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Light-induced short-term ion flux responses and associated transport mechanisms in leaf tissues of pea (Pisum sativum L. cv. Argenteum)

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Light-induced short-term ion flux responses and associated transport mechanisms in leaf tissues of pea (*Pisum sativum* L. cv. *Argenteum*)

By

Bin Yan

AN ABSTRACT OF A DISSERTATION

Submitted to
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2001

Professor Irvin E. Widders

ABSTRACT

LIGHT-INDUCED SHORT-TERM ION FLUX RESPONSES AND ASSOCIATED TRANSPORT MECHANISMS IN LEAF TISSUES OF PEA (*PISUM SATIVUM* L. CV. *ARGENTEUM*)

By Bin Yan

The study was conducted to investigate light-induced rapid flux responses of Ca^{2+}, H^+, K^+ and Cl^- and associated transport mechanisms in leaf tissues of pea (*Pisum sativum* L. cv. *Argenteum*). Light stimulation (450 μmol m^{-2} s^{-1}) resulted in a transient increase in net Ca^{2+} influx of about 50 nmol m^{-2} s^{-1} in mesophyll tissues. The light-response lag time of Ca^{2+} is the most fast (13 s), whereas net influxes of H^+, Cl^- and K^+ were also rapidly induced to increase within approximately 29, 45, and 48 s by light exposure, respectively. After this initial response to light, a rapid Post-Initial Flux Response (PIFR) was measured for Ca^{2+} and Cl^- within 2-3 min, due most likely to an increase in efflux. In contrast, PIFR of H^+, K^+ was slower. The light-induced alkalinization of mesophyll apoplast (0.4-0.5 pH increase) which occurred within initial 5-6 min could be explained by either CO_2 uptake into photosynthesizing tissues or increased K^+ and Cl^- uptake mediated by H^+/K^+ and H^+/Cl^- symports, respectively.

$LaCl_3$ (0.1 mM), a Ca^{2+} channel blocker, and or EGTA (1.0 mM), a carion/Ca^{2+} chelator inhibited the light-induced initial net Ca^{2+} influx, and lead to reduction of the light-induced PIFRs of Cl^- and K^+ around the mesophyll tissues over time. In contrast, the addition of Ca^{2+} ionophore A23187 (5 μM) or high Ca^{2+} concentration (2.0 mM) in the bathing solution produced an opposite effect. Therefore, Ca^{2+} influx resulting from

increased Ca^{2+} channel activity may be an initial light response which could contribute to membrane depolarization. The plasma membrane H^+-ATPase inhibitors, Na_3VO_3 (1.0 mM) and DCCD (0.1 mM), inhibited the light-induced initial response in net H^+ influx by 50-60 % and eliminated the PIFR of H^+, whereas fusicoccin (0.01 mM) significantly increased the light-induced PIFR of H^+ and the initial response in net Cl^- influx. This suggests that H^+-ATPase in the plasma membrane may not be directly involved in the initial light response in mesophyll tissues. In addition, the K^+ outwardly-directed channels and the R-type anion channels are thought to play a role in the light-induced PIFR of K^+ and Cl^-, respectively, whereas Cl^- PIFR is Ca^{2+}-dependent.

Light-induced changes in net Ca^{2+}, H^+, K^+ and Cl^- flux from mesophyll tissues increased in a saturable manner with increasing fluence. However, high fluence (1800 and 2800 $\mu mol\ m^{-2}\ s^{-1}$) enhanced the temperature of the bathing solution and produced a possible heat effect on ion flux. White light (WL) of 450 $\mu mol\ m^{-2}\ s^{-1}$ stimulated significant changes in net flux of Ca^{2+}, H^+, K^+ and Cl^- from mesophyll tissues. Blue-light (BL) and red-light (RL) of similar fluence induced similar time course changes in net Ca^{2+}, H^+, K^+ and Cl^- fluxes as WL. There were no significant differences in the magnitude of change in net Ca^{2+}, H^+, K^+ and Cl^- fluxes between BL and RL. The result suggests that BL and RL play a similar role in rapid ionic responses to light.

To my parents and my aunt who believe in me

and whom I love.

ACKNOWLEDGEMENTS

I express sincere gratitude to my committee members, Dr. Irv Widders, Dr. Ken Poff, Dr. Eunice Foster and Dr. Eric Hanson, for the guidance and encouragement they have given me during my Ph.D. study. Their great helps made me finish the graduate program. First of all, I would like to thank my major professor, Dr. Irv Widders, for his didactic advise and tremendous contribution of his knowledge. His patience and supports are indispensable to my research. I extend my appreciation to Dr. Poff who enhanced my understanding of light signal transduction and gave me confidence. Drs. Foster and Hanson paid a lot attention and contribution to data analysis and experimental suggestion. Without their support my dissertation might be still an elementary step.

I wish to give a special thanks to Drs. Ian Newman, Sergy Shabala and Olga Babourina from University of Tasmania, Australia. They provided great help with MIFE technique. A very warm thanks to Dr. Frank Dennis and Dr. Jose Saunders who edited my writing. I would like to thank Richard Wolthuis for his assistance in building wonderful chambers. Bob Forbes made a nice Faraday cage for the MIFE system.

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LIST OF ABBREVIATIONS

A-9-C: Anthracene-9-carboxylic acid
ANOVA: analysis of variance
BL: blue light
Ca^{2+}: calcium ion
Cl^-: chloride ion
CsCl: Cesium chloride
d: day
DCCD: N-N'-dicyclohexil carbodiumide
DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea
D_i: diffusion coefficient
DIDS: Diisothiocyanatostilbene
DSMO: dimethylsulfoxide
E_n: Nernst potential
EGTA: Ethyleneglycol-bis(β-aminoethylether)N, N, N',N'-tetraacetic acid
F: Faraday constant
FC: Fusicoccin
H^+: hydrogen ion
HEPES: N-(2-Hydroxyrthyl)piperazine-N'-(2-thanesulfonic acid)
K^+: potassium ion
$LaCl_3$: Lanthanum chloride
LSD: Least Significant Difference
MIFE: Microelectrode Ion Flux Estimation
min: minus
Na_3VO_4: sodium vanadate
PIFR: Post-Initial Flux Response
R: gas constant
RL: red light
s: second
SE: standard error
T: temperature
TEA: tetraethylammonium
ΔV: electrochemical potential gradient
VDI: valid data interval
WL: white light
z: valency

INTRODUCTION

GENERAL INTRODUCTION

Plants are constantly exposed to fluctuations in various abiotic and biotic factors such as light, temperature, water, micro-organisms, and to mechanical and chemical stresses. Most of these signals evoke morphological, biochemical and physiological responses, indicating that there must be mechanisms in plants to perceive these external stimuli, and to elicit and transduce signals.

In recent years, a great deal of progress has been made toward understanding signal transduction in plants (Felle, 1993; Leigh, 1993; Verhey and Lomax, 1993; Gilroy and Trewavas, 1994; Yang, 1996; Blumwald et al., 1998; Ebel and Mithöfer, 1998; Grabov and Blatt, 1998; Genoud and Métraux, 1999). Most studies indicate that plants have developed multiple pathways to perceive and transduce signals. The inter-related molecular, cellular and tissue level events involved in triggering final responses have become important areas of research in plant science. However, the biochemical and biophysical mechanisms are not yet fully understood.

External stimulation leads to changes in membrane potential and induce rapid electrical signal propagation either locally or systematically (Davies, 1987a; Wildon et al., 1992; Thain and Wayne, 1993; Rhodes et al., 1996). Most researchers agree that the electrical signalling involve ion fluxes across membrane systems (Beilby, 1984; Davies, 1987a; Felle, 1993; Wayne, 1993; Ward and Schroeder, 1997; Grabov and Blatt, 1998).

Therefore, environmental stimulation most certainly induces changes of ion flux across cellular membranes.

Calcium, a powerful second messenger, presumably plays a central role in signal transduction in plants. Recent investigations on stimuli-induced changes in cytosolic Ca^{2+} concentration have enhanced the understanding of the mode of Ca^{2+} action in plants (Bush, 1995; Webb et al., 1996; Blatt, 1999; Sanders et al, 1999; Trewavas, 1999). The identification of Ca^{2+}- or Ca^{2+}/calmodulin-dependent protein kinases in plant cells suggests that Ca^{2+} functions is a critical signalling ion. Further investigation is needed, however, to examine the role of Ca^{2+} in plants.

Of the many environmental stimuli affecting plant growth and development, light may be the most important. Light has two principal functions in plants, energizing photosynthesis and photomorphogenesis. Plants have developed complex and sophisticated photosensory machinery that enable them to sense, transduce and respond to the direction, duration, quality, and quantity of light. Light-induced signalling systems in higher plants have been demonstrated by several authors (Chamovitz and Deng, 1996; Batschauer, 1998; Kurana et al, 1998). The light responses of leaves involve at least two photoreceptors, a phytochrome and a blue/UV-A photoreceptor.

An early response by leaf cells to light stimulation is a rapid change in the electrical potential across the plasma membrane (Bentrup, 1974; Cheeseman et al., 1982; Elzenga et al., 1995; Ermolayeva et al., 1996; Trebacz and Sievers, 1998). Changes in membrane potential indicate the involvement of ion fluxes in light signal transduction. Studies have attempted to determine if ion transporters, ion pumps, carriers and ion channels, in the

plasma membrane are involved in light-induced electrical signals (Mummert and Gradmann, 1991; Ermolayeva et al., 1996; Elzenga and van Volkenburgh, 1997; Szarek and Trebacz, 1999). Four inorganic ions were most highly correlated with light-induced bioelectrogensis, Ca^{2+}, H^+, K^+ and Cl^-. Despite extensive research, it is unclear what role ion transport mechanisms play in the electrical response to light, which ions are important in electrical signalling, or whether Ca^{2+} is a key ionic messenger, and which photoreceptors may be linked to ion transporters and thus regulate their activity in cellular membrane. The interesting questions in need of answer include, how do leaves transduce light-elicited signals, what is the role of ion fluxes in the transduction of signals in photo-responses.

In previous reports, pea leaves displayed rapid membrane depolarization, possibly involving H^+, Cl^- and Ca^{2+} (Elzenga and Van Volkenburgh, 1993; Elzenga et al., 1995; Stahlberg and Van Volkenburgh, 1999). However, there were no experimental evidence of ion fluxes for this conclusion. The Microelectrode Ion Flux Estimation (MIFE) technique has been introduced to investigate ion flux changes in root and leaf tissues (Lucas and Kochian, 1986; Newman et al., 1987; Shabala et al., 1997; Shabala and Newman, 1999). This noninvasive approach provides a direct tool to monitor ion dynamics around the plasma membrane of pea leaf cells, and to reveal possible ionic transport mechanisms. Ion-selective microelectrodes are utilized broadly in plant cell biology (Felle, 1992).

This study proposes three hypotheses at the outset: (1) rapid light-induced electrical signalling results from ions fluxes driven by ion transporters localized in the plasma

membranes; (2) Ca^{2+} plays an initial role in affecting activity of other ion transporters within the plasma membrane; and (3) ion fluxes across the plasma membrane are influenced by light quantity and quality. Thus, the first goal of this research was to characterize light-elicited rapid changes in ion (Ca^{2+}, K^+, H^+ and Cl^-) fluxes in leaf tissues, and investigated their relationships to early light electrical signals. Subsequently, we sought to identify the transport mechanisms involved in regulatory ion flux across the plasma membranes as well as the role of Ca^{2+} in light-induced ion flux responses. Finally, we measured the effects of light quantity and quality on the fluxes of Ca^{2+}, K^+, H^+ and Cl^-. This research should provide experimental evidence to elucidate photosensory mechanisms in leaves.

LITERATURE REVIEW

1. Generality of Signal Transduction

Like other biological organisms, plants can sense, transduce and respond to external signals via the built-in signalling systems. When stimulated, specific receptors induce one or several intracellular or intercellular sequences of biochemical and /or biophysical event leading to the modification of metabolic activities or the regulation of specific genes, which in turn produce a final response. Thus, sensory systems of plant cells typically involve three steps: perception, signal elicitation and transduction, and a localized or systemic plant response.

Juniper (1976) thought that "perception" is an altered physical state, which is assumed to pass the first physiological phase. The term represents the initial step of

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signalling and involves interaction between the stimulus and the receptors in a plant cell. However, stimulus-perception may involved a complex of physical, biochemical and molecular processes. Genoud and Métraux (1999) imaged that perception is analogous to a group of interacting algorithms, including an input layer, an output layer, and an intermediate layer linking the output and the input. Thus, they suggested a Boolean network model. "Signal transduction" infers sequence from stimulus perception to final response, and may involve conversion of energy, alteration of metabolic pathways, and changes of pH and water potential. Ray (1999) thought that "signal transduction" is the common term used to define a diverse topic that encompasses a large body of knowledge about the biochemical mechanisms that regulate cellular physiology. "Response" involves downstream steps in signal transduction chains, including morphological, biochemical, or physical changes.

Research on signal transduction in plants follows animal models. Some important progress in plants has been made, including the identification of the ethylene receptor (Bleecker and Schaller, 1996), phytochromes (Bowler, 1997), and detection of certain intermediate proteins, such as receptor-like protein kinases, GTP-binding proteins (G proteins), phospholipase C and Ca^{2+}/calmodulin-dependent kinases (Gilory and Trewavas, 1994; Yang, 1996). To study plant sensory systems, molecular genetic, biochemical and biophysical approaches are used widely in plant research. Signal transduction mechanisms have been proposed in almost all environmental and hormonal signals, including gravity (Chen, 1999), water (Bray, 1997), pathogens (Blumwald et al., 1998, Ebel and Mithöfer, 1998), light (Chamovitz and Deng, 1996; Khurana et al., 1998), mechanical stimuli (Verhey and Lomax, 1993), and abscisic acid (ABA) (Leung and

Giraudat, 1998). However, the mechanistic base of signal transduction in higher plants remains a "black box".

With the improvement of research technology, progress has been made toward understanding plant signal transduction processes. Some proposed models have been established to explain the biochemical pathways of plant response to external and internal signals (Gilory and Trewavas, 1994; Yang, 1996; Chang and Stewart, 1998). After perception by a specific receptor, the primary signal (first messenger) interacts and is transformed into transduction chains. G proteins serve a key regulatory role in initiating signal transduction. Next, the secondary messengers amplify the first messenger. Finally, the amplified signals drive and link cellular signalling pathways that result in final responses. Many secondary messengers/signalling elements are suggested in plants, including Ca^{2+}, H^{+}, lipids and cAMP (for reviews see Felle, 1993; Verhey and Lomax, 1993; Leckie et al., 1998). Among these second messengers, Ca^{2+} is best studied and accepted as a main signalling component. The Ca^{2+} messenger is thought to connect many signalling networks in plant cells. However, plant-signalling systems are being found to be highly complex, as new details are discovered. Interaction between signal transduction pathways may also occur when plants are stimulated concurrently by multiple factors.

2. Member potential and electrical signalling

A voltage potential is defined as a voltage difference across a membrane, which is created by the net separation of positive charges from negative charges (Wayne, 1993). The voltage potential represents a quantitative expression of membrane potential. In plant

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cells, positive charges are carried by cations, e.g. K^+, Ca^{2+}, Na^+ and H^+, and negative charges are carried by anions, e.g. Cl^-, and organic acids. When a plant cell is not stimulated, the measured membrane potential is considered a resting potential with range from -100 to -200 mV.

Membrane potential changes whenever the given membrane is perturbed or stimulated. When plasma membrane depolarization occurs in plant cells, there is a shift from the normally negative resting potential to less negative potentials. Either a net positive charge movement into a cell or a net negative charge movement out of a cell is necessary to effect membrane depolarization. Conversely, membrane hyperpolarization is a process of either a net positive charge movement out of a cell or a net negative charge movement into a cell, which shifts membrane voltage potential to more negative. Many environmental stimuli can alter membrane potential resulting in depolarization or hyperpolarization (for reviews see Davies, 1987a; Thain and Wildon, 1993).

Electrical signals involving membrane potential changes are thought to "control" many responses, for instant, plant cell growth (Pickard, 1971; Hush et al., 1991), metabolic changes associated defense to biotic factors (Wildon et al., 1989), and activation of ion channels, such as Ca^{2+}, K^+, and anion channels. Two classes of electrical signalling, action potentials and slow-wave potentials, potentially perform local or long-distance communication (Stankovic and Davies, 1998; Stankovic et al., 1998). Local electrical signalling occurs when electrical potential changes occur across membranes within a cell, whereas long-distance signalling involves the systemic transmission and propagation of voltage potential changes along membrane systems between cells or

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organs. Many stimuli induce local membrane potential changes. However, the evidence of systemic transduction of voltage potential changes seems to be limited to wound-induced plants (Wildon et al., 1992; Rhodes et al., 1996).

Action potentials in plants are characterized by a large, transient change in the membrane potential. Slow-wave potentials or variation potentials differ from action potentials by their amplitude and slower rate of propagation (Frachisse and Desbiez, 1989; Thain and Wildon, 1993; Stahlberg and Cosgrove, 1997). Action potentials are observed in a wide range of plant cells and thought to play a major role in intercellular and intracellular communication and in the regulation of ion transport and turgor at the cellular and organell levels (Retivin and Opritov, 1987; Fromm, 1991). A remarkable example is *Chara* algae cell, which exhibits a large and transient action potential (Hope and Findlay, 1964). Many higher plant species generate action potentials in response to light (Trebacz and Zawadzki, 1985; Trebacz and Sievers, 1998), cold (Shiina and Tazawa, 1986; Pyatygin et al., 1992) and wounding (Wildon et al., 1992; Stankovic et al., 1998).

Action potentials are considered to be the most rapid electrical phenomena in plants. The electrical signalling suggests that changes in ion flux across the plasma membrane occur. Wayne (1993) and Davies (1987a) proposed that effluxes of Cl^- and K^+ and influx of Ca^{2+} give in part rise to the action potentials. Fromm and Spanswick (1993) provided an indirect evidence in willow (*Salix viminalis*) using ion transporter inhibitors. In light-induced liverwort (*Conocephalum conicum*), Cl^- channels are involved in the depolarization event associated with action potentials (Trebacz et al., 1994).

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3. Ion fluxes and signal transduction

In biology, ion flux is a quantitative expression of the net movement of ions from one site in a cell to another over a specific period of time and area. Ion transport across the plasma membrane is either by passive or active pathways. Passive transport, through ion channels, protein carriers and facilitated diffusion pathways, depends on electrochemical gradients of ions across the membrane. In contrast, active transport is an energy-dependent process.

Membrane potential changes mediated by ion fluxes may be considered primary events in signal transduction. Ca^{2+}, H^+, K^+ and Cl^- are believed to be primary ions involved in effecting changes in membrane potential. Stimulus-induced changes in membrane potential could be coupled to perception and transduction of extracellular signals. Consequently, ion fluxes across the plasma membrane may be linked with cellular signalling pathways. Guard cells provide a valuable model system for study of photoreception, ion transport, and osmoregulation in plant cells (Serrano and Zeiger, 1989). Stomatal movement is operated through coordination of signalling elements in guard cell ion channels, and is regulated by ABA and auxin (Grabov and Blatt, 1998). Membrane depolarization in guard cells is evoked by ABA and auxin and is accompanied by Ca^{2+} influx and cytosolic alkalinization (McAinsh et al., 1997). In addition, outward-rectifying K^+ channels are activated by membrane depolarization and a rise in cytoplasmic Ca^{2+} concentration and pH (Plieth et al., 1998; Roelfsema and Prins, 1998; Blatt, 1999). Opening of anion channels leads to ion efflux and further depolarization

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(Ward and Schroeder, 1997). The massive ion fluxes across the guard cells control turgor and thus stomatal aperture. This indicates that ion fluxes are involved in both signalling and achievement of the physiological response in guard cells. However, the similar model of stimuli-response systems in other leaf tissues are still lacking.

The ionic mechanism is based on the observation of ion influxes or effluxes across membranes. However, ion fluxes may be controlled by various transport mechanisms. Davies (1987b) has already underlined that "ions (...) are likely to play a major role, especially as local (intracellular) signals", and that "electrical signals (...) need to attain respect from a greater number of plant researchers, as candidates for major intercellular communication devices". Ion-distribution and ion-channel blocking experiments suggest that the fluxes of Ca^{2+}, K^+, H^+, and Cl^- across membrane systems may be involved in specific electrical signalling events in a transduction pathway.

3.1. Ca^{2+} flux and Ca^{2+} signalling

Calcium, as a second messenger, plays a central role in signal transduction in plants. When the primary stimuli perceived by receptors are transduced to intracellular messengers (e.g., Ca^{2+}), the second messenger amplifies primary signals associated with modification of many enzymes and brings about changes in biochemical and physiological responses. Numerous reviewers have implicated Ca^{2+} function in plant signal transduction (Bush, 1995; Blatt, 1999; Sanders et al., 1999; Trewavas, 1999). Cytoplasmic Ca^{2+} and Ca^{2+}-dependent protein kinases have been studied to elucidate signalling mechanisms.

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A huge Ca^{2+} concentration gradient exists across the plasma membrane. In resting cells, cytosolic Ca^{2+} concentrations are maintained within the range of 0.1-1.0 μM, whereas the level of apoplastic Ca^{2+} for land plants is approximately 1000 μM (Bjorkman and Cleland, 1991; Harker and Venis, 1991). This produces a typical concentration difference of about 10^{-4} to 10^{-3} fold. Estimation of cytosolic Ca^{2+} concentration is based on some reliable techniques, including Ca^{2+}-selective electrodes, fluorescent dyes and luminescent protein aequorin (Read et al., 1993; Roos , 2000). In general, the electrical potentials across the plasma membrane are -100 to -200 mV, and the resultant electrochemical difference in Ca^{2+}, calculated from the Nernst equation, is approximately 50 kJ mol^{-1} which is comparable to the free energy for ATP hydrolysis, i.e. approximately -44 kJ mol^{-1}, in living cells (Miller et al., 1990). The electrochemical gradients for Ca^{2+} across other cellular membranes, indicating those found the endoplasmic reticulum (ER) and tonoplast, are also large and rarely reversed (Bush, 1993).

Calcium movement across membranes is facilitated by Ca^{2+}-permeable channels and Ca^{2+} pumps. Passive influx of Ca^{2+} occurs mainly through Ca^{2+}-permeable channels found in the plasma membrane. Considering ion selectivity, White (1998) suggested that there are at least two major classes of Ca^{2+} channels in the plasma membrane. The first class is relatively non-selective with respect to cations, and the other has a high single-channel conductance and is called a maxi-cation channel (White, 1993; White, 1994). The second class is referred to as voltage-dependent channels with greater selectivity for cation (White, 1994; Pineros and Tester, 1995; Pineros and Tester, 1997). In addition, the third Ca^{2+}-channel has been reported, a stretch-activated channel, which can be activated by membrane tension (Cosgrove and Hedrich, 1991; Ding and Pickard, 1993).

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Among the Ca^{2+}-permeable channels characterized in the plasma membrane of plant cells, voltage-activated Ca^{2+} channels may contribute to calcium signalling. Membrane depolarization causes activation of Ca^{2+}-channels. Direct measurement using the patch clamp technique indicated that depolarization of the plasma membrane in carrot protoplasts involves activated Ca^{2+}-permeable channels (Thuleau et al., 1994). Similar observation has been made in maize shoots (White, 1997). Such voltage gating which activates the channel may be critical during cellular signal transduction. Numerous stimuli, including blue light (Spalding and Cosgrove, 1992b), red light (Ermolayeva et al., 1996) and fungal elicitors (Kuchitsu et al., 1993), are known to induce rapid membrane depolarization. The resultant increase in Ca^{2+} channel activity and influx would be expected to enhance cytosolic Ca^{2+} concentration by influx. Concurrently, the increase in Ca^{2+} influx results in membrane depolarization. Therefore, voltage-gated Ca^{2+} channels provide a connection between stimuli and Ca^{2+} influx across the plasma membrane. However, compared with other ion channels (e.g. K^+ channels), there is limited direct evidence that Ca^{2+} channels operate in the plasma membrane of leaves. Most conclusions are based on pharmacological and cell biological studies. These results only provide indirect evidence that voltage-dependent Ca^{2+} channels in the plasma membrane are important for the initiation of plant responses to external signals (Hepler and Wayne, 1985; Leonard and Helper, 1990; Thion et al, 1996).

Two energy-dependent Ca^{2+} transport systems have been shown to function. Ca^{2+}-ATPase provides high affinity Ca^{2+} transport whereas proton-coupled antiporters provide low affinity Ca^{2+}-transport (Evans and Williams, 1998). Calcium efflux from the plasma membrane is mediated by Ca^{2+}-ATPase or ATP-hydrolyzing pumps and calcium

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exchangers, which catalyze an exchange of Ca^{2+} with H^+ (Evans et al., 1991; De Michelis et al, 1992). The nH^+/Ca^{2+} carriers have been reported in the plasma membrane (Rasi-Caldogno et al., 1987), tonoplast (Schumaker and Sze, 1986), and chloroplast thylakoid membrane (Ettinger et al., 1999). The plasma membrane Ca^{2+}-ATPase itself was considered to function as a ATP-dependent nH^+/Ca^{2+} antiporter (Rasi-Caldogno et al., 1987). However, there is no further evidence supporting this. The Ca^{2+}-ATPase is a primary efflux transporter functioning against a Ca^{2+} concentration gradient. Like the plasma membrane H^+-ATPase, Ca^{2+}-ATPase is also inhibited by vanadate (Williams et al., 1990; Carnelli et al., 1992).

The regulation of cytoplasmic Ca^{2+} involves a coordination of Ca^{2+} channels and the plasma membrane Ca^{2+}-ATPase activity in plant cells. Low cytosolic Ca^{2+} concentrations are required for the stability and activity of many enzymes, and appear to play a critical role in protein synthesis in the secretory pathways (Rudolph et al., 1989; Gill et al., 1996). Evans et al. (1991) suggested that the plasma membrane Ca^{2+}-ATPase plays a central role in the regulation of Ca^{2+} homeostasis of the cell, and, in particular, the restoration of low cytosolic Ca^{2+} concentrations after a stimulus-induced increase in concentration. Some intracellular organelles, including vacuoles, endoplasmic reticulum, mitochondria, and even chloroplasts, are able to store and sequester Ca^{2+} effectively. The vacuole occupies 90% of the mature plant cell volume. It contains a high Ca^{2+} concentration (DuPont et al., 1990) and represents a major intracellular Ca^{2+} pool. The voltage-dependent or voltage-gated Ca^{2+} channels in tonoplasts contribute to the regulation of cytoplasmic Ca^{2+} concentrations (Sanders et al., 1999). These channels are sensitive to inositol-1,4,5-triphosphate (IP_3) concentrations, which induce a release of

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Ca^{2+} from the vacuole (Schumaker and Sze, 1987; Alexandre et al., 1990; Gilroy et al., 1990). Afterward, cyclic ADP-ribose (cADPR) was found to activate tonoplast Ca^{2+} channels (Allen et al., 1995), thereby indicating that tonoplast Ca^{2+} channels are ligand-regulated. IP_3 is also proposed to be an important signalling messenger in cellular signal transduction (Gilory and Trewavas, 1994; Leckie et al., 1998). Calmodulin (CaM), upon binding to Ca^{2+}, is another unique protein which interacts with a number of key enzymes, and other structural proteins called CaM-binding proteins (Roberts et al., 1986). The plasma membrane Ca^{2+}-ATPase, a CaM-binding protein, can be activated by CaM (Colbran and Soderling, 1990; Hsieh et al., 1991). The Ca^{2+}-binding-CaM enhances the capability of the plasma membrane Ca^{2+}-ATPase to respond to an increase of cytoplasmic free Ca^{2+} concentrations.

The spatial and temporal changes in cytosolic Ca^{2+} indicate that Ca^{2+} is involved in a wide range of cellular signalling networks, and Ca^{2+} oscillations are crucial to the specificity of Ca^{2+} signalling mechanisms (McAinsh and Hetherington, 1998). Increases in cytosolic free Ca^{2+} concentrations have been widely observed in response to a number of stimuli, including that of ABA (McAinsh et al., 1990), mechanical stress and cold (Knight et al., 1991; Knight et al., 1992), auxin (Felle, 1988a), red light (Shacklock et al., 1992), and during phototropism and gravitropism (Gehring et al., 1990). The role of Ca^{2+} in action potentials has been demonstrated (Beilby, 1984; Wayne, 1993). Calcium signalling is involved in cell growth and development (Brownlee et al., 1999). Ca^{2+}-/CaM-dependent proteins and protein kinase C, driven by increased cytosolic Ca^{2+}, regulate protein phosphrylation/dephosphorylation in plant cells (Poovaiah and Reddy,

1993; Trewavas and Malhó, 1998), a primary mechanisms of signal transduction. Changes of in intra- and intercellular Ca^{2+} concentrations are considered to be a possible key component of cellular signalling pathways.

Coordination of Ca^{2+} transporters in membranes is necessary to achieve the dramatic changes of cytosolic Ca^{2+} concentration, and time course in concentration oscillations. Trewavas (1999) suggested that the function of cytoplasmic Ca^{2+} oscillation may lie in constructing a cellular "intelligence". Determination of changes in Ca^{2+} fluxes may be an important approach to the study of Ca^{2+} signalling mechanisms. Some common Ca^{2+}-related signalling components, such as Ca^{2+}-dependent protein kinase (CDPK), and Ca^{2+}/CaM-dependent protein kinases, support the role of Ca^{2+} in cellular signal transduction. However, Ca^{2+}-independent pathways or other signalling systems are accepted as existing in plants (MacRobbie, 1992; Allan et al., 1994).

3.2. H^+ fluxes

Plant cells maintain a pH gradient across the plasma membrane of about 1.5 - 2.0 pH units, i.e., cytoplasm with pH 7.0-7.5 and apoplast with pH 5.0-5.5. The proton gradient is believed to energize many important transport systems through H^+-coupled co-transporters, including (1) solute uptake into the cells against concentration gradients mediated by symporters, and (2) loading of organic compounds into the phloem for transport to sink tissues (Palmgren and Harper, 1998). Thus, this electrochemical gradient, known as the proton driving motive force (*dmf*), represents the sum of the membrane potential and the pH difference across a given membrane. The *dmf* provides

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energy for secondary active proton-driven solute transport across the plasma membrane, e.g. K^+ (Marré et al., 1989) and sugars (Serrano, 1985). Mitchell's chemiosmotic theory hypothesized that H^+ gradients across biological membranes function as a source of energy for cellular work (Mitchell, 1961;, 1976;, 1985).

Proton fluxes across the plasma membrane are chiefly catalyzed by the plasma membrane H^+-ATPase (proton pump) and H^+-coupled transporters. The plasma membrane H^+-ATPases generate a chemiosmotic H^+-gradient and are the main pathway for H^+ efflux across the plasma membrane. Studies have demonstrated that the plasma membrane H^+-ATPase is a proton pump and is electrogenic (i.e., it establishes a negative membrane potential that is positive on the outside, and negative on the inside) (Sze, 1985). The enzyme pumps outwardly H^+ across the plasma membrane into the apoplast. The resulting extracellular acidification, i.e. efflux of positively charged H^+, causes an initial repolarization of membrane potential. On the contrary, the inward movement of H^+ produces a membrane depolarization and cytoplasmic acidification. Biochemical and molecular research indicates that the enzyme is involved in many physiological roles (Palmgren and Harper, 1998; Sze et al., 1999).

A major function of the plasma membrane H^+-ATPase is the establishment of a pH gradient across the plasma membrane. Although the enzyme plays an indirect role for nutrient uptake into the cell, it actually serves as a main energy source in plant cells. For example, from the Nernst equation, $E_n = 58.2 \log [Ion]_{out}/[Ion]_{in}$. A Nernst membrane potential (E_n) of -175 mV (3× 58.2), $[Ion]_{out}/[Ion]_{in}$ would create a 1000-fold concentration gradient in a monovalent cation such K^+; i.e., the cell can maintain 100

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mM K^+ inside by virtue of a channel protein even though K^+ is present at only 100 μM in the soil solution (Stein, 1990).

Proton influx into the cytoplasm is mediated by symporters, such as H^+/K^+ (Rodriguez-Navarro et al., 1986; Netting , 2000) and H^+/Cl^- (Sanders et al., 1985) in the plasma membrane. Both carriers also facilitate K^+ and Cl^- uptake by cells. However, the rate of transport through symporters is much slower than via K^+ and anion channels (Palmgren and Harper, 1999). This is because solute fluxes through symporters are energetically coupled with H^+ transport. The symport enzyme requires energy transformation for conformational changes. Clearly, both H^+-ATPase and H^+ coupled symports regulate proton fluxes across the plasma membrane. Experimental evidence about H^+/K^+, H^+/Cl^- or other symporters remain insufficient.

A high cytoplasm pH is of utmost importance for maintaining cellular metabolic activities. A number of transporters capable of maintaining a stable cytoplasmic pH are well characterized (Sze et al., 1999). Except the plasma membrane H^+-ATPase, the H^+-ATPase and H^+-pyrophosphase in the tonoplast are also important pathways contributing to cytoplasmic 'pH stat' (Sze et al., 1992; Rea and Poole, 1993; Zhen et al., 1997). Moreover, the regulation of cytoplasmic pH regulation is thought to be linked to K^+ channels (Blatt, 1992; Ilan et al., 1994), and anion channel activity (Johannes et al., 1998). In corn root tissue, Felle (1998) found that cation channels may contribute to apoplastic pH regulation. Several other mechanisms are involved in pH regulation, including the production and consumption of malate via the biochemical pH stat (Leigh, 1993).

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pH regulation in plant cells may reflect a dynamic equilibrium in ion fluxes across membranes. Protons are considered to be another second messenger in plants because of the above pH-dependent processes (Felle, 1989b; Felle, 1993; Grabov and Blatt, 1998; Leckie et al., 1998). The most remarkable observation is that ABA functions in the regulation of stomatal movement. The ABA action in stomata is strictly associated with cytoplasmic alkalinization by 0.2-0.4 pH (Iring et al., 1992; Blatt and Thiel, 1993). Blatt and Armstrong (1993) confirmed the key role of H^+ as a messenger in regulating stomata aperture when they found that ABA-induced stomatal closure was blocked by low cytosolic pH. Another hormone, auxin, may also play a role in cytoplasmic pH signalling. Auxin at low concentrations can elicit stomata opening (Marten et al., 1991) and reduce cytosolic pH (Irving et al., 1992). This is because auxin stimulates proton extrusion by enhancing the activity of plasma membrane H^+-ATPases (Felle, 1993). The resulting cytoplasm alkalinization activates a K^+-inward rectifier channel, which leads to osmotic changes in guard cells and ultimate stomatal opening (Thiel and Wolf, 1997). In contrast, the key role of the H^+ messenger for stomatal closure is achieved by activation of a K^+-outward rectifier channel (Lemtiri-Chlieh and Macrobbie, 1994; Grabov and Blatt, 1997) and anion channels (Blatt and Grabov, 1997).

The experimental evidence for the role of H^+ as second messenger is limited to guard cells so far. The cytoplasm pH regulation is known to link many ionic transport mechanisms. It thus is reasonable to assume that H^+ messenger plays a similar role in other tissues as guard cells. The regulation of plasma membrane ATPase may provide an connection for H^+ fluxes and stimuli. Auxin has been demonstrated to activate gene

expression of H^+-ATPase (Theolois, 1986). This enzyme was also phosphorylated *in vitro* (Xing et al., 1996). A high-affinity fusicoccin binding protein has also been identified in different plant tissues (Weiler et al., 1990). In addition, white light (Linnemeyer et al., 1990) and blue light (Shimazki et al., 1993) stimulate proton efflux, suggesting that H^+-ATPase may be regulated by external factors.

3.3 K^+ flux

Potassium ion (K^+) has been demonstrated to serve both biophysical and biochemical functions in plant cells. In the cytoplasm, K^+ within the 50-500 mM range, is an indispensable cytosolic component in plant cells, a major contributor to osmotic potential of plant cells (Haschke and Lüttge, 1975). Because of the high concentration gradient across the plasma membrane, K^+ also exerts a dominant influence on the diffusion potential component of transmembrane electrical potential. Additionally, high cytoplasmic K^+ serves as a catalyst for a large number of enzymes (Evans and Wildes, 1971) and is a requirement for protein synthesis (Leigh and Wyn Jones, 1984; Memon et al., 1985). K^+ fluxes across the plasma membrane are essential for many physiological functions, e.g., stomatal opening (Humbel and Hsiao, 1970) and leaf movements (Lee, 1990). The regulation of K^+ transport and cytosolic K^+ homeostasis is accomplished by K^+ channels and ion-coupled K^+ symport or antiport. High affinity uptake via a symport mechansim has been postulated in *Neurospora* (Blatt, 1987). The symport of H^+/K^+ results in K^+ accumulation in the cytoplasm against its concentration gradient. The influxes of K^+ and H^+ depolarize the membrane and render the apoplast alkaline (Netting ,

2000). In contrast, a K^+/H^+ antiport facilitates H^+ efflux coupled to K^+ influx. This mechanism has been referred to as a capable pump-independent pathway of short-term pH regulation (Marré et al., 1987; Felle, 1988b; Felle, 1989a).

Two classes of K^+ channels have been characterized; an outwardly-directed K^+ channel facilitating K^+ efflux and an inwardly-directed K^+ channel facilitating or K^+ influx. The activity of both channels depend strongly upon their membrane potentials, various effectors, channel blockers, as well as their respective gating mechanisms (Schroeder, 1992; Blatt and Thiel, 1993). Known K^+ channels are highly permeable to K^+, which is larger than for other monovalent cations, such as Pb^+, NH^+, and Na^+ (Bentrup, 1990). Compared as to other cation channels, important progress has been made in understanding the molecular mechanisms and regulation of K^+ channels (Czempinski et al., 1999). Two types of inwardly rectifying K^+ channels (KTA1, AKT1) from *Arabidopsis thaliana* have been cloned (Anderson et al., 1992; Sentenac et al., 1992).

Potassium inward rectifiers, or inwardly-directed K^+ channels were originally demonstrated in guard cells (Schroeder et al., 1987; Blatt, 1992). These channels were shown to be activated in a voltage- and time-dependent manner when membranes are hyperpolarized from -50 mV to -200 mV (Blatt, 1992). Thus the inwardly-directed K^+ channels are considered to represent a pathway for K^+ uptake by plant cells. Another pathway for K^+ influx is K^+-H^+ symport. However, it is not easy to distinguish symporters from K^+ inward rectifiers (Hedrich and Schroeder, 1989).

Another class of K^+ channels, outward rectifiers, or outwardly-directed K^+ channels,

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represent a main mechanism for K^+ efflux across the plasma membrane. This type of K^+ channel is activated by depolarization of the membrane potential (Iijima and Hagiwara, 1987; Moran et al., 1988; Schroeder, 1989). In guard cells, outwardly-directed K^+ channels play a major role in stomatal closure (Thiel and Wolf, 1997). The same K^+ outward currents were also observed in *Arabidopsis thaliana* mesophyll cells (Miedema et al. , 2000). The efflux of K^+ mediated by outwardly rectifying K^+ conductance may result in repolarization of action potentials in both algae and higher plant cells (Sikaoka, 1966; Beilby and Coster, 1979; Simons, 1981) and takes part in motor cell movement of *Samanea saman* (Satter et al., 1988).

In general, K^+ fluxes mediated by K^+ channels are thought to possibly be involved in signalling in guard cells, or participate in electrical signalling in excitable plants. Both classes of K^+ channel are sensitive to changes in apoplastic pH and Ca^{2+} concentration (Herich et al., 1990; Blatt and Armstrong, 1993; Grabov and Blatt, 1997; Roelfsema and Prins, 1998). Cytoplasmic acidification reduces the conductance of the outward rectifier but increases that of the inward rectifier. Increased cytoplasmic Ca^{2+} is known to inactivate the K^+ inward rectifier (Kelly et al., 1995) but to activate another one, i.e., the K^+ outward rectifier (Schroeder and Hegiwara, 1989). The involvement of K^+ channels in sensing numerous stimuli suggests that K^+ fluxes or channels play a potential role in certain signalling pathways similar to that found in guard cells. Assmann (1996) hypothesized that G-protein regulation of K^+ channels may be a main ABA-signal transduction pathway. Further regulation of K^+ channel gating may be accomplished by G proteins and protein phosphorylation (Fairley and Assmann, 1991; Kelley et al., 1995).

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Therefore, K^+ fluxes or channels may be involved in many processes, including regulation of pH and Ca^{2+}, and hormone signalling.

3.4. Cl^- fluxes

Chloride ion (Cl^-) is a predominant inorganic anion in plant cells. A large electrochemical gradient for Cl^- exists across the plasma membrane. For example, a positive potential difference of 300-340 mV is typically present across the plasma membrane in *Chara* (Smith and Walker, 1976). The concentration gradient drives passive Cl^- efflux from the cytoplasm. Chloride efflux also play an important role in maintaining a negative membrane potential. It thus can be anticipated that anion channels are a primary route of Cl^- efflux from the cytoplasm, while the influx or uptake for Cl^- is mediated by a carrier mechanism (Assmann and Zeiger, 1987). Zeiger et al. (1978) hypothesized that Cl^- enters via a Cl^-/H^+ symport or a Cl^-/OH^- antiport.

Chloride influx requires the input of a considerable amount of metabolic energy. The pH gradient across the plasma membrane, or proton motive driving force, is thought to provide this energy. Chloride import through a $2H^+/Cl^-$ symport against an opposing Cl^- concentration gradient has been proposed and has been demonstrated experimentally in *Chara* (Smith and Walker, 1976; Sanders, 1980; Beilby and Walker, 1981; Sanders et al., 1985) and root-hair cell of *Sinapis alba* (Felle, 1994). However, experimental proof in higher plants has remained somewhat scant so far.

Anion channels have higher selectivity for Cl^- than for malate (Hedrich and Martin, 1993; Schmidt and Schroeder, 1994). Many elements, such as nucleotides (Herdich et al., 1990), pH (Tyermen et al., 1986), malate (Hedrich and Marten, 1993), and

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phosphorylation/dephosphorylation events (Terry et al, 1991; Hedrich and Marten, 1993), affect the activity of anion channels. Cytoplasmic Ca^{2+} has been shown to regulate anion channels (Hedrich et al, 1990; Schroeder and Hagiwara, 1990). A Ca^{2+}-activated Cl^- channel has been observed in an inside-out membrane patch of *Chara* plasma membrane (Okihara et al., 1991), whereas Cl^- efflux in perfused *Chara* cells is sensitive to Ca^{2+} influx (Mimura and Shimmen, 1994). An anion-conducting chloride channel family (CIC) has been cloned (Lurin et al., 1996; Jentsch and Günther, 1997).

Two modes of anion channel activity have been identified in guard cells: slow-type (S-type) and rapid-type (R-type) (Schroeder and Keller, 1992; Schroeder, 1995). The slow and sustained activation of S-type anion channels would produce a long-term depolarization which last a range of several minutes or longer (Schroeder and Hagiwara, 1989; Linder and Raschke, 1992). ABA and Ca^{2+} cause long-term depolarization (Bisson and Gutknecht, 1980; Findlay, 1982; Blatt, 1987), leading to the suggestion that the S-type anion channels may be activated by either ABA or Ca^{2+}. On the other hand, the rapid anion channels (R-type), produce a short, transient depolarization of the plasma membrane (Hedrich et al., 1990). In light of voltage-dependent, anion channels can also be divided into at least two major classes: depolarization- (Tazawa et al., 1987; Keller et al., 1989; Schroeder and Hagiwara, 1989) and hyperpolarization-activated (Tyerman et al., 1986; Terry et al., 1991). Another class of anion channels can be activated by stretching of membrane (Falke et al., 1988; Schroeder and Hedrich, 1989; Cosgrov and Hedrich, 1991).

Tyerman (1992) and Schroeder (1995) suggested that anion channels play key roles in controlling cellular functions, including turgor- and osmoregulation, stomatal movements

and anion transport. Most results in studies of guard cells indicate that anion channels in the plasma membrane may provide a control mechanism for stomatal closure. The proposed model suggests that anion channel activation and the resulting anion efflux from guard cells cause further membrane depolarization, thereby activating the non-selective Ca^{2+} channel which ultimately driving K^+ efflux through outwardly-directed K^+ channels required for stomatal closure (Schroeder and Hagiwara, 1989; Ward and Schroeder, 1997; Asamann and Shimazaki, 1999). Therefore, in guard cells, anion channels may be involved in ABA-induced stomatal movement by linking the Ca^{2+} messenger, Cl^- and K^+ effluxes. Furthermore, anion channels-controlling Cl^- efflux constitutes one of main ionic components in leaf movement (Satter, 1988; Coté, 1995). Sensitivity of anion channels to a wide range of stimuli provides a possible role for involvement of Cl^- flux in cellular signal transduction. Johannes et al (1998) proposed that anion channels play a central role in cytosolic pH regulation in plants, in addition to their established roles in turgor/volume regulation and signal transduction.

4 Light Signal Transduction

4.1 Photoreceptors

Light plays a primary role in regulating the growth and development of plants. Two important processes in plants, photomorphogenesis and photosynthesis are driven directly by light. Light also provides plants with many different kinds of information about their environment, such as quantity, quality, and direction. This information, known as light signals, is perceived and integrated by a complex of sensory systems, and used to optimize a variety of physiological and morphological responses. Recently, much

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attention has focused on photosensory mechanisms in plants, and several light signalling pathways have been proposed (Chamovitz and Deng, 1996; Batschauer, 1998; Khurana et al., 1998). The most important progress has been identifying photoreceptors.

Three classes of photoreceptor systems have been suggested for the photoperception in plants. These include phytochromes, blue/UV-A photoreceptors, and UV-B photoreceptors. The presence of multiple photoreceptors provides an approach to explore photosensory machinery.

Among these photoreceptors, phytochromes are the best characterized with specific sensitivity for red/far red light in plants. Five phytochrome genes (phyA ~ phyE) are present in *Arabidopsis* (Sharrock and Quail, 1989; Quail et al., 1991; Neuhaus et al., 1993; Clark et al., 1994). Light absorption is mediated by a chromophore covalently bound to an apoprotein, and each phytochrome can exist in two photo-interconvertible forms: a red (R) absorbing Pr form and a far-red (FR) absorbing Rfr form (Bowler, 1997). Several models of phototransduction mediated by phytochromes have been hypothesized (Bowler et al., 1994b, Chamovitz and Deng, 1996). Biochemical and molecular research suggested that GTP binding proteins (G-proteins) are major components of signal machinery (Clark et al., 1993). Ca^{2+} and cyclic GMP (cGMP) are utilized as second messengers in phytochrome signalling (Neuhaus et al., 1993; Bowler et al., 1994a). However, the primary mechanism of phytochrome action remains to be determined.

Progress in understanding photoregulation of plant processes activated by blue-light has until recently lagged far behind the phytochrome work (Briggs and Liscum, 1997). Four blue-light responses in higher plants have receiving concentrated attention (Kaufman, 1993). Two responses occur in the leaf (blue-light-induced increase in

stomatal aperture and blue-light-induced change in gene expression) and two responses occur in the stem (blue-light-induced suppression of stem elongation, blue-light-induced curvature of the stem, i.e. phototropism). However, blue-/UV-absorbing photosensory systems are still elusive. Higher plant blue-light photoreceptor have not been definitively isolated nor characterized (Short and Briggs, 1994). Based on their absorption properties, flavins, proteins or carotenoids have been proposed as blue-light receptors (cryptochromes) in higher plants (Galland and Senger, 1991; Horwitz and Berrocal, 1997). Phototropism is representative of blue-light-induced responses in plants. Briggs and co-workers have identified in maize coleptiles and etiolated pea seedlings a plasma-membrane-associated protein of about 120 kDa, which is connected to protein phosphorylation following exposure to blue light (Gallagher et al., 1988; Short and Briggs, 1990; Short et al., 1991; Palmer et al., 1993). Quarmby (1994) and Chamovitz and Deng (1996) have proposed that Ca^{2+} and cAMP have a role in blue-light signalling pathways. However, to our knowledge, the mechanism of blue-light signal transduction is still unclear.

4.2 Light effects on membrane potentials and ion fluxes

An early response by leaf cells to light stimulation is a transient change in the membrane potential. Bentrup (1974) found that light induces changes in transmembrane electrical potential in green tissues. The transition from light to dark usually reduces the extracellular membrane potential prior to a subsequent increase, while the reverse transition has an opposite effect in *Tradescantia albbiflora* leaves (Giroldini, 1988). A similar transient depolarization of membrane potential were found in light-induced

mesophyll cells of *Dionaea muscipula* Ellis (Trebacz and Sievers, 1998), pea (Elzenga et al., 1995), the intact leaves of *Atriplex* and *Chenopodium* (Lüttge and Pallaghy, 1969), *Tradescantia* and *Commelina* (Cheeseman et al, 1982), *Arabidopsis* (Spalding et al, 1992), as well as in several other aquatic and terrestrial plants (Novak and Ivankina, 1975). Most electrical responses to light involve membrane depolarization. However, light-induced membrane hyperpolarization has also been observed (Mimura and Tazawa, 1986; Takeshige et al., 1992).

The changes in membrane potential indicate that light induces underlying changes in ion flux across membranes and infer the involvement of ion transporters. In the *Characean* algae, the action-potential-like depolarization results from activation of Ca^{2+} (Weisenseel and Ruppert, 1977) and Cl^- channels (Lunevsky et al, 1983). Chloride pumps as well as K^+ and Cl^- channels were involved in action potentials in *Acetabularia* (Mummert and Gradmann, 1991). In gametophytes of *Asplenium trichomances*, light-induced depolarization was suppressed by Cl^- channel blockers (Szarek and Trebacz, 1999). Blom-Zandstra et al. (1997) reported transient light-induced changes in ion channel and proton pump activities in tobacco mesophyll protoplasts, and suggested that these ion transporters in the plasma membrane were involved in light-induced transient changes in the membrane potential. In *Arabidopsis* mesophyll, white light induced an increase in transient membrane depolarization and in K^+ channel activity within the plasma membrane (Spalding et al., 1992). A light-induced anion channel in the plasma membrane of pea mesophyll cells has been identified by Elzenga and Van Volkenburgh (1997). Shabala and Newman (1999) reported a light-induced flux response involving

H^+, Ca^{2+}, Cl^- and K^+ around bean mesophyll tissues. Light increased Ca^{2+} flux into xylem exudate from excised tomato petioles within less than 4 s after light-on or light-off treatments (Ries et al., 1995). Light also stimulated changes in the pH of the apoplasm of pea leaves (Van Volkenburgh and Cleland, 1980; Staal et al., 1994; Stahlberg and Van Volkenburgh, 1999). Similar result was reported in the cytoplasm of the unicellular green alga *Ermosphaera viridis* (Bethmann et al., 1998).

Both red and blue light are thought to stimulate stomatal opening (Assmann and Shimazaki, 1999). Red light, matching the absorption spectrum of chlorophyll, stimulates an outward electrical current that may be carried by the plasma membrane H^+-ATPase in *Vicia* guard cell protoplasts (Serrano et al., 1988). Blue light presented as a single pulse stimulates stomatal opening (Zeiger et al., 1987; Zeiger, 1990) and induces proton extrusion from guard cell protoplasts (Assmann et al., 1985; Shimazaki et al., 1993). In addition, since an anion channel in *Arabidopsis* hypocotyl is activated by blue light (Cho and Spalding, 1996), blue light would probably affect anion channels in guard cells. Using calcium-sensitive fluorescent dyes, Shacklock et al. (1992) have shown that red light induces proplast swelling and causes a transient Ca^{2+} increase followed by a rapid reduction in cytoplasmic Ca^{2+} to below resting levels. The blue-light-induced membrane depolarization in etiolated cucumber hypocotyls involves inhibition of the proton pump and influx of calcium (Spalding and Cosgrove, 1992b), whereas K^+ channels are activated by both phytochromes (Lew et al, 1990) and blue-light activated (Suh et al., 2000).

Clearly, light elicits transient electrical signals facilitated by ion fluxes across the

plasma membrane of plant cells. These changes may be one of rapid pathways of light signal transduction. Athough biochemical and molecular technology are becoming dominant approaches for the investigation of signalling intermediates and to elucidate the proposed signal transduction pathways, the ionic approach is still an important and powerful tool to understand photosensory mechanisms.

Chapter I.

CHARACTRIZATION OF LIGHT-INDUCED CA^{2+}, H^+, K^+ AND CL^- FLUXES FROM MESOPHYLL AND EPIDERMAL TISSUES OF PEA LEAF TISSUES.

ABSTRACT

Peeled mesophyll and epidermal tissues from excised leaflets of pea (*Pisum sativum* L. cv. *Argenteum*) bathed in 0.5 mM $CaSO_4$ and 1.0 mM KCl at pH 5.2 were exposed to an approximately 30 min light (450 μmol m^{-2} s^{-1}) period. The net flux rates of Ca^{2+}, H^+, K^+ and Cl^- around mesophyll and epidermal tissues were measured simultaneously by noninvasive ion-selective microelectrodes. Light stimulation resulted in a transient net Ca^{2+} influx an increase in influx of about 50 nmol m^{-2} s^{-1} within 1 min in mesophyll tissue. The light-response lag time of Ca^{2+} is the most fast (13 s), whereas net influxes of H^+, Cl^- and K^+ were also rapidly induced to increase within approximately 29, 45, and 48 s by light exposure, respectively. A light-induced extracellular alkalinization (pH increase of 0.4 - 0.5) was observed that lasted 5 - 6 min. Following the light-induced initial flux response, a rapid post-initial flux response occurred for Ca^{2+} and Cl^- which led to a net efflux within 3 - 4 min. When light was turned off, net flux of all four ions decreased and then gradually exhibited a small change in magnitude. In contrast, in the epidermis, light only induced slight changes in the flux of Ca^{2+}, H^+, K^+ and Cl^-. Compared to pH 5.2, changing pH in the bathing solution to a more acidic conditions (pH 4.0) increased further the light-induced initial response in net K^+ and Cl^- influx, whereas alkalinization of external medium (pH 6.0) produced an opposite effect on K^+ influx and enhanced the light-induced Post-Initial Flux Response of Cl^- (i.e. a net efflux). Inhibition of ion flux by DCMU (0.05 mM) indicates that the light-induced change in ion fluxes is dependent upon photosynthesis. These results suggest that the light-induced increase in net Ca^{2+} influx is an initial response that may contribute to membrane depolarization.

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Light-induced initial apoplastic alkalinization could be explained as either CO_2 uptake by mesophyll tissues or increased uptake of K^+ and Cl^-.

INTRODUCTION

Light-induced signalling systems have been found to exist in higher plants. One of the most remarkable light responses is a rapid change of membrane potential. Almost all green plant cells investigated react to sudden illumination by light-induced electrical signals. Because of the complexity of the effect and differences among plant species, either hyperpolarization (Mimura and Tazawa, 1986; Takeshige et al., 1992) or depolarization (Elzenga et al., 1995; Szarek and Trebacz, 1999) has been measured. Most results, however, showed that light induces a transient depolarization.

The mechanism of light-induced electrical signalling is not yet entirely understood. Membrane potential represents a voltage difference between cations and anions across a given membrane. Membrane potential changes indicate that changes in ion flux across membrane systems occur. Therefore, it is possible that light also induces changes in ion fluxes across the plasma membrane of plant cells. Four ions, Ca^{2+}, H^+, K^+ and Cl^-, were shown to correlate with the light-induced bioelectrogensis. Some researchers tried to look for whether ion transporters in the plasma membrane are involved in light-induced electrical signalling (Mummert and Gradmann, 1991; Ermolayeva et al., 1996; Stahlberg and Van Volkenburgh, 1997; Szarek and Trebacz, 1999).

Opinions in previous reports about the ionic basis involved in the light-induced electrical response are still controversial, and possibly there are somewhat different mechanisms in different species and even different tissues of the same species. In

addition, experimental technology is often different in some reports. The characterization of the light-induced ionic responses, the magnitude of the responses, the specific ions involved and time responses depend strongly on the ionic compositions (Elzenga et al., 1995; Johannes et al., 1997) and pH (Prins et al., 1982; Remis et al., 1994; Shabala et al., 1997) of the medium environment. Pea leaves have been shown to display a rapid membrane depolarization followed by a slower membrane repolarization (Elzenga and Van Volkenburgh, 1993; Elzenga et al., 1995). These reports suggest that H^+, Ca^{2+} or Cl^- fluxes across the plasma membrane may be involved in the light-induced electrical signals of pea mesophyll. However, there is an incomplete understanding about which ion is acting as the depolarizing agent in the initial phases of plasma membrane depolarization and how the ions are involved in the event.

To study light-induced ionic responses, some research groups have used ion-selective microelectrodes to measure membrane potential changes. Ion-selective microelectrodes have been utilized broadly in plant cell biology. The Microelectrode Ion Flux Estimation (MIFE) technique was introduced to investigate ion fluxes around root (Lucas and Kochian, 1986; Newman et al., 1987; Shabala et al., 1997) and leaf tissues (Shabala and Newman, 1999). This non-invasive approach provides a direct tool to monitor ion dynamics around the plasma membrane of pea leaf cells, and to reveal rapid photosensory mechanisms. Shabala and Newman (1999) found that light induced a rapid increase in Ca^{2+} influx in bean mesophyll tissues and proposed that Ca^{2+} influx was involved in light-induced depolarization. However, Cl^- efflux has also been proposed to be involved in the depolarization response to light stimulation (Spalding and Cosgrove, 1992; Elzenga et al., 1995).

Reports about the role of proton flux in plasma membrane electrical signalling are also contradictory. Plasma membrane H^+-ATPase activity was reported to increase following light illumination (Marré et al, 1989; Linnemeryer et al., 1990; Remis et al., 1994). The enzyme can mediate H^+ efflux from leaf cells which leads to apoplastic acidification. However, it has been observed in many cases that light causes an initial extracellular alkalinization in leaf tissues (Prins et al., 1982; Shabala and Newman, 1999; Stahlberg and Van Volkenburgh, 1999). The reason for this contradiction could be that there has not been direct evidence to explain the experimental relationship between H^+ fluxes and extracellular pH under light exposure. One can expect that external pH influences ion transports across the plasma membrane. Therefore, measurement of ion fluxes from pea mesophyll in the changing external pH environment may provide insights into pH and H^+ flux responses induced by light.

The first investigation presented in this chapter deals with the characterization of light-induced ion fluxes from mesophyll and epidermal tissues of pea leaves. To explain the ionic basis of the light-induced transience of membrane depolarization of pea leaf tissues, the kinetics of Ca^{2+}, K^+, H^+ and Cl^- fluxes across the plasma membrane was measured using the non-invasive MIFE technique. External pH effects on light-induced ion flux changes are discussed.

MATERIAL AND METHODS

Plant Material

Pea (*Pisum sativum* L. cv. *Argenteum*) seeds were soaked in distilled water for 6-8 hours,

and then germinated in an artificial clay shale (Turface manufacture) at room temperature (23±1°C). After 4-5 days of germination, the young seedlings were transplanted and transferred to a growth chamber with a 16 hour photoperiod (200 μmol m^{-2} s^{-1} at the top of the canopy), day/night temperatures of 20/25 °C and 75% relative humidity. The plants were cultured in a modified Hoagland solution (Johnson et al., 1957) following an increase in concentration from 0 → ¼ → ½ at 4-5 d intervals after transplanting.

Ion-Selective Microelectrode

Glass capillaries (catalog no. MIB 150-6; World Precision Instruments, Inc., Sarasota, FL, USA) were pulled using a two stage puller (PC-10, Narishige, Tokyo, Japan) to produce micropipettes with a tip diameter of about 2 μm. After pulling, the micropipettes were dried in the oven at 200°C for 4-5 h, and silanized with N-dimethyltrimethyldiylamine (catalog no. 41716, Fluka, Milwaukee, WI). Cooled microelectrodes were back-filled with 500 mM $CaCl_2$ for Ca^{2+}, 500 mM KCl for K^+ and Cl^-, and 15 mM NaCl plus 40 mM KH_2PO_4 (adjusted to pH 6.0 with NaOH) for H^+. Electrode tips were then front-filled with a 50-80 μm-long column of the appropriate ion-selective sensor, including H^+ (no. 95291), Ca^{2+} (no. 21048), K^+ (no. 60031) and Cl^- (no. 24902) cocktails (all from Fluka, Milwaukee, WI). Silanized micropipettes were used within one week of preparation, while the filled microelectrodes only were used within one-day period.

Microelectrodes were calibrated for each ion using a known set of standard solutions before and after use. The standard solutions contained a pH 4.0 -7.0 for H^+, 0.1 - 2.0 mM

$CaSO_4$ for Ca^{2+}, or 0.1-5.0 mM KCl for K^+ and Cl^-. The average slope was 52-55 mV / pIon for monovalent ions and 26-29 mV / pCa^{2+} for Ca^{2+} electrodes.

An Ag/AgCl reference electrode was prepared using a 4-5 cm section of 2 mm diameter polyethylene tubing filled with 2 % agar in 1 M KCl. A section of silver wire (diameter 0.38 mm) was coated in 0.2 M HCl. The coated silver wire was then inserted into one end of the tubing containing 2 % agar and wrapped with parafilm to fix in position. The reference electrode was stored in 1 M KCl solution and only used 1 d. During measurements, the end of the reference electrode with silver wire was connected to the preamplifier, while the open end of the tubing was bathed into solution being measured.

MIFE system

Ion concentrations at the surface of the leaf mesophyll cells were measured using a non-invasive Microelectrode Ion Flux Estimation (MIFE) system (MIFE, Unitas Consulting, Hobart, Australia) (Shabala and Newman, 1997; Shabala et al., 1997). From time course data obtained by the MIFE system, the directionality and rates of ion flux could be estimated. Additional information on the MIFE is available at http://www.phys.utas.edu .au/physics /biophys.

The set-up of the MIFE system is illustrated in Figure 1 and 2. An inverted microscope (Leitz Wetzlar, Germany) was placed on an anti-vibration table that was installed inside a Faraday cage to minimize electrical and magnetic noise. Three ion-selective electrodes were mounted on a coarse multi-microelectrode holder/manipulator

with three-dimensional positioning ability. After a tissue ample/solution chamber (8.0× 2.5 × 1.5 cm) was fastened to its holder, the electrodes were positioned at a 50 μm distance parallel to the tissue surface. The distance between adjacent electrode tips was about 4-5 μm. For fine positioning of the microelectrodes, the holder supporting the tissue sample/solution chamber was controlled by a three way hydraulic micromanipulator (WR 88, Narishige, Tokyo, Japan) driven by a computer-controlled stepper motor (MO61-CE08, Superior Electric, Bristol, CT). Thus, the ion-selective electrode could be moved to any programmed position through computer control of the stepper motor. In this study the electrodes were moved vertically from 50 to 90 μm above the leaf surface in a 20-s square-wave cycle with a frequency of 0.1 Hz, i.e. 40 μm for each movement (a half cycle) with 10 s pass at each position.

The three ion-selective electrodes and the reference electrode were connected to a pre-amplifier inside the Faraday cage. The pre-amplifier was then linked to a main amplifier outside the Faraday cage. A personal computer with an A/D card was used to monitor and record all measurements (Figure 1, 2).

A small Plexi-glass curved mount in the shape of arc with a diameter 1.2 cm was positioned at the bottom of the measuring chamber. This mount caused a gentle bending of the leaf segment mounted in the chamber. Two braces were used in the chamber to prevent dislocation of the leaf segment from the mount. This set-up allowed for a clear view of leaf cells for electrode positioning through the inverted microscope. The microscope light position, tangential to leaf surface, provided background illumination of approximately 50 μmol m^{-2} s^{-1} (Figure 1). The microscope lamp was turned off prior to

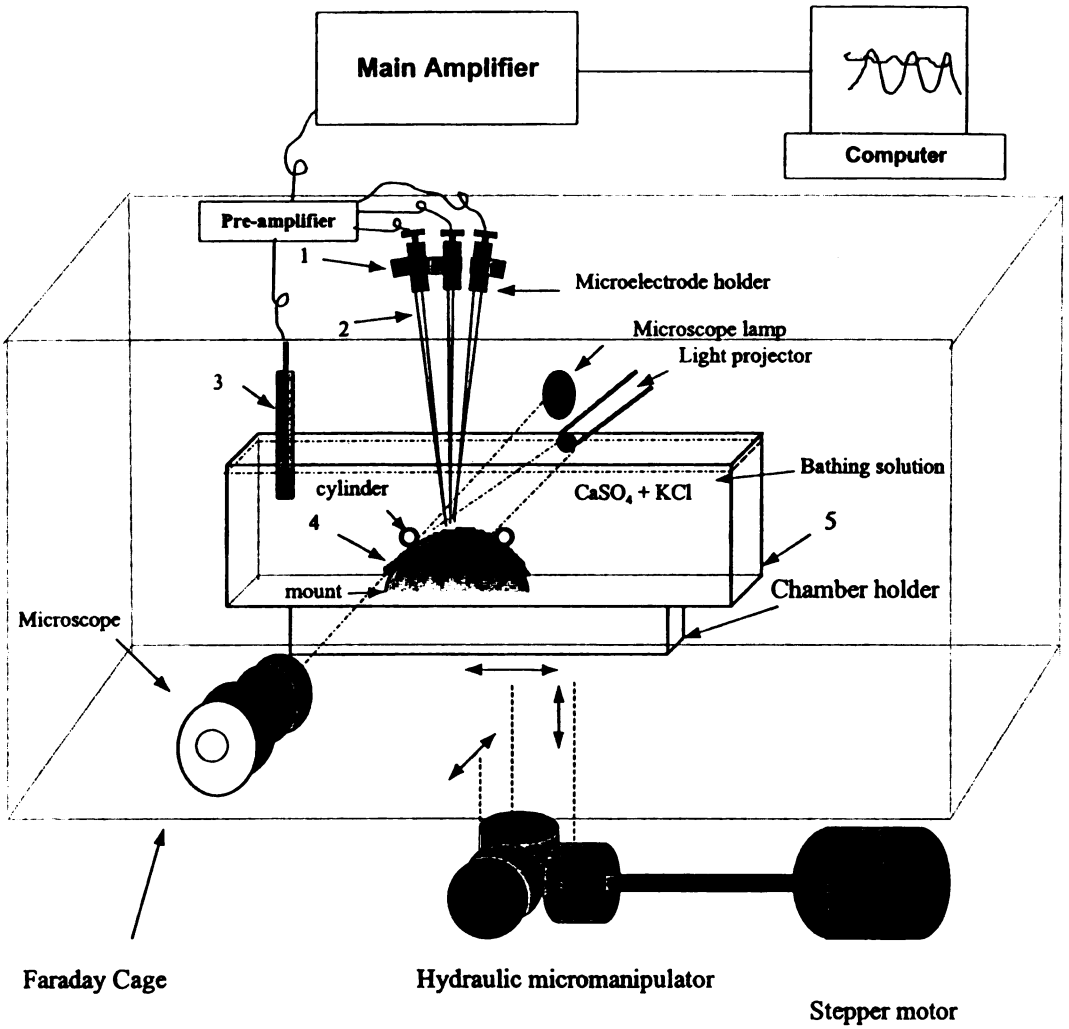


Figure 1. Schematic illustration of the experimental set-up, the MIFE system and the position of the light source

1: Micromanipulator; 2: Microclectrodes; 3: Ag/AgCl reference electrode;
4: leaf segment; 5: Measuring chamber.

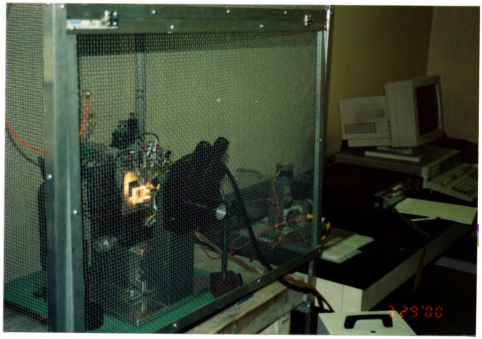
initiating any experiments in order to allow the tissue to equilibrate the new environment.

A fiber-optic light (450 μmol m^{-2} s^{-1}) was used as a light source (Figure 2). The 250 W lamp was from GS Edmumd Scientific Company (Barrington, NJ). A lens (no L-16-16, World Precision Instruments, Inc., Sarasota, FL) was fixed to the top of a light guide to focus the beam. Light intensity was measured using a quantum sensor (model LI-189, LI-COR, Lincoln, NE).

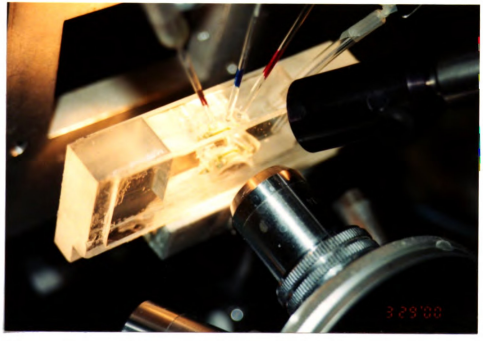
Flux measurements

The MIFE system utilizes slowly vibrating-specific ion microelectrodes, enabling concurrent measurement of multiple ions. The MIFE data recording system and flux calculation protocol have been described by Shabala et al. (1997). In brief, a software CHART package (Unitas Consulting, Hobart, Australia) provides an automatic record, control all measurement processes in a real-time display on the computer screen (Figure 1 and 2). After measurements are completed, the recorded data files can be transferred by CHART to MS-Excel files. These programs were then used to calculate average ion concentrations and mean net fluxes at 10-s intervals (each half-cycle of manipulator movement).

The concentration of each ion in the bathing solution was calculated for each position by measurement of voltage potential on the basis of Nernst equation. Ion concentrations close to the leaf were calculated from the calibration data and from the average mV value for the two positions. The magnitude and the directionality of the difference in estimated ion activity between the two positions, perpendicular to the leaf tissue surface, the



The MIFE system



the microelectrode and light source

Figure 2. The MIFE system and the position of the microelectrode and light source

concentration gradient, was the basis for estimating both the rate and directionality of the ion fluxes.

The estimation methods for ion flux using ion-selective microelectrodes have been described by Newman et al. (1987), Kochian et al. (1992), and Shabala et al. (1997). Basically, ion fluxes were calculated using Fick's first law of diffusion assuming a planer diffusion profile:

$$J_i = \frac{D_i (C_1 - C_2)}{\Delta r} \qquad (1)$$

where J_i is the net ion flux, D_i is the self-diffusion coefficient for the ion in equation (in $cm^{-2}\ s^{-1}$); C_1 and C_2 are the ion activities at the two positions, Δ r is the amplitude of electrode movement. In this study, Δ r is 40 μm. The net ion flux is affected by D_i, temperature, leaf shape, Δr and electrochemical potential gradient. Temperature influences the Nernst equation according to $E_n = RT/zF \ln (C/C_e)$, where T is temperature, z is valency, F is Faraday constant (96,487 J mol^{-1} $volt^{-1}$), R is gas constant, and C/C_e is concentration difference between the membrane. The temperature was ignored due to a constant room temperature (23 °C). D_i was used for Ca^{2+} (8.0×10^{-2} $cm^{-2}\ s^{-1}$), H^+ (9.17×10^{-5} $cm^{-2}\ s^{-1}$), Cl^- (2.032×10^{-5} $cm^{-2}\ s^{-1}$) and K^+ (1.9×10^{-5} $cm^{-2}\ s^{-1}$). Leaf shape could be considered as a planar system. The general equation for estimation of planar diffusion based upon measurements using the MIFE system is:

$$J = c\ u\ F\ (58/\text{Nernst slope})\ (dv/dx) \qquad (2)$$

where c = ion concentration; u = mobility, and dv/dx is voltage gradient over distance x.

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The Nernst slope is obtained from a calibrated curve over a range of ionic concentration. The mobility u is specific for each ion and is related to the diffusion coefficient D_i. Because D_i equals uRT, one can appreciate that D_i depends on temperature T (Noble, 1991). Therefore, the net ion flux is dependent on the voltage potential gradient (dv) when other conditions are constant. The ion flux values were expressed in terms of nmol m^{-2} s^{-1}.

The calculation procedure for electrochemical potential gradient is illustrated in Figure 3. The electrometer's ΔV is considered as the electrical voltage equivalent of the electrochemical potential difference over the distance Δ r. At 30 s the manipulator moved electrodes to position 1 (90 μm from leaf surface) within 10 s. The electrochemical potential measured by the electrometer was about 2.3 mV. At 40 s, the manipulator moved the electrodes to position 2 (40 μm from leaf surface). The electrochemical potential decreased to about 1.4 mV within 10 s. Position 3 had a higher mV (about 2.8) that when electrodes were positioned more distant from the leaf surface. The high-low potential was cycled each 20 s period. Higher mV at position 1 more distance from leaf surface indicates a higher H^+ concentration at this position. Hydrogen ion movement down its electrochemical potential gradient is toward the leaf cell, i.e. influx. An opposite movement is an efflux, against the electrochemical gradient.

During flux estimation, the first step in the calculation of ion flux was the determination of voltage potential at the extreme position of each manipulator movement. Since only ion activities at two positions perpendicular to the sample surface were measured, only net flux could be estimated. The net flux represents a balance between

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influx and efflux. The first 4 s (2 s for electrode movement and 2 s for electrochemical settling of the electrodes) of each half cycle (10 s) were not used for concentration and flux estimation. This was considered to be a delay time necessary to obtain a stable measurement following movement of the microelectrodes. Instability was thought to be due to solution imbalance and vibration noise following movement. The remaining 6 s at a position is defined as the valid data interval (VDI). Mean voltage potential during three consecutive VDIs, i.e. VDI_1, VDI_2 and VDI_3 (Figure 3), at position 1, 2, and 3, respectively, were determined (2.31, 1.40 and 2.80 mV, respectively). The function for the line connecting the mean mV values for VDI_1 and VDI_3 was established. The ΔV was the difference between the mean mV midtime (T_2) value for VDI_2 and also at T_2 to this line function as shown in Figure 3.

Ten s later, the next VDIs were repeated to create the next triplet, to be used to calculate ΔV again in similar manner. The electrochemical potential gradients (ΔV) were calculated by this process at 10 s intervals which thus reflected running averages for 30 s period. Finally, net flux was estimated from the ΔV, i.e. dv in equation (2) A positive estimated flux value indicates a net influx, while negative one is a net efflux.

The above procedure makes reasonable considerations for the settling of voltage at the electrode tip after each movement. It also allows for drift of the signal, as seen Figure 3, which is to be expected when the tissue flux causes changes in the concentration of the ion being measured.

Experimental Protocol

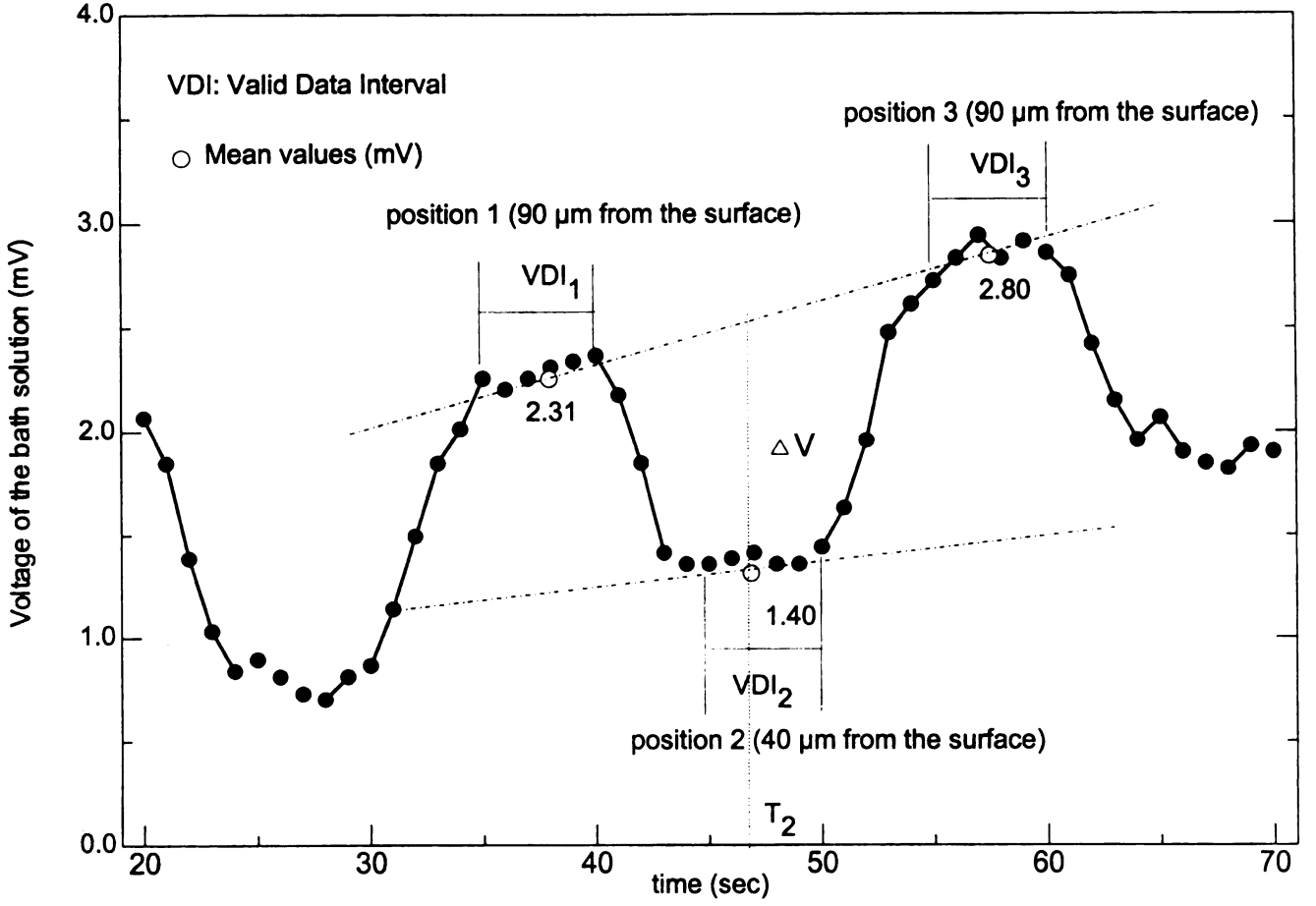

Figure 3. A segment of H^+ flux data record from pea mesophyll to illustrate the voltage change from which the fluxes were estimated using the MIFE system.

Young expanding leaflets (leaf age about 2 weeks) were harvested from pea plants with a razor blade. For studies involving flux from mesophyll tissues, leaf sections of approximately 15 × 10 mm (L×W) with limited veinal tissue were excised from the leaflet lamina. Using a fine forceps, a small strip of epidermal tissue was then detached from the abaxial surface of the lamina in such a manner so as to avoid major veins and to minimize mechanical damage. Isolated leaf segments were floated on buffer solution (0.5 mM $CaSO_4$, 1.0 mM KCl, 2.5 mM HEPES-HCl, and 10 mM sucrose at pH 5.2) under 60 W m^{-2} illumination (Philips lamp). After 2.0-2.5 h, the peeled lamina segments were mounted within the sample chamber and bathed in 6-7 mL unbuffered solution (containing 0.5 mM $CaSO_4$, 1.0 mM KCl, pH 5.2). The bathing solution was replaced 3 - 5 times. The sample tissue was then pretreated for 50-60 min in the dark. The dark treatment allowed for stabilization of ion flux across the plasma membrane as a result of equilibration of the tissue with the new environment. After dark pre-treatment, the leaf tissue was ready for measurement.

Preliminary experiments showed that ion fluxes can vary significantly from one position to another over the surface of the lamina tissues. To minimize the time course variability of flux measurements, regions with stable flux were sought by moving manually the hydraulic manipulator stepper. After a suitable site was selected, ion fluxes were measured for a minimum 5-10 min before the treatment light was turned on. Data recording for flux estimation was continued for an average 30 min after the onset of illumination. The light source was then turned off, and data recording continued for an additional 10-15 min. The sample chamber was then removed and replaced with another

chamber with a fresh leaf sample. All experiments were conducted in unbuttered solution at room temperature (23±1°C). Arif et al. (1995) explained the reasons for not using buffers in the bath. This is because some of the H^+ crossing the tissue boundary diffuse as protoned buffer whose flux is not including in the flux calculated from the H^+ electrochemical gradient (Arif et al., 1995). Heat emission from the light source was negligible (was discussed in Chapter III).

The pH treatments were achieved by adding 0.1 mM HCl / NaOH to the unbuffered bathing solution. Photosynthetic inhibitor, DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, from SIGMA) was prepared in 0.5 % (v/v) DMSO and then dissolved in unbuffered solution adjusted to pH 5.2. DMSO of 0.5 % had no discernible effect on ion flux. To test effects of DCMU on ion flux, the control (an unbuffered solution) was replaced with DCMU solution (0.5 mM $CaSO_4$, 1.0 mM KCl, 0.05 mM DCMU, pH 5.2) during the course on an experiment using a single leaf sample.

Data statistical analysis

By using ion-specific microelectrodes, net ion fluxes from leaf cells can be measured directly. One of the major concerns has been whether any artifacts were associated with ion flux measurement using the MIFE technique. No significant artifacts could be identified in this study. In most cases, changes in extracellular ion concentrations were consistent with changes in net ion flux. Secondly, light illumination did not cause significant changes in ion fluxes from leaf tissues when electrodes were lifted far (at least 1500-2000 μm) from leaf surface (Figure 4). Additionally, heat effects induced by light

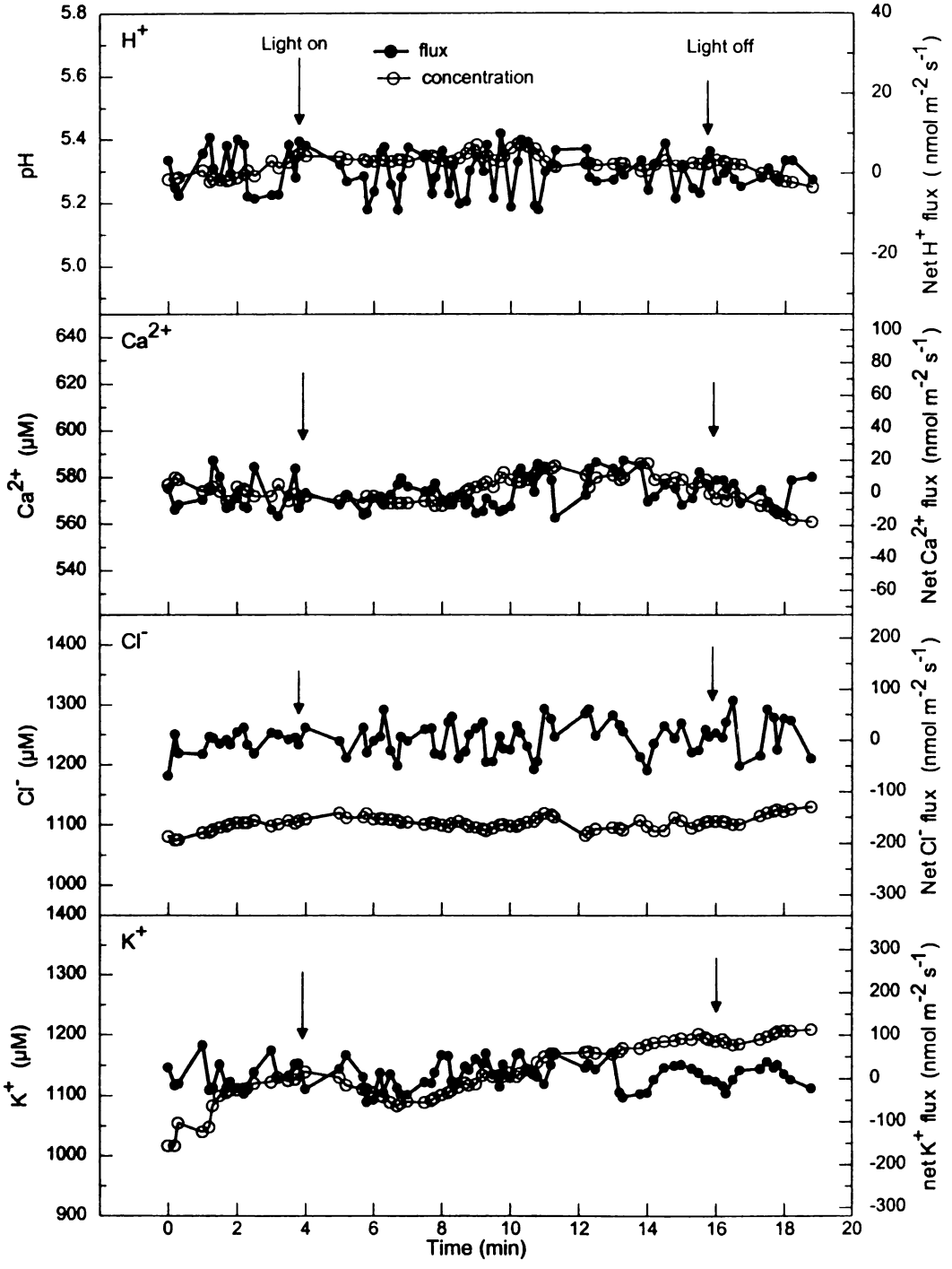

Figure 4. Time course of net ion fluxes and extracellular ion concentrations of H^+, Ca^{2+}, Cl^- and K^+ at 1500-2000 µm from pea leaf surface using the MIFE system.

treatment were considered to be negligible (see Chapter III). Fluctuations in ion flux over time are thought to reflect normal ion transport processes and dynamics across the plasma membrane of leaf cells. Thus, the extracellular ion concentration changes measured by the MIFE system were considered to be results of real changes in net fluxes across the plasma membrane.

Over 30 replicated leaf samples were conducted for the control. For analysis of ion flux response to light stimulation, there was concern about simply averaging replicate time-course measurements. The problem of averaging multiple replicates is that important time course changes in flux elicited by the light/dark treatments would be masked. For example, if two different replicate leaf samples exhibit a similar response in ion flux, but with a shift in time of several seconds or minutes, those shifts would result in a much difference response trend if the data were averaged. This, thus, would cause a loss of valuable information. For this reason, representative individual replicates were selected and shown in Figure 6 and 7 to best illustrate a typical response.

In order to smooth the treatment data, and to reduce some of the noise for analysis of the responses, data (including concentration and flux) over 1 min interval were averaged. The data could be reproduced for replicates of leaf samples.

One-way Analysis of variance (ANOVA) of the data was conducted using SAS 8 (SAS Institute Inc, Cary, NC). To determine if specific treatments (i.e. pH, DCMU) or comparisons among the four ions resulted in statistically significant differences in the light-responses for flux and extracellular ion concentration, LSD test (Fisher) at 0.05 was used. ANOVA results appear in the text and in the figures.

Figure 5 illustrated how important features of the light-response for net H^+ flux and

extracellular pH was calculated. The analysis for other ions (Ca^{2+}, K^+ and Cl^-) could also be made in a similar manner. The following terms were used in this study:

F_0 or ***C_0***: Five min average net ion flux or extracellular ion concentration before light-on.

***Average change during the entire light period*:** the averaged net ion flux or extracellular ion concentration during the entire light period minus F_0 or C_0, respectively.

***Average change after 10 min light-off*:** the averaged net ion flux or extracellular ion concentration during the entire light period minus F_f or C_f, where F_f or C_f is 10 min average net fluxes or extracellular ion concentrations from light-on to light-off, respectively.

Ion flux and extracellular ion concentration responses:

Initial flux response (net influx): initial maximum net influx or minimum net efflux after light-on minus F_0 (Figure 5). A positive value indicates a net influx. ***Initial concentration response (decrease)***: initial minimum extracellular ion concentration after light-on minus C_0 (Figure 5). However, pH response is an increase (positive), i.e. H^+ concentration decrease.

Maximum flux increase (net influx): maximum net influx or minimum net efflux during the entire light period minus F_0. A positive value indicates increase in net influx. ***Maximum extracellular ion concentration decrease***: minimum extracellular ion concentration during entire light period minus C_0.

Change during the first min: the average of net ion flux or extracellular ion concentration during the first min after light-on minus F_0 or C_0. ***Change during the first***

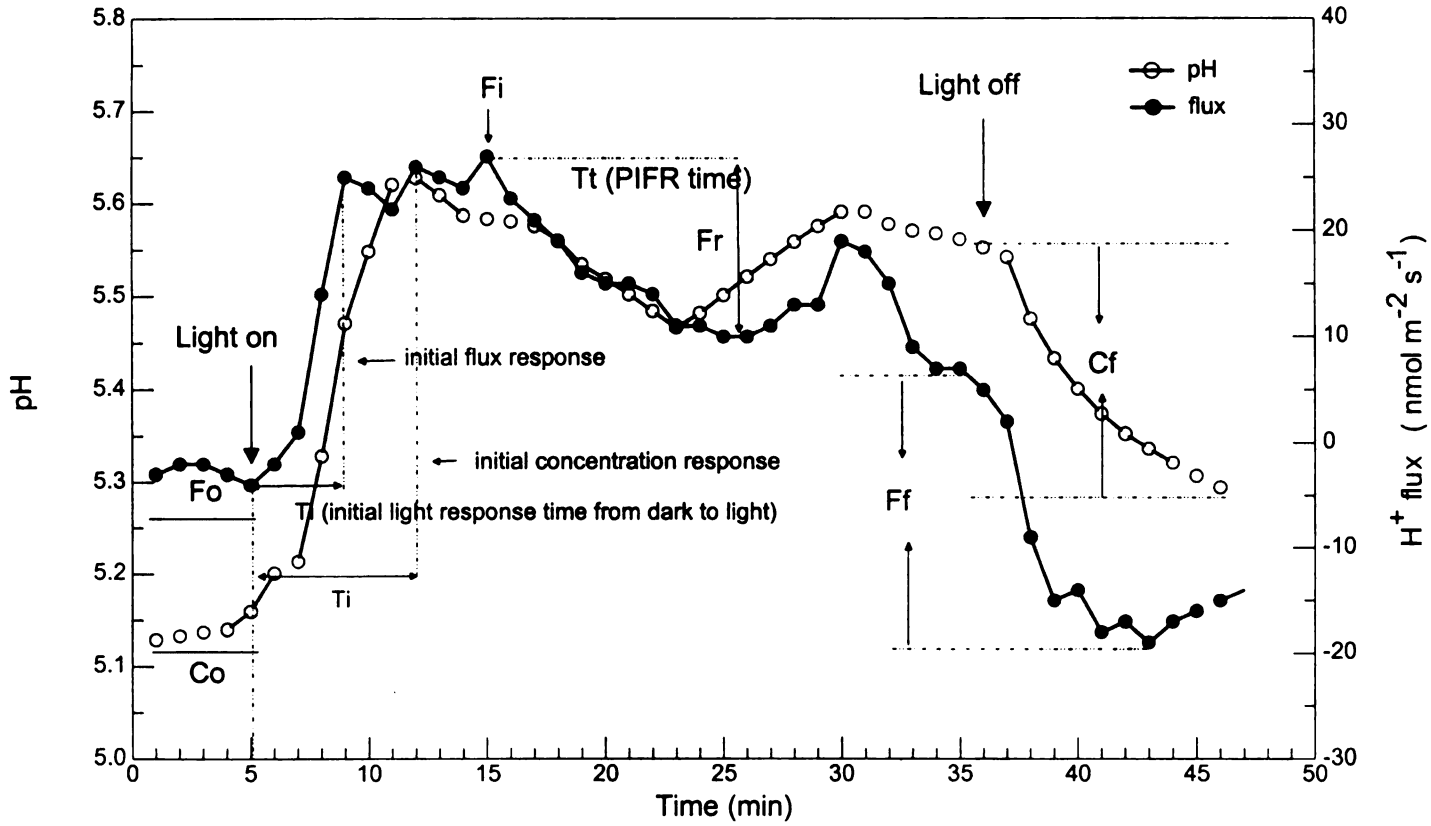

Figure 5. Calculation of light-induced change in net H^+ fluxes and extracelluler pH around pea mesophyll tissues. Fo or Co is 5 min average of net fluxes or extracellular ion concentrations, respectively, before light-on. Fr is amplitude of light-induced Post-Initial Flux Response (PIFR). Fi is initial net ion flux or extracellular ion concentrations before PIFR. Ff or Cf is initial flux or concentration response, respectively, after light-off.

five min: the average value of net ion flux or extracellular ion concentration during the first 5 min after light-on minus F_0 or C_0.

Amplitude of light-induced Post-Initial Flux Response (PIFR): the lowest influx or the highest efflux after Fi minus Fi (Figure 5), where F_I is the net flux when the light-induced initial increase in net influx started to decline. PIFR is a negative value.

Time response:

Response lag time (second): the time between the light-on or light-off and change in net ion fluxes or extracellular ion concentrations.

Initial light response time (min): the time to reach initial maximum changes for ion fluxes or for extracellular ion concentrations after light-on (from dark to light) (Figure 5).

$T_{50\%}$ for initial change (min): the time to reach 50 % of initial response in net influx or in extracellular ion concentration after light-on.

Time to maximum change (min): the time to reach maximum increase in net influx or maximum decrease in extracellular ion concentration during the entire light period.

PIFR time: the time for whole PIFR process or the time from Fi to lowest influx or highest efflux after Fi (Figure 5).

RESULTS

Ion flux change of mesophyll tissues in darkness

In the dark, net fluxes (a balance between influx and efflux) of H^+, Ca^{2+}, Cl^- and K^+ from pea mesophyll tissues in a bathing solution containing 0.5 mM $CaSO_4$ plus 1.0 mM KCl,

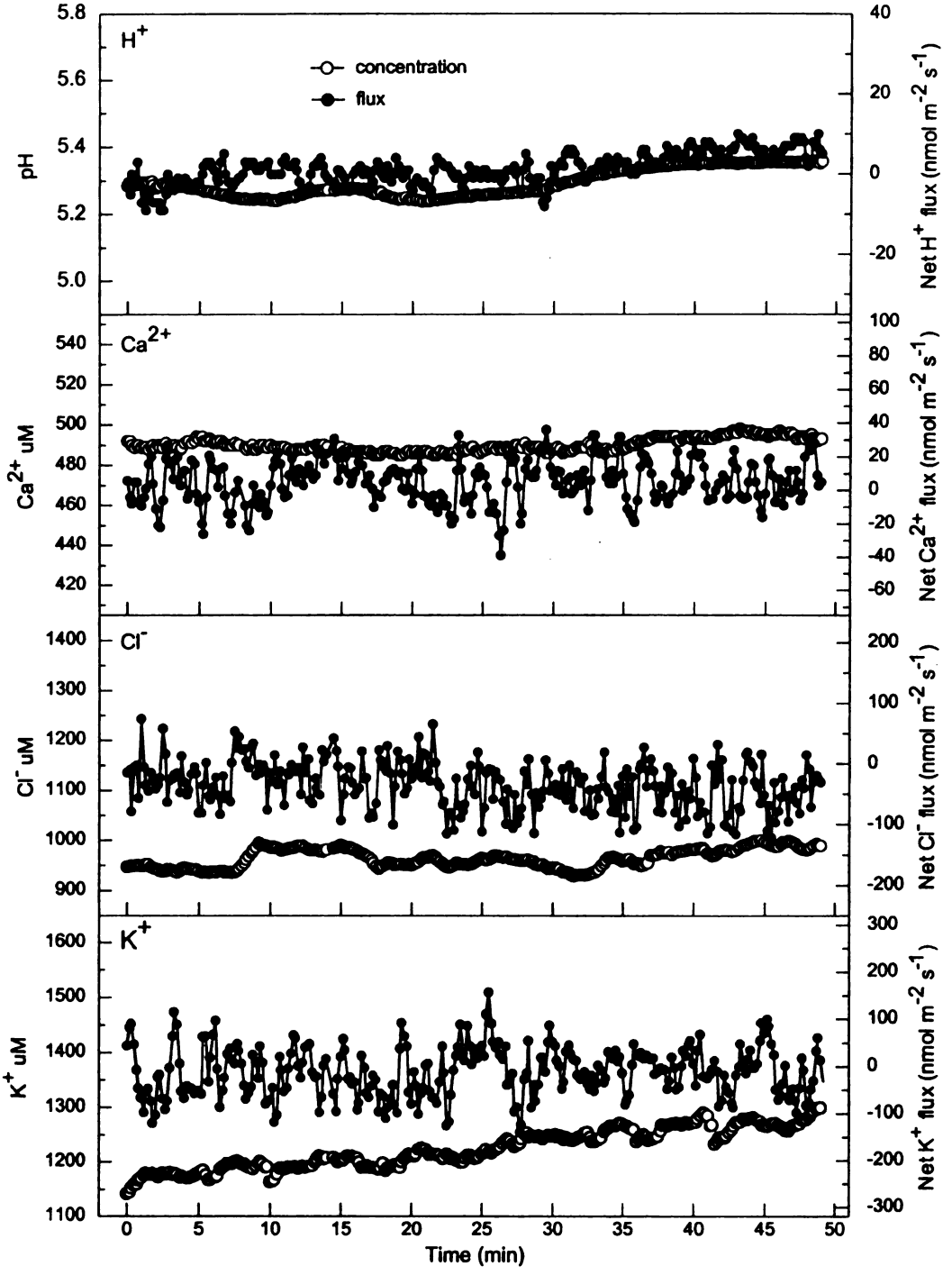

Figure 6. Time course of net fluxes and extracellular concentrations of H^+, Ca^{2+}, Cl^- and K^+ around pea mesophyll tissues of one leaf sample under dark.

at pH 5.2, exhibited small fluctuation over time (Figure 6). The extracellular concentrations of these ions were stable and fluctuated only slightly. Estimation of mean rates for the entire dark period for H^+, K^+ and Cl^- fluxes were -4, -51, -89 nmol m^{-2} s^{-1}, respectively. Thus, a net efflux of these ions occurred under dark conditions. In contrast, a net Ca^{2+} influx (+9 nmol m^{-2} s^{-1}) was observed during the dark period, indicating Ca^{2+} uptake by the mesophyll.

Ion flux response of mesophyll tissues in light

When pea mesophyll tissues were stimulated by exposure to light in the same bathing solution (0.5 mM $CaSO_4$, 1.0 mM KCl, at pH 5.2), H^+, Ca^{2+}, K^+ and Cl^- exhibited different patterns of change in net fluxes and extracellular ion concentration over 30 min (Figure 7). In spite of some variability in the light-induced ion influx responses, especially of K^+ and Cl^-, most replicate leaf samples showed similar light responses for each ion. In general, light induced a rapid increase in net flux (positive) and a decrease in extracellular ion concentration (negative). After a rapid large initial flux response, a reduced or slower net flux change was observed over time. The process, called the Post-Initial Flux Response (PIFR) in this study, typically involved a gradual return to flux rate closer to or lower than that measured prior to light stimulation. Since only net flux could be measured using the MIFE system, the PIFR could theoretically involve either independent changes or concurrent changes in both influx and efflux. Both Ca^{2+} and Cl^- exhibited a rapid PIFR, whereas H^+ and K^+ maintained a net influx for an extended period, which was then followed by a slow PIFR. Thus, the PIFR is a negative value. When the light was turned off, the net flux rate for each ion declined leading ultimately to

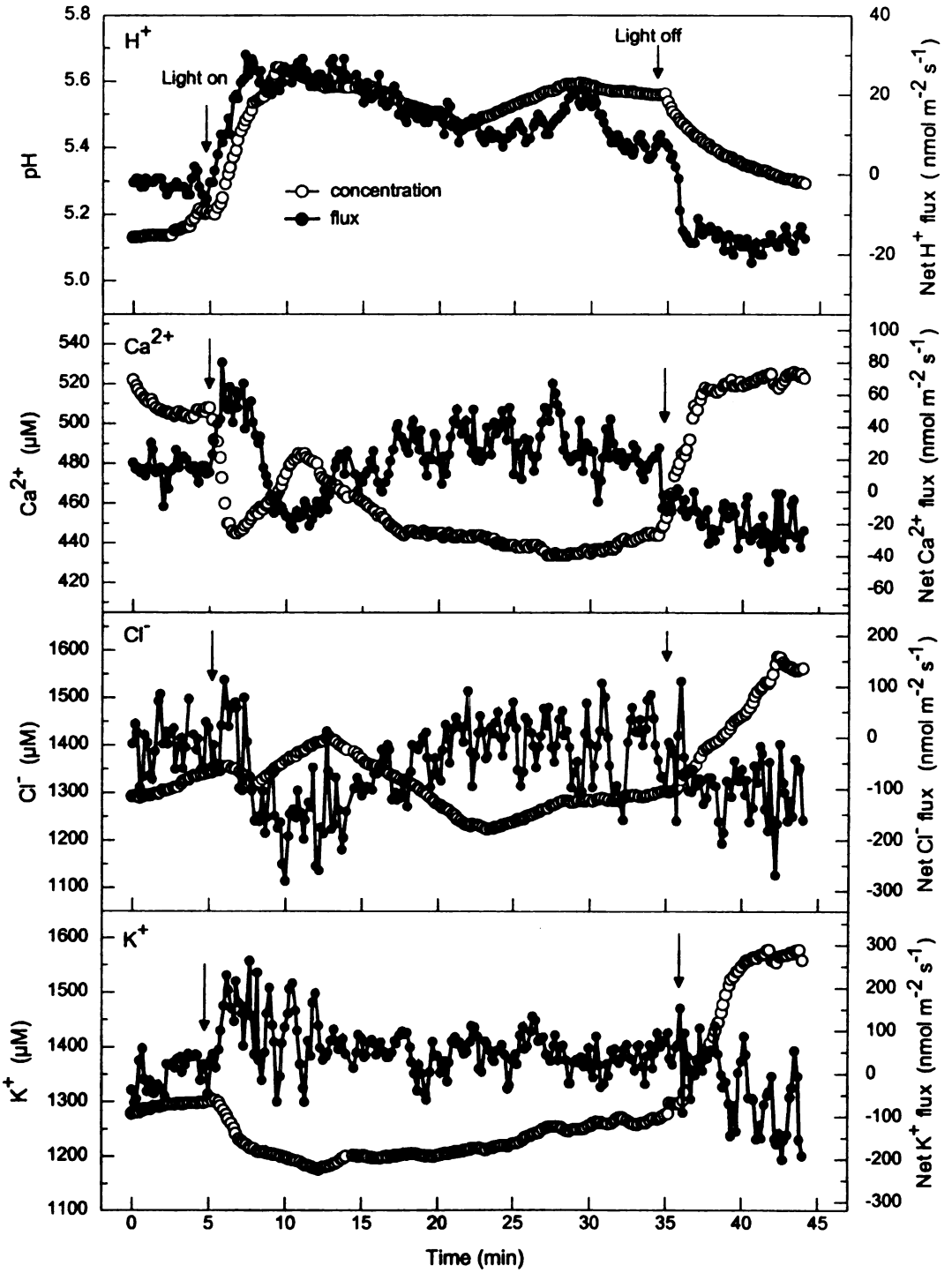

Figure 7. Light-induced time course in net fluxes and extracellular concentrations of H^+, Ca^{2+}, Cl^- and K^+ around pea mesophyll tissues of one leaf sample.

a net efflux. The flux change was small over time as compared to the response to light. The corresponding apoplastic ion concentrations increased, where pH decreased (Figure 7).

The quantitative features for each ionic response are summarized in Table 1 and Table 2. A simple averaging of different periods (the entire period from dark to light or from light to dark) for each ion resulted in a smaller magnitude of change in either ion flux or extracellular ionic concentration (Table 1a, 1b). For H^+, the average flux change over the entire light period was +11 nmol m^{-2} s^{-1}, whereas the light-induced initial net flux change was +26 nmol m^{-2} s^{-1} and maximum increase in net influx was +29 nmol m^{-2} s^{-1} (Table 1a). Thus, the average change over the entire light period was not used as a parameter to evaluate light-induced ionic responses. The light-induced initial responses in net flux and ion concentration were similar to the light-induced maximum changes for each ion (Table 1a and 1b).

By comparison, Ca^{2+} flux exhibited the most rapid response to light. A transient increase in net Ca^{2+} influx of 48 nmol m^{-2} s^{-1} occurred after light exposure (Figure 7 and Table 1a). This increase is considered a result of the increase in Ca^{2+} influx and not a decrease in Ca^{2+} efflux, because the initial Ca^{2+} flux before light exposure (F_0) is a net influx. The net fluxes of H^+, K^+ and Cl^- changed initially from a net efflux to a net influx with a magnitude of 26, 174, and 119 nmol m^{-2} s^{-1}, respectively, following exposure to light (Table 1a). Extracellular concentration for these ions surrounding mesophyll tissues also decreased, consistent with the development of their net influxes. The extracellular pH around mesophyll tissues increased by 0.4 - 0.5 during the 30 min (Table 1b). However, the average change in net Cl^- flux during the first 5 min after illumination was

Table 1. Quantitative characteristics of the light-induced net fluxes and extracellular concentrations of H^+, Ca^{2+}, K^+ and Cl^- in pea mesophyll tissues bathed in 0.5 mM $CaSO_4$ and 1.0 mM KCl at pH 5.2.

a: ion fluxes

	H^+	Ca^{2+}	K^+	Cl^-
		(nmol m^{-2} s^{-1})		
F_0[§]	-3	+8	-51	-61
Average change during the entire light period	+[†]11	+14	+84	+31
Average change after 10 min after light-off	-8	-21	-43	-46
Initial flux response (net influx)	+26	+48	+174	+119
Change during the first min	+6	+25	+20	+31
Change during the first five min	+14	+31	+55	-56
Maximum increase (net influx)	+29	+50	+208	+190
Amplitude of PIFR[‡]	-20	-52	-176	-171

b: extracellular ion concentrations

	H^+	Ca^{2+}	K^+	Cl^-
	pH	μM	μM	μM
C_0[§§]	5.02	562	1172	1126
Average change of the entire light period	+[†]0.36	-37	-45	-30
Average change after 10 min after light-off	-0.21	+31	+208	+213
Initial concentration response (decrease)	+0.43	-49	-109	-108
Change during the first min	+0.04	-9	+11	-15
Change during the first five min	+0.24	-27	-50	+38
Maximum extracellular ion concentration decrease	+0.47	-59	-128	-132

All data are means with 30 replicates.

[§] F_0: five min average of net ion flux before light-on, where + or – indicate net ion influx or net ion efflux, respectively.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

[§§] C_0: five min average of extracellular ion concentration before light-on.

negative (-56 nmol m^{-2} s^{-1}, Table 1a), and extracellular Cl^- concentration increased during the same period (Table 1b). This result indicates that a net Cl^- efflux response occurred.

Some ions responded more quickly to light than others. Compared to other ions (H^+, K^+ and Cl^-), Ca^{2+} flux was observed to have the smallest $T_{50\%}$ (1.5 min), the time for 50% change in initial net ion flux. The time to the maximum increase (influx) was also the most rapid for Ca^{2+} (3.5 min) (Table 2a). Chloride flux had a similar initial light response time (2.3 min) compared to Ca^{2+} (2.2 min), and both were significantly shorter than H^+ and K^+. In considering light-response lag time, Ca^{2+} flux response was the most rapid within 13 s, then followed H^+ (29 s) and by Cl^- (45 s) and K^+ (48 s). Similar results were also observed in extracellular ion concentration (Table 2b).

The PIFR time for Ca^{2+} (3.4 min) and Cl^- (4.1 min) were significantly shorter than for H^+ (8.4 min) and K^+ (10.3 min) (Table 2a). When light was turned off, the response lag time for Ca^{2+} flux from light to dark was also significantly shorter (19 s) than other ions.

The qualitative characteristics of the measured light-induced ionic responses are presented in Table 3. Changes in net flux and apoplastic ion concentration occurred concurrently following light stimulation when compared Table 1a with Table 1b. For instant, light exposure quickly stimulated Ca^{2+} uptake by the mesophyll within 13 s, and also caused a reduction in apoplastic Ca^{2+} concentration within 13 s. Thus, the evidence is strong that the Ca^{2+} flux response was not an artifact of some other factors.

Light-induced ion fluxes from epidermal tissues

Table 2. Quantitative characteristics of the light-induced time responses of H^+, Ca^{2+}, K^+ and Cl^- in pea mesophyll tissues bathed in 0.5 mM $CaSO_4$ and 1.0 mM KCl at pH 5.2.

a: ion fluxes

	H^+	Ca^{2+}	K^+	Cl^-
Response lag time (s) (from dark to light)	29 b	13 c	48 a	45 a
Response lag time (s) (from light to dark)	26 b	19 c	50 a	45 a
Initial light response time (min)	4.6 a	2.2 b	4.3 a	2.3 b
$T_{50\%}$ † (min)	2.0 b	1.5 c	2.7 a	1.9 c
Time to maximum change (min)	5.9 b	3.5 c	10.1 a	9.3 a
PIFR‡ time (min)	8.4 a	3.4 b	10.3 a	4.1 b

b: extracellular ion concentrations

	H^+	Ca^{2+}	K^+	Cl^-
Response lag time (s) (from dark to light)	32 b	13 c	51 a	45 a
Response lag time (s) (from light to dark)	28 b	18 c	45 a	43 a
Initial light response time (min)	5.3 a	3.3 b	5.9 a	3.4 b
$T_{50\%}$ † (min)	2.6 a	1.6 b	2.7 a	2.6 a
Time to maximum change (min)	15.1 a	8.7 b	7.0 b	8.1 b

All data are means with 30 replicates.

Different letters indicate significant difference across rows according to LSD test at $p < 0.05$.

† $T_{50\%}$: time to reach 50 % initial response of net ion flux or extracellular ion concentration, respectively.

‡ **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. PIFR time: the time for whole PIFR process.

Table 3. Qualitative characteristics of light-induced ionic responses of pea mesophyll tissues bathed in 0.5 mM $CaSO_4$ and 1.0 mM KCl at pH 5.2

Table 3. Qualitative characteristics of light-induced ionic responses of pea mesophyll tissues bathed in 0.5 mM $CaSO_4$ and 1.0 mM KCl at pH 5.2.

	H^+	Ca^{2+}	K^+	Cl^-
Initial light-responses				
flux	rapid change from net efflux to net influx long duration	transient net influx increase short duration	rapid change from net efflux to net influx long duration	rapid change from net efflux to net influx short duration
concentration	rapid pH increase long duration	transient decrease short duration	slow decrease long duration	rapid decrease short duration
Light-induced post-initial response				
flux	net influx decrease or efflux increase or both slow response	net influx decrease or net efflux increase or both rapid response	net influx decrease or net efflux increase or both slow response	from net influx decrease to net efflux increase rapid response
concentration	pH decrease slow and small response	increase rapid response	increase slow response	increase slow response
After light-off				
flux	rapid net influx decrease then net efflux increase stable	rapid net influx decrease then net efflux increase stable	rapid net influx decrease then net efflux increase variable	rapid net influx decrease then net efflux increase variable
concentration	rapid pH decrease	rapid increase	rapid increase	rapid increase

An isolated strip of epidermal tissue (about 5 × 15 mm) was mounted in the same measuring chamber and bathed in the same solution as the mesophyll tissue. The experimental protocol was similar to that described previously. Unlike mesophyll tissues, light did not elicit a large flux change for any ion (Figure 8). No significant difference in PIFR were observed during about 25-30 min measurement. The data of Table 4 presented a possible characterization of the light-induced ion fluxes and extracellular ion concentrations around the epidermal tissues. After light stimulation, net Ca^{2+} influx increased slightly, net H^+, K^+ and Cl^- effluxes decreased slowly (H^+) or changed toward a net influx (K^+ and Cl^-) during the first min or during the first 5 min after illumination (Table 4). Although net flux of each ion was estimated to have positively increased, the magnitude of increase in epidermal tissues (Table 4a) was much smaller than that of mesophyll tissues (Table 1a). In addition, light only induced a small initial increase in extracellular pH (Figure 8, Table 4b).

External pH effects on light-responses of mesophyll tissues

The mesophyll tissue was pretreated under dark for 2.0 - 2.5 h in a buffered solution with either pH 4.0 or 6.0. The net ion fluxes from mesophyll tissues were measured under light illumination in the unbuffered solution (containing 0.5 mM $CaSO_4$ and 1.0 mM KCl) at the same pH as each buffered solution. Light period (30 min) was the same as at the control (pH 5.2). Compared to the control, a higher pH (6.0) of the bathing solution resulted in a reduced light-induced initial response in net influx of both H^+ and K^+ by about 45 %, in particular, in a significant decrease in net flux change over 5 min

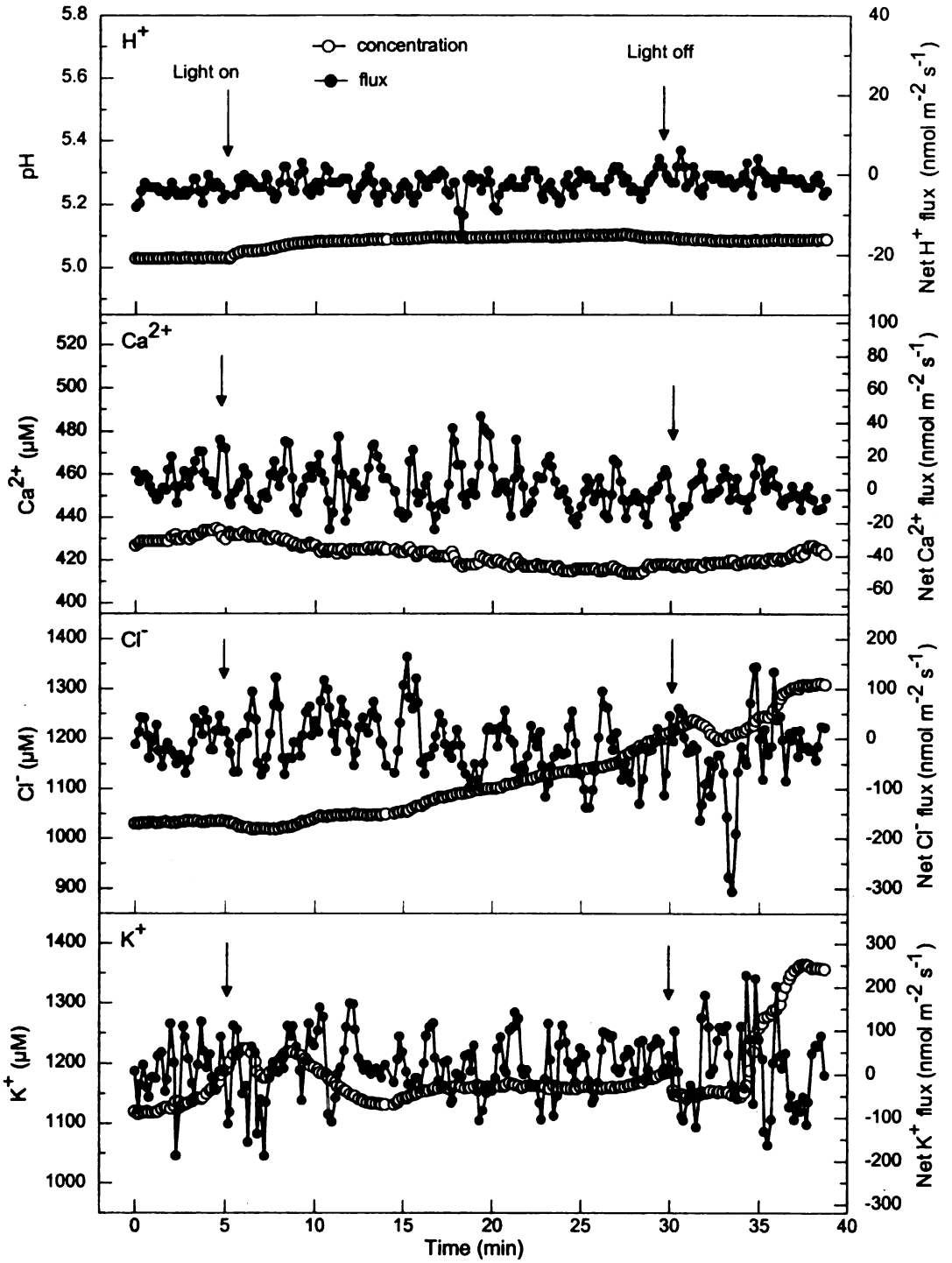

Figure 8. Light-induced time course of net fluxes and extracellular concentrations of H^+, Ca^{2+}, Cl^- and K^+around pea epidermal tissues of one leaf sample.

illumination (Table 5). The alkaline medium also declined the light-induced PIFR of both ions (50 % for H^+ and 36 % for K^+). However, the increase in external pH did not alter the light-induced initial response in extracellular pH (Table 6), as well as in net influxes of Ca^{2+} and Cl^- (Table 5).

Acidification of the bathing solution (pH 4.0) produced a different response compared to the pH 5.2 treatment. Net H^+ flux was always positive (net influx) while mesophyll were being illuminated. It started with a large influx (156 nmol m^{-2} s^{-1}) under dark conditions (Table 5). Light treatment initially induced a large increase in net influx (+44 nmol m^{-2} s^{-1}) which subsequently declined during the remainder of the light period (Table 5). Thus, the light-induced PIFR of H^+ was a decrease in net influx (-108 nmol m^{-2} s^{-1}) which was significantly larger than at both pH 5.2 and 6.0 (Table 5). Under pH 4.0, the light-induced initial increase in extracellular pH was much slower and reached to 0.35 within 19.5 min (Table 6). In contrast, the lower external pH resulted in a larger light-induced initial response in net influxes of K^+ and Cl^- as compared to pH 5.2 (Table 5). During 5 min after illumination, the net flux of Cl^- still increased positively (54 nmol m^{-2} s^{-1}) at pH 4.0. However, that of the pH 5.2 decreased negatively (-74 nmol m^{-2} s^{-1}) compared with at pH 4.0 (Table 5). The similar difference was observed in change in extracellular Cl^- concentration (Table 6). The light-induced PIFR of K^+ was not influenced by lower pH (Table 5). However, the PIFR of Cl^- was reduced by a large magnitude from 148 to 29 nmol m^{-2} s^{-1} (Table 5).

The pH 4.0 treatment did not affect the magnitude of light-induced Ca^{2+} initial response. However, the PIFR of Ca^{2+} and its initial light response time in either flux or

Table 4. Quantitative characteristics of the light-induced induced net ion fluxes and extracellular ion concentrations in pea epidermal tissues bathed in 0.5 mM $CaSO_4$ and 1.0 mM KCl at pH 5.2.

a: ion fluxes

	H^+	Ca^{2+}	K^+	Cl^-
	nmol m^{-2} s^{-1}			
F_0[§]	-10 (9)[‡]	2 (8)	-9 (9)	-1 (10)
Initial flux response (net influx)	+[†]6 (9)	+18 (8)	+96 (8)	+81 (8)
Change during the first min	-5 (9)	+5 (8)	+23 (8)	+36 (8)
Change during the first five min	-3 (9)	+3 (8)	+3 (8)	+2 (8)
Maximum increase (net influx)	+9 (9)	+23 (8)	+121(8)	+104 (8)

b: extracellular ion concentrations

	H^+	Ca^{2+}	K^+	Cl^-
	pH	μM	μM	μM
C_0[§§]	5.04 (9)	529 (8)	1126 (9)	1093 (8)
Initial concentration response (decrease)	+[†]0.08 (9)	-28 (8)	-75 (7)	-36 (8)
Change during the first min	+0.01 (9)	0 (8)	+45 (7)	+4 (8)
Change during the first five min	+0.03 (9)	-7 (8)	-11 (7)	-11 (8)
Maximum extracellular ion (concentration decrease)	+0.10 (9)	-38 (8)	-75 (7)	-47 (8)

[§] F_0: five min average of net ion flux before light-on, where + or – indicate net ion influx or net ion efflux, respectively.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] The numbers in parentheses indicate the number of replicates.

[§§] C_0: five min average of extracellular ion concentration before light-on.

Table 5. Effect of external pH in bathing solution on the light induced net fluxes flux of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues.

pH in bathing solution	H^+	Ca^{2+}	K^+	Cl^-
	nmol m^{-2} s^{-1}			
F_0[§]				
4.0	+156(8)[§§] a	+21(8)	-102 (8) a	-232 (7) a
5.2	-0.4 (9) b	5 (12)	-81 (8) ab	-72 (8) ab
6.0	-7 (8) b	0 (9) ns	24 (7) b	-26 (5) b
Initial flux response (net influx)				
4.0	+[†]44 (8) a	+50 (8)	+272 (8) a	+174 (7) a
5.2	+22 (9) ab	+46 (12)	+167 (8) b	+101 (8) b
6.0	+12 (8) b	+41 (9) ns	+92 (5) b	+131 (5) ab
Change during the first min				
4.0	-15 (8) a	+28 (8)	+69 (8)	+115 (7) a
5.2	+2 (9) b	+25 (12)	+39 (8)	+17 (8) b
6.0	+1 (8) b	+18 (9) ns	+28 (5) ns	+31 (4) ab
Change during the first five min				
4.0	-3 (8) a	+15 (8)	+89 (8) a	+54 (7) a
5.2	+11(9) b	+29 (12)	+62 (8) a	-74 (8) b
6.0	+2 (8) a	+12 (9) ns	+1 (5) b	-10 (5) ab
Amplitude of PIFR[‡]				
4.0	-108 (8) a	-74 (8) a	-208 (8) a	-29 (7) b
5.2	-22 (9) b	-59(12) ab	-203(8) a	-148 (8) a
6.0	-11 (8) b	-42 (9) b	-131(5) b	-195 (5) a

Different letters indicate significant difference between external pHs within columns according to LSD test at $p < 0.05$.

"ns" indicates no significant difference.

§ F_0: five min average of net ion flux before light-on, where + or – indicate net ion influx or net ion efflux, respectively.

§§ The numbers in parentheses indicate the number of replicates.

† - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

‡ **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

Table 6. Effect of external pH in bathing solution on the light-induced extracellular concentrations of H^+, Ca^{2+}, K^+ and Cl^- and time responses in pea mesophyll tissues.

pH in the bathing solution C_0[§]	H^+ (pH)	Ca^{2+} (μM)	K^+ (μM)	Cl^- (μM)
4.0	4.46(8)[§§]c	608 (8)	1159 (8) ab	1168 (8)
5.2	5.00 (9) b	558 (12)	1200 (8) a	1065 (8)
6.0	5.40 (8) a	541 (9)ns	1015 (7) b	1175 (5) ns
Initial concentration response (decrease)				
4.0	+[†]0.35 (8)	-46 (8)	-192 (8)	-192(6) a
5.2	+0.42 (9)	-46(10)	-128(8)	-111(8) ab
6.0	+0.30 (8) ns	-44 (9) ns	-92 (5) ns	-66 (5) b
Change on the first five min				
4.0	+0.07 (8) b	-24 (8)	-82 (8)	-135 (8) a
5.2	+0.21 (9) a	-37 (12)	-63 (8)	-29 (8) b
6.0	+0.16 (8) ab	-18 (9) ns	-30 (5) ns	-21 (5) b
Initial light response time (min)				
4.0	19.5 (8) a	3.1 (8) b	8.3 (8) a	4.4 (8) a
5.2	5.3 (9) b	2.6(12) b	4.6 (8) b	2.3 (8) b
6.0	6.3 (8) b	5.4 (9) a	4.0 (5) b	3.4 (5) ab

Different letters indicate significant difference between external pHs within columns according to LSD test at $p < 0.05$.

"ns" indicates no significant difference.

[§] C_0: five min average of extracellular ion concentration before light-on.

[§§] The numbers in parentheses indicate the number of replicates.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

extracellular concentration were significantly larger at pH 4.0 than pH 6.0 (Table 5, 6). The lower pH medium prolonged the initial light response time of extracellular K^+ and Cl^- concentration compared with pH 5.2 (Table 6).

Effect of photosynthetic inhibitor on light-induced ion fluxes

Photosynthetic inhibitor, DCMU can block electron transfer. This inhibitor at 0.05 mM was added to the bathing solution (0.5 mM $CaSO_4$ and 1.0 mM KCl) and the pH adjusted to 5.2 with HCl. The addition of DCMU significantly inhibited the light-induced initial response in net influxes of H^+, Ca^{2+} and K^+ around mesophyll tissues (Figure 9), and elonged the light response lag time of H^+ and Ca^{2+} fluxes (Table 7). However, the light-induced initial response in net Cl^- influx were not significantly influenced by DCMU application (Figure 9).

Sum of light-induced net ion fluxes

When the magnitude and the directionality of net charge flux, based upon the combined net fluxes of H^+, Ca^{2+}, K^+ and Cl^- (Figure 7), the light-induced net charge flux of the four ions increased transiently, lasting for about 5 min, and then declined (Figure 10 and 11). The light-induced initial increase in net influx (about 300 nmol m^{-2} s^{-1}) indicates that a positive net charge move to the cytoplasm and would be expected to contribute to a membrane depolarization. Compared with mesophyll tissues, however, net charge flux rates in epidermal tissues did not exhibit any response to light (Figure 11).

Table 7. Effect of DCMU on the light-induced the time responses of H^+, Ca^{2+}, K^+ and Cl^- in pea mesophyll tissue.

	H^+	Ca^{2+}	K^+	Cl^-
		(s)		
Light response lag time				
-DCMU (Control)	28 (5)** b	13 (6) b	46 (6)	42 (5)
+ 50 μM DCMU	57 (6) a	32 (6) a	47 (5) ns	46 (5) ns

Different letters indicate significance between the control and DCMU treatment according to LSD test at $p< 0.05$. "ns" indicates no significance.

* Response lag time is the time between the light-on or light-off and change in net ion fluxes.

** The numbers in parentheses indicate the number of replicates.

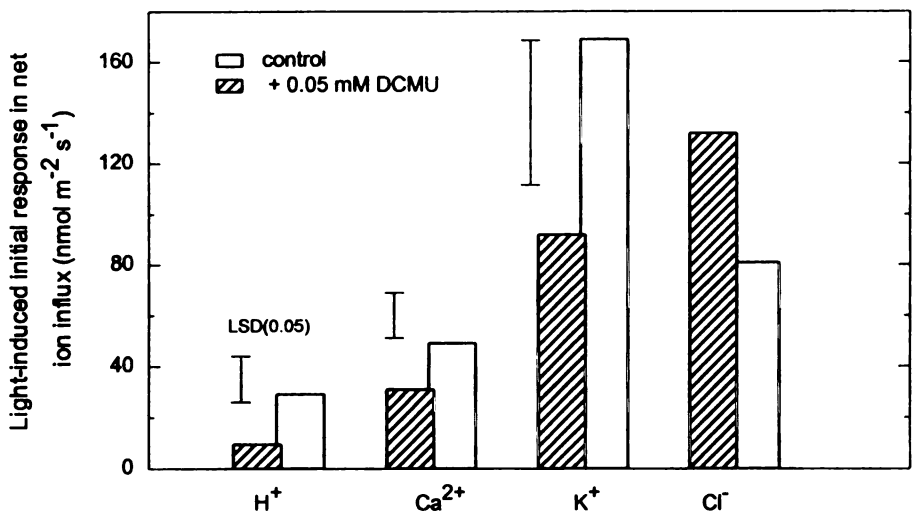

Figure 9. Effect of a photosynthetic inhibitor, DCMU, on light-induced initial response in net ion influx from pea mesophyll tissues. Data represents an average of 6-9 replicates. Vertical bars represent LSD test result at $p<0.05$.

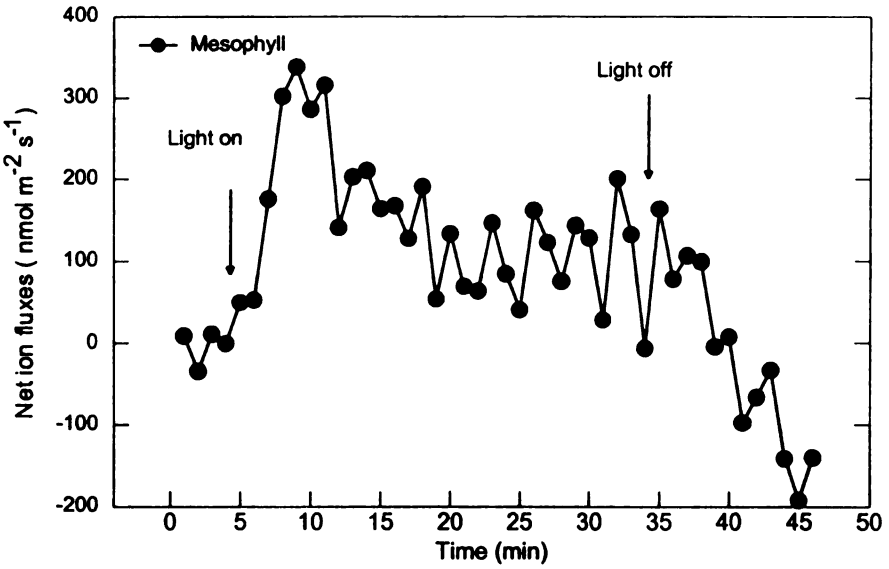

Figure 10. Light-induced time course of net ion flux from pea mesophyll tissues. Data points represent a leaf sample. Net charge flux is a sum of H^+ + Ca^{2+} + K^+ - Cl^- fluxes

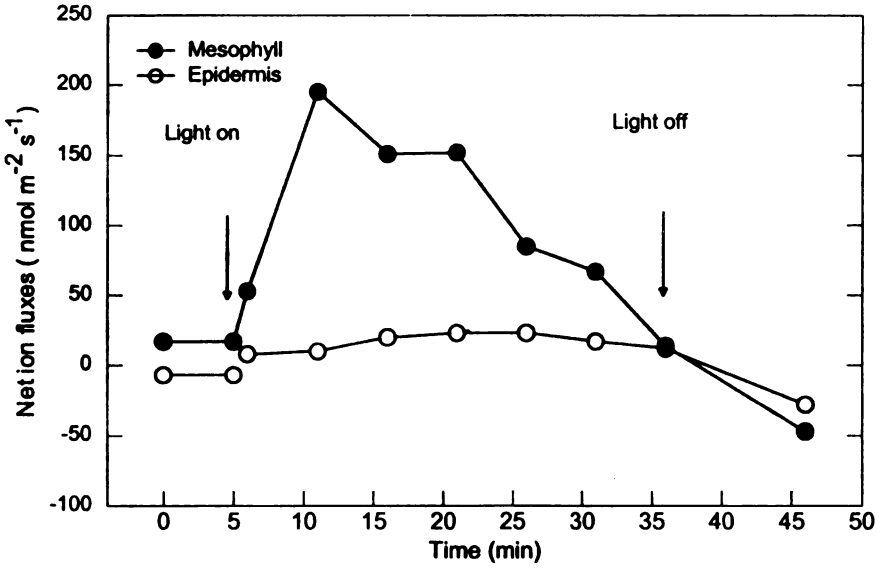

Figure 11. Light-induced time course of net ion flux from pea mesophyll and epidermal tissues. Data points represent an average of 7-12 leaf samples. Net charge flux is a sum of H^+ + Ca^{2+} + K^+ - Cl^- fluxes.

DISCUSSION

Which ion is the initial depolarizing agent?

Exposure of leaf mesophyll cells to light has been shown to result in a transient depolarization of the membrane potential, followed by a slow repolarization (Elzenga et al., 1995). Similar observations have been reported in other species including in *Arabidopsis* (Lewis et al., 1997), *Phaseslus vulgaris* (Nishizaki, 1992), *Asplenium trichomanes* (Szarek and Trebacz, 1999), etc. The membrane depolarization responses, however, have varied in their magnitude of membrane potential and the time responses, etc. In general, changes in membrane potential are considered to be the result of ion fluxes across the plasma membrane. Thus, the ionic component of light-induced membrane depolarization seems to be a foundation for understanding electrical signalling. Many authors suggested that Ca^{2+}, H^+, K^+ and Cl^- play a major role in stimuli-induced electrical signalling. Cation influx or anion efflux from leaf cells results in membrane depolarization. However, the identify of the ions involved in transient plasma membrane depolarization is still controversial in the literature.

Elzenga et al. (1995) demonstrated that the light-induced plasma membrane depolarization (about 25 mV) in pea mesophyll tissues occurred at about 1-2 min after stimulation, which was followed by a slow repolarization. Assuming that four ions (Ca^{2+}, H^+, K^+ and Cl^-) are primarily involved in light-induced membrane potential changes, the summed net charge flux rates (Figure 10) measured in the present study exhibited a similar time course trend as the light-induced membrane potential response reported previously (Elzenga et al., 1995; Stahlberg and Van Volkenburgh, 1999). Therefore, we propose that the change in the net charge flux involving Ca^{2+}, H^+, K^+ and Cl^- following

light exposure could potentially be responsible for the reported light-induced membrane potential response. A light-induced transient increase in the net charge flux could be expected to depolarize the plasma membrane. Thus, the findings in this study provide indirect experimental evidence for the involvement of Ca^{2+}, H^+, K^+ and Cl^- fluxes in light-induced electrical signalling.

Shabala and Newman (1999) suggested that Ca^{2+} is a depolarizing ion based upon direct measurement of light-induced ion fluxes in bean mesophyll tissues. Their conclusion was based on time-course data following illumination, i.e., Ca^{2+} flux exhibited a more rapid (5 s) change than H^+, K^+ and Cl^- fluxes. The result in pea mesophyll (Figure 7 and Table 1, 2) agrees with their results in bean leaf tissues. However, they did not evaluate the transition from light to dark. Our results indicate that the light-induced flux changes of Ca^{2+}, H^+, K^+ and Cl^- declined and returned gradually to the rate which existed before light treatment when the light was turned off (Figure 7). Considering the time of the response to light, net Ca^{2+} influx increased rapidly to the maximum magnitude within 3.5 min after light exposure. The maximum increase in net H^+ and K^+ influx, however, occurred after a 5.9-min and a 10.1-min delay, respectively (Table 2a). The initial light response time, $T_{50\%}$ and light response lag time of Ca^{2+} were also relatively short (Table 2a). Among the four different ions measured, Ca^{2+} should be considered as a main candidate for membrane depolarization in pea mesophyll. After about 1 min of illumination, net Ca^{2+} influx increased by approximately 25 nmol m^{-2} s^{-1} near mesophyll tissues. This result should be explained as an increase in Ca^{2+} influx, and not a decrease in Ca^{2+} efflux.

According to the calculation of Nobel (1991), charge transfer (Q) = capacitance of

membrane (C) × voltage generated (ΔE), i.e. Q = CΔE. The C of most biological membranes are approximately are 1μF cm^{-2} (Lüttge and Pitman 1976). If membrane potential change (ΔE) is 25 mV, the Q required is 25×10^{-9} Coulombs cm^{-2}. One mole of monovalent ions has charge of 96500 Coulombs, or a mole of divalent ions has approximately 2×10^5 Coulombs. Therefore, a net transfer (Q) of 0.125 pmol of Ca^{2+} can realize 25 mV membrane change. In this study, an increase in Ca^{2+} influx of 25 nmol m^{-2} s^{-1} or 2.5 pmol cm^{-2} s^{-1} in pea mesophyll was observed within 1 min after light exposure. Actual measured membrane depolarization resulting from light exposure has been reported to rang between 25 and 35 mV (Elzenga et al., 1995; Stahlberg and Van Volkenburgh, 1999). In addition, the initial response time for transient Ca^{2+} influx (about 2 min) measured in this study, thus, is also consistent with light-induced depolarization (2-3 min) reported by Elzenga et al. (1995). Therefore, Ca^{2+} can be considered to be an initial depolarizing ion.

However, one should not exclude other ions from possible involvement in the light-induced membrane depolarization. A light-induced *Arabidopsis* plasma membrane conductance change for Cl^- has been suggested by Spalding et al. (1992). In pea mesophyll, Elzenga et al. (1995) found that a transient depolarization depended upon external Cl^- concentrations and was unaffected by changing external Ca^{2+} or K^+ concentration. On the other hand, light-induced membrane depolarization of pea epidermal cells was enhanced by increasing Ca^{2+} concentration in the bathing solution. Thus, these investigators concluded that the ionic mechanism for light-induced depolarization differs between epidermis and mesophyll. The membrane potential

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changes in the epidermis were explained as being due to changes in Ca^{2+} flux and activity of the plasma membrane H^+-ATPase (Elzenga at al., 1995). However, their conclusion was based on experiments in which the ionic concentrations of Ca^{2+} and Cl^- were modified. Also ion flux was not measured directly. Because of numerous interactive and feedback regulatory mechanisms for ion transporters in the plasma membrane, the measurement of only membrane potential is not sufficient to reveal involvement of the specific ion fluxes.

Only a few groups have directly measured light-induced ion flux changes in leaf tissues. Johannes et al. (1997) observed that Ca^{2+}, K^+ and anion-permeable channels were open at the peak of light-induced membrane depolarization in red-light-induced moss *Phycomitrella patens*. While Ca^{2+} influx and anion efflux coincided with the depolarizing phase (2-15 s), K^+ influx occurred only during the first 30 s after light stimulation. Their results indicated that a transient K^+ efflux appeared later and induced the plasma membrane repolarization. However, their conclusions on ion flux dynamics are questionable because they measured ion fluxes using 30 s or 1 min intervals which is longer than the time necessary to attain the peak of membrane depolarization (2-15 s).

Shabala and Newman (1999) rejected Cl^- efflux and K^+ influx as depolarizing agents after measuring ion fluxes using the MIFE system. They found both large Cl^- and K^+ influxes after a 2 min delay between the onset of light stimulation and the onset of flux changes in bean mesophyll. In this study, changes in net Cl^- and K^+ influx did not occur until approximately 50 s (response lag time) after illumination (Table 2a). The magnitudes of increase in net influx from pea mesophyll were smaller than that from bean mesophyll (about 1000 nmol m^{-2} s^{-1}). After 2.3 min, the net Cl^- influx declined to a net

efflux below the F_0 (Table 2a). Thus, this process, i.e. the PIFR, could be considered to be a result of an increase in Cl^- efflux not a decrease in Cl^- influx. This is because the amplitude of PIFR (-171nmol m^{-2} s^{-1}) was greater than the magnitude of light-induced initial increase in net Cl^- influx (119 nmol m^{-2} s^{-1}) (Table 1a). The anion efflux could be expected to be involved in membrane depolarization. Elzenga and Van Volkenburgh (1997) tested anion channels by using patch-clamp techniques and found high anion channel activity in pea mesophyll cells after a 30 s light treatment. They concluded that Cl^- efflux is involved in light-induced membrane depolarization. In this study, however, light induced a rapid initial response in net Cl^- influx. This net Cl^- influx could be involved in the repolarization process and might counteract the light-induced depolarization. However, summation of net charge fluxes (including Ca^{2+}, H^+, K^+ and Cl^-) in Figure 10 and Figure 11 suggests that light still caused a net influx increase that contributes to a transient depolarization. Thus, Cl^- efflux is involved in light-induced membrane depolarization but not in the initial transient depolarization event.

The K^+ exerts a dominating influence on the diffusion potential component of transmembrane electrical potential differences by virtue of its relatively high permeability in biological membranes. Even under dark conditions, K^+ maintained a variable net flux rate around ±100 nmol m^{-2} s^{-1} which is larger than H^+ and Ca^{2+} (Figure 6). Among Ca^{2+}, K^+ and Cl^-, which the electro-chemical potential for K^+ is the lowest at the unperturbed membrane voltage of approximately -150 mV. This is because the equilibrium voltage for K^+ (E_K) is probably near -100 mV (Leigh and Wyn Jones, 1984). Spalding and Cosgrove (1992) put forward that K^+ is not a major depolarizing ion in blue-light-induced cucumber hypocotyls because TEA, an inhibitor of K^+ channels, did not affect the onset

of depolarization. In most cases, K^+ is considered as a charge balancing ion, moving passively to compensate for light-induced ion movement of Cl^- or H^+. However, membrane depolarization of *Desmodium motorium* appeared to be associated with K^+ efflux (Antkowiak et al., 1991). This conclusion was not based on direct flux measurement. In the present study and in bean mesophyll (Shabala and Newman, 1999), direct K^+ measurement using the MIFE system indicated that K^+ does not function in initial light-induced electrical signalling. The data also suggested that H^+ is not an initial depolarizing ion. Net H^+ influx and apoplast pH exhibited only relatively small increases within 1 min after light stimulation (Table 1a). The time-course response of H^+ was slower than that of Ca^{2+} (Table 2). The delay between light application and the beginning of the H^+, K^+ and Cl^- flux responses could be explained as the time required to elevate cytosolic free Ca^{2+} concentrations to a level that can elicit a cellular signalling cascade response. This observation is in agreement with the hypothesis that Ca^{2+} is a major ion initiating the plasma membrane depolarization in pea mesophyll.

Light-induced alkalinization and photosynthetic effects

Initial extracellular alkalinization in response to light was observed in leaf tissues of many species including sunflower (Hoffmann and Kosegarten, 1995), pea (Stahlberg and Van Volkenburgh, 1999), and bean (Muhling et al., 1995; Shabala and Newman, 1999). The same result was observed in Figure 7. A light-induced increase in H^+ influx occurred after about a 30 s lag time (Table 2a), whereas Shabala and Newman (1999) found a lag time of 1-2 min in bean mesophyll. The magnitude of the increase in apoplastic pH, 0.4 - 0.5, is similar to the finding of Stahlberg and Van Volkenburgh (1999). In addition, they

also reported an extracellular acidification after 15 min of light exposure with the parallel occurrence of a gradual hyperpolarization of membrane potential. They concluded that growth stimulation of pea leaves is due to H^+ efflux mediated by a plasma membrane H^+-pump. Unfortunately, however, H^+ fluxes were not measured in their study. In the present experiment, direct H^+ flux measurement does not support their speculation. Light treatment caused a rapid increase in net H^+ influx, not an efflux. Light-induced alkalinization was slowed down and extracellular pH started to decline after approximately 5-6 min, whereas net H^+ influx was decreased or reversed to net efflux or both, i.e. PIFR. These results seem to correspond to the "acidification" response described by Stahlberg and Van Volkenburgh (1999). Their observation, however, occurred as a result of a 1.0-1.5 h light treatment and the "acidification" was due to a decrease in apoplastic pH from the maximum initial light-induced pH. It should be noted though that the reported reduction in pH was not lower than the original level measured before light was turned on. Termination of light exposure resulted in a decrease of apoplastic pH (Figure 7) in agreement with Stahlberg and Van Volkenburgh (1999).

In bean mesophyll tissues, Shabala and Newman (1999) showed a light-induced initial net H^+ efflux but not a net influx, even though extracellular pH increased. They explained that this apparent inconsistency could be a result of rapid CO_2 uptake by photosynthesizing tissues. Another possible consideration is the effect of other ion transporters which regulate extracellular pH. Several mechanisms can be involved in apoplastic pH regulation (Leigh, 1993), including production and consumption of malate via the biochemical pH 'stat', transport of protons to and from the vacuole, and fluxes of

H^+ at the plasma membrane. Other ions, such as K^+, Ca^{2+} and anions may be involved in cytoplasmic acidification or extracellular alkalinization which contribute to an initial depolarization of membrane potential (influx of positively charged H^+) followed by a repolarization as the H^+ pump is activated (Franchisse and Desbiez, 1989). Gating of the K^+-inward and K^+-outward rectifiers in guard cells is sensitive to external pH (Thiel and Wolf, 1997). Therefore, it is possible that the activity of other ion transporters in the plasma membrane also influence H^+ flux and extracellular pH.

The photosynthetic inhibitor DCMU is often used to test the effects of photosynthesis on light-induced electrical signalling in leaf tissues (Nishizaki, 1992; Elzenga et al., 1995; Stahlberg and van Volkenburgh, 1999; Szarek and Trebacz, 1999). In pea mesophyll, 0.05mM DCMU inhibited to a large extent membrane depolarization (Stahlberg and van Volkenburgh, 1999). The presence of DCMU at the same concentration inhibited the light-induced initial response in net H^+, Ca^{2+} and K^+ influxes (Figure 9) and delayed the initial light response (Table 7). This result experimentally demonstrates the correlation between photosynthesis and light-induced ion flux change.

Carbon dioxide (CO_2) uptake was thought to be one reason for light-induced apoplast alkalinization (Gotow et al., 1985; Shimazaki and Zeiger, 1987; Shabala and Newman, 1999). In lower plants, light-induced pH increase is due to the uptake of HCO^- ions in (Merrett et al., 1996). Formation of HCO^- ions and CO_2 is expected from the following equilibrium: $CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO^-_3 \Leftrightarrow CO^-_3 + 2H^+$. Reduction in either CO_2 or HCO^-_3 results in a decline H^+ concentration or pH increase in solution. In the bathing solution, CO_2 reacts with H_2O to form HCO^-_3 with a combined pK of 6.3

(Neumann and Levine, 1971). In this study, when light was turned on, the uptake of CO_2 by mesophyll cells shifted the equilibrium switch toward production of H_2CO_3. This resulted in a decrease in H^+ concentration and thus alkalinization. Therefore, DCMU inhibition of photosynthesis which lowers CO_2 uptake may significantly inhibit light-induced H^+ influx as suggested by this study (Figure 9, Table 7).

Additional evidence for the light-induced alkalinization comes from external pH effects. High extracellular pH (7.0) initiated further alkalinization (0.6-0.7 pH unit, data not shown) compared with pH 5.2 (0.4 - 0.5 pH unit) of Table 1b. Enhanced pH shifts the equilibrium toward H_2CO_3 formation (Yin et al., 1996) resulting in a further increase in pH. On the other hand, a shift to a more acidic environment (pH 4.0) would be expected to reduce H_2CO_3 formation and inhibit the rapid pH rise illustrated in Table 6. After 5 min of illumination, only a small pH increase (0.07) was observed in the acidic bathing solution (Table 6), whereas at pH 7.0, extracellular pH was transiently increased by 0.4 - 0.5 during the same period (data not shown). Similar observations were made in light-induced *Cyanidium cadarium* (Kura-Hotta and Enami, 1981) and *Dunaliella acidophia* (Remis et al., 1994). Therefore, it seems reasonable to suggest that CO_2 uptake might cause apoplastic alkalinization in the light-induced pea mesophyll tissues. Moreover, the results using different external pH treatment indicate that the regulation of extracellular pH is not solely dependent on H^+ flux across the plasma membrane. The high net H^+ flux under pH 4.0 was similar to the increase in extracellular pH as under pH 6.0 and pH 5.2 (Table 6).

Furthermore, another possible explanation may be that ion uptake is driven by the proton motive potential. H^+ influx into cytoplasm is mediated by symporters within the plasma membrane, e.g. H^+/K^+ (Hedrich and Schroeder, 1989; Netting, 2000) and H^+/Cl^- (Sanders et al., 1985). The uptake of H^+ by plant cells is energetically favorable due to the negative membrane potential and a low cytosolic H^+ concentration in the cytoplasm compared to the apoplast. Thus, H^+ fluxes are believed to serve as the energy source for membrane conformational changes that accompany solute transport involving symporters. The influx or uptake of Cl^- is mediated by active transports (Assmann and Zeiger, 1987), and hypothesized to occur via a Cl^-/H^+ symport or a Cl^-/OH^- antiport (Zeiger et al., 1978). The Cl^- import requires the input of a considerable amount of metabolic energy. To meet this requirement, $2H^+/Cl^-$ co-transport against an opposing Cl^- gradient has been postulated which has indeed been demonstrated and kinetically modeled for *Chara* (Sanders et al., 1985) and root-hair cell of *Sinapis alba* (Felle, 1994). The experimental evidence in higher plants, however, has remained somewhat scant to date. In pea mesophyll, a light-induced initial response in net Cl^- influx was observed within 2-3 min (Table 1a, 2a). Extracellular Cl^- concentration declined following the net Cl^- influx (Table 1b). With decreasing pH or increasing H^+ concentration in the bathing solution (4.0), the light-induced initial response in net Cl^- influx was significantly enhanced as compared to that at pH 5.2 and pH 6.0 (Table 5). However, high external pH (6.0) resulted in a higher light-induced PIFR of Cl^- than at pH 4.0 (Table 5). Similar results were observed for the K^+ response to external pHs (Table 5). Therefore, Cl^-/H^+ and H^+/K^+ symport might be hypothesized to mediate light-induced apoplastic

alkalinization of pea mesophyll. More experimental evidence is required to clarify this issue though.

No direct attempt has been made to experimentally determine the influence of extracellular pH on Ca^{2+} flux from leaf cells so far. The H^+/Ca^{2+} antiporter is a likely candidate for H^+ - Ca^{2+} exchange which in principle mediates transport of Ca^{2+} against its concentration gradient and requires a H^+/Ca^{2+} stochiometry of at least three (Schumaker and Sze, 1987). However, activity of H^+/Ca^{2+} activity frequently was associated with membranes of mitochondria and chloroplast and only rarely with the plasma membrane (Bush, 1995). In this experiment, external pH did not influence the light-induced initial Ca^{2+} flux change (Table 5). Compared with pH 4.0, the alkalinizing environment (pH 6.0) inhibited the light-induced PIFR of Ca^{2+} (Table 5). Presumably changes in external pH alter the pH gradient across the plasma membrane or affect feedback regulation by cytoplasmic pH, thus influencing cytosolic Ca^{2+} homeostasis.

The light-induced PIFR is evidenced in the current study. Shabala and Newman (1999) did not discuss this event. The PIFR may reflect a combined response of Ca^{2+}, H^+, K^+ and Cl^- flux in leading to membrane repolarization which could be a cellular mechanism to regulate external pH. Another possible function of the PIFR could be to regulate a cytoplasmic Ca^{2+} messenger which is involved in light-induced signal transduction. Further investigation on the interaction between ion transporters in the plasma membrane is needed.

Light-induced ion fluxes from epidermal cells.

Shabala and Newman (1999) only measured a relatively small change of net ion fluxes

from bean epidermal strip when exposed to light. They suggested that the epidermis is an effective barrier to ion flux but not for CO_2 diffusion into the leaf. Elzenga et al. (1995) proposed that the ionic mechanisms of light-induced depolarization for mesophyll and epidermis of pea are different. In the current research, the light-induced initial ion flux changes in epidermal tissues were also smaller than in mesophyll (Figure 8, Table 4). Light initially induced a small decrease in net H^+ efflux resulting in apoplastic alkalinization (0.08 pH unit), and a reduction in extracellular Ca^{2+} concentration (28 μM) as well. The summation of light-induced net flux Ca^{2+}, H^+, K^+ and Cl^- in epidermal tissues was smaller than in mesophyll tissues (Figure 11). This result was similar to the relatively small light-induced membrane potential change reported by Elzenga et al. (1995). Although light-induced ion flux changes were observed, it is impossible to speculate on the ionic mechanism for electrical signalling in epidermal tissue.

The results in this study explain in part the ionic basis for light-induced bioelectrogenesis. However, more evidence is needed on the specific ion transporters involved in electrical signal transduction. Further investigations should also focus on the role of ion channels and proton pumps in light responses.

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Chapter II.

POSSIBLE MECHANISM OF LIGHT-INDUCED CA^{2+}, H^+, K^+ AND CL^- FLUXES FROM MESOPHYLL TISSUES.

ABSTRACT

Mechanisms of light-induced rapid changes in net Ca^{2+}, H^+, K^+ and Cl^- flux around mesophyll tissues from excised leaflet of pea (*Pisum sativum* L. cv. *Argenteum*) were analyzed. Calcium channel blocker $LaCl_3$ (0.1 mM) or chelator EGTA (1.0 mM) inhibited 42-44 % of the light-induced initial response of net Ca^{2+} influx and the light-induced Post-Initial Flux Response (PIFR) of Cl^- and K^+ over time. In contrast, the Ca^{2+} ionophore A23187 (5 μM) and increased exogenous Ca^{2+} (2.0 mM) significantly enhanced the light-induced (PIFR) of Cl^-, whereas low external Ca^{2+} (0.1 mM) produced an opposite effect. The evidence suggests that Ca^{2+} influx, resulting from increased Ca^{2+} channel activity, might be an initial step in the rapid response by leaf mesophyll to light. The plasma membrane H^+-ATPase inhibitors, Na_3VO_4 (1.0 mM) and DCCD (0.1 mM), inhibited the light-induced initial response in net H^+ influx by 50 - 60 % and eliminated the PIFR of H^+. The presence of Na_3VO_4 in the bathing solution also significantly suppressed the light-induced increase in net K^+ influx. However, these inhibitors did not affect the light-induced response in net Ca^{2+} influx. Furthermore, fusicoccin (0.01 mM) significantly increased the light-induced PIFR of H^+ and the initial response in net Cl^- influx. These results suggest that H^+-ATPase in the plasma membrane is not involved directly in the initial light response, whereas H^+-K^+ and H^+-Cl^- symports may contribute to the light-induced initial extracellular alkalinization and the uptake of K^+ and Cl^- by mesophyll tissues. In addition, TEA (5.0 mM) significantly blocked most of the light-induced PIFR of K^+. The R-type anion channel blocker DIDS (0.1 mM) completely suppressed light-induced net Cl^- efflux within the first 5 min of illumination, but the S-type blocker A-9-C (0.1 mM) did not. These observations indicate that the outwardly-

directed K^+ channels and the R-type anion channels may play a role in light-induced PIFR of K^+ and Cl^-, respectively, whereas that of Cl^- is Ca^{2+}-dependent.

INTRODUCTION

Rapid light-induced changes in membrane potential are ubiquitous in higher green plants. This bioelectrogenesis can be explained as light-induced changes in ionic fluxes across the plasma membrane. Light has been shown to elicit rapid changes in ion flux in leaves of *Dunalliella acidophila* (Remis et al., 1994), *Physcomitrella patens* (Johannes et al., 1997), bean (*Vicia fava*) (Shabala and Newman, 1999). These reports propose that ion transport across the plasma membrane is highly regulated, involving specific transport mechanisms during periods of light exposure. However, few studies have related ion transport activity and regulatory mechanisms to responses to light.

In general, plant physiological responses to light depend on cellular signalling systems that link light perception to terminal responses. Therefore, light-induced ion flux changes may link one or several signalling pathways. Ion fluxes across cell membrane systems are controlled by many ion channels, carrier proteins, and electrogenic ion pumps. These ion transporters regulate transport of ions across the plasma membranes and maintain ion homeostasis, and large chemical potential gradients. Light stimulation may activate or up/down regulate certain mechanisms that mediate changes of ion fluxes across membranes of plant cells. Light-induced leaf movement has been shown to involve K^+, Cl^- and H^+ fluxes mediated by K^+ channels and proton pumps in extensor and flexor tissues (Coté, 1995; Mayer et al., 1997). Other studies have focused on stomatal guard cell changes in turgidity controlled by ion fluxes across the plasma membrane of guard

cells (Grabov and Blatt, 1998; Assmann and Shimazaki, 1999). The dominant ions involved in signal transduction in guard cells include H^+, Ca^{2+}, K^+ and Cl^-. Ion channels (such as Ca^{2+}-, inward K^+ rectifier and outward K^+ rectifier and anion-channels) and H^+-ATPase are considered to play important roles in light-responses of guard cells. However, research is lacking on the regulation and responsiveness of ion transport mechanisms in other leaf tissues to environmental stimuli. Data on the ionic basis of light-induced membrane potential changes are also controversial and vary with plant species and experimental conditions.

To date, it is not fully understood which ion transporters are associated with light-induced ion flux responses in leaf tissues. Many possibilities exist because multiple ions are involved in the regulation of plasma membrane electrical potential. Shabala and Newman (1999) measured directly ion fluxes from bean mesophyll tissues and suggested that light-induced Ca^{2+} influx is a main depolarizing agent. They did not, however, explain the relationship between Ca^{2+} channel activity and the light-induced transient net Ca^{2+} influx. The results of Johannes et al. (1997) support a role for Ca^{2+} in the red-light-induced flux change in *Physcomitrella patens*. Blom-Zandstra et al. (1997) reported transient light-induced changes in ion channel and proton pump activities in the plasma membrane of tobacco mesophyll protoplasts, and suggested that ionic transporters in the plasma membrane might be involved in the light-induced transient changes in membrane potential. In *Arabidopsis* mesophyll, white light induced an increase in K^+ channel activity in the plasma membrane and a transient membrane depolarization (Spalding et al., 1992). In pea mesophyll cells, the light-controlled anion channels in the plasma membrane were proposed as a mechanism for membrane depolarization (Elzenga and

Van Volkenburgh, 1997). Further investigation indicated that the plasma membrane H^+-ATPase is not directly involved in rapid light-induced membrane depolarization (Stahlberg and Van Volkenburgh, 1999). They also suggested that several different mechanisms participate in light-induced membrane depolarization in the mesophyll and the epidermis (Elzenga at al., 1995). However, ion flux in leaf cells was not directly measured in the above studies. In Chapter I, the light-induced rapid changes in the net flux of H^+, Ca^{2+}, K^+ and Cl^- were demonstrated in pea mesophyll tissues. The transient net Ca^{2+} influx and the concomitant apoplastic alkalinization were thought to involve Ca^{2+} channels, or H^+-K^+ or H^+-Cl^- symporter, as well as or H^+-ATPase in the plasma membrane. The light-induced Post-Initial Flux Response (PIFR) of Cl^- from a net influx to a net efflux suggests that anion channels may also play a role whereas the function of K^+ fluxes in light response has still not been confirmed in pea mesophyll. Differential changes in ionic flux reveal that there are several ion transporters involved in the cellular mechanisms regulating light signal transduction. Therefore, further research is necessary to determine which transport mechanisms are associated with the light-induced ion flux changes.

Specific chemicals that inhibit or activate ion channels or ion pumps are used widely to determine the effect of environmental stimuli on specific ion transporters in the plasma membrane. However, most previous studies have only measured light-induced membrane potential change. The role of ion transporters cannot be elucidated from only membrane potential data alone. To test our hypothesis, two different approaches were used. The first consisted of treating pea mesophyll tissues with selective inhibitors or activators of H^+, Ca^{2+}, K^+ and Cl^- transporters in the plasma membrane before light

illumination. Light-induced dynamic ion flux changes of H^+, Ca^{2+}, K^+ and Cl^- were then monitored using the Microelectrode Ion Flux Estimation (MIFE) system. The second approach was to observe ionic flux responses to light in different external ionic solution environments. In this way, one is able to study possible mechanisms involved in the ionic response to light.

MATERIALS AND METHODS

Plant Material

Seeds of pea (*Pisum sativum* L. cv. *Argenteum*) were germinated 4-5 d and then transferred to a growth chamber with a 16 h photoperiod, day/night temperatures of 25/25 °C, 75% relative humidity, and a radiance of 200 μmol m^{-2} s^{-1} at the top of the canopy. The plants were cultured using modified Hoagland's solution (Johnson et al., 1957), gradually increasing the concentration from 0 to ¼ to ½ strength at 4-5 d intervals.

Flux measurements

Ion fluxes and concentrations at the surface of the leaf mesophyll were measured using the MIFE system. Technical details were generally as described in Chapter I and Shabala et al. (1997). Additional information on the MIFE system is available at http://www.phys. utas.edu.au/physics/biophys. In brief, micropipettes with a tip diameter of 2-3 μm were pulled from 1.5-mm-diameter borosilicate glass capillaries (catalog no. MIB 150-6; World Precision Instruments, Inc., Sarasota, FL) with a horizontal electrode puller (PC-10, Narishige, Tokyo, Japan). After silanization, cooled microelectrodes were back-filled with 500 mM $CaCl_2$ for Ca^{2+}, 500 mM KCl for K^+ and Cl^-, and 15 mM NaCl

plus 40 mM KH_2PO_4 (adjusted to pH 6.0 with NaOH) for H^+. Electrode tips were then front-filled with a liquid-membrane-type of the appropriate ion-selective sensor, H^+ (no. 95291), Ca^{2+} (no. 21048), K^+ (no. 60031) and Cl^- (no. 24902) cocktails (all from Fluka, Milwaukee, WI), until a 50- to 80- μm long column of ionophore was produced. Microelectrodes were calibrated for each ion using a known set of standards before and after use (see Chapter I). The Ag/AgCl reference electrode was made using a plastic tube containing 1000 mM KCl in 2% agar.

Calibrated ion-selective electrodes were positioned over the desired cells in a leaf segment using a micromanipulator with three-dimensional control. The set-up was mounted on the stage of an inverted microscope (Leitz Wetzlar, Germany) which provides a clear view for electrode positioning. The sample chamber with mounted leaf segment was placed on a three-way hydraulic micromanipulator (WR 88, Narishige, Tokyo, Japan) driven by a computer-controlled stepper motor (MO61-CE08, Superior Electric, Bristol, CT). The MIFE system allows for measurement of three ions simultaneously. Three microelectrodes can be slowly vibrated at any angle in a two-dimensional plane by separately controlling the amplitude of the vibration of the micromanipulator.

The MIFE system measured the voltage differences at two extreme positions of a 50 μm vibration above the leaf tissue surface. The magnitude and the directionality of the difference in estimated ion activity between the two positions was the basis for estimating both the rate and directionality of the ion fluxes. In this research the electrodes were moved in a 20-s square wave cycle between 50 to 90 μm above the leaf surface at a low frequency (0.1 Hz). The concentrations of ions were calculated from their

electrochemical potentials at each position. The flux for each specific ion was calculated from the measurements of the difference in the electrochemical potential between these positions (Shabala et al., 1997, or see Chapter I). During analysis, the first 4 s of each half cycle (10 s duration) were not used for concentration and flux estimation. This 4 s period accounted for the time for electrode movement to a new position and for voltage potential stability.

Experimental protocol

Young expanding leaflets (leaf age about 2 weeks) were harvested and a leaf section of approximately 15 × 10 mm (L×W) with limited veinal tissue was cut surgically from the lamina. A small strip of epidermal tissue was peeled from the abaxial surface of the lamina of each section so as to prevent mechanical damage to the underlying veinal and mesophyll tissue (Long and Widders, 1990). Isolated leaf segments were floated on the buffered solution (0.5 mM $CaSO_4$, 1.0 mM KCl, 2.5 mM HEPES-HCl, and 10 mM sucrose at pH 5.2) under 60 W m^{-2} illumination (Philips lamp). After 2.0-2.5 h, the cut segment was mounted in a sample chamber. The sample chamber contained 6-7 mL unbuffered solution or bathing solution (0.5 mM $CaSO_4$, 1.0 mM KCl, pH 5.2), and the segments then were exposed to 50-60 min of darkness. After 5-10 min of measurement, the light was turned on. Flux measurement was continued for an average 30 min after the start of illumination. After light was turned off, the measurement was conducted for another 10-15 min. The sample chamber was then removed and a new leaf sample was installed.

A fiber-optic light source (delivering 450 μmol m^{-2} s^{-1}) as measured by a quantum sensor, model LI-189, LI-COR, Lincoln, NE) was used with a 250 W halogen lamp (GS Edmumd Scientific Company, Barrington, NJ). A lens (no L-16-16, World Precision Instruments, Inc., Sarasota, FL) was fixed on the top of light guide to focus the beam.

Chemicals

Chemical treatments were selected according to their effects on ion transporters. These treatments included a Ca^{2+} channel blocker ($LaCl_3$), a Ca^{2+} chelator (EGTA), a Ca^{2+} ionophore A23187, H^+-ATPase inhibitor (vanadate and DCCD), a H^+-ATPase activator fusicoccin (FC), K^+ channel blockers (TEA and CsCl), and anion channel blockers (A-9-C and DIDS). Vanadate (Fisher, Lawn, NJ), CsCl, EGTA, and $LaCl_3$ (SIGMA, St. Louis, MO) were dissolved directly in an unbuffered solution (0.5 mM $CaSO_4$, 1.0 mM KCl, pH 5.2). Others including FC, DCCD, A-9-C, DIDS, Ca^{2+} ionophore A23187 and TEA (from SIGMA, St. Louis, MO) were prepared in DMSO as stock solutions.

To test the effects of these chemicals (Vanadate, DCCD, FC, EGTA, $LaCl_3$, Ca^{2+}-ionophore A23187, A-9-C, DIDS, TEA, CsCl) on ion fluxes under light stimulation (450μmol m^{-2} s^{-1}), all chemicals from their stock were dissolved with the unbuffered solution adjusted to pH 5.2. The final DSMO concentration was below 0.5 % (v/v) in the bathing medium. The leaf segment was incubated in the unbuffered bathing solution (containing one of these chemicals) under darkness for 50-60 min. Following the procedure of experimental protocol described above, ion flux was measured over an average 30 min period of light exposure.

Vanadate, DCCD, FC, EGTA, $LaCl_3$, Ca^{2+} ionophore A23187, A-9-C, DIDS, TEA,

CsCl were added to bathing solution, respectively, to test then individual effects on a specific ion flux under low fluence (50μmol m^{-2} s^{-1}).

Different external concentrations of Ca^{2+} (0.1 and 2.0 mM), K^+ (0.2, 5.0 and 10 mM) and Cl^- (0.2, 2.0, and 10 mM) were achieved by changing the salt concentrations in the bathing solution.

Data statistical analysis

Data analysis has been described in Chapter I. Analysis of variance (ANOVA) of the data was conduced using SAS 8 (SAS Institute Inc, Cary, NC).

RESULTS

Effects of H^+-ATPase inhibitor and FC.

To test the possible involvement of a plasma membrane proton pump in the light-induced flux change, two plasma membrane H^+-ATPase inhibitors, DCCD (at 0.1 mM) and Na_3VO_4 (at 1.0 mM), as well as a stimulator of H^+-ATPase, Fusicoccin (FC) at 0.01 mM, were applied to the bathing solution (0.5 mM $CaSO_4$, 1.0 mM KCl, adjusted to pH 5.2). The FC is a powerful effector that stimulates the plasma membrane H^+-ATPase and is used as an experimental tool to investigate the mechanism of physiological modulation of this enzyme (Marré, 1979). Under low background light (50 μmol m^{-2} s^{-1}), the net H^+ flux from mesophyll tissues of the control (Figure 1) exhibited a small change around zero. However, the addition of DCCD and Na_3VO_4 inhibited H^+ efflux and resulted in a high net H^+ influx of 16-20 nmol m^{-2} s^{-1}. In contrast, FC stimulated H^+ efflux and resulted in decrease in net H^+ flux of 18 nmol m^{-2} s^{-1} after approximately 30 min (Figure 1).

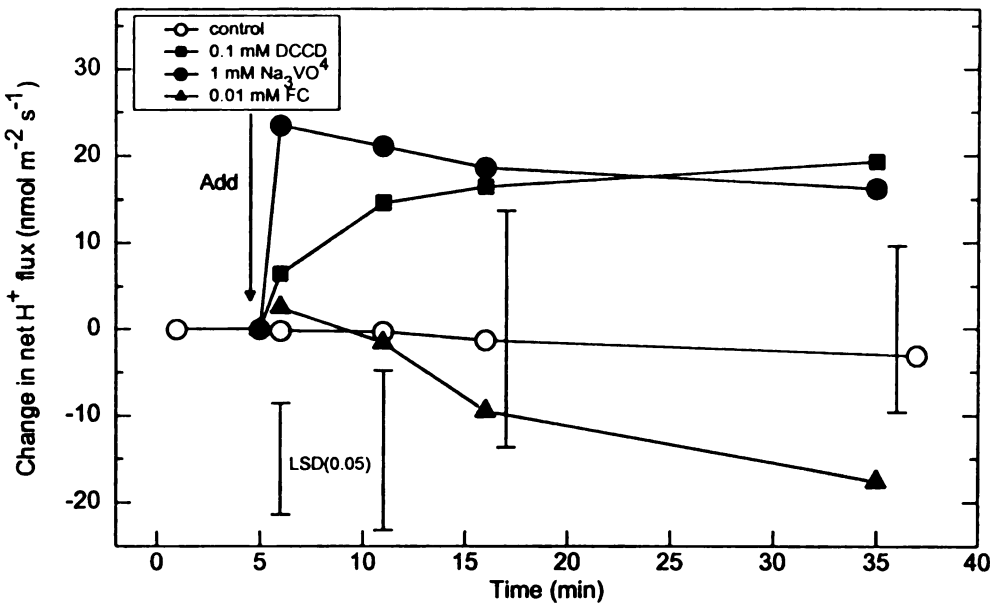

Figure 1. Effect of H^+-ATPase inhibitors, vanadate (Na_3VO_4) and N, N-dicyclohexi cabodiamide (DCCD), and the ATPase stimulator Fusicocin (FC) on net H^+ flux under fluence (50 µmol m^{-2} s^{-1}). Data points represent an average of 4-6 replicates. Vertical bars represent LSD values at $p<0.05$.

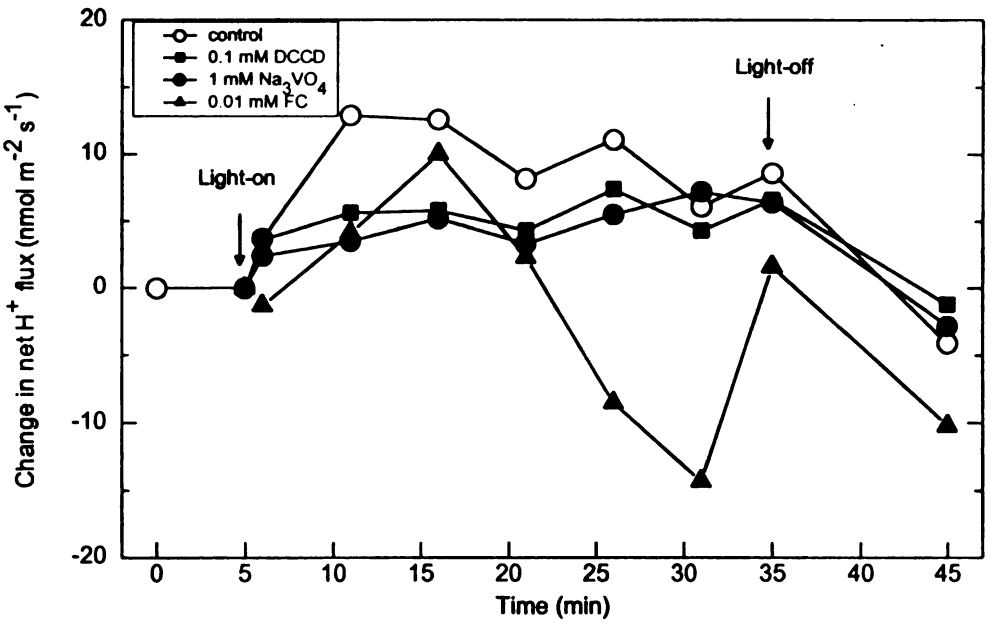

Figure 2. Effect of H^+-ATPase inhibitors, vanadate (Na_3VO_4) and N, N-dicyclohexicabodiamide (DCCD), and the ATPase stimulator Fusicocin (FC) on the time course of light (450 µmol m^{-2} s^{-1}) -induced change in net H^+ flux from pea mesophyll tissues. Data points represent an average of 6-10 replicates.

After DCCD, Na_3VO_4 and FC were added (at the same concentrations as in low light) to the bathing solution (0.5 mM $CaSO_4$, 1.0 mM KCl, adjusted to pH 5.2), the leaf tissue was incubated for 50-60 min in the dark. Net H^+ flux and extracellular pH around the pea mesophyll tissues were then measured following light stimulation (450 μmol m^{-2} s^{-1}). Light induced a rapid increase in both net H^+ influx and extracellular pH. After 5-10 min this influx (the control) decreased or changed to a net efflux, which is known as the light-induced PIFR (Figure 2, Table 1). The PIFR could involve a gradual return to flux rate closer to or lower than that measured prior to light stimulation. Either decrease in influx or increase in efflux (or both) caused the PIFR. Addition of DCCD and Na_3VO_4 resulted in a significant inhibition of the light-induced initial response (61% and 54% of the control respectively) in net H^+ influx (Table 1) and extracellular pH (data not shown). A 50-60 % reduction in H^+ PIFR was observed in the presence of DCCD or Na_3VO_4 (Table 1). The FC also reduced the light-induced initial response in net H^+ influx within 5 min of exposure to light and suppressed the light-induced extracellular alkalinization. As compared to the control (-22 nmol m^{-2} s^{-1}), FC caused a higher amplitude of PIFR (-46 nmol m^{-2} s^{-1}) from net influx to net efflux (Table 1), indicating that FC had stimulated H^+ efflux from the plasma membrane.

As discussed previously in Chapter I, light induced a transient increase in net Ca^{2+} influx. Both DCCD and Na_3VO_4 treatments significantly reduced the light-induced PIFR of Ca^{2+} (38 % of the control), whereas Na_3VO_4 significantly inhibited the light-induced initial response (28 %) in net K^+ influx (Table 1). FC increased light-induced initial response in net Cl^- influx (240 %) but inhibited that of net Ca^{2+} influx (Table 1). During the first 5 min of light treatment, the light-induced net Cl^- flux change was 27 nmol m^{-2} s^{-1}

Table 1. Effects of H^+-ATPase inhibitors and FC on light-induced net fluxes of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues.

	H^+	Ca^{2+}	K^+	Cl^-
		(nmol m^{-2} s^{-1})		
Initial flux response (influx)				
control	+†26(10)§a	+48(10) a	+171 (8) a	+101 (8) b
1.0 mM Na_3VO_4	+12 (10) b	+57 (7) ab	+132 (8) b	+72 (7) b
0.1 mM DCCD	+12 (8) b	+52 (8) ab	+139 (7) ab	+67 (7) b
0.01 mM FC	+24 (7) a	+30 (6) b	+200 (6) a	+242 (6) a
Flux change during the first min				
control	+4 (10)	+26 (10) a	+24 (8) a	+13 (8) ab
1.0 mM Na_3VO_4	+2 (10)	+35 (9) a	+19 (8) a	-9 (7) b
0.1 mM DCCD	+4 (8)	+7 (10) ab	+27 (7) a	-20 (7) b
0.01 mM FC	-1 (8) ns	+1 (8) b	-62 (6) b	+69 (6) a
Flux change during the first five min				
control	+13 (10)	+29 (10) a	+67 (8)	-60 (8)
1.0 mM Na_3VO_4	+4 (10)	+41 (8) a	+16 (7)	-55 (7)
0.1 mM DCCD	+6 (8)	+17 (10) ab	+17 (7)	-36 (7)
0.01 mM FC	+4 (8) ns	-13 (6) b	+9 (6) ns	+27 (6) ns
Amplitude of PIFR‡				
control	-22 (10)a	-63 (10) a	-188 (8) ab	-151 (8)
1.0 mM Na_3VO_4	-9 (8) c	-40 (8) b	-138 (8) a	-213 (5)
0.1 mM DCCD	-10 (8) c	-49 (8) b	-200 (7) b	-173 (7)
0.01 mM FC	-46 (6) b	-53 (6) ab	-181 (6) ab	-207 (6) ns

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.

"ns" indicates no significant difference.

§ The numbers in parentheses indicate the number of replicates.

† - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

‡ **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

under FC, indicating a net influx. However, in other treatments, net Cl^- flux changes were negative, indicating a net efflux. Additionally, the light-induced PIFR of Cl^- (-207 nmol m^{-2} s^{-1}) occurred after 5.3 min, whereas the control only needed 2.1 min. FC did not affect the light-response of K^+ flux. These results indicated that the inhibition of H^+-ATPases caused a decrease in the light induced initial influxes of H^+ and K^+, and in the PIFRs of H^+ and Ca^{2+}. On the other hand, an increase in H^+-ATPase activity enhanced the light induced initial influxes of Cl^- and PIFR of H^+.

Effects of Ca^{2+} channel blocker and chelator

The Ca^{2+} channel blocker ($LaCl_3$) or chelator EGTA were used to evaluate their effects on Ca^{2+} channel activity. The Ca^{2+}-ionophore A23187 is thought to mediate $Ca^{2+}/2H^+$ exchange across the membrane (Pressman, 1976). When at the same low light background (50 μmol m^{-2} s^{-1}), net Ca^{2+} flux from pea mesophyll tissues in the control only displayed a small change (Figure 3). However, the net Ca^{2+} influx was inhibited by both $LaCl_3$ (0.1 mM) and EGTA (1.0 mM) but increased by Ca^{2+}-ionophore A23187 of 5 μM (Figure 3).

Light (450 μmol m^{-2} s^{-1}) elicited a transient increase in net Ca^{2+} influx followed by a PIFR (Figure 4, or see Chapter I). Compared with the control, both $LaCl_3$ and EGTA significantly reduced the light-induced initial response in net Ca^{2+} influx (by 42-44% of the control) and corresponding response in extracellular Ca^{2+} concentration (64% and 39%, data not shown), as well as the light-induced PIFR of Ca^{2+} around pea mesophyll (27 % and 37 %, respectively). Its initial light response time also was delayed by both

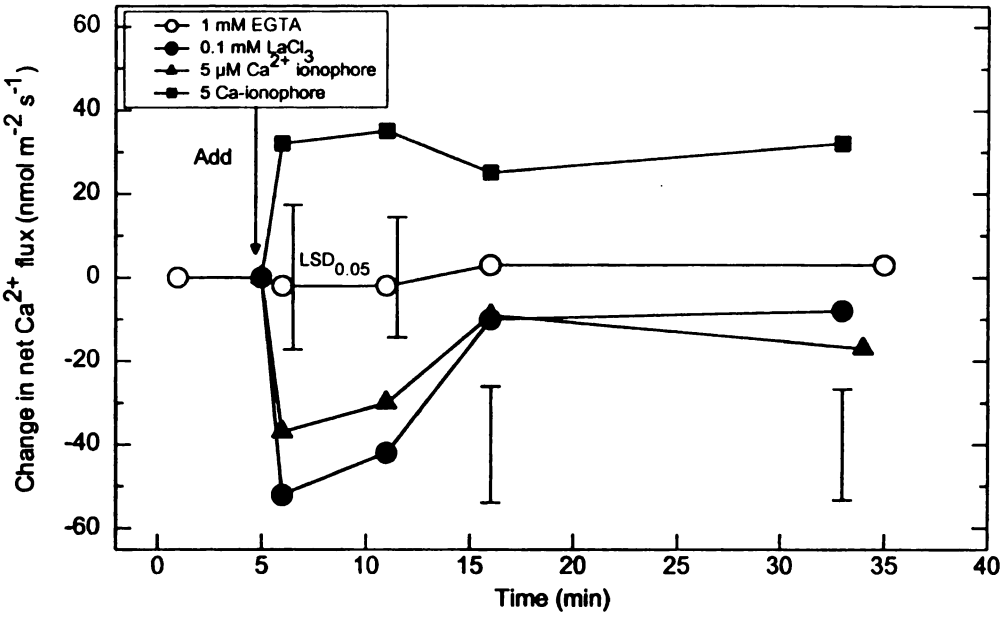

Figure 3. Effects of Ca^{2+} channel blocker ($LaCl_3$), chelator (EGTA) and ionophore A23178 on net Ca^{2+} flux from pea mesophyll tissues under low fluence (50 µmol m^{-2} s^{-1}). Data points represent an average of 6-8 replicates. Vertical bars represent LSD values at $p<0.05$.

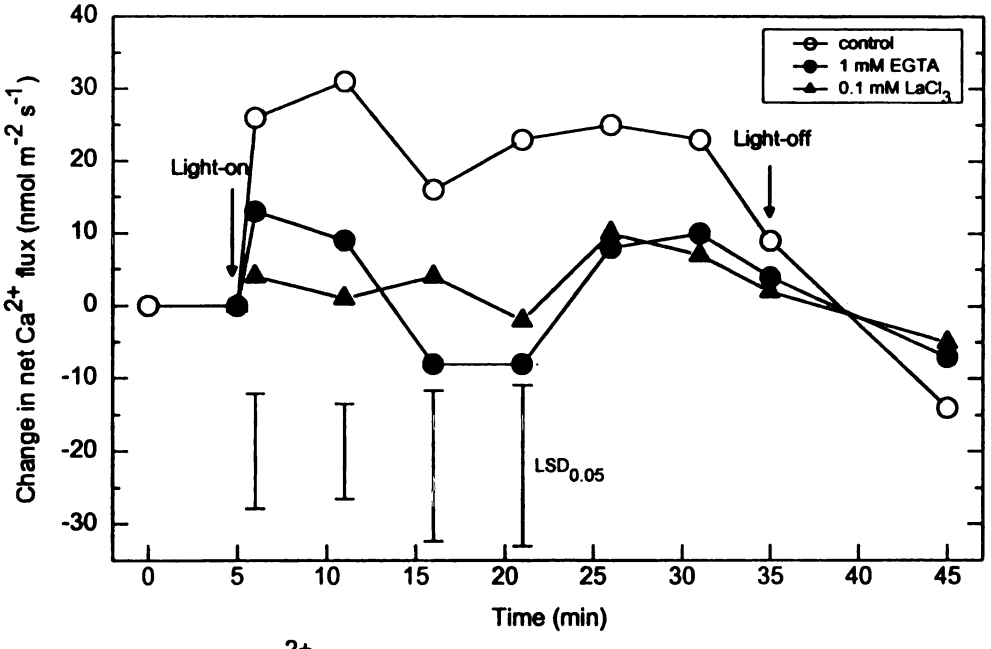

Figure 4. Effect of the Ca^{2+} chelater (EGTA) and channel blocker ($LaCl_3$) on the time course of light (450 µmol m^{-2} s^{-1})-induced change in net Ca^{2+} flux from pea mesophyll tissues. Data points represent an average of 6-10 replicates. Vertical bars represent LSD values at $p<0.05$.

Table 2. Effects of Ca^{2+} channel blocker and chelator on light-induced fluxes of H^+, Ca^{2+}, K^+ and Cl^- and time response in pea mesophyll tissues.

	H^+	Ca^{2+}	K^+	Cl^-
Initial flux response (influx)		(nmol m^{-2} s^{-1})		
control	+[†]26 (10)[§] a	+45 (8) a	+169 (8) a	+96 (8)
1.0 mM EGTA	+7 (8) b	+25 (9) b	+121 (8) b	+138 (8)
0.1 mM $LaCl_3$	+13 (8) b	+26 (10)b	+75 (8) c	+85 (9) ns
Flux change during the first min		(nmol m^{-2} s^{-1})		
control	+3(10)	+24 (8) a	+22 (8)	+10 (8)
1.0 mM EGTA	+1 (8)	+13 (9) b	+52 (8)	+71 (7)
0.1 mM $LaCl_3$	+3 (8) ns	+4 (10) b	+29 (8) ns	+12 (9) ns
Flux change during the first five min		(nmol m^{-2} s^{-1})		
control	+13 (10) a	+32(8) a	+63 (8)	-57 (8) b
1.0 mM EGTA	+1 (8) b	+9 (9) b	+30 (8)	+61 (7) a
0.1 mM $LaCl_3$	+8 (8) a	+1(10) c	+13 (8) ns	-4 (9) c
Amplitude of PIFR[‡]		(nmol m^{-2} s^{-1})		
control	-26 (10) a	-60 (8) a	-201 (8) a	-143 (8) a
1.0 mM EGTA	-18 (8) ab	-38 (9) b	-121 (8) b	-34 (4) b
0.1 mM $LaCl_3$	-11 (7) b	-44(10) b	-99 (8) b	-50 (9) b
Initial light response time		(min)		
control	3.1(10)	2.1 (8) b	3.9 (8)	2.3 (8) b
1.0 mM EGTA	4.5 (8)	3.7 (9) a	6.6 (8)	4.8 (8) b
0.1 mM $LaCl_3$	3.4 (8) ns	3.8(10) a	4.4 (8) ns	5.6 (9) a

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.

"ns" indicates no significant difference.

[§] The numbers in parentheses indicate the number of replicates.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

(Table 2). This indicates that Ca^{2+} channel blocker and chelator inhibit the light response in net Ca^{2+} influx.

Simultaneously, the light-induced initial responses of net H^+ and K^+ influx were also significantly suppressed in the presence of $LaCl_3$ and EGTA (Table 2). Neither Ca^{2+} influx inhibitor affected the light-induced initial response of net Cl^- influx. However, the light-induced PIFR of Cl^- was greatly inhibited by $LaCl_3$ and EGTA (Table 2). During the first 5 min after illumination, a negative change in net Cl^- flux, a net efflux, had been observed in the presence of EGTA. The change was significantly higher than that of $LaCl_3$ and EGTA. Like Cl^-, $LaCl_3$ and EGTA also produced a decrease in the light-induced PIFR of K^+ and H^+ (only $LaCl_3$) (Table 2).

Effects of K^+ channel blocker

Tetraethylammonium (TEA) is a powerful blocker for both classes of K^+ channels, inward- and outward-rectifiers (Bentrup, 1990). CsCl blocks inward K^+ current (Moran et al., 1990). To identify whether K^+ channels are involved in the light-induced K^+ flux change, TEA-Cl^- (5.0 mM) and CsCl (2.0 mM) were added to the bathing solution (0.5 mM $CaSO_4$, 1.0 mM KCl, adjusted to pH 5.2). In general, light stimulation produced a rapid increase in net K^+ influx followed by a PIFR which may be due to a decrease in net K^+ influx or an increase in net K^+ or both (Figure 5 or see Chapter I). Compared to the control, TEA significantly blocked the light-induced initial response (35 % of the control) in net K^+ influx and the PIFR of K^+ (66 %) (Table 3). The effects of CsCl on the light-induced K^+ flux change were similar but smaller in the magnitudes as compared to that of TEA.

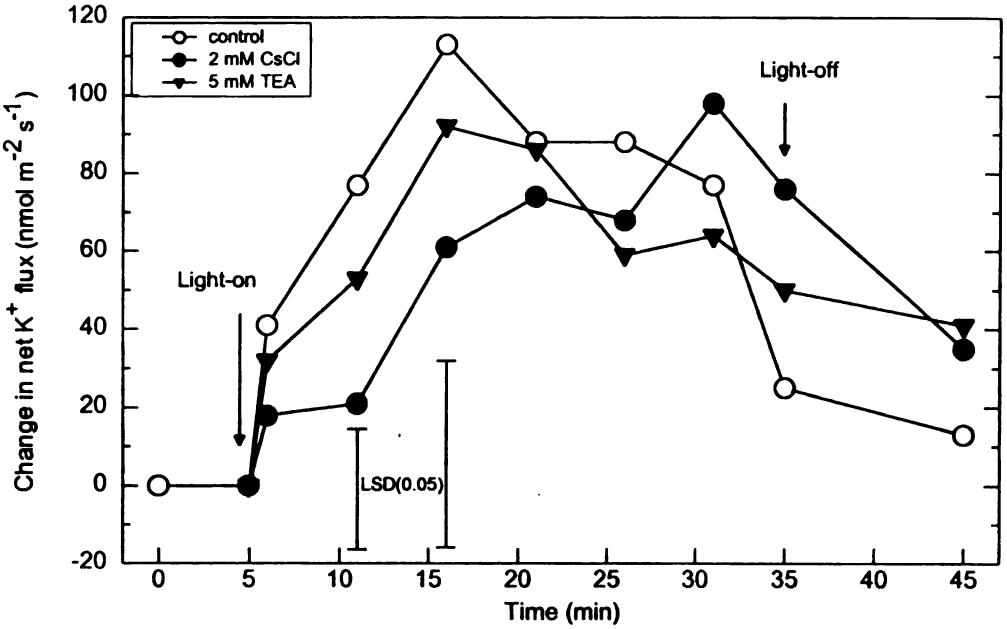

Figure 5. Effect of K^+ channel blockers tetraethylammonium (TEA) and Cesium chloride (CsCl) on the time course of light (450 µmol m^{-2} s^{-1})-induced change in net K^+ flux from pea mesophyll tissues. Data points represent an average of 6-10 replicates. Vertical bars represent LSD values at $p<0.05$.

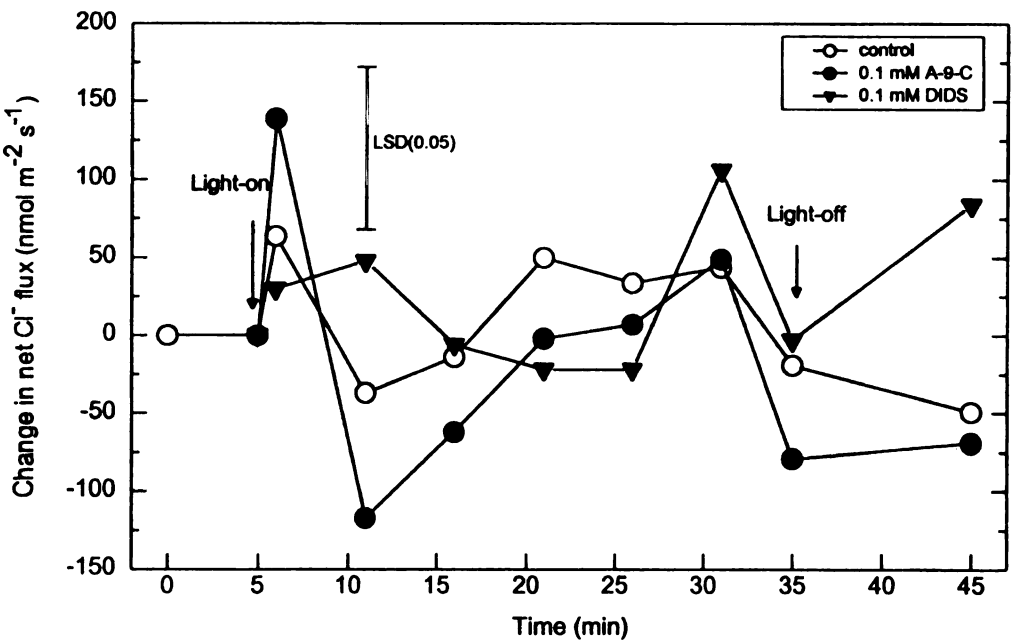

Figure 6. Effect of Cl^- channel blockers diisothiocyanatoslibene (DIDS) and anthracene-9-carboxylic (A-9-C) on the time course of light (450 µmol m^{-2} s^{-1})-induced change in net Cl^- flux from pea mesophyll tissues. Data points represent an average of 6-10 replicates. Vertical bar represents LSD values at $p<0.05$.

Table 3. Effects of K^+ channel blocker on light-induced net fluxes of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues.

	H^+	Ca^{2+}	K^+	Cl^-
		(nmol m^{-2} s^{-1})		
Initial flux response (influx)				
control	+[†]25 (10)[§] a	+47 (8)	+183 (10)a	+91 (8) b
5.0 mM TEA	+16 (8) b	+42 (6) ns	+120 (9) b	+246 (7) a
2.0 mM CsCl	+12 (5) b	n.m.	+142 (8) ab	n.m.
Flux change during the first min				
control	+4 (10)	+21 (8)	+41 (10)	+15 (10) b
5.0 mM TEA	+5 (8)	+30 (6) ns	+18 (9)	+148 (7) a
2.0 mM CsCl	+3 (5) ns	n.m.	+32 (8) ns	n.m.
Flux change during the first five min				
control	+11 (10) a	+21 (10)	+77 (10)	-34 (8) b
5.0 mM TEA	+9 (8) ab	+10 (6) ns	+24 (9)	+93 (7) a
2.0 mM CsCl	+4 (5) b	n.m.	+53 (8) ns	n.m.
Amplitude of PIFR[‡]				
control	-18 (10)	-57 (8)	-189(10) a	-149 (8) a
5.0 mM TEA	-20 (8)	-65 (6) ns	-64 (9) b	-39 (3) b
2.0 mM CsCl	-11 (5) ns	n.m.	-95 (8) b	n.m.

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.

"ns" indicates no significant difference.

[§] The numbers in parentheses indicate the number of replicates.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

Both TEA and CsCl also significantly reduced the light-induced initial response (36 and 52 %, respectively) (Table 3) and maximum increase (38 and 50 %, respectively, data not shown) in net H^+ influx. The blocker TEA further increased the light-induced initial response of net Cl^- influx, possibly because of high Cl^- concentrations in the bathing solution. However, it did not affect the light responses of net Ca^{2+} flux (Table 3).

Effects of Cl^- Channel blocker

Two anion channel blockers, A-9-C (for the Slow-type channels) and DIDS (for the Rapid-type channels), were used to determine whether anion channel activity was correlated with the light-induced Cl^- flux change. Light elicited a rapid and short response in net Cl^- influx and followed by a PIFR (Figure 6, Table 4, or see Chapter I). The A-9-C treatment did not significantly affect the light-induced initial response of net Cl^- influx (Table 4). In contrast to A-9-C and the control, DIDS did not cause a net Cl^- efflux but a net influx (48nmol m^{-2} s^{-1}) during the first 5 min of illumination (Table 4). A PIFR of Cl^- occurred after 6.3 min in the presence of DIDS, whereas the control only needed 2.3 min of light exposure. When the light was turned off, the net Cl^- flux changed to a net efflux for the control whereas A-9-C and DIDS increased the net Cl^- influx (Figure 6).

Both A-9-C and DIDS, when applied at the same concentration (0.1 mM), did not significantly influence the light-induced initial response of net influx of H^+, Ca^{2+} and K^+ (K^+ data for DIDS not measured experimentally). By comparison to the control, DIDS significantly reduced the light-induced response of H^+ and Ca^{2+} influxes during the first 5 min of illumination and extended the initial light response time of Ca^{2+} (Table 4).

Table 4. Effects of Cl^- channel blocker on light-induced net fluxes of H^+, Ca^{2+}, K^+ and Cl^- and time response in pea mesophyll tissues.

	H^+	Ca^{2+}	K^+	Cl^-
Initial flux response (influx)		(nmol m^{-2} s^{-1})		
control	+[†]27 (8)[§]	+48 (6)	+161 (8)	+110 (8)
0.1 mM A-9-C	+23 (11)	+59 (7)	+171 (8) ns	+164 (7)
0.1 mM DIDS	+18 (8) ns	+45 (7) ns	n.m.	+201 (5) ns
Flux change during the first min		(nmol m^{-2} s^{-1})		
control	+8 (8)	+22 (6) a	+35 (8) a	+64 (8)
0.1 mM A-9-C	+7 (11)	+21 (7) a	-43 (8) b	+139 (7)
0.1 mM DIDS	+4 (8) ns	-7 (7) b	n.m.	+30 (7) ns
Flux change during the first five min		(nmol m^{-2} s^{-1})		
control	+13 (8) a	+22 (6) a	+68 (8) a	-30 (8) ab
0.1 mM A-9-C	+12 (11) a	+22 (7) b	+25 (8) b	-99 (5) b
0.1 mM DIDS	+6 (8) b	+2 (7) b	n.m.	+48 (7) a
Initial light response time		(min)		
control	4.1 (10)	2.2 (6) b	4.3 (8) b	2.3 (8) b
0.1 mM A-9-C	3.2 (11)	3.0 (7) b	8.0 (8) a	1.7 (7) b
0.1 mM DIDS	3.5 (8)ns	7.7 (7) a	n.m.	6.3 (7) a

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.

"ns" indicates no significant difference.

"n.m.". not experimentally measured.

[§] The numbers in parentheses indicate the number of replicates.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

Effects of Ca^{2+} ionophore and external Ca^{2+}, K^+ and Cl^- concentrations

Treatment with the Ca^{2+}-ionophore A23187 (5 μM) significantly stimulated the light-induced initial response in net Ca^{2+} influx from 47 to 67 nmol m^{-2} s^{-1} (Figure 7). Compared to the control, the light-induced initial response of net H^+, K^+ and Cl^- influx were not significantly influenced by the Ca^{2+}-ionophore (K^+ and Cl^- data not shown) (Figure 7). In the same treatment, the light-induced PIFR of Cl^- increased significantly from -144 to -241 nmol m^{-2} s^{-1}, whereas the PIFR of K^+ was not significantly affected (Figure 7). Therefore, the Ca^{2+}-ionophore and $LaCl_3$ or EGTA produce different effects on light-induced H^+, Ca^{2+}, Cl^- and K^+ flux change in pea mesophyll tissues.

When the Ca^{2+} concentration in the external bathing solution was increased to 2.0 mM (2.0 mM $CaSO_4$, 1.0 mM KCl, pH 5.2), a significant increase in net Ca^{2+} influx response to light was observed (Figure 8). The light-induced PIFR of Cl^- was also significantly enhanced by the higher Ca^{2+} concentration within the bathing solution (Figure 8). Compared with the control (0.5 mM Ca^{2+}), the light-induced initial response in net H^+, K^+ and Cl^- influxes were not significantly affected (K^+ and Cl^- data not shown). However, when the external Ca^{2+} concentration was experimentally set at 0.1 mM, the PIFR of K^+ and Cl^- declined significantly.

The light-induced initial response in net K^+ influx increased at higher external K^+ concentration (5.0 mM) in the bathing solution and decreased at the lower concentration (0.2 mM K^+) (Table 5a). Thus, the initial light response of K^+ flux appears to be concentration-dependent. However, changing the K^+ concentration did not significantly affect the light-induced initial response nor the PIFR of H^+, Ca^{2+} and Cl^- (Table 5a).

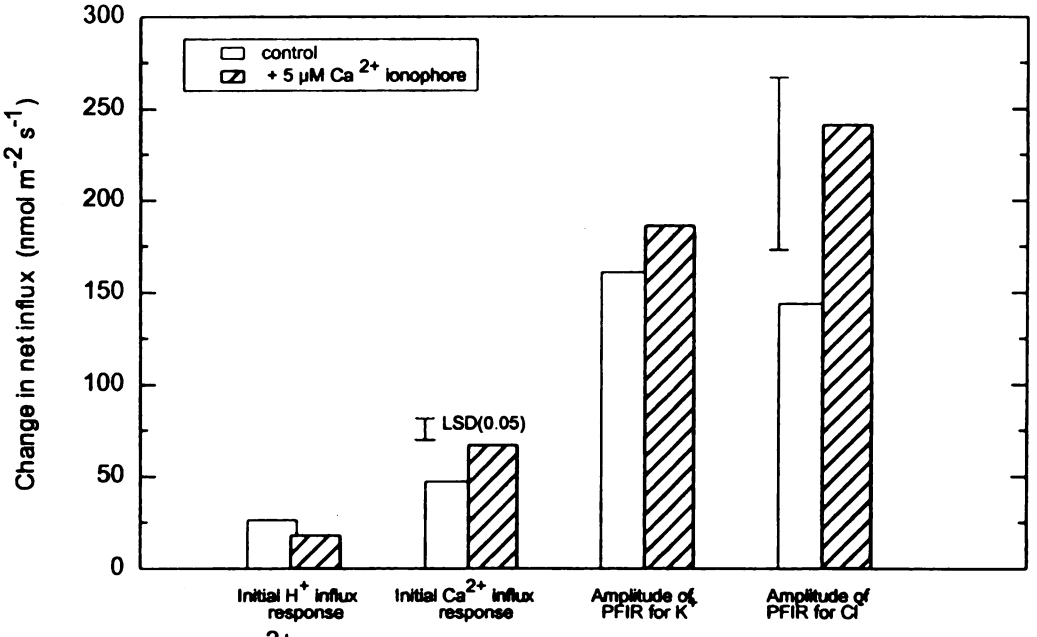

Figure 7. Effect of Ca^{2+} ionophore A23187 on the light-induced ion flux response of pea mesophyll tissues. Changes in net K^+ and Cl^- flux indicate the amplitude of light (450 μmol m^{-2} s^{-1})-induced Post-Initial Flux Response (PIFR), negative values. Data represents an average of 6-9 replicates. Vertical bars represent LSD values at $p<0.05$.

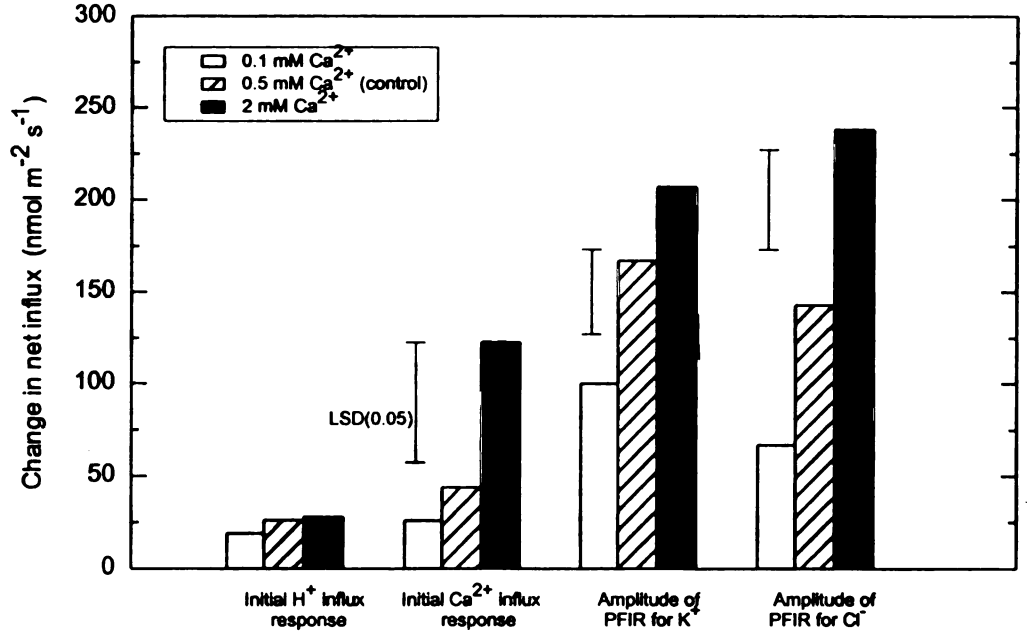

Figure 8. Effect of external Ca^{2+} concentration on the light-induced ion flux response of pea mesophyll tissues. Changes in net K^+ and Cl^- flux indicate the amplitude of light (450 μmol m^{-2} s^{-1})-induced Post-Initial Flux Response (PIFR), negative values. Data represents an average of 6-9 replicates. Vertical bars represent LSD values at $p<0.05$.

Table 5. Effects of external K^+ and Cl^- concentration on light-induced net fluxes of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues.

a: external K^+ concentrations

	H^+	Ca^{2+}	K^+	Cl^-
		(nmol m^{-2} s^{-1})		
Initial flux response (influx)				
control(1.0 mM K^+)	+[†]21(7)[§]	+48 (8)	+153 (6) b	+91 (7)
0.2 mM K^+	+19 (7)	+43 (9)	+49 (7) c	+106 (7)
5.0mM K^+	+25 (6) ns	+38 (6) ns	+255 (7) a	+153 (6) ns
Amplitude of PIFR[‡]				
control(1.0 mM K^+)	-16 (7)	-51 (8)	-181 (6) a	-143 (7)
0.2 mM K^+	-19 (7)	-52 (9)	-82 (7) b	-119 (6)
5.0 mM K^+	-11 (6) ns	-51 (6) ns	-262 (7) ab	-111 (4) ns

b: external Cl^- concentrations

	H^+	Ca^{2+}	K^+	Cl^-
		(nmol m^{-2} s^{-1})		
Initial flux response (influx)				
Control(1.0mM Cl^-)	+27 (8) a	+48 (8) a	+176 (6) b	+103 (8) b
0.2 mM Cl^-	+17 (5) a	+28 (6) b	+107 (8) b	+101 (6) b
2.0 mM Cl^-	+20 (7) a	+43 (6) a	n.m.	+160 (5) b
10 mMKCl	+8 (6) b	+37 (7) ab	+739 (8) a	+312 (7) a
Amplitude of PIFR				
Control(1.0mM Cl^-)	-24 (8) a	-59 (8)	-193 (6) b	-184 (8)
0.2 mM Cl^-	-17 (5) a	-48 (5)	-155 (8) b	-112 (6)
2.0 mM Cl^-	-23 (7) a	-45 (6)	n.m.	-126 (5)
10 mMKCl	-8 (5) b	-51 (7) ns	-509 (8) a	-157 (6) ns

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.

"ns" indicates no significant difference.

"n.m." not experimentally measured.

[§] The numbers in parentheses indicate the number of replicates.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

If the Cl^- concentration in the bathing solution was lowered to 0.2 mM (did not alter Ca^{2+} and K^+ concentration), the light-induced initial response in net Ca^{2+} influx was significantly inhibited (Table 5b). Doubling the K^+ and Cl^- concentrations in the external bathing medium to 10 mM KCl dramatically reduced the light-induced initial response in net H^+ influx and significantly enhanced net K^+ and Cl^- influx in response to light (Table 5b).

DISCUSSION

Ca^{2+} fluxes and Ca^{2+} transporters

Increase in cytosolic free Ca^{2+} concentrations is considered to be necessary for cellular signal transduction in response to external stimuli. Passive influx of Ca^{2+}, facilitated by the opening of Ca^{2+}-permeable channels in the plasma membrane, is one of the primary means of increasing cytosolic free Ca^{2+}. Numerous physiological, pharmacological and Ca^{2+} flux studies have provided indirect evidence that Ca^{2+} channels in the plasma membrane of higher plant cells play a central role in the initiation of various signal transduction sequences, including, plant-pathogen interactions (Bach et al., 1993), light-induced leaf movement (Mayer et al., 1997), and hormone- and light-regulated stomatal responses (Ward and Schroeder, 1997; Grabov and Blatt, 1998). However, direct evidence for the light-induced Ca^{2+} channels in the plasma membrane of mesophyll cells has not been obtained. Accurate measurement of Ca^{2+} influx across the plasma membrane is quite difficult. In the current research, net Ca^{2+} flux was measured by a noninvasive, ion-selective, slowly vibrating microelectrode. A light-induced transient increase in net Ca^{2+} influx was identified and proposed as an initial depolarizing agent in

Chapter I, suggesting that an increase in Ca^{2+} influx not a decrease in Ca^{2+} efflux is one major reason for the initial Ca^{2+} influx response (see Chapter I). Although H^+-Ca^{2+} exchanges occurred in the cell wall of *Chara australis* cells (Ryan et al., 1992), the result of net Ca^{2+} flux mainly represents free Ca^{2+} movement within the apoplast of the tissues. Reid and Smith (1992) did not find significant effects on Ca^{2+} influx following changes in external pH or following changes in internal pH by separating the cell wall from cell contents of *Chara corallina*, indicating that exchanges between H^+-Ca^{2+} are relatively constant under a certain range of pH in the bathing solution. Therefore, it is reasonable to believe that the light-induced net Ca^{2+} influx is the result of Ca^{2+} uptake facilitated by Ca^{2+}-channels in the plasma membrane.

Further evidence for the role of putative Ca^{2+} channels is obtained by examining the effects of a channel blocker and a Ca^{2+} chelator. Both $LaCl_3$ and EGTA significantly suppressed the light-induced transient increase in net Ca^{2+} influx (Table 2), demonstrating the relationship between Ca^{2+} influx and Ca^{2+} channel activity.

At least two major classes of Ca^{2+} channels reside in the plasma membrane (White, 1998). The first class includes those that are relatively non-selective with respect to cations and possess a high single-channel conductance; these are known as maxi-cation channels (White, 1993; White, 1994). The second class is relatively more selective for cations, and exhibits a smaller single-channel conductance. Since the addition of $LaCl_3$ and EGTA also resulted in a significant reduction of the light-induced initial response of net K^+ influx (Table 2), the influx of both Ca^{2+} and K^+ is assumed to occur by diffusion through non-selective cation channels. The same finding was reported in *Samanea saman* protoplasts (Moran and Satter, 1990) and during light-induced swelling of

Phaseolus coccineus protoplasts (Mayer et al., 1997). The Ca^{2+} or K^+ influx across the putative cation-channels was dependent upon the external concentration of Ca^{2+} or K^+ but not upon each other (Table 2, 5). However, an organic Ca^{2+} channel blocker, verapamil (0.1 mM), did not affect light-induced Ca^{2+} influx or other ion fluxes (data not shown). Reid and Smith (1992) also made similar observations in *Chara*. In animal cells, La^{3+} can block at least two types (L- and N-type) of voltage-activated Ca^{2+} channels, whereas verapamil only blocks the L-type (Tsien et al., 1987). Apparently, not all types of cation-channel are responsive to light in pea leaf tissues.

The effect of Ca^{2+} on the light-induced PIFR of Cl^- provides more evidence for Ca^{2+} influx as an initial response to light. Clearly, increased cytoplasmic Ca^{2+} has been shown to trigger anion channels and to precede Cl^- efflux (Schroeder, 1995). The Cl^- efflux across the plasma membrane was activated by intracellular free Ca^{2+} in *Chara* cells (Mimura and Shimmen, 1994; Johannes et al., 1998). A rapid light-induced PIFR of Cl^- after 2-3 min of illumination was detected and explained as a result of an increase in Cl^- efflux (See Chapter I). This time allows for Ca^{2+} activation of anion channels and a change in the directionality of the anion flux. Three facts support the role of Ca^{2+} in regulating Cl^- efflux: (a) $LaCl_3$ and EGTA significantly blocked the light-induced PIFR of Cl^- (Table 2); (b) the addition of Ca^{2+}-ionophore A23187 (Figure 7) or (c) increasing Ca^{2+} concentration in the bathing medium resulted in a significant increase in the light-induced PIFR of Cl^- (Figure 8). Nevertheless, the light-induced Ca^{2+} influx was not influenced by Cl^- channel blockers (Table 4).

Both $LaCl_3$ and EGTA dramatically inhibited the light-induced initial response in net H^+ influx (Table 2). Felle (1998) hypothesized that cation channels may contribute to the

regulation of the apoplastic pH in roots. He also observed that extracellular Ca^{2+} was apparently less effective in influencing apoplastic pH. Using the Ca^{2+}-ionophore A23187 as a Ca^{2+}/H^+ exchanger did not significantly affect the light-induced H^+ influx (Figure 7). This result is different from that of $LaCl_3$ and EGTA treatments. A possible explanation is that proton pumps are deactivated when cation channels are blocked. Kinoshita et al. (1995) reported that the activation of H^+-ATPase required elevated cytoplasmic Ca^{2+} concentration in guard cells. Under the light, perhaps the interaction of Ca^{2+} and H^+ (and other ions, such as K^+, Cl^-) with H^+-ATPase in the plasma membrane stimulates its phosphorylation/dephosphorylation, as occurs under stresses (Netting, 2000). No work seems to have been done on the possible mechanisms for such a light-induced response. The present result might constitute a linkage between Ca^{2+}-channels or Ca^{2+} messenger and H^+ flux across the plasma membrane during light signal transduction. All considerations taken together, the simplest interpretation is that light-induced transient Ca^{2+} influx facilitated by Ca^{2+} channels could initiate other ionic responses in pea mesophyll.

It is interesting to note that external Ca^{2+} concentrations influenced ion flux responses to light. The first effect of apoplastic Ca^{2+} is the light-induced initial response in net Ca^{2+} influx which was significantly increased by higher Ca^{2+} concentration (2.0 mM), whereas the initial flux responses in other ions were not significantly influenced by changing extracellular Ca^{2+} concentrations (Figure 8) (K^+ and Cl^- data not shown). However, the light-induced PIFR of K^+ and Cl^- was significantly influenced by apoplastic Ca^{2+} concentrations (Figure 8). These results suggest that apoplastic K^+ also play an important role in regulating ionic responses to light.

It is remarkable that light immediately stimulated a rapid PIFR of Ca^{2+} following a transient Ca^{2+} influx (see Chapter I,). Perhaps the PIFR is associated with Ca^{2+} efflux and reflects light-regulation of cytosolic Ca^{2+} homeostasis. When cytosolic Ca^{2+} concentration increases, active Ca^{2+}-transport systems work to restore the resting cytoplasmic Ca^{2+} to submicromolar levels (Trewavas, 1999). Vanadate is not completely specific for the plasma membrane H^+-ATPase inhibition. The plasma membrane Ca^{2+}-ATPase also is inhibited by vanadate (Palmgren and Harper, 1999). Thus, it is not surprising that vanadate and even DCCD significantly reduced the light-induced PIFR of Ca^{2+} (Table 1) and enhanced the light-induced maximum increase in net Ca^{2+} influx (data not shown). On the other hand, Ca^{2+} channel blocker and EGTA also reduced the light-induced PIFR of Ca^{2+} (Table 2). Therefore, the decrease in Ca^{2+} influx is not a contributor to this process. Ca^{2+} efflux across the plasma membrane mediated by Ca^{2+}-ATPase in the plasma membrane would be expected to be a main pathway for the light-induced PIFR of Ca^{2+}.

Cl^- fluxes and Cl^- transporters

Elzenga and Van Volkenburgh (1997) suggested that light-induced transient depolarization across the plasma membrane of pea mesophyll cells is at least due in part to an increased plasma membrane conductance to anions. They found that one of the anion channels identified by the patch clamp technique is characterized with a single-channel conductance of 32 picasiemens as a light-induced candidate. What is the role of Cl^- efflux and anion channels? Anion channels have higher selectivity for Cl^- than for malate (Hedrich, 1994; Schroeder, 1995). In plant cells, anion channels regulate Cl^-

efflux from cytoplasm into the extracellular space, driven by the anion-gradient across the plasma membrane and negative membrane potentials. In two types of anion channels, the S-type anion channels would produce a long-term and sustained depolarization, whereas the R-type anion channels would be involved in short and transient depolarization. The voltage-dependence and kinetics of R-type anion channels are also reminiscent of voltage-dependent Ca^{2+} and K^+ channels in excitable membranes (Bisson, 1984). The data presented showed that the two anion channel inhibitors displayed different effects on the light-induced change in net Cl^- flux. The S-type inhibitor, A-9-C, did not affect the light-induced PIFR of Cl^- (an increase in net Cl^- efflux), which was eliminated by the R-type blocker DIDS (Figure 8). A-9-C did not affect the light response in net influx of other ions (H^+, Ca^{2+} and K^+). By comparison, DIDS significantly reduced the influx of H^+ and Ca^{2+} influx during the first 5 min of illumination (Table 4). Thus, R-type, and not S-type anion channels, appear to mediate Cl^- efflux during the light response.

Most previous evidence supports the model that Cl^- efflux catalyzed by the opening of anion channels is a component of light-induced depolarization (Spalding and Cosgrove, 1992; Trebacz et al., 1994; Elzenga and Van Volkenburgh, 1997). However, Cl^- flux was not measured in their studies. The Cl^- ion can move inwardly or outwardly across the plasma membrane. Indeed, light yielded an initial and short increase in net Cl^- influx; in other words, Cl^- influx exceeds Cl^- efflux during the initial light response (see Chapter I). On the other hand, this result provides some evidence that H^+/Cl^- symport might be a pathway for the light-induced influx of both Cl^- and H^+ as discussed in Chapter I. Fusicoccin (FC) significantly increased the light-induced initial response in net Cl^- influx (Table 1) which also was enhanced in acidic bathing medium (see Chapter I). Thus, Cl^-

efflux mediated by anion channels might be involved, but is probably not the initial depolarizing component.

Calcium-dependent activation of anion channels seems to be one of its main pathways in cellular signal transduction. Conversely, Cl^- efflux in response to activation of anion channels produces further depolarization, which, in turn, leads to rapid signal amplification as additional anion channels respond to increased depolarization (Ward and Schroeder, 1997). Ward and Schroeder (1997) hypothesized that anion efflux induced by cellular stimulation results in membrane depolarization which in turn triggers the activation of voltage-dependent Ca^{2+}-channels that mediate Ca^{2+} influx. In this study, low Cl^- concentration (0.2 mM) in the bathing solution significantly limited the light-induced response in net Ca^{2+} influx. At the same low concentration of exogenous Cl^-, the light-induced initial response in net Cl^- influx significantly decreased (Table 5b), suggesting a possible relationship between Ca^{2+} influx and Cl^- fluxes or concentrations during the light response. Elzenga et al. (1995) showed that light-induced depolarization of pea mesophyll was eliminated by high Cl^- concentrations (about 10 mM, but they did not test Cl^- concentration below 1.0 mM). High external KCl concentrations, 10 mM, greatly increased the light-induced response in net Cl^- and K^+ influx but affected H^+ influx much less (Table 5b). This provides an explanation for their results. However, the possibility that high ion concentrations in the bathing medium may generate large variable ion flux changes cannot be excluded.

K^+ fluxes and K^+ transporters

As shown previously, the first K^+ response to light is a rapid increase in net K^+ influx

across the plasma membrane of pea mesophyll tissues (Figure 5). Uptake of K^+ across the plasma membrane is dominated by three possible pathways. One of these is K^+ influx through a K^+ inward rectifier. When a K^+ inward channel blocker (CsCl) was added to the bathing solution, the light-induced initial response in net K^+ influx was reduced slightly, but the maximum increase in net K^+ influx was inhibited later. Seemingly, whether the K^+ inwardly rectifier is involved in the initial light response in net K^+ influx is still unclear. Another candidate is the non-selective cation channels which are permeable for both Ca^{2+} and K^+. Both $LaCl_3$ and EGTA resulted in significant reductions in the light-induced net K^+ influx response (Table 2), which were also dependent on external K^+ concentration (Table 5a). The final possibility for K^+ influx is active K^+-H^+ symport. The proton motive force (pH gradient) generated by the H^+ pump can energetically drive K^+ uptake through a carrier (Hedrich and Schroeder, 1989; Netting , 2000). Two facts support the view that light increases K^+-H^+ symport across the plasma membrane of pea mesophyll. First, changing apoplastic pH caused a significant change in the light-induced initial response in net K^+ influx (See Chapter I). Acidic external bathing solution increased the K^+ influx. Second, the plasma membrane H^+-ATPase inhibitor, Na_3VO_4, decreased the light-induced response in net K^+ influx (Table 1). Roelfsema and Prins (1998) reported that light stimulated proton extrusion and resulted in apoplastic acidification enhancing the conductance of K^+ inward rectifying channels. Hedrich and Schroeder (1989) tried to compare the H^+-K^+ symporter with the K^+-inward rectifier. However, the flux measurement of the present experiment could not distinguish between these alternative pathways. To complicate things further, both K^+ channel blockers also significantly reduced the light-induced initial response of net H^+ influx

(Table 3), seemingly supporting the existence of a H^+-K^+ symporter. As discussed previously, the light-induced K^+ influx is not a main depolarizing ionic event. Considering light-induced transient apoplastic alkalinization and the CsCl effect on the initial response of net K^+ influx, the light-induced H^+-K^+ symport is proposed as an initial pathway for K^+ influx.

Unlike Ca^{2+} and Cl^-, light only elicited a slow PIFR of K^+ following the initial response in net K^+ influx (see Chapter I). This PIFR may be due to a decrease of K^+ influx or an increase of K^+ efflux mediated by K^+ outward rectifiers. In pea mesophyll, the K^+ channel blocker TEA eliminated most the light-induced PIFR of K^+ (Table 3), indicating that outwardly-directed K^+ channels are a main pathway for K^+ efflux and contribute to the PIFR. However, the PIFR of K^+ probably counteracts Cl^- efflux and thus results in a membrane repolarization. Ca^{2+}-activated K^+ channels were found in higher plants or green algae (Elzenga and Van Volkenburgh, 1993; Johannes et al., 1997; Bauer et al., 1998). Exogenous $LaCl_3$ and EGTA or low external Ca^{2+} concentration (0.1 mM) reduced the light-induced PIFR of K^+ (Table 2, Figure 8). However, high external Ca^{2+} concentration (2.0 mM) or addition of Ca^{2+}-ionophore did not affect the PIFR of K^+ (Figure 7, 8). A possible reason is related to the decrease in net K^+ influx when inward K^+ rectifiers were deactivated by elevated cytosolic Ca^{2+} concentrations (Lemtiri-Chlieh and MacRobbie, 1994; Blatting, 1999). In general, the K^+ outward rectifiers are activated by membrane depolarization and up-regulated by cytoplasmic pH increase, but are virtually insensitive to cytosolic Ca^{2+} (Lemtiri-Chlieh and MacRobbie, 1994; Grabov and Blatting, 1997). Keunecke and Hansen (2000) found a different pH-dependence of K^+ channel activity between bundle sheath and mesophyll cells of maize leaves.

Alternatively, as in guard cells (Allan et al., 1994), Ca^{2+}-independent pathways are assumed to also exist during the light-induced PIFR of K^+ in pea mesophyll. This process may be associated with the regulation of H^+ homeostasis and membrane potentials, which also involve other ions. The regulation of K^+ channel gating in guard cells may be accomplished by G proteins and protein phosphorylation (Thiel and Wolf, 1997). K^+ fluxes in mesophyll do not have the clearly defined specialized functions that are obvious in guard cells (Assmann, 1996). The regulation of K^+ channel activation in mesophyll cells still needs to be investigated.

The plasma membrane H^+-ATPase is not directly involved in light-induced H^+ influx and apoplastic alkalinization

Transient apoplastic alkalinization in mesophyll tissues has been demonstrated (Elzenga et al., 1995; Shabala and Newman, 1999; or see Chapter I). Stahlberg and Van Volkenburgh (1999) proposed that the plasma membrane H^+-ATPase does not directly take part in the light-induced membrane depolarization and alkalinization of pea mesophyll cells. However, previous results indicated that the plasma membrane H^+-ATPase activity increased following illumination (Marré et al., 1989; Linnemeryer et al., 1990; Remis et al., 1994). The resulting apoplastic acidification is thought to be coupled with H^+ efflux from the leaf cells. As proposed previously, CO_2 uptake by mesophyll cells and H^+-K^+ or H^+-Cl^- symport are possible reasons for the alkalinization (see Chapter I). In the present study, light induced an initial increase in extracellular pH (data not shown) and in net H^+ influx both of which were inhibited by H^+-ATPase inhibitors (Figure 2). Why did the H^+-ATPase inhibitor also reduce alkalinization? Possibly

because the inhibition of the H^+ pump by vanadate or DCCD decreased H^+ extrusion from the cytoplasm thereby reducing the proton motive force, the H^+ gradient across the plasma membrane. The resulting decrease in the light-induced net K^+ influx could be related to the blockage of H^+-ATPase (Table 1). Considering the consistent change of H^+ flux and extracellular pH, it is assumed that the plasma membrane H^+-ATPase may contribute to the light-controlled apoplastic alkalinization. If light activates the plasma membrane H^+-ATPase and H^+ extrusion, or if H^+-ATPase is a primary channel for H^+ transport, the net flux rate should be shifted to efflux. In fact, however, a transient increase in H^+ influx occurred after the onset of light stimulation, indicating that more H^+ moves inwardly or H^+ efflux was inhibited at least. Even though in the presence of FC, which hyperpolarizes the plasma membrane by activating H^+ extrusion and simultaneously enhancing K^+ influx (Ullrich and Guern, 1990), light still elicited a rapid increase in net H^+ influx (Figure 2, Table 1). Remarkably, H^+ influx is larger than its efflux; in other words, at least H^+-ATPase does not function as a direct agent in the light-induced initial response. Therefore, the present results support the conclusion of Stahlberg and Van Volkenburgh (1999).

The function of H^+-ATPase may involve feedback regulation of ion flux. Cytoplasmic pH homeostasis is attributed to the regulation of H^+ pumps and other ionic transporters, including K^+ channels and anion channels such as in guard cells (Grabov and Blatting, 1998). Stahlberg and Van Volkenburgh (1999) observed a long period of apoplastic acidification immediately following light-induced transient alkalinization of pea mesophyll, whereas vanadate (0.5 mM) eliminated this acidification but did not influence the light-induced transient depolarization. Light induced a PIFR of H^+ after a

rapid initial increase in net H^+ influx (Figure 2). An increase in H^+ efflux or a decrease in H^+ influx resulted in this process, which may contribute possibly to extracellular acidification. Treatment with FC significantly increased the PIFR of H^+, which was eliminated by vanadate or DCCD (Table 1), suggesting that the H^+ pump could be involved in this response by facilitating H^+ efflux. Proton signalling has been proposed as a second messenger in guard cells (Leckie et al., 1998). Alternatively, it is possible that H^+ flux or pH, another candidates as signals, may also be involved in light responses in pea mesophyll through a Ca^{2+}-independent pathway. Dynamic cytoplasmic H^+ changes may link Ca^{2+}-dependent pathways, or share some common signalling components in cellular signal transduction. The role of the H^+ messenger remains to be investigated.

A model: ionic pathways for light response

Figure 9 presents a schematic model integrating the observed and hypothetical events in light-induced ionic signal transduction in pea mesophyll. Upon activation of putative photoreceptors, Ca^{2+} influx through non-selective cation channels would play an initial role in transmitting the light signal and depolarizing the membrane. K^+ and Cl^- uptake coupled with H^+ influx contribute to apoplastic akalinization. Light-induced initiation of Ca^{2+} channels drives several differential secondary signalling elements, e.g., intracellular Ca^{2+} and pH, K^+- and anion-channels. A common feature for all ions measured is that light induced PIFR of ion fluxes from pea mesophyll. The processes are associated with the activities of ionic transporters (e.g., Ca^{2+}-ATPase, H^+-ATPase, K^+ channels and anion channels) in the plasma membrane or intracellular membrane systems, which contribute to membrane repolarization.

However, this research does not exclude the possibility of Ca^{2+}-independent pathways. Proton signalling coupled with ion flux changes in cellular signal transduction challenges the hypothesis above. Identification of some specific ion transporters, e.g., inward or outward K^+ rectifiers, anion channels, Ca^{2+}-ATPase, can allow discrimination among specific pathways that are presumably involved in the rapid light response. Direct measurement of dynamic changes of cytosolic ion concentration will provide more valuable evidence. Further investigation will need to link the observed early events to downstream steps, such as protein phosphorylation/dephosphorylation, Ca^{2+}/calmodulin dependent-kinases and gene expression involved in photoreception.

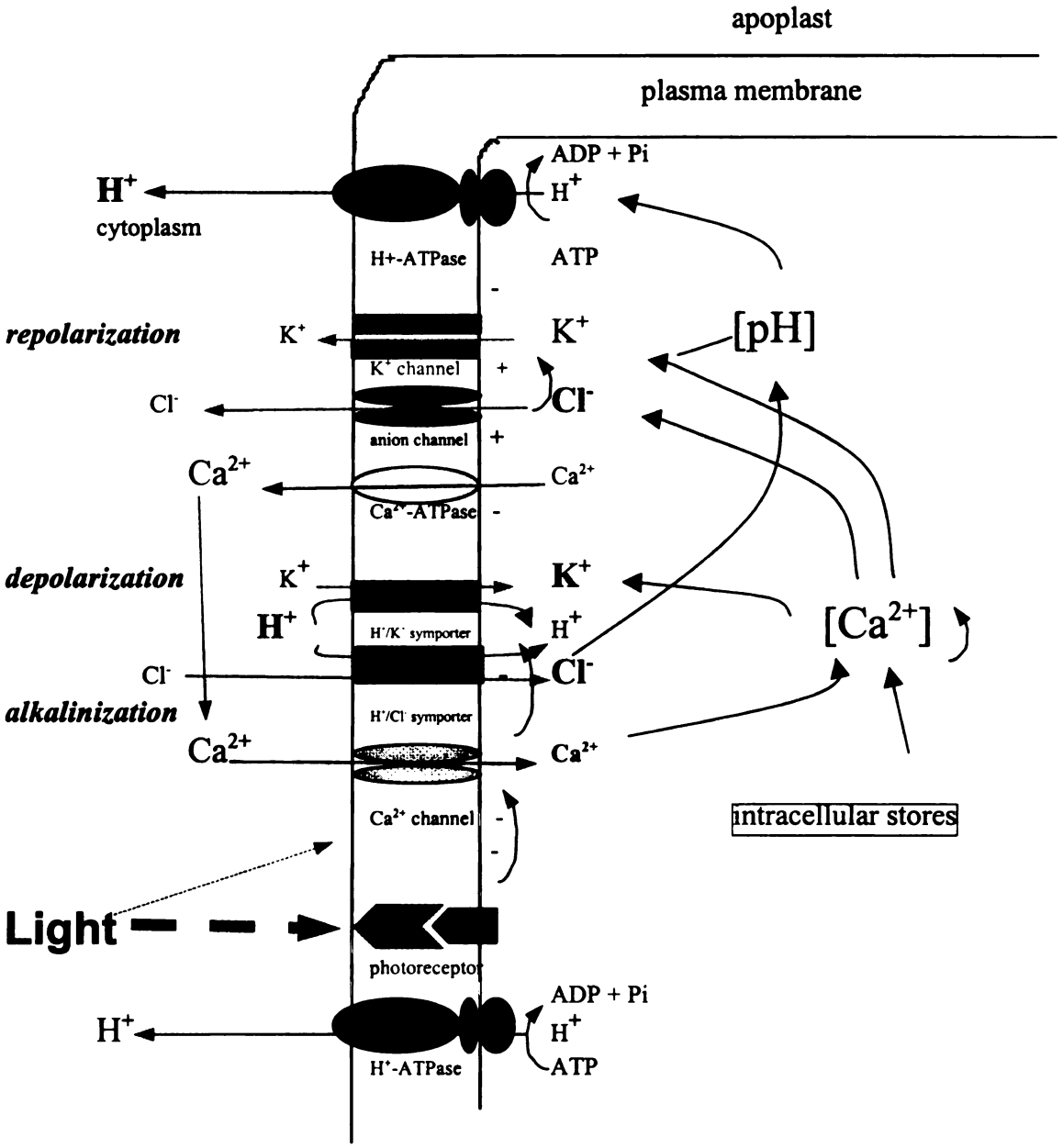

Figure 9. A hypothetical model for light-induced ion flux response in pea mesophyll cells

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Chapter III

Effects of light intensity and quality on ion flux from pea mesophyll tissues.

ABSTRACT

Mesophyll tissues from excised leaflet of pea (*Pisum sativum* L. cv. *Argenteum*) were exposed to different light intensity treatments. Five fluence rates (225, 450, 900, 1800 and 2800 μmol m^{-2} s^{-1}) were used to test fluence-response of ion flux. Light-induced changes in net Ca^{2+}, H^+, K^+ and Cl^- flux increased in a saturable manner with increasing fluence. However, high fluence increased the temperature of the bathing solution, which may have caues a heat effect on ion flux. White light (WL) of 450 μmol m^{-2} s^{-1} stimulated significant changes in net flux of net Ca^{2+}, H^+, K^+ and Cl^- from mesophyll tissues. When leaf tissues were exposed to the blue-light (BL) and red-light (RL) of similar intensity, the change in net Ca^{2+}, H^+, K^+ and Cl^- flux exhibited a similar pattern to WL. At an intensity of 225 μmol m^{-2} s^{-1}, the RL-induced initial increase in net K^+ influx was more rapid than under BL. However, there were no significant differences in the magnitude of change in net Ca^{2+}, H^+, K^+ and Cl^- fluxes between BL and RL. The BL- and RL-induced flux responses of all ions were saturated at intensities higher than 16 μmol m^{-2} s^{-1} (BL) and 75 μmol m^{-2} s^{-1} (RL), respectively. The result suggests that BL and RL play a similar role in rapid ionic responses to light.

INTRODUCTION

Light provides plants with many different kinds of information, as a result of variations in light fluence, quantity, direction and duration. Plants sense light signals through a complex sensory system consisting of different photoreceptors, which detect different light qualities over a wide spectral range, but with overlapping action spectra (Batschauer, 1998). Light energy is then used either directly or indirectly to optimize a

variety of physiological and morphological processes. Recently much attention has focused on photosensory mechanisms in plants and several light signalling pathways have been proposed (Chamovitz and Deng, 1996; Batschauer, 1998; Khurana et al., 1998). The most important aspect of understanding the regulation of photosensory mechanisms is to elucidate the function of photoreceptors. At least three classes of photosensory pigments (photoreceptors) have been implicated in the perception of light signals in plants, including phytochromes, blue/UV-A photoreceptors, and UV-B photoreceptors. The multiple photoreceptor classes imply the presence of a highly responsive, intricate, and yet finely tuned, cellular signal transduction machinery (Batschauer, 1998). However, the chemical structure and function of receptors are not fully understood.

Early events in the transduction of light-mediated signals in plants are often associated with transient changes of membrane potential. This bioelectrogensis reflects changes in ion fluxes across the plasma membrane. Light-induced rapid ion flux changes have been demonstrated in many plant species, such as *Dunalliella acidophila* (Remis et al., 1994), *Physcomitrella patens* (Johannes et al., 1997), and *Vicia fava* (Shabala and Newman, 1999). Both red and blue lights have been reported to elicit electrical signalling or ionic flux changes, especially for guard cells (Serrano and Zeiger, 1989; Assmann and Shimazaki, 1999). Red light matching the absorption spectrum of chlorophyll, stimulates an outward electrical current that may be carried by the plasma membrane H^+-ATPase (Serrano et al., 1988). Blue light given in a single pulse stimulates stomatal opening (Zeiger et al., 1987) and induces proton extrusion from guard cell protoplasts (Assmann et al., 1985; Shimazaki et al., 1993). In addition, since an anion channel in *Arabidopsis* hypocotyl tissue was shown to be activated by BL (Cho and Spalding, 1996), it is likely

that BL would affect similar anion channels in guard cells. Shacklock et al. (1992) demonstrated that RL induces protoplast swelling and causes a transient Ca^{2+} increase followed by a rapid reduction in cytoplasmic Ca^{2+} concentrations to below resting levels. The BL-induced depolarization in etiolated cucumber hypocotyls involves the inhibition of a proton-pumping ATPase and an influx of Ca^{2+} (Spalding and Cosgrove, 1992). However, the reports on pulvinar movements are complicated by the fact that phytochromes, blue-light photoreceptors, and underlying circadian rhythms all play roles in regulating turgor changes (Briggs and Liscum, 1997). Recently, Suh et al. (2000) characterized BL activated K^+ efflux channels in flexor cells from *Samanea saman*. Lew et al. (1990) also had reported phytochrome-activated K^+ channels in *Mougeotis* protoplasts.

Clearly, light elicits transient electrical signalling which is mediated by ion fluxes across the plasma membranes of plant cells. However, the identity of the light quality which plays a main role in regulating ion flux remains to be investigated. In previous reports, the light-induced rapid flux changes of Ca^{2+}, H^+, K^+ and Cl^- had been demonstrated in pea mesophyll. Some specific ion transporters were proposed to be involved in the light-induced ionic flux responses (See Chapter II). Based upon the differential reports above, we hypothesize that either BL or RL, or both, may be involved in the regulation of light-induced ion flux responses. In addition, light intensity would be expected to influence ion flux changes in pea mesophyll cells. To test our hypotheses, BL and RL, were used to evaluate the effects of light quality on ion fluxes. Different fluence treatments were also imposed to determine ionic responses.

MATERIALS AND METHODS

Plant materials

Seeds of pea (*Pisum sativum* L. cv. *Argenteum*) were germinated for 4-5 d. Plants then were transferred to a growth chamber with a 16 h photoperiod, day/night temperatures of 20/25 °C, 75% relative humidity, and a fluence of 200 μmol m^{-2} s^{-1} at the top of the canopy. The pea seedlings were cultured using modified Hoagland's solution (Johnson et al., 1957), gradually increasing the concentration from 0 to ¼ to ½ strength at 4-5 d intervals.

Flux measurements

The ion fluxes and extracellular ion concentrations at the surface of the leaf mesophyll were measured using the MIFE system. Technical details of this system are similar to that generally as described in Chapter I and by Shabala et al. (1997). From time course data obtained by the MIFE system, the directionality and rates of ion flux could be estimated. Additional information on the MIFE is available at http://www.phys.utas.edu.au/ physics/biophys. In brief, micropipettes with a tip diameter of 2 - 3 μm were pulled from 1.5 mm-diameter borosilicate glass capillaries (catalog no. MIB 150-6; World Precision Instruments, Inc., Sarasota, FL) with a horizontal electrode puller (PC-10, Narishige, Tokyo, Japan). After silanization, cooled microelectrodes were back-filled with 500 mM $CaCl_2$ for Ca^{2+}, 500 mM KCl for K^+ and Cl^-, and 15 mM NaCl plus 40 mM KH_2PO_4 (adjusted to pH 6.0 with NaOH) for H^+. Electrode tips were then front-filled with a liquid-membrane-type of the appropriate ion-selective sensor, H^+ (no. 95291), Ca^{2+} (no. 21048), K^+ (no. 60031) and Cl^- (no. 24902) cocktails (all from Fluka,

Milwaukee, WI), until a 50- to 80- μm long column of ionophore was produced. Microelectrodes were calibrated for each ion using a known set of standards before and after use. The Ag/AgCl reference electrode was prepared using a plastic tube containing 1000 mM KCl in 2% agar.

After calibration, the ion-selective electrodes were slowly vibrated by a micromanipulator which provided three-dimensional positioning. The microelectrode/ micromanipulator set-up was mounted on the stage of an inverted microscope (Leitz, Wetzlar, Germany). A measuring chamber with leaf sample was placed on a holder that was connected to a three-way hydraulic micromanipulator (WR 88, Narishige, Tokyo, Japan) driven by a computer-controlled stepper motor (MO61-CE08, Superior Electric, Bristol, CT). The MIFE system allows measurement of three ions simultaneously. Three microelectrodes can be vibrated at any angle in a two-dimensional plane by separately controlling the amplitude of the slow vibration of the micromanipulator.

The MIFE system measured the voltage differences at the two extreme positions of vibration, which provided the basis for estimation of both the rate and directionality of net ion fluxes. In this study the electrodes were moved in a 20-s square wave cycle between 50 to 90 μm above the leaf surface at a low frequency (0.1 Hz). Ion concentration of ions was calculated from the voltage potentials at each position using the calculation curve for the specific microelectrode. The net flux for each specific ion was estimated from the measurements of the difference in the electrochemical potential between these positions (Shabala et al., 1997). The first 4 s of each half cycle (10 s) were not used for concentration and flux estimation. This was considered to be the minimum delay time necessary to obtain a stable measurement following movement of the

microelectrodes. Instability was thought to be caused by solution turbulence and physical vibrational noise following movement.

Light sources

Light sources were provided by a fiber-optic light projector (CUDA Products Corporation, Jacksonville, FL) with a 250 W halogen bulb (GS Edmumd Scientific Company, Barrington, NJ). A lens (no L-16-16, World Precision Instruments, Inc., Sarasota, FL) was fastened on the top of a light guide to focus the beam. The blue light (BL) and red light (RL) treatments were achieved by a blue glass filter (5-60, thickness 4.5 mm, Kopp Glass Inc., Pittsburgh, PA) and a red glass filter (2-61, thickness 4.5 mm, Kopp Glass Inc., Pittsburgh, PA), respectively, which were placed in front of the optics in the lens. The spectra of white-, blue- and red-light sources were determined using Li-1800 spectroradiometer (LI-COR, Lincoln, NE). The white light delivered a broad spectra between 400-1000 nm (Figure 1A). However, most of light lies between 500-800 nm. The long wavelength (above 800 nm) fluence was decreased by 45-47 % after filtering with distilled water (Figure 1). The blue source emitted light of wavelengths between 375-525 nm with peak emission at around 450 nm (Figure 1). The output of the red source included light of wavelengths between 610-1000 nm. The peak of light emission was between 650-740 nm (Figure 1). The blue- and red-light fluence rates were measured using a quantum sensor (model LI-189, LI-COR, Lincoln, NE).

In this study, White-light (WL) of 450 μmol m^{-2} s^{-1} was used as light treatment. When the WL was filtered by either the blue- or red-glass, the BL of 32 μmol m^{-2} s^{-1} and the RL of 225 μmol m^{-2} s^{-1} were generated, respectively.

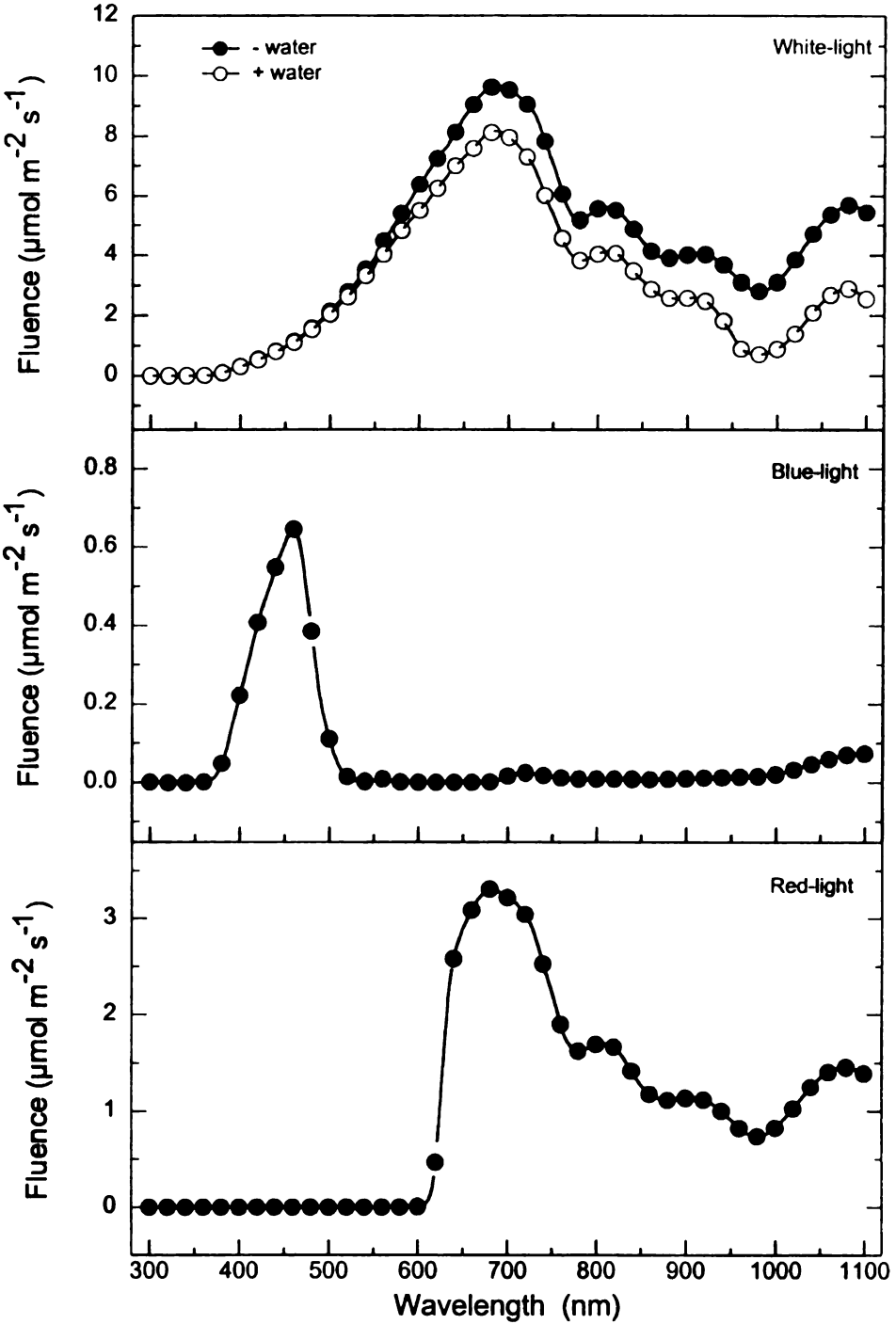

Figure 1. Fluence of white, blue and red lights over a range of wavelengths.

Experimental protocol

Young expanding leaflets were harvested and a leaf section with limited veinal tissue of approximately 15 × 10 mm (L×W) was surgically cut from the lamina. A small strip of epidermal tissue was peeled from the abaxial surface of the lamina of each section so as to avoid incurring mechanical damage to the underlying veinal and mesophyll tissue (Long and Widders, 1990). Isolated leaf segments were incubated in a buffered solution (0.5 mM $CaSO_4$, 1.0 mM KCl, 2.5 mM HEPES-HCl, and 10 mM sucrose at pH 5.2) under 60 W m^{-2} illumination (Philips lamp). After 2.0-2.5 h, the cut segment was mounted in a measuring chamber. The measuring chamber contained about 6-7 mL of unbuffered solution or bathing solution (0.5 mM $CaSO_4$, 1.0 mM KCl, pH 5.2), and the segments then were exposed to 50 - 60 min of darkness before light treatment.

All experiments were conducted in unbuffered solution at room temperature (23±1°C). Flux measurement was continued for an average of 30 min from the onset of illumination and then following the onset of darkness for 10-15 min.

Solution temperature

The temperatures of the solutions during illumination were measured using a thermocouple thermometer (Model 600-2050, Barnant Company, Barrington, IL). To increase the solution temperature, a warmer bathing solution was expelled into the bathing solution (23±1°C) and then repeatedly sucked and expelled with a mL pipette for 5 - 6 times. The period of time during which warmer solution had been added, mixed and equilibrated was ignored when analyzing ionic concentration and flux data. This

represented about 3-4 min total. Change of temperature of the bathing solution could be maintained within a range of 1-2 °C during 15-20 min measurement. All solutions were unbuffered (0.5 mM $CaSO_4$, 1.0 mM KCl, pH 5.2).

Data statistical analysis

Data analysis has been described in Chapter I. Analysis of variance (ANOVA) of the data was conducted using SAS 8 (SAS Institute Inc, Cary, NC).

RESULTS

Effects of light intensity on ion fluxes

Since net flux is a balance between influx and efflux, a positive net influx response is the result of enhanced influx or reduced efflux of the ion in question. In this study, five fluence rates of White-light (WL) (225, 450, 900, 1800 and 2800 μmol m^{-2} s^{-1}) was used to test the effects of light intensity. Under different light intensities, the net fluxes of Ca^{2+}, H^+, K^+ and Cl^- around pea mesophyll exhibited a similar response trend, i.e. a light-induced initial response in net ion influx (more positive net flux) followed by a Post-Initial Flux Response (PIFR) (see Chapter I). The PIFR could theoretically involve either independent changes or concurrent changes in both influx and efflux. Therefore, either a decrease in influx or an increase in efflux or both can elicit a negative PIFR. In general, the light-induced initial responses in net influx of all ions were the smallest at the lowest intensity (225 μmol m^{-2} s^{-1}). Further increase in light intensity did not produce a significant difference in the responses of net H^+ and Cl^- influx between intensities from 450 to 2800 μmol $m^{-2}s^{-1}$ (Figure 2). Net Ca^{2+} and K^+ fluxes exhibited saturation kinetics

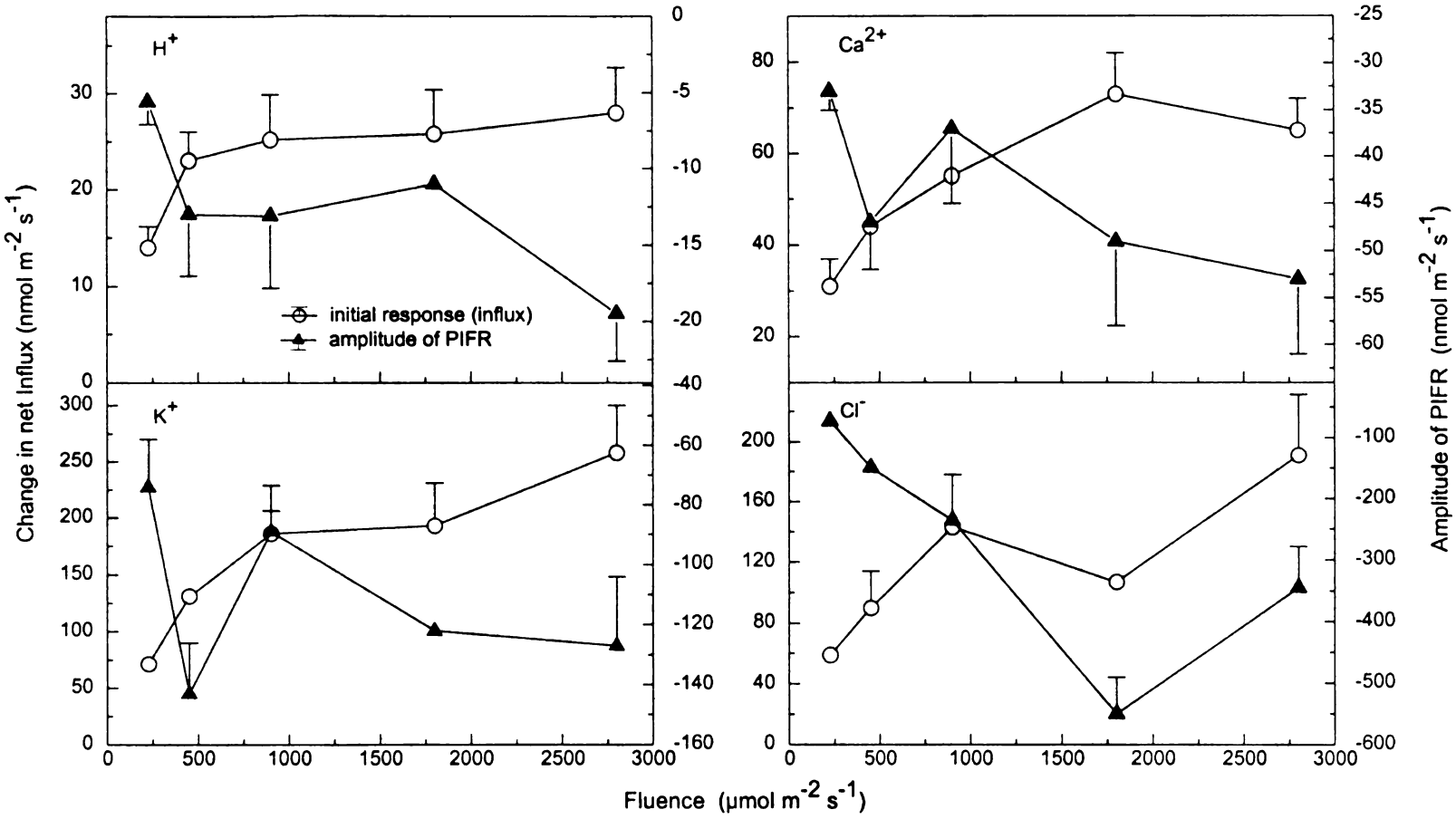

Figure 2. Effect of white-light intensity on flux responses of H^+, Ca^{2+}, K^+ and Cl^- around pea mesophyll tissues. Data points represent an average with 3-5 replicates. Vertical bars represent the standard error of the mean. Change in net ion influx indicates the light-induced initial response (a positive value). Amplitude of light-induced Post-Initial Flux Response (PIFR) indicates a decrease in influx or an increase in efflux or both (a negative value).

in the initial influx response at light intensities above 900 μmol m^{-2} s^{-1}. The light-induced PIFRs of H^+, Ca^{2+} and K^+ were saturated above 450 μmol m^{-2} s^{-1}. In contrast, high light intensities (1800 - 2800 μmol m^{-2} s^{-1}) enhanced dramatically the amplitude of PIFR of Cl^- (Figure 2).

Effects of solution temperature on ion fluxes

When the temperature of the bathing solution increased by 1.0-1.5 °C or 1.5-2.5 °C, the net fluxes of Ca^{2+}, H^+, K^+ and Cl^- from pea mesophyll did not change significantly in darkness during 15-20 min of measurement (Figure 3). A 30 min illumination of 225 and 450 μmol m^{-2} s^{-1} generated an increase in solution temperature of 0.6 °C and 1.3 °C, respectively (Figure 4). This indicates that the heat effect of these two light intensities is not consequential to ion flux. However, when light intensity was further increased to 900, 1800 and 2800 μmol m^{-2} s^{-1}, the temperature of the bathing solution was enhanced by 2.6, 3.8 and 5.0 °C with 20 - 30 min, respectively (Figure 4). At 2800 μmol m^{-2} s^{-1}, in particular, the increase of temperature was quite rapid. Red-light at intensities of 450, 1170 and 1490 μmol m^{-2} s^{-1} also heated up the bathing solution by 1.1, 2.1 and 3.2 °C within 30 min, respectively (Figure 4). The highest fluence of red-light (1490 μmol m^{-2} s^{-1}) produced a rapid increase in solution temperature. A large flux change for Ca^{2+}, H^+, K^+ and Cl^- was observed when the temperature was increased by 3 - 4 or 4 - 5 °C in darkness within 15-20 min (Figure 3). Therefore, high fluence is expected to cause heat effects on ion fluxes during experiments of 30 min.

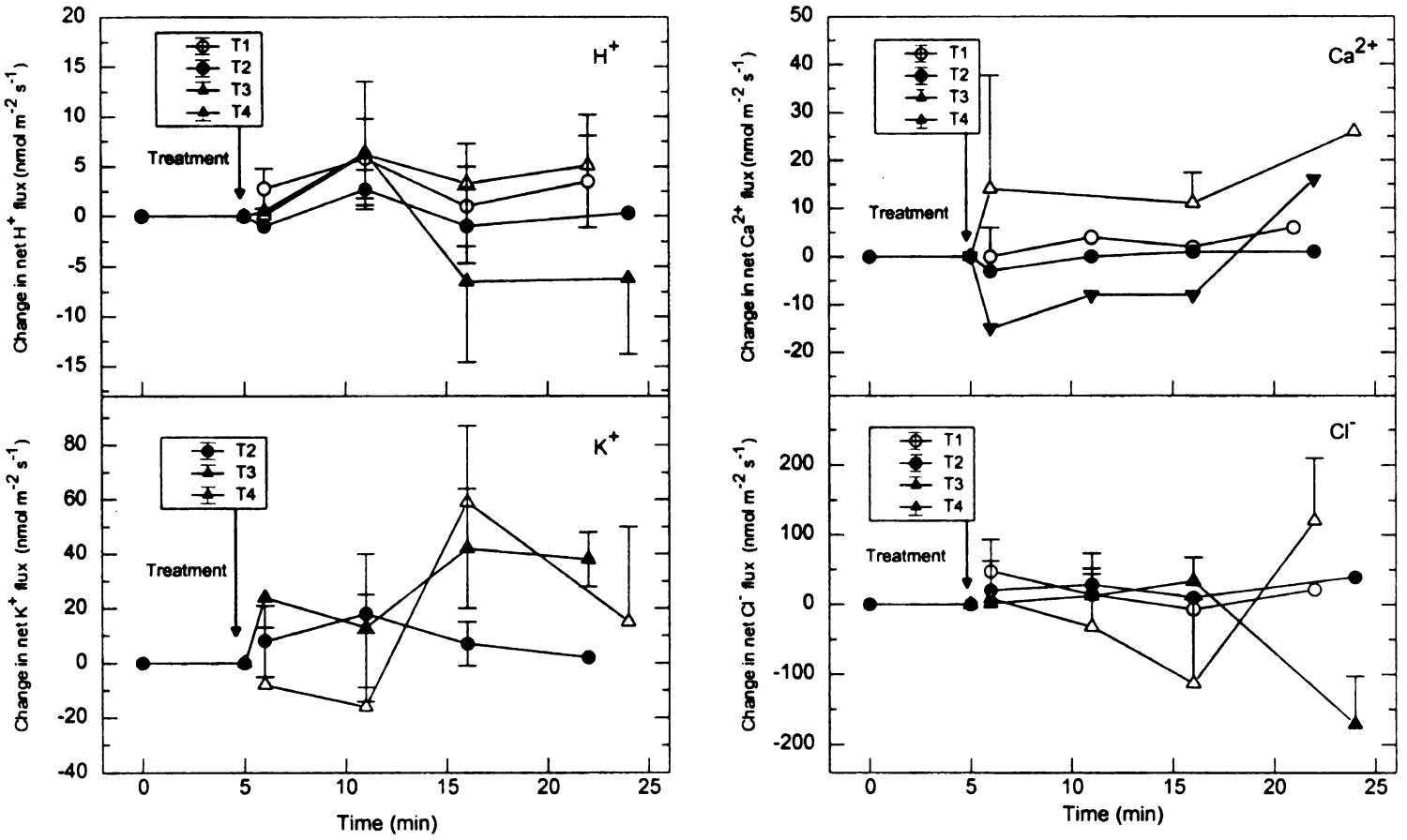

Figure 3. Effects of increasing solution temperature on net fluxes of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues. Data points represent an average of 2-5 replicates. Vertical bars represent standard error of the mean. Treatment temperature increases: T1 = 1-1.5 °C; T2 = 1.5-2.5 °C; T3 = 3-4 °C; T4 = 4-5°C. The base temperature was 23±1°C.

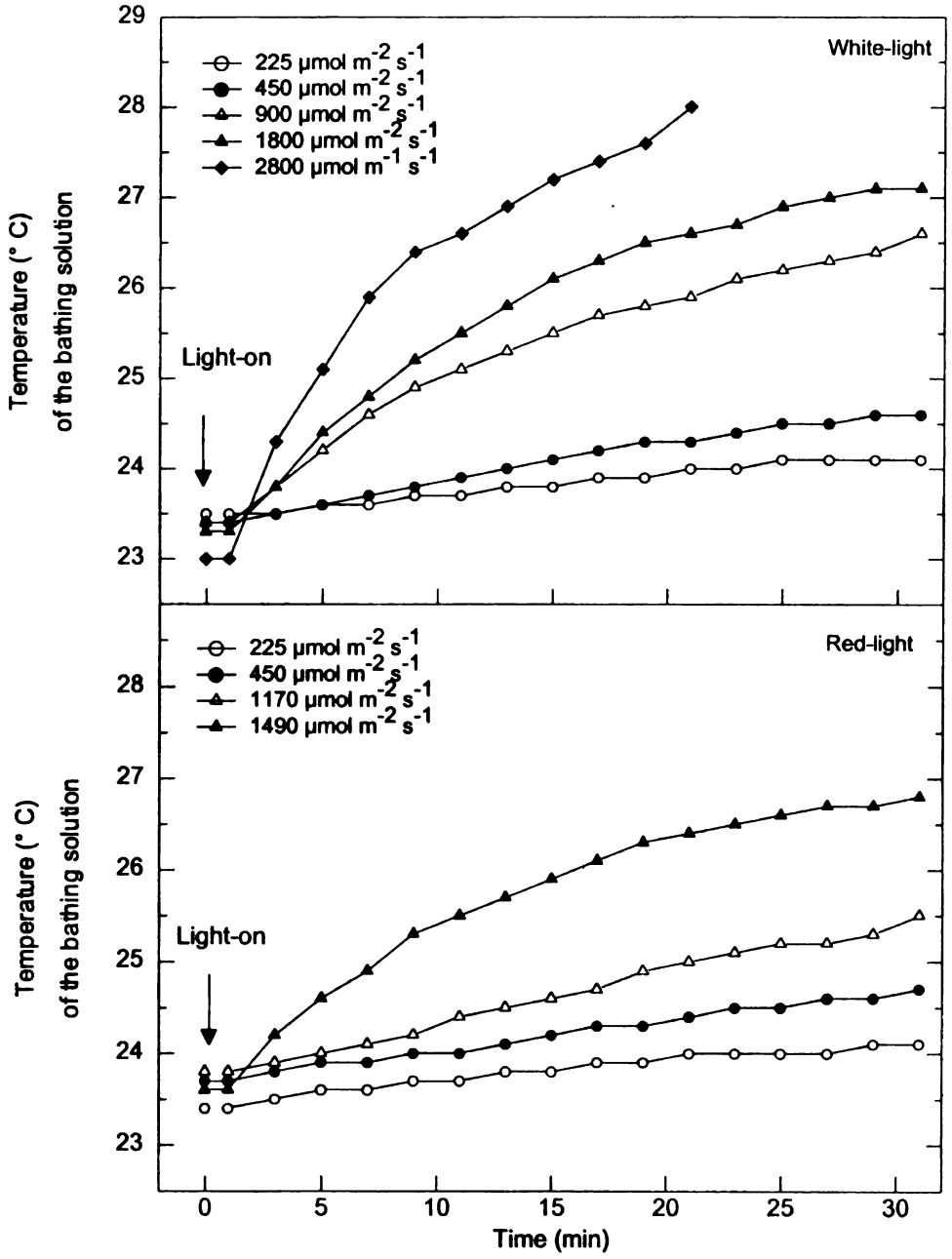

Figure 4. Effect of white and red lights on temperature of the bathing solution. Data points represent an average of 3 replicates.

Effects of blue-light on ion fluxes

Like WL, a BL exposure of 32 μmol m^{-2} s^{-1} induced an initial response in net H^+, Ca^{2+}, K^+ and Cl^- influxes (Figure 5). This was followed by Post-Initial Flux Responses (PIFRs) of H^+, Ca^{2+}, K^+ and Cl^-. The net fluxes of all ions decreased to a net efflux (negative value) or returned to the original value before the BL treatment when BL was turned off (Figure 5). Compared with the WL of 450 μmol m^{-2} s^{-1}, the magnitude of BL-induced initial response in net influx of H^+ and Ca^{2+} was significantly smaller (Table 1). The BL-induced changes in net H^+ flux during the first min declined as compared to WL. In particular, the BL-induced change in net K^+ flux was much smaller than WL-induced during the first 5 min (Figure 5). The BL-induced PIFRs for Ca^{2+}, K^+ and Cl^- decreased significantly, especially for K^+ and Cl^- which were 58 % and 74 % lower than the WL-induced PIFR, respectively. During the first 5 min, the flux change of Cl^- was positive, i.e., BL caused a significant increase in net Cl^- flux. In contrast, WL caused a decrease in net Cl^- flux (Figure 5). The BL-induced initial responses of net K^+ and Cl^- flux were approximately 3-4 min and 2-3 min slower, respectively, than the WL-induced initial response (Table 1).

To examine the BL-fluence-response relationships of ion flux, five BL intensities were used: 16, 32, 70, 160, and 225 μmol m^{-2} s^{-1}. These BL treatments elicited a similar ion flux response as shown in Figure 5. The initial response of net ion influx was the smallest under the low BL intensity (16 μmol m^{-2} s^{-1}) for all ions (Ca^{2+}, H^+, K^+ and Cl^-) (Figure 6). Above 32 μmol m^{-2} s^{-1}, the BL-induced initial response in net H^+, K^+ and Cl^- influx increased and exhibited light saturation kinetics. For Ca^{2+}, higher BL intensities (above 70 μmol m^{-2} s^{-1}) generated larger initial response in net influx (Figure 6). With

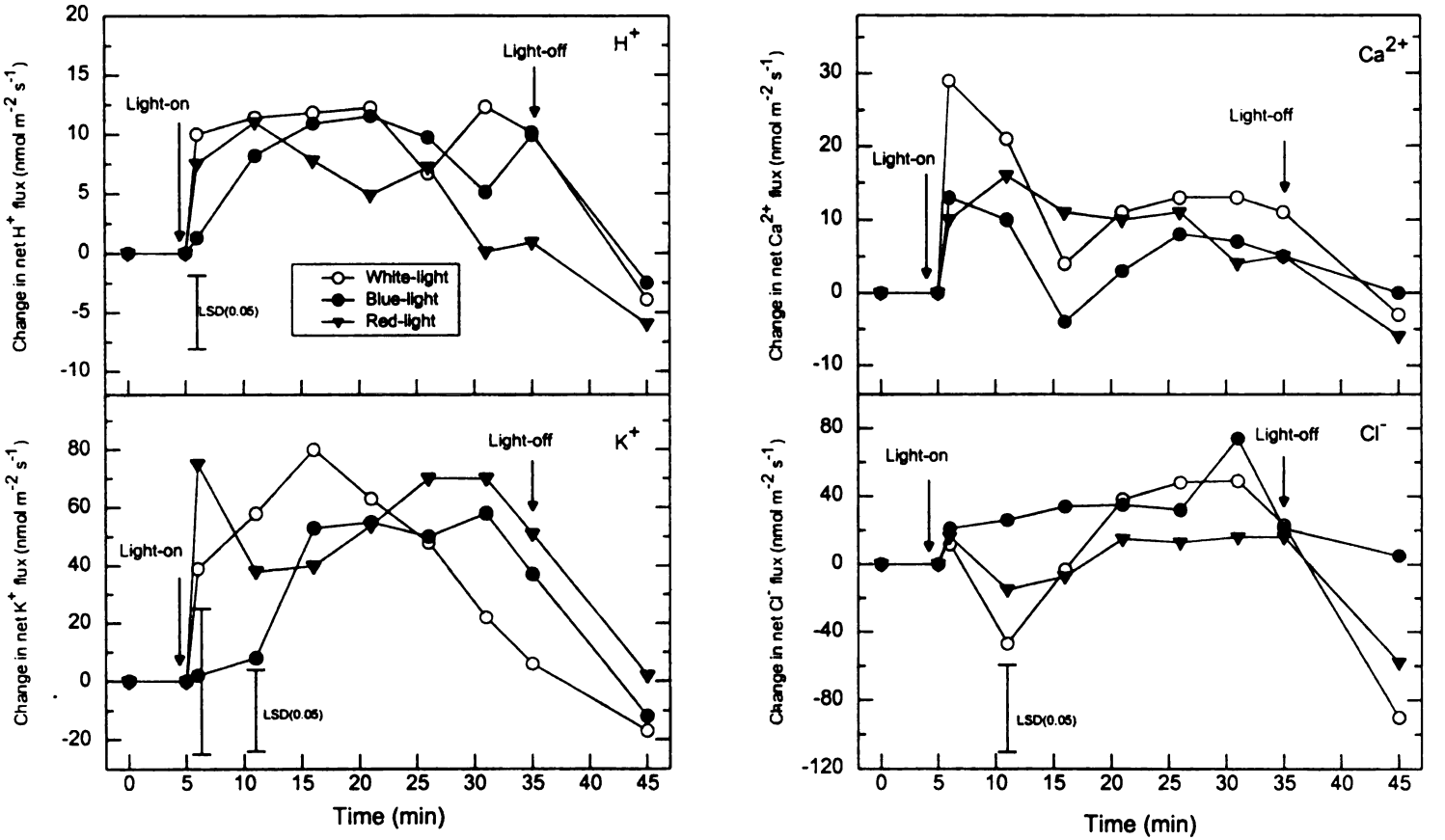

Figure 5. Effect of light quality on time course of net flux of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues. Data points represent an average of 8-10 replicates. Vertical bars represent LSD test result at $p<0.05$. Light intensity of white-light (WL), blue-light (BL) and red-light (RL) are 450, 32 and 225 μmol m^{-2} s^{-1}, respectively.

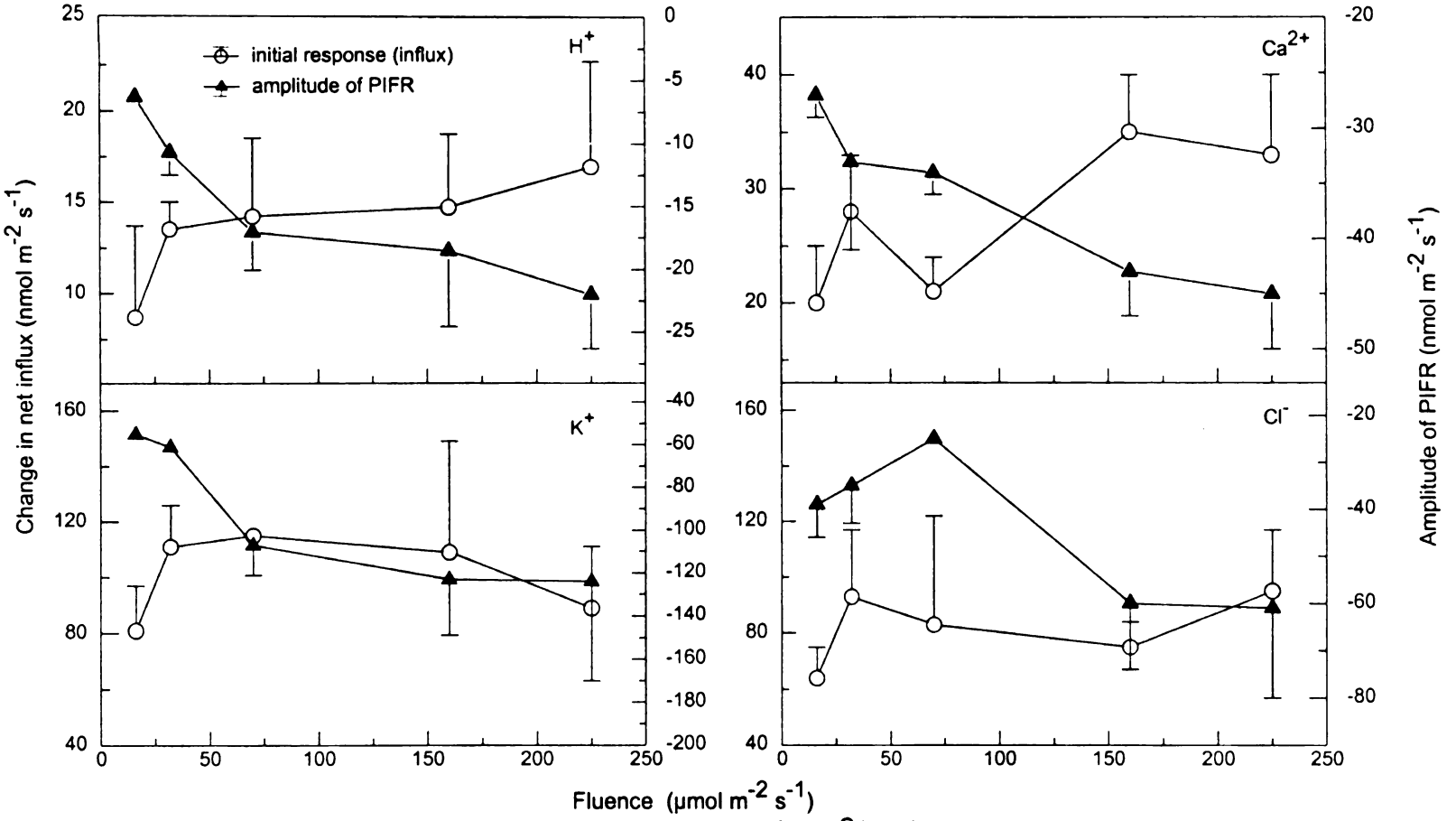

Figure 6. Effect of blue-light intensity on flux responses of H^+, Ca^{2+}, K^+ and Cl^- around pea mesophyll tissues. Data points represent an average with 3-5 replicates. Vertical bars represent the standard error of the mean. Change in net ion influx indicates the light-induced initial response (a positive value). Amplitude of light-induced Post-Initial Flux Response (PIFR) indicates a decrease in influx or an increase in efflux or both (a negative value).

increasing intensity, the BL-induced PIFR of the four ions increased but was saturated above 70 μmol m^{-2} s^{-1} (for H^+ and K^+) or above 160 μmol m^{-2} s^{-1} (for Ca^{2+} and Cl^-) (Figure 6).

Effects of red-light on ion fluxes

Red-light of 225 μmol m^{-2} s^{-1} produced similar net flux responses for H^+, Ca^{2+}, K^+ and Cl^- as observed following WL or BL exposure. A return to dark conditions caused a decrease in net ion flux (negative) similar to WL and BL (Figure 5). Compared with the WL of 450 μmol m^{-2} s^{-1}, the initial net influx responses of H^+, Ca^{2+}, K^+ and Cl^- were not significantly influenced by RL of 225 μmol m^{-2} s^{-1} (Table 1). However, the RL-induced PIFRs of K^+ and Cl^- were significantly lower than WL (Table 1). In most cases, there were no significant differences between RL- and BL-effects. The significant difference was that RL induced a rapid net K^+ influx (75 and 38 nmol m^{-2} s^{-1}, respectively) which was larger than BL (2 and 8 nmol m^{-2} s^{-1}, respectively) during the first min and during the first 5 min after illumination (Figure 5). In addition, RL caused a decrease in net Cl^- flux (-15 nmol m^{-2} s^{-1}) whereas BL still increased net Cl^- flux (+26 nmol m^{-2} s^{-1}) during the first 5 min of illumination (Figure 5). The initial RL response time for Cl^- (3.0 min) was significantly shorter than BL (5.5 min) (Table 1).

Five RL intensities, 75, 225, 450, 1170 and 1490 μmol m^{-2} s^{-1}, were tested to evaluate the RL fluence-response on ion flux. The highest two intensities caused an increase in solution temperature of approximately 3-4 and 4-5 °C with in 20 - 30 min (Figure 4). Therefore, these RL exposures would be expected to generate heat effects on ion flux. However, low fluence (below 450 μmol m^{-2} s^{-1}) did not cause significant heating of the

Table 1. Effects of white-, blue- and red-light on net fluxes of H^+, Ca^{2+}, K^+ and Cl^- from pea mesophyll tissues.

	H^+	Ca^{2+}	K^+	Cl^-
Initial flux response (influx)		(nmol m^{-2} s^{-1})		
White light	+[†]22 (8)[§] a	+44 (8) a	+142 (10)	+124 (8)
Blue-light	+14 (8) b	+35 (8) b	+118 (8)	+93 (8)
Red-light	+19 (8) ab	+41 (8) ab	+145 (8) ns	+80 (8) ns
Amplitude of PIFR[‡]		(nmol m^{-2} s^{-1})		
White light	-17 (8)	-59 (8) a	-196 (10) a	-161 (8) a
Blue-light	-11 (8)	-41 (8) b	-82 (8) b	-39 (8) b
Red-light	-19 (8) ns	-45 (8) ab	-111 (8) b	-85 (8) c
Initial light response time		(min)		
White light	4.1 (8)	2.4 (8)	4.3 (10) b	2.8 (8) b
Blue-light	4.3 (8)	3.6 (8)	8.4 (8) a	5.5 (8) a
Red-light	3.0 (8) ns	4.0 (8) ns	2.5 (8) b	3.0 (8) b

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.
"ns" indicates no significant difference.

White-light fluence is 450μmol m^{-2} s^{-1}. Blue- and red-light fluences are 32 and 225 μmol m^{-2} s^{-1}, respectively, as a result of filtering the white light.

[§] The numbers in parentheses indicate the number of replicates for each treatment.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

solution. Like WL, all RL intensities exhibited a similar response in net ion fluxes as shown in Figure 5. There were no significant differences of the initial response of net H^+, Ca^{2+}, and Cl^- influx among all RL intensities except at the lowest (75 μmol m^{-2} s^{-1}) (Figure 7). The RL-induced initial response of net K^+ influx was intensity-dependent below 450 μmol m^{-2} s^{-1} but became saturated when RL intensity was further increased. Higher RL fluence rates (1170 and 1490 μmol m^{-2} s^{-1}) also enhanced the initial H^+ influx response (Figure 7). This again was possibly due to heat effects. The RL-induced PIFR by the four ions increased in magnitude but became saturated above 225 μmol m^{-2} s^{-1} for H^+, Ca^{2+} and K^+. However, the higher fluence (1490 μmol m^{-2} s^{-1}) caused a large increase in the PIFR of Cl^- which is also thought to be due to the heating effects (Figure 7).

Comparison between blue- and red-light effects on ion fluxes

Comparison was conduced when all lights were a similar fluence of 225 μmol m^{-2} s^{-1}. Blue-light produced a smaller change in net Ca^{2+} flux than WL did during the first min (Table 2a). The PFIR of H^+ induced by both BL and RL were significantly larger than the WL-induced PIFR. However, no significant differences in the initial response of net H^+ influx were observed between the three lights. In the same manner, the WL, BL and RL did also not produce a significant difference in either the light-induced initial flux response or the light-induced PIFR of Cl^- and K^+ (Table 2a).

When both BL and RL intensities were reduced to 70-75 μmol m^{-2} s^{-1}, no significant differences of ionic light response were observed between BL and RL. Both BL and RL

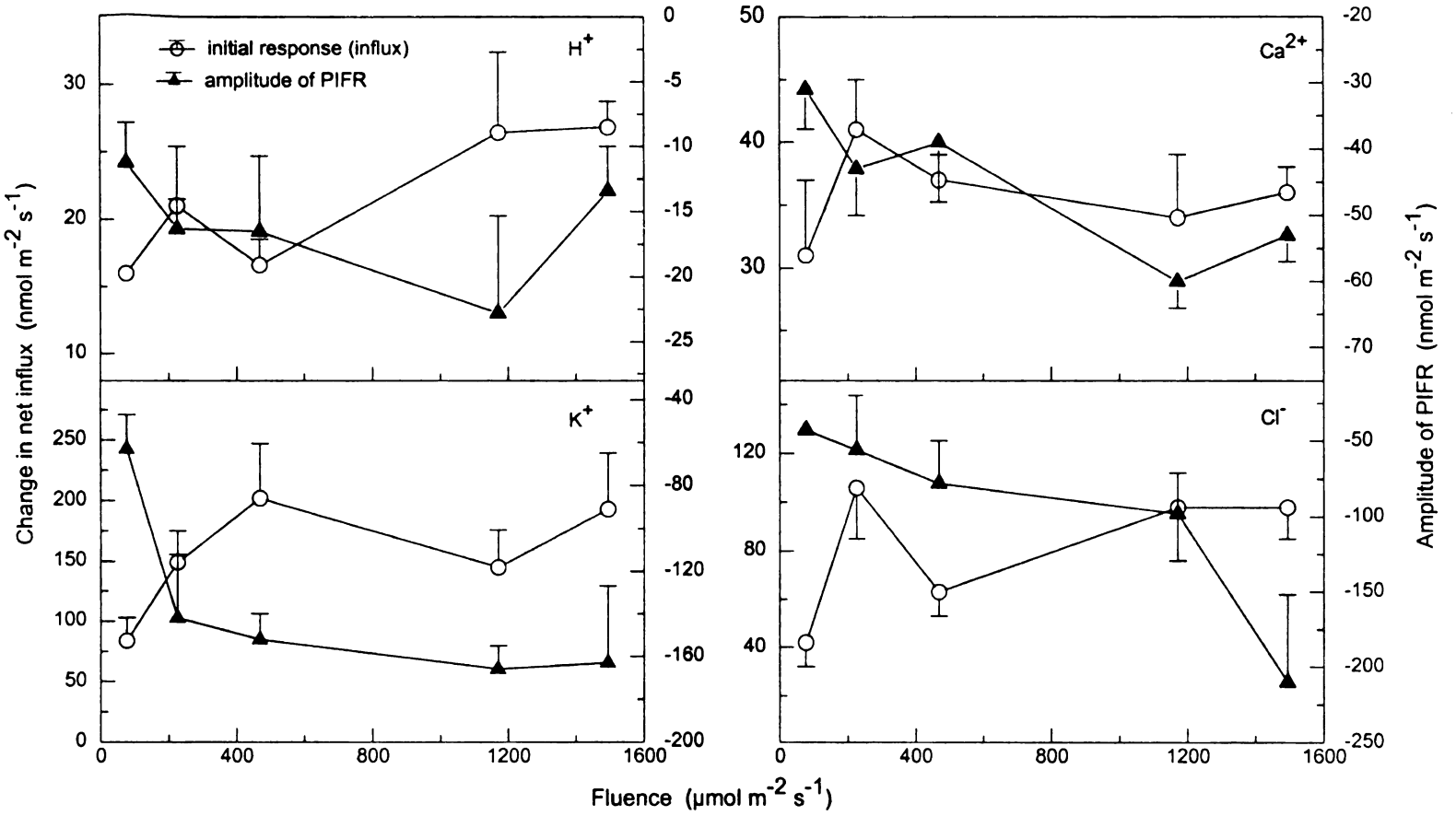

Figure 7. Effect of red-light intensity on flux responses of H^+, Ca^{2+}, K^+ and Cl^- around pea mesophyll tissues. Data points represent an average with 3-5 replicates. Vertical bars represent the standard error of the mean. Change in net ion influx indicates the light-induced initial response (a positive value). Amplitude of light-induced Post-Initial Flux Response (PIFR) indicates a decrease in influx or an increase in efflux or both (a negative value).

Table 2. Effects of white-, blue- and red-light on ion flux rate in pea mesophyll tissues at similar light fluences.

a: at 225 μmol m^{-2} s^{-1}

	H^+	Ca^{2+}	K^+	Cl^-
	(nmol m^{-2} s^{-1})			
Initial flux response (influx)				
White light	+[†]14(5)[§]	+31 (5)	+71 (3)	+27 (3)
Blue-light	+17 (5)	+26 (3)	+89 (4)	+95 (4)
Red-light	+21 (3) ns	+41 (4) ns	+113 (4) ns	+106 (4) ns
Flux change during the first min				
White light	+4 (5)	+24 (5) a	+42 (3)	+3 (3)
Blue-light	+11 (5)	-8 (3) b	+5 (4)	+12 (4)
Red-light	+2 (5) ns	+5 (4) ab	+92 (4) ns	+14 (5) ns
Amplitude of PIFR[‡]				
White light	-6 (5) b	-33 (5)	-74 (3)	-73 (3)
Blue-light	-22 (3) a	-45 (3)	-124 (4)	-61 (2)
Red-light	-16 (5) a	-43 (4) ns	-114 (4) ns	-56 (3) ns

b: at 70-75 μmol m^{-2} s^{-1}

	H^+	Ca^{2+}	K^+	Cl^-
	(nmol m^{-2} s^{-1})			
Initial flux response (influx)				
Blue-light	+14 (4)	+21(4)	+115 (4)	+83 (3)
Red-light	+16 (4) ns	+31(5) ns	+84 (4) ns	+42 (4) ns
Amplitude of PIFR				
Blue-light	-18 (4)	-34 (4)	-96 (4)	-25 (2)
Red-light	-11 (4) ns	-31 (5) ns	-63 (4) ns	-43 (3) ns

Different letters indicate significant difference between treatments within columns at $p < 0.05$ according to LSD test.
"ns" indicates no significant difference.

[§] The numbers in parentheses indicate the number of replicates.

[†] - or + indicates decrease or increase of net ion flux and extracellular ion concentration, respectively.

[‡] **PIFR**: Light-induced Post-Initial Flux Response, a result of either a decrease in net influx or an increase in net efflux, or both. This value is negative.

produced similar initial flux responses or amplitudes of PIFR of H^+, Ca^{2+}, K^+ and Cl (Table 2b).

DISCUSSION

Effects of light intensity

Previous studies on light-induced ionic or electrical responses in plants have used an intermediate fluence within the range of 200 - 400 μmol m^{-2} s^{-1}. Only a few studies have evaluated light responses to different light intensities. Elzenga et al. (1995) found that light-induced depolarization in pea mesophyll tissues depended, in a saturable way, on light intensities between 50-1000 μmol m^{-2} s^{-1}. Lower intensities were observed to result in less depolarization of the plasma membrane and a delay in the light response. The light-induced polar pH reaction in *Elodea canadensis* was also found to be reduced at lower light intensities (Elzenga and Prins, 1989). In the current study, the light responses in the net fluxes of Ca^{2+}, H^+, K^+ and Cl^- were limited at the lowest WL fluence (225 μmol m^{-2} s^{-1}) (Figure 2). Although higher fluence (450 - 1800 μmol m^{-2} s^{-1}) produced a higher magnitude of net influx changes for all ions, that of net H^+ and Cl^- influx appeared to saturate. Under 450 μmol m^{-2} s^{-1}, the light-induced PIFR of Ca^{2+}, H^+ and K^+ also were saturated (Figure 2). Elzenga et al. (1995) also reported that light-induced depolarization of pea mesophyll was saturated at approximately 400 μmol m^{-2} s^{-1}.

However, the increase in solution temperature during light exposure constitutes a potential problem in measurement of ion flux at high fluence. Both factors, light intensity and solution temperature, should be considered when one evaluates the effect of

light on ion flux. Most researchers ignore or do not consider the light-induced heat effects on leaf tissues at lower radiance levels (Stahlberg and Van Volkenburgh, 1999, Shabala and Newman, 1999). High fluence was found to increase bathing solution temperature by approximately 4 - 5 °C within 20 min (Figure 4). Therefore, temperature effects caused by high fluence on ion flux must be considered and calculated. Bathing solution temperature increase of 3 - 5 °C clearly increase net flux kinetics of Ca^{2+}, H^+, K^+ and Cl^- under darkness. By contrast, smaller increases in temperature (1.0 - 2.5 °C) did not affect net ion flux in a measurable manner (Figure 3). Therefore, high fluence-induced changes of ion fluxes could be expected to result from two factors, light intensity and heat. In other words, ion flux changes induced by high intensities (1800 and 2800 μmol m^{-2} s^{-1}) should not be only explained on the basis of light fluence, but also include possible heat effects. Temperature directly affects the kinetics of ion transport across the membranes in biological systems. For example, Lee and Satter (1988) found that temperature had a large influence on H^+ release from extensor cells of *Samanea saman* pulvini. In this study, it is difficult to differentiate between light- and temperature-effects at high fluence. The present work indicates that radiance of 450 of μmol m^{-2} s^{-1} is enough to stimulate ionic responses to light.

Effects of light quality

Light (WL) induced a rapid increase in net H^+ influx from pea mesophyll tissues (see Chapter I). Both RL and BL also elicited a similar time-course change of net H^+ (Figure 5). At the same fluence, no significant differences in the initial response in net H^+ influx

was observed among the three light treatments (Table 2). However, the BL- and RL-induced PIFR for H^+ were significantly larger than that of WL- at 225 μmol m^{-2} s^{-1} (Table 2a). As discussed in Chapter II, the plasma membrane H^+-ATPase may contribute to the light-induced PIFR of H^+ causing apoplastic acidification due to increased proton efflux from mesophyll cells. Both lights stimulate proton efflux (Assmann et al., 1985; Serrano et al., 1988). However, several contradictory opinions exist in the literature. Nishizaki (1992) found that BL and RL elicited membrane depolarization and hyperpolarization, respectively. He proposed that a pulse of BL inhibited the activity of the proton-ATPase in pulvinar motor cells. Spalding and Cosgrove (1992), on the other hand, suggested that the mechanism of BL-induced membrane depolarization of cucumber hypocotyls initially involves inhibition of a H^+-ATPase. This study provides indirect evidence for the role of both BL and RL in stimulating proton efflux. Additionally, the fluence-responses to both BL and RL were similar for the initial response of net H^+ influx (Figure 6, 7).

It is well established that light induces a transient increase in net Ca^{2+} influx. Like H^+, the effects of both BL and RL on net Ca^{2+} flux were similar to that of WL (Figure 5). Elevated concentrations of cytosolic Ca^{2+} were found in RL- (Shacklock et al., 1992; Neuhaus et al., 1993; Ermolayeva et al., 1996) and BL-treated cells (Christie and Jenkins, 1996; Sharma et al., 1997). The role of Ca^{2+} as a cellular messenger, has been suggested to play a role in both BL and RL signal transduction. However, evidence for a direct link between Ca^{2+} transport and light quality is still lacking. The present results support the hypothesis that both light qualities might initially increase net Ca^{2+} influx. The most likely pathway for Ca^{2+} influx into cytoplasm would be through Ca^{2+} channels in the plasma membrane (see Chapter II) resulting in a transient increase in cytosolic free Ca^{2+}

concentration.

Both BL and RL stimulated K^+ uptake by guard cells (MacRobbie, 1988) and by bean mesophyll (Blum et al., 1992). In pea mesophyll, RL induced a rapid and large increase in net K^+ influx from pea mesophyll (Figure 5). In comparison, the BL-induced change in net K^+ influx was slower and smaller during the first min of illumination. However, such result was not observed at low RL fluence of 75 μmol m^{-2} s^{-1} (Table 2b). Similarly, no difference between both BL and RL existed in the magnitude of the initial response in net K^+ influx (Table 2). These differences are mainly reflected in the initial light response time. Furthermore, the BL- and RL-induced PIFRs of K^+ were also similar (Table 2). As proposed in Chapter II, the light-induced PIFR of K^+ flux change may be associated with a enhanced net K^+ efflux mediated by K^+ channels. Both light qualities were found to stimulate K^+ channel activity. For example, BL activated K^+ efflux channels in flexor cells of *Samanea saman* (Suh et al. 2000), whereas phytochrome activated K^+ channels in *Mougeotis* protoplasts (Lew et al., 1990). Subsequent research demonstrated that one or two Ca^{2+}-activated K^+ channels were involved (Lew et al., 1992). According to the present results in pea mesophyll, BL and RL should be considered to play a role in the light-induced K^+ flux response in a similar manner.

Blue-light-activated anion channels have been identified in leaves of *Arabidopsis* (Cho and Spalding, 1996; Lewis et al., 1997). These authors speculated that Cl^- efflux, mediated by anion channels, is responsible for BL-induced membrane depolarization. However, the activity of anion channels does not explain fully the change of Cl^- flux across the plasma membrane. Except efflux through anion channels, Cl^- can move

inwardly into cytoplasm through a H^+/Cl^- symport mechanism (Sanders et al., 1985). The net flux reflects a balance between influx and efflux across the plasma membrane in pea mesophyll. In the present study, both BL and RL stimulated similar changes in net Cl^- influx and the PIFR of Cl^- at the same fluence (Table 2). The light-induced PIFR of Cl^- has been considered to be the result of Cl^- efflux catalyzed by anion channels within the plasma membrane (see Chapter II).

Taken together the present data suggest that both BL and RL all induced flux responses of H^+, Ca^{2+}, K^+ and Cl^- in a similar manner. The same phenomenon was also found in effects of BL and RL on the activity of ion transport across the plasma membrane of guard cells (Assmann et al., 1985; Zeiger et al., 1987; Serranno et al., 1988; Shimazaki et al., 1993). This indicates that BL + RL regions of the light spectrum can elicit ionic flux responses in pea mesophyll tissues. The similarity in ionic responses to BL and RL are consistent with the conclusion in Chapter I that ion flux responses in pea mesophyll are photosynthesis dependent. This is supported by the finding that DCMU, a photosynthestic inhibitor, also inhibited the light-induced initial responses in net ion flux (see Chapter I) and removed light-induced membrane depolarization (Stahlberg and Van Volkenburgh, 1999) in pea mesophyll. The current study provides a starting point for further investigations on potential involvement of photoreceptor.

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SUMMARY

Light signalling is becoming a main area of research in plant science. Plant scientists try to elucidate photosensory mechanisms through genetic, biochemical and biophysical approaches. Membrane potential changes are commonly observed responses to many stimuli. However, the ionic basis for such stimulus-elcited membrane potential changes remains to be determined. Ion transport mechanisms and kinetics are frequently not understood. It is known that ion activity gradients and fluxes exist across cellular membranes, and this fact provides a potential target for studying electrical signalling. Because electrical signals are short-lived, it is difficult to use biochemical and molecular techniques for investigation of such responses. In contrast, ion-microelectrode technology can measure rapid ion kinetics in a time-course manner around plant cells without tissue damage.

Microelectrode Ion Flux Estimation (MIFE) is a slowly vibrating ion-selective microelectrode system developed by Shabala and Newman (Shabala et al., 1997; Shabala and Newman, 1999). The MIFE has three advantages. First, it can measure the simultaneous flux of three ions, thus one can observe multiple ion movements across mesophyll plasma membrane. Second, it provides a stable and anti-noise environment during an experiment. In most cases, changes in extracellular ion concentration with are consistent with changes in net ion flux, the enabling the identification and exclusion of artifacts. Third, this system contains a software package to automatically record all

measurements and to estimate flux. Therefore, the MIFE allows direct, dynamic and continuous quantitative estimation of ion kinetics around cells.

To focus on mesophyll cells, a small strip of epidermal tissue was gently removed from pea leaflets with little or no wounding. One cannot fully discount the possibility, however, that peeling may elicit some type of long-term wound response which may predispose the tissue to responding to light in a certain manner and thus give rise to artifacts. On the other hand, extensive research with peeled leaf lamina tissue indicates that ion flux kinetics is similar to intact tissue. The peeled mesophyll cells can easily be viewed with an inverted microscope. This configuration ensures that measured ion fluxes are predominately from mesophyll cells.

Although Elzenga et al. (1995) observed a rapid light-induced membrane depolarization (25 mV) in pea mesophyll cells, they did not provide further evidence for the ionic basis of bioelectrogenesis. This dissertation demonstrates a significant, short-term, light-induced ion flux response of pea mesophyll tissues. Rapid initial increases in net Ca^{2+}, H^+, K^+ and Cl^- flux from mesophyll cells were measured after onset of illumination. The response of Ca^{2+} flux was the most rapid (13 s), whereas H^+, Cl^- and K^+ flux responses started within approximately 29, 45, and 48 s, respectively, after stimulation. Following the initial response, a light-induced Post-Initial Flux Response (PIFR) occurred, which led to a decrease in net flux. The PIFR time of Ca^{2+} and Cl^- was shorter (3-4 min).

Ion transport across the plasma membrane can alter membrane potentials. Assuming that Ca^{2+}, H^+, K^+ and Cl^- are primary ions involved in light-induced changes in membrane potential, this study showed that the time course of summed net charge flux was similar

to that of the light-induced membrane potential reported previously (Elzenga et al., 1995; Stahlberg and Van Volkenburgh, 1999). A light-induced initial increase in the net charge flux would be expected to depolarize the plasma membrane. Therefore, this dissertation presents a effective and useful method to estimate the involvement of ion fluxes in electrical signalling.

Calcium functioning as a second messenger is thought to play a critical role in cellular signal transduction in plant cells. An increase in free Ca^{2+} concentrations in the cytoplasm occurs in most stimulus-responses. The present results support Ca^{2+} influx as an initial depolarizing agent in the light-induced membrane depolarization (Spalding et al., 1992; Shabala and Newman, 1999). However, other researchers have not yet provided experimental evidence for a Ca^{2+} influx mechanism. This study showed that the light-induced initial net Ca^{2+} influx was significantly inhibited by 42-44 % in the presence of the Ca^{2+} channel blocker $LaCl_3$ and the chelator EGTA. Apparently, the light-induced initial response in net Ca^{2+} flux is the result of an increase in influx mediated by Ca^{2+} channels, rather than a decrease in efflux. The increased Ca^{2+} influx would be expected to elevate cytoplasmic Ca^{2+} concentrations, thus initiating cellular signal transduction. The effects of Ca^{2+} influx on other ion flux responses also provide additional evidence for this hypothesis. Both $LaCl_3$ and EGTA significantly reduced the light-induced initial H^+ and K^+ response and PIFR of Cl^-. The light-induced PIFR of Cl^- were also significantly enhanced by both Ca^{2+} ionophore A23187 and high exogenous Ca^{2+} concentration. Therefore, this dissertation may be the first to experimentally demonstrate that Ca^{2+} influx through Ca^{2+} channels represents an initial step in light-induced membrane depolarization.

A light-induced extracellular alkalinization (pH increase of 0.4 - 0.5) was observed in the current study. The results were anticipated due to CO_2 uptake and H^+-mediated K^+ or Cl^- uptake by mesophyll cells. Shabala and Newman (1999) also concluded that CO_2 uptake of bean mesophyll cells was a reason of light-induced increase in apoplast pH. However, the plasma membrane H^+-ATPase inhibitors, Na_3VO_4 and DCCD, inhibited the light-induced initial response in net H^+ influx by 50-60 % and eliminated the PIFR of H^+. The presence of Na_3VO_4 in the bathing solution also significantly suppressed the light-induced increase in net K^+ influx. In contrast, the ATPase activator, Fusicoccin significantly increased the light-induced PIFR of H^+ and the initial response in net Cl^- influx. In addition, external pH in the bathing solution affected further the light-induced response in net K^+ and Cl^- influx. These results suggest that H^+-ATPase in the plasma membrane is not directly involved in the initial light response. The enzyme is involved in mediating H^+/K^+ and H^+/Cl^- symports, which contribute to light-induced initial extracellular alkalinization.

Anion channels may also be involved in membrane potential changes. Elzenga and Van Volkenburgh (1997) also reported increased activity of an anion channel in pea mesophyll cells in response to light. However, they did not measure Cl^- flux, which was a main reason for the light-induced PIFR of Cl^- in my study. Testing with anion channel blockers indicated that rapid-type anion channels play a role in increasing Cl^- efflux, but anion channels are not involved in light-induced initial Cl^- flux response. This result is different from that of Elzenga and Van Volkenburgh (1997).

Another contribution of this dissertation is the demonstration that the light-induced PIFR is associated with ion transporters, including Ca^{2+}-ATPase, H^+-ATPase, K^+ channels

and anion channels in the plasma membrane. Differential alteration in ion transporter activity suggests that multiple ion transport pathways function during short-term light responses. To date, such ion flux evidence has not been reported. Most research has just focused on the effects of ion transporter inhibitors or activators on membrane potentials or on ion concentration change in bulk solution surrounding plant tissues.

In this dissertation, I proposed a model for short-term light responses, based on experimental evidence, and provided a possible explanation for bioelectrogenesis. Ion flux responses represent an important component of cellular signalling systems in plants to external stimuli. This model also provides a potential approach for future research in signal transduction of plants.

Both blue light and red light display a similar action in controlling stomotal movement (Serranno and Zeiger, 1989; Assmann and Shimazaki et al., 1999). Similar effects of both wavelengths on ion fluxes in pea mesophyll cells were found in the present study. The fact that DCMU treatment inhibited the light-induced H^+ and Ca^{2+} flux responses suggests that the ionic responses to light are photosynthesis-dependent. The wide action spectra of the ion flux response introduces a further question; namely whether photoreceptors are ever involved in the light-induced ion flux response? Combining MIFE technology with specific mutants will enhance the possibility of ascertaining the involvement of specific photoreceptors in the future research.

Although the MIFE system can estimate net ion flux dynamics in the apoplast, the technique does not directly measure intracellular ion concentration change. Some attempts have been made to detect cytosolic ion concentrations and pH with improved ion-selective microelectrode techniques, such as the double-barreled electrode and the

pH-electrode (Felle, 1993). In further studies, the MIFE system might be used to test Ca^{2+} concentration and pH in the cytoplasm with improved microelectrodes.

The present study does not exclude the possibility of Ca^{2+}-independent mechanisms. Hydrogen signalling, coupled with ion flux changes may constitute an alternate mechanism for cellular signal transduction. Further research is needed to test such a hypothesis. A combination of both MIFE and patch-clamp techniques would provide further discrimination among specific ion transporters, for instance, inward or outward K^+ rectifiers and anion channels.

My data in this dissertation and studies by others (Shabala et al., 1997; Shabala and Newman, 1999) indicate that, among the ion microelectrode techniques, the MIFE system is a most powerful tool to measure ion flux in plant cells. The further improvement of the MIFE system will open many new interesting avenues of research, including the elucidation of signal transduction pathways in response to pathogen infection, salinity, cold, wounding, etc. It would also be interesting to investigate localized ion flux changes in remote tissues of whole plants when stimuli have been shown to elicit systemic response in plants, thus indicating the occurrence of long-distance signal systems.

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