake or CO_2 evolution were of the same order for the two types of protoplasts (on a protein basis excluding RuBPCase), pointing toward a comparable mitochondrial metabolism in darkness.

Major differences appear when respective CO_2 metabolisms are considered. CO_2 fixation by MCPs was typical of C3 metabolism with a concomitant O_2 uptake probably due partially to respiratory processes. O_2 uptake was even higher when CO_2 level in the medium was decreased (data not shown). This is characteristic of photorespiration, as already described in C3 cells (26). In contrast, guard cell protoplasts were able to fix CO_2 in darkness and light enhanced this capability by 50%. On one hand, this enhancement may be due to photosynthetic carbon fixation resulting from RUBPCase activity which has been found in GCPs (13, 25). On another hand, this data also agrees with PEPCase activity. This last hypothesis accords with previous studies which indicate a metabolism based on malate and a twofold increase in PEPCase activity after illumination (17).

The parallel variations in O_2 evolution and CO_2 uptake were also very different for each protoplast type. With MCPs, O_2 evolution and CO_2 uptake were closely correlated, reflecting the functioning of the Calvin cycle in the chloroplast. With guard cells the two processes appeared disconnected. CO_2 fixation in light presented a high induction time and was always far lower than O_2 evolution. Nevertheless, the higher rate of O_2 evolution was only obtained when CO_2 fixation rate was also maximal. This may indicate an overreduction of photosystems due to the lack of redox equivalent consumption. Altogether, these results suggest a PEPCase fixation, with a time lag due to the transfer of energy between different cellular compartments. In addition, our data are not inconsistent with the intervention of photosynthetic carbon reduction pathway: 3-PGA exported from the chloroplast to the cytoplasm could be converted to PEP and provide additional substrate for PEPCase.

MCPs seem to be autotrophic, net CO_2 fixation being largely positive under light. However, GCPs appear deficient in relation to CO_2 fixation which never compensates CO_2 evolution from respiratory processes. This may be specific to isolated protoplasts and due to metabolism alterations caused by enzymic treatment or may reflect the actual physiology of guard cells. This question has recently been discussed by

Table II. *Reported Rates of O_2 Uptake in Darkness and O_2 Evolution under Light of GCPs and MCPs Determined by Different Methods*

Plant Material	Dark O_2 Uptake	Light O_2 Evolution	Method	Reference
	$\mu mol\ O_2\ mg^{-1}\ Chl\ h^{-1}$			
GCP				
C. communis	127	162	Polarography	7
	1259	—[a]	Cartesian-diver	3
	113	—	Polarography	21
	100/82	61/103	MS/polarography	This report
P. sativum	130	—	—	24
V. faba	175	150	Polarography	30
	—	290	Polarography	11
	—	100–240	Polarography	32
MCP				
As. sprengi	6.9	34.4	Polarography	4
Av. sativa	5.9	—	Polarography	15
C. communis	6.6	48	Polarography	7
	8.1	73	MS	This report
N. tabacum	5.4	30.2	Polarography	18
N. plumbaginifolia	10	53.6	MS	26
P. sativum	7	—	—	24
V. faba	6	100	Polarography	30

[a] Not specified.

Table III. *CO_2 Uptake Rates in GCPs and MCPs in Darkness and under Light Determined by Different Methods*

Plant Material	Dark	Light	Method	Reference
	μmol CO_2 mg^{-1} Chl h^{-1}			
GCP				
C. communis	14.2	14.6	^{14}C	2
	27	40	MS	This report
P. sativum	9.9	32.2	^{14}C	25
V. faba	35	49	^{14}C	27
	3.5	—[a]	^{14}C	5
	13.9	35.2	^{14}C	12
	—	111–345	ΔpH	32
	3.7	23.3	^{14}C	13
MCP				
7 species	—	84/90[b]	^{14}C	29
C. communis	−0.2	74	MS	This report
N. tabacum	—	13.7	^{14}C	18
P. sativum	0.43	20.7	^{14}C	25
V. faba	—	80	^{14}C	13

[a] Not specified. [b] Palisade cell protoplasts/spongy cell protoplasts.

Reckmann *et al.* (25) who observed a different pattern of ^{14}C labeling between sugars and organic acids in isolated guard cells or GCPs. Some experiments indicate that organic substrates, precursors of malate formation and normally provided by mesophyll, may be limiting in GCPs (25, 27). Experiments are now in progress on the effects of different substrates (PEP, pyruvate, sucrose, etc.) on CO_2 fixation by GCPs. First results show an activation by external PEP and an inhibition by malate.

In any case, our results are very different from those obtained by Shimazaki *et al.* (32) who attributed the medium alkalinization under red light to a large CO_2 fixation by GCPs. This may result from differences between species and also to the specific experimental conditions (use of an open oxygen electrode in ref 32).

MS also provided original results on CO_2 evolution and O_2 uptake under light. In MCPs, the increase in O_2 uptake induced by light may be attributed to an enhancement in respiration and to the starting of O_2 consumption processes in chloroplasts. The decrease in CO_2 evolution could be due to a control of mitochondrial decarboxylation rate by reducing power and ATP export from chloroplasts (1, 9). The pattern was quite different with GCPs. Light only triggered a slight decrease in O_2 uptake and a rapid drop in CO_2 evolution which resumed after illumination (Fig. 2A). The slight decrease in O_2 uptake may be due to inhibition of respiration by an increase in the energy charge due to photophosphorylations. Considering the very low level of RuBPCase in guard cells (19), the absence of photorespiration seems logical. More surprising is the large decrease in CO_2 evolution under light. Two interpretations may be given. A cycling of CO_2 between mitochondria and chloroplasts could occur, CO_2 evolved by respiration being refixed by PEPCase. However, this hypothesis implies that the cycling must be three times the rate of fixation of external CO_2 at a saturating level. Moreover, recent experiments with *Dianthus caryophyllus* L. cells and mathematical modeling seem to discount a major role of cycling in this process (1). The second hypothesis is a decrease in substrate decarboxylation due to an inhibition of the TCA cycle. Such an inhibition was shown to occur in *Chlamydomonas reinhardtii* cells deprived of rubisco activity (9). The inhibitory process could depend on the ATP/ADP level (9) or on an export of reducing power from the chloroplast to mitochondrion. In this case, NAD(P)H could then be used in the mitochondria to produce ATP directly. Since CO_2 fixation appears limited in guard cells, the contribution of NAD(P)H production has been questioned many times (24, 32). NAD(P)H production could be lowered by photoreduction of oxygen in chloroplasts (Mehler reactions) as proposed in C4 mesophyll cells and *C. reinhardtii* cells deprived of Rubisco activity (9). NAD(P)H could also be transferred from chloroplasts by an OAA/malate shuttle or the phosphate translocator (33) toward cytosol to supply a plasmalemma oxydoreduction system with electrons. Via exchange systems, *e.g.* an OAA/malate shuttle, reducing power could also be oxidized in the mitochondrion to produce ATP in order to sustain the plasmalemma-H^+-ATPase activity involved in ion transport. This could explain the inhibition of stomatal opening under light by respiratory inhibitors. In conclusion, it appears that mitochondria play an important role in the bioenergetic processes of guard cells even under light, and that close relations between chloroplastic and mitochondrial activity are implied.

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