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Topical Review

Ion Channel Gating in Plants: Physiological Implications and Integration for Stomatal Function

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Introduction

Primary evidence of ion channel activity in plant cells can be traced back to the first half of this century. Cole and Curtis (1938), in their pioneering studies of neuronal action potentials, made use of giant Characean algal cells in the winter months when squid axons were not available. They noted the parallel to the action potential of the nerve membrane; like the nerve, Characean action potentials were accompanied by a 30- to 50-fold rise in membrane conductance. Subsequent work by Gaffey and Mullins (1958), Hope (1961), Findlay (1962) and Kishimoto (1965) established the ionic nature of the Characean action potential and fundamental features of the transient, especially its gating, the voltage dependence of which gives rise to the self-propagating wave of the action potential. Similar observations could be drawn from higher plants, including the insectivorous plant *Dionaea,* albeit in much less detail (*see* Sibaoka, 1969). These studies fostered a view (*cf.* Simons, 1981; Hill & Findlay, 1981) that in plants, as in animals, gated ion fluxes can serve to conduct and transduce signals between stimulus and response. Nonetheless, the plant kingdom offers relatively few examples of such excitability and, in the case of the Characean algae, its physiological significance (but not its scientific value) remains obscure. Thus, until recently, ion channels were generally seen as curiosities well removed from the dominant themes of transport relations in plant cells.

The advent of the patch electrode, planar bilayer, and associated recording techniques has had as great (if not greater) an impact on our understanding of plant cell membranes as it has on that of animal cells. Now at the end of the decade which saw the first single-channel recordings from plant protoplasts (Moran et al., 1984; Schroeder, Hedrich & Fernandez, 1984), we recognize the diversity and, more still, the ubiquity of ion channels in excitable as well as nonexcitable membranes of plants. Ion channels have been found in higher plant cells, algae and fungi, and in all the major membranes of the cell, including the plasma membrane, tonoplast, mitochondrial and chloroplast thylakoid membranes (*cf.* Hedrich & Schroeder, 1989; Tester, 1990; also the many abstracts in Dainty et al., 1989).

Only recently has research been directed beyond a simple compilation of ion channels to questions of ion-channel interaction, channel regulation and their implications for physiological function in plants. Many of the gross biophysical features exhibited by plant ion channels do find counterparts in those of animal membranes, including ion selectivities, voltage and ionic dependencies, sensitivities to a range of channel agonists and antagonists, and mechanisms for ion permeation (e.g. Schroeder, 1988; Tester, 1988*a,b*; Boult et al., 1989; Moran, Fox & Satter, 1990; Thiel & Blatt, 1991; *see also* Tester, 1990). Even so, there is an obvious danger to the logic that channel behavior in plant cells must therefore anticipate the established themes for channel function (Hedrich & Schroeder, 1989); the habits of plants often entail different roles, and possibly divergent structural features for ion channels. Already documented are some subtle, but fundamental distinctions in channel gating apparently unique to plants (*cf.* Tyerman, Findlay & Paterson, 1986*a,b*; Iijima & Hagiwara, 1987; Blatt, 1988*a*, 1991*a*; Schroeder, 1988; Ketchum, Shrier & Poole, 1989) and more surprises are undoubtedly in store. Thus the challenge lies not only in detailing the biophysical and biochemical characteristics of these channels, but in understanding their integration within the physiology of plant membrane transport as a whole.

Key Words stomatal guard cell · abscisic acid · ion channel activation · signal transduction · transport control · H^+-ATPase

This review focuses on aspects of channel gating as it bears on known or postulated roles for ion channels in plants cells, and specifically on channel function in transport across the guard cell plasma membrane. Much of our present understanding of ion channels at the physiological level in plants has developed from work with stomatal guard cells, and sufficient detail is available now for an integrated view of channel behavior in stomatal function; it is here, too, that fundamental gaps in our knowledge of plant ion channels are most conspicuous. A number of reviews on guard cells have appeared in the literature (Outlaw, 1983; Willmer, 1984; MacRobbie, 1987, 1988*a,b*; Raschke, 1987; Hedrich & Schroeder, 1989), and readers will be interested also in related developments on turgor regulation in giant algae (Okazaki & Tazawa, 1990) as well as more general aspects of ion channel and transport characteristics of plant membranes (Baker & Hall, 1988; Sanders, 1990; Tester, 1990).

Experimental Approaches

New technologies frequently overtake research for a time, both within and peripheral to the purposes for which they are originally designed. Electrophysiologists working with plant cells have sought, in the new patch electrode methodologies, a means to overcoming a number of obstacles unique to these tissues. In large measure, these techniques have proven successful; but, like intracellular microelectrode recording, there remain important, albeit distinctive limitations on the scope for measurements and, hence, on the information which can be obtained. Thus, a few notes on these complementary recording techniques in relation to plant cell structure are in order.

For the electrophysiologist, three features of plant cells are outstanding. (1) Plant cells typically are characterized by a thin (2–15 μm) layer of cytoplasm bounded, on the outside, by the plasma membrane and cell wall and, on the inside, by the tonoplast and central vacuole. (2) Plant cells maintain hydrostatic (turgor) pressures of several atmospheres on the wall; the osmotic contents of the cells generally fall in the range of 300–500 mOsm, while the immediate environment of the higher-plant and fresh-water algal cell can be described as a dilute solution of simple salts for most purposes. (3) In higher-plant tissues, the cytoplasmic compartments of individual cells commonly interconnect via tens or even hundereds of junctions—plasmodesmata—each of which is large enough to permit the free diffusion of molecules with molecular weights of several hundred Daltons (*cf.* Terry & Robards, 1987; Tucker, Mauzerall & Tucker, 1989; also, in relation to guard cells, Erwee, Goodwin & Van Bel, Palevitz & Hepler, 1985).

Electrical Geometries and Intracellular Recording

Regardless of the approach, channel and other transporter currents must be measured under conditions in which membrane voltage is brought under experimental control. In conventional electrophysiological recording, using intracellular microelectrodes to record voltage and to pass current for voltage clamping, this requires some knowledge of the relevant electrical "geometry" of the cell; in the final analysis, an accounting for the temporal and spatial characteristics of the current distribution over the entire surface of the membrane is essential. For intact plant cells, this means that electrical coupling via plasmodesmata must be taken into consideration. The situation is compounded, too, by potential problems of recording serially across two membranes, the tonoplast and the plasma membrane.

Because of uncertainties in ascribing conductances between two or more membranes in series, it is the latter issue which has often been given a high profile in work with intracellular microelectrodes (Findlay & Hope, 1976; Goldsmith & Goldsmith, 1978; Hedrich & Schroeder, 1989). In practice, the emphasis is misplaced and irrelevant to most recording situations; there is now convincing evidence that successful impalements of plant cells leave the microelectrode tip in the cytoplasm (Blatt, 1987*a*; Miller & Sanders, 1987; Felle, 1988; Blatt, Thiel & Trentham, 1990*a*; Gilroy, Read & Trewavas, 1990; McAinsh, Brownlee & Hetherington, 1990), so obviating any concern about currents passing across both plasma membrane and tonoplast. Even in the event of serial recording across both membranes, the errors introduced would likely be much less severe than might be anticipated (*cf.* Spray, Harris & Bennett, 1981; Neyton & Trautmann, 1985); the period during action potentials excluded, the conductance of the tonoplast is sufficiently high that measurements would be dominated by the characteristics of the plasma membrane (*see* Smith, 1983; Bentrup et al., 1986; Hedrich & Neher, 1987; Tester, Beilby & Shimmen, 1987). The well-documented distinctions between cytoplasmic and vacuolar recordings in *Chara* (*see* Beilby, 1990; *also* Findlay & Hope, 1964) indicate that the errors incurred are minimal and limited to the voltage extremes.

In fact, electrical coupling between cells presents the most severe limitation to intracellular recordings from all but a few higher-plant cells (*cf.*

Goldsmith & Goldsmith, 1978; Spanswick, 1981; Blatt, 1991*b*). The difficulties of current passage within syncitia are not unique to plants (*cf.* Eisenberg & Johnson, 1970; Eisenberg, Barcilon & Mathias, 1979; Jack, Noble & Tsien, 1983), but few plant tissues show a spatial homogeneity which might lend itself to any simplifying assumptions (Eisenberg et al., 1979; Parsons & Sanders, 1989). It is hardly surprising, then, that the voltage-clamp studies crucial to quantitative analyses of charge transport have been restricted to a few, geometrically tractable preparations, such as fungi (*cf.* Gradmann et al., 1978; Sanders, 1988), rhizoids (Felle & Bentrup, 1976) and, among higher-plant cells lacking functional plasmodesmata, stomatal guard cells (Blatt, 1987*b*).

Patching and Protoplasts

The principal advantage of the patch electrode lies in its application to single-channel enzymology; but, until recently (*cf.* Bertl & Gradmann, 1987; Laver & Walker, 1987; Bertl, Klieber & Gradmann, 1988; Bertl, 1989; Laver, 1990), attention in work with plant cells has been drawn rather to the "peripheral" benefits in controlling solute compositions on both sides of the membrane and in affording direct access to endomembranes such as the tonoplast. Its potential for broad-range application to higher-plant cells is also an advantage of the patch electrode. Current distribution is not an issue in patch-electrode measurements with plant cells. The requirement for an exposed membrane surface means that measurements are generally carried out with isolated protoplasts after removing the cell wall enzymatically. Furthermore, effective control of membrane voltage can be expected, even when recording in the "whole-protoplast" mode with protoplasts from large mesophyll cells (Schauf & Wilson, 1987; Moran et al., 1988); the specific resistivity of the plant plasma membrane is typically 10-fold higher than that of most animal cells (Sanders & Slayman, 1989), thereby facilitating a low relative access resistance via standard patch electrodes.

These methods, too, are subject to some important trade-offs. From the standpoint of physiological analyses, dialysis of the cytoplasm against the electrode solution is clearly a two-edged sword (*see* Schroeder, 1988; Schroeder & Hagiwara, 1989; Blatt, 1990; Tester, 1990); it offers a means to manipulating both transport currents and putative intermediates of signalling cascades but leaves questions about their significance in vivo unanswered. For plant cells, such issues are further compounded by the need to isolate and maintain protoplasts under osmotically neutral conditions, thus making extrapolations to the membrane characteristics of the intact plant cell all the more difficult. These are problems which will need special attention, notably in relation to the functioning of stretch-activated channels in plants.

Transport and Gating Characteristics

Two Classes of K^+ Channels Predominate

Potassium channels are a prominent feature of the plasma membrane in guard cells and, generally, in higher-plant cells and algae (*cf.* Tester, 1990). Of these, attention so far has been drawn primarily to the two predominant classes of K^+ channels which exhibit appreciable voltage sensitivities, rectifying either outward or inward. A much wider spectrum of K^+ channels can be anticipated, however, in light of evidence for phenotypically heterogeneous K^+ channels in animal cells which arise from splicing variants within a gene "super-family" (Butler et al., 1989; Chandy et al., 1990; Schwarz et al., 1990). Schroeder (1989) mentions the occurrence of a second, low conductance K^+ channel (5 pS in 35 mM K^+_o : 210 mM K^+_i) which rectifies outward in *Vicia* guard cell protoplasts. The channel is muted to have a very low open probability, presumably contributing little to the overall K^+ conductance of the plasma membrane. Hosoi, Ino and Shimazaki (1988) also reported a low conductance (4–5 pS) channel of unknown selectivity. Finally, recent studies have revealed a stretch-activated K^+ channel of low unitary conductance in *Vicia* guard cell protoplasts (Cosgrove & Hedrich, 1990).

K^+ Outward Rectifier

As a class, outward-rectifying K^+ channels are the best characterized to date and appear most widely distributed. For reasons which will be outlined later, these channels are responsible for the K^+ conductance of the guard cell membrane at free-running potentials near E_{K+}. In *Vicia* guard cell protoplasts, the principal outward rectifier has shown unitary conductances of 10–25 pS with 175 and 11 mM K^+ on the cytoplasmic and extracellular sides of the membrane, respectively (Schroeder, Raschke & Neher, 1987), or with symmetric 50 mM K^+ solutions (Hosoi et al., 1988). A direct comparison based on channel conductance is not possible, because of the often widely varying experimental conditions; nonetheless, outward rectifiers at the plasma membranes

of several higher-plant species lie in the range of ca. 5–50 pS with K^+ concentrations about 100 mM (Schauf & Wilson, 1987; Iijima & Hagiwara, 1987; Bush et al., 1988; Moran et al., 1988, 1990; Ketchum et al., 1989; *see also* Fairley & Walker, 1989; Stoeckel & Takeda 1989).

The guard cell outward rectifier—like the majority of K^+ channels, animal or plant—is blocked reversibly by tetraethylammonium chloride (Blatt, 1988*a*) and Ba^{2+} (Schroeder et al., 1987). In *Vicia* guard cell protoplasts, it shows a moderate to high selectivity for K^+ over other alkali metal cations, based on bi-ionic equilibria with cytoplasmic K^+ (Schroeder, 1988); in the intact guard cells channel selectivity for K^+, at least over Na^+, may be even more pronounced (Blatt, 1988*a*). Interestingly, Na^+ will enter the K^+ channel from the cytoplasmic side of the membrane before becoming lodged and blocking the channel pore; block by intracellular Na^+ exhibits an anomalously strong voltage dependence, suggesting that the channel functions as a long, multi-ion pore and that block normally occurs with additional ions present in the channel (Thiel & Blatt, 1991). Such behavior is common among animal K^+ channels (*cf.* Hille & Schwarz, 1978; Hille, 1984; French & Shoukimas, 1985; Latorre, 1986) and is likely to prove so also for K^+ channels of plants (Tester, 1988*b*; Bertl, 1989; *see also* Tester, 1990). Along with voltage-dependent block, it might also account for the apparent voltage saturation of channel conductance at positive voltages (*see* Fig. 1*B, inset*) and the decline in K^+ current itself often seen at these potentials (*see* Blatt, 1988*a*; *compare also* Beilby, 1986*a*, for a similar phenomenon in *Chara*).

Gating of the guard cell outward rectifier is moderately voltage sensitive. Few details of the single-channel characteristics have been published to date (but *see* Hosoi et al., 1988); rather, kinetic analyses of channel behavior in guard cell protoplasts have drawn primarily on the macroscopic current(s). In "whole-protoplast" records (Schroeder, 1988, 1989) the K^+ current was observed to activate sigmoidally over periods up to 300–500 msec, with halftimes essentially independent of voltage. By contrast, deactivation was voltage dependent but much faster, with time constants near 15 msec at clamp voltages negative of −100 mV. No evidence for time- or voltage-dependent inactivation[1] of the current was found, and steady-state conductance-voltage characteristics were consistent with a minimum gating charge of 2.

An outward-rectifying K^+ current is evident also in the intact *Vicia* guard cells (Blatt, 1988*a*, 1990), and may be carried by this ca. 20 pS channel. The relationship between the two channel currents nonetheless remains uncertain in the face of quantitative (total membrane current) as well as qualitative (gating kinetics) differences. Notably, current activation was 3- to 10-fold faster in the intact cells and showed an appreciable voltage dependence. Possibly, these differences are the consequence of protoplast isolation or dialysis by the patch electrode of cytoplasmic constituents that control channel gating; the explanation would also account for the comparatively small currents recorded from the protoplasts.

Gating of the K^+ Outward Rectifier Depends on External K^+. Remarkably, outward-rectifier gating in the intact guard cells (Blatt, 1988*a*, 1990, and *in preparation*) and, to a lesser extent, in the protoplasts (Schroeder, 1988) is also sensitive to the extracellular K^+ concentration. For the intact cells (Blatt, 1988*a*), activation halftimes rose with K^+_o so that gating followed the K^+ equilibrium potential (E_{K+}), i.e., gating depended on the voltage difference $V - E_{K+}$ rather than on V alone; K^+_o had little or no effect on deactivation. The net effect of raising K^+_o was to shift the steady-state current- and conductance-voltage characteristics with E_{K+} to the right along the voltage axis (Fig. 1).

The K^+_o dependence is novel to the K^+ outward rectifier (*cf.* Hogdkin, Huxley & Katz, 1952; Hille, 1984), but analogous to the situation in neuromuscular and egg cell membranes for which K^+_o affects the gating of the anomalous (inward) rectifier (*compare* Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara & Yoshii, 1979; *see also* Hille, 1984). Significantly, this dependence has appeared in the outward rectifiers as a class, rather than in the inward rectifiers of plant plasma membranes so far (below; but *see* Bush et al., 1988). Similar K^+_o sensitivities may be deduced from currents of a K^+ outward rectifier in *Zea* (Ketchum et al., 1989) and of a 5 pS K^+ channel in *Dionaea* mesophyll protoplasts (Iijima & Hagiwara, 1987). The observations imply an additional (allosteric?) interaction between the cation and K^+ channel, complementary to the voltage dependence of gating but distinct from ion permeation itself; channel opening is influenced by K^+ availability on the outside, although net current through the channel draws on K^+ from the cytoplasmic side of the membrane (*cf.* Iijima & Hagiwara, 1987).

The action of K^+_o is kinetically distinguishable from that of extracellular Ca^{2+} which appears to affect only current deactivation (Blatt, 1991*a*; and *in preparation*). In this respect, a parallel can be drawn to the classic delayed (outward) rectifier of the squid

[1] The time dependence to inactivation observed at a constant voltage may vary with the choice of this voltage.

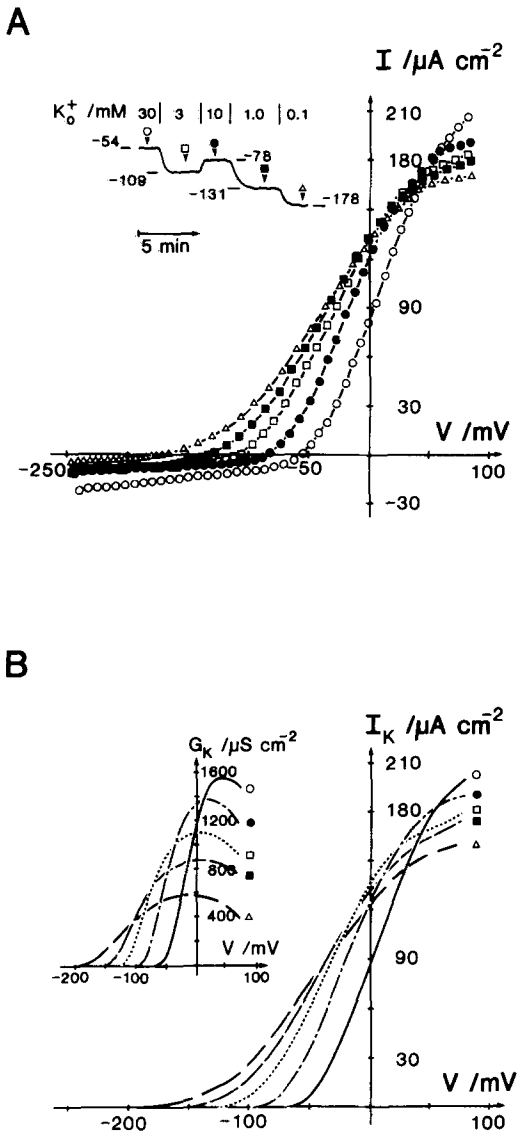

Fig. 1. Dependence of K^+ outward-rectifier gating on extracellular K^+. Steady-state current-voltage (*I-V*) and conductance-voltage (*G-V*) relations from one *Vicia* guard cell bathed in 5 mM Ca^{2+}-HEPES, pH 7.4, with 0.1–30 mM KCl are shown. Currents were recorded using a bipolar-staircase protocol (for details, *see* Blatt, 1987*b*, 1988*a*, 1990). (*A*) Shown are whole-cell *I–V* curves with symbols cross-referenced to times of *I–V* scans as marked on the voltage trace (*inset*, with voltages in mV; *I–V* scans masked from trace). (*B*) Corresponding K^+ current (leak subtracted) and conductance [*inset*, calculated as $I_{K+}/(V - E_{K+})$] showing the apparent Nernstian shift in gating with E_{K+}.

axon (*see* Armstrong & Matteson, 1986; Armstrong & Lopez-Barneo, 1987). Nonetheless, Hodgkin-Huxley formalisms (*see* Schroeder, 1989) are clearly insufficient to account for the K^+_o dependence and its predominance in activation; in the future it will be necessary to invoke more complex Markovian serial models (*cf.* Blatt & Clint, 1989).

K^+ Inward Rectifier

Like the outward rectifiers, K^+-selective channels that activate on (negative-going) hyperpolarization are likely to prove wide spread at the plant plasma membrane; among higher plants, they have been found in guard cells (Schroeder et al., 1987; Schroeder, 1988; Blatt et al., 1990*a*), in barley aleurone (Bush et al., 1988) and pulvinar mesophyll protoplasts (Moran et al., 1988; Moran & Satter, 1989), and there are indications of channels with similar characteristics in the Characean algae (Sokolik & Yurin, 1986; *see also* Katsuhara, Mimura & Tazawa, 1990). Again, single-channel conductances lie in the range 5–50 pS with "near-physiological" solute concentrations on either side of the membrane (Schroeder et al., 1987; Bush et al., 1988; Moran & Satter, 1989; Katsuhara et al., 1990); but details are generally sparce, precluding more than this superficial comparison.

In *Vicia* guard cells, inward and outward rectifiers are differentiable on the basis of several physical characteristics, but, as Tester (1990) notes, blockade of the inward rectifier with the unilateral applications of Al^{3+} used early on (Schroeder et al., 1987; Schroeder, 1988) is not diagnostic. Among other criteria (below), the guard cell inward rectifier showed a significantly higher selectivity (17:1) for K^+ over Na^+ (Schroeder, 1988) and lower unitary conductance (4–8 pS; Schroeder & Hagiwara, 1989) compared with the outward rectifier. Three additional features of the inward rectifier are outstanding; these relate to its gating and sensitivities to cation concentrations on either side of the membrane.

Gating of the K^+ Inward Rectifier is Insensitive to K^+_o. In the intact guard cells, both current activation and deactivation were found to be roughly exponential and K^+_o independent (Blatt et al., 1990*a*; Blatt, 1991*a*). Activation halftimes were only weakly voltage dependent with values of about 100 msec; deactivation was rapid ($\tau \cong 8$ msec) and voltage independent positive of approx. −50 mV, with a marked voltage dependence at more negative potentials. Characteristics of the inward rectifier in guard cell protoplasts were comparable (*see* Schroeder et al., 1987; Schroeder, 1988), and in neither case was there evidence of time- or voltage-dependent inactivation.[2] Thus, in both preparations, steady-state current was observed on hyperpolarization to voltages negative of approx. −120 mV, irrespective of K^+_o (3–100 mM). Extracellular K^+ affected the in-

[2] *See* footnote 1, p. 98.

ward rectifier only magnitude, the current becoming vanishingly small at K_o^+ concentrations below 1–3 mM (Blatt et al., 1990*a*; Blatt, 1991*a*); a similar inactivation of the tunicate egg inward rectifier has been documented in the absence of external K^+ (Ohmori, 1978).

Analogous to the situation with the outward rectifier, the effect of K_o^+ on gating distinguishes this guard cell K^+ channel from its inward-rectifier counterparts in neuromuscular and egg cell membranes. Activation in these latter cases typically follows E_{K+} with a fixed voltage offset (*cf.* Hagiwara et al., 1976; Hagiwara & Yoshii, 1979; Hille, 1984). The K_o^+-independent conductance-voltage profile of the guard cell inward rectifier is characteristic, rather, of many delayed (outward) rectifiers in animals (above). It is as if the guard cell plasma membrane, or at least its dominant K^+ channels, were functionally inside-out!

Gating of the K^+ Inward Rectifier is Sensitive to Ca^{2+}. Schroeder and Hagiwara (1989) first reported inactivating the K^+ inward rectifier in guard cell protoplasts by buffering the cytoplasmic free Ca^{2+} concentration at supramicromolar levels. Single-channel conductance was not affected; instead, the response was evident in the open channel probability, yielding both a reduced current amplitude and a negative-going shift of the steady-state current-voltage characteristic. The most pronounced effect occurred between free Ca^{2+} concentrations of 0.1 to 1.5 μM. An appreciable Ca^{2+} signal gain may be inferred, but, as yet, there is little quantitative information which could bear on a stoichiometry for Ca^{2+}-channel interaction. Some uncertainty must remain, also, about the degree to which free Ca^{2+} in the cytoplasm can be controlled (Pusch & Neher, 1988) and the possibility that free Ca^{2+} levels may vary significantly within the cell, notably in the peripheral cytoplasm near the membrane (Tillotson & Gorman, 1980; Foskett et al., 1989; Petersen & Wakui, 1990). Nonetheless, these and additional results (*see* Blatt et al., 1990*a*; Gilroy et al., 1990; *also Stomatal Closing* below) leave no doubt about the physiological importance of Ca^{2+}-dependent current inactivation.

Extracellular Ca^{2+}, too, is able to modulate the K^+ inward rectifier, but the concentrations required are in excess of 1 mM and the effect is probably indirect, mediated by Ca^{2+} entry across the plasma membrane (Busch, Hedrich & Raschke, 1990; Blatt, 1991*a*). This same explanation applies to Ba^{2+} block of the current (Schroeder et al., 1987); Blatt (1991*a*) found that block with submillimolar Ba^{2+} concentrations in the bath showed a voltage dependence counter to that expected if block reflected the driving force for cation entry from the bath into the channel pore.

It is noteworthy that Ca^{2+}-dependent activation of K^+ channels in many animal cells (Barrett, Magleby & Pallotta, 1982; Petersen & Maruyama, 1984; Blatz & Magleby, 1987) is also consequent on a negative-going shift in the channel gating (conductance-voltage) characteristic. Of course, the effect for the guard cell inward rectifier is to shift the activating voltages left along the voltage axis and *beyond* the normal physiological voltage range, by contrast with the situation for the Ca^{2+}-activated channels in the animal cells.

Gating of the K^+ Inward Rectifier is Sensitive to H^+. In recent studies, Blatt (1991*a*) found that K^+ inward-rectifier current of intact *Vicia* guard cells was promoted by H^+ outside. Acid pH_o affected primarily activation, accelerating the current rise at any one voltage. For transitions from pH 7.4 to 5.5, the result was to boost steady-state current five- to sevenfold at voltages near −200 mV and to shift the conductance-voltage profile to the right (positive-going) along the voltage axis. Current titrations suggested the response was mediated by H^+ binding to a single site within the membrane electric field but distinct from the channel pore itself, and the H^+ dependence could be related to the extracellular $[H^+]$, distinct from any influence of pH_i. A kinetic analysis of gating, based on global parameter optimizations (Balser, Roden & Bennett, 1990), yielded satisfactory results with a three-state Markov series. The analyses predicted a channel with voltage-dependent bursting behavior and mean open lifetime ($\cong$ 6 msec near −200 mV), and with a pH-dependent closed lifetime.

The pK_a (= 6.8 near −150 mV, indicating a $K_d \cong 200$ nM) of the current highlights a sensitivity comparable to that for intracellular free Ca^{2+} and carries implications for channel function *in situ* (below). It also accounts for the general absence of the current from earlier studies with the intact guard cells (*cf.* Blatt, 1988*a*, 1990) and, possibly, also with guard cell protoplasts (Hosoi et al., 1988). Again, the H^+ sensitivity of the current poses a striking contrast to known effects of H^+ on several ion channels—including K^+ channels—in animal cells for which H^+ is an often potent blocker (*see* Hille, 1984; Moody, 1984; Yellen, 1987, for reviews; also Hagiwara et al., 1978; Cook, Ikeuchi & Fujimoto, 1984; Christensen & Zeuthen, 1987; Tytgat, Nilius & Carmeliet, 1990). An inward-rectifying Cl^- channel in the *Chara* plasma membrane is known to display similar characteristics (Tyerman et al., 1986*a*,*b*), and the ATP sensitivity of K^+ channels in skeletal muscle is relieved at low pH_i (Davies, 1990). The K^+

inward rectifier clearly does not show characteristics of high-affinity, H^+-coupled K^+ uptake, such as has been described for *Neurospora* (Rodriguez-Navarro, Blatt & Slayman, 1986; Blatt, 1987); the current reversal (equilibrium) potential was independent of $[H^+]$ (Blatt, 1991*a*), thus discounting an earlier suggestion of a link with the H^+ circuit of the plasma membrane (Hedrich & Schroeder, 1989).

Cl^- (Anion) Channels are Ca^{2+}-Sensitive

There is a long history for Cl^- channels and their control by Ca^{2+} in various plant action potentials (*cf.* Findlay & Hope 1964; Lunevsky et al., 1983; Kataev, Zherelova & Berestovsky, 1984; Caldwell, Van Brunt & Harold, 1986; Shiina & Tazawa, 1988; Thiel, MacRobbie & Hanke, 1990; *see also* Tester, 1990); only very recently has attention turned to the presence of Cl^- channels in traditionally nonexcitable higher-plant cells (Falke et al., 1987, 1988; Schauf & Wilson, 1987; Fairley & Walker, 1989; *see also* Okazaki & Tazawa, 1990). It is all the more striking, therefore, that a dominant theme among the recent discoveries is the role of Ca^{2+} in activating or modulating channel current (Boult et al., 1989; Schroeder & Hagiwara, 1989; Hedrich, Busch & Raschke, 1990).

In fact, the issue of Ca^{2+} control was a focus of contention following the initial studies with *Vicia* guard cell protoplasts which showed a Ca^{2+}-activated Cl^- conductance (Schroeder & Hagiwara, 1989) and a Ca^{2+} insensitive, 39 pS Cl^- channel (Keller, Hedrich & Raschke, 1989). Hedrich et al. (1990) have since demonstrated a requirement for nucleotide in preconditioning for Ca^{2+}-sensitive channel activity. Nonetheless, some question remains about the relationship between the two data sets, not the least of which stems from the voltage dependence of the currents; there is good reason to anticipate the presence of other anion channels, and it is clear that much more work is needed to follow up Schroeder and Hagiwara's observations.

The guard cell Cl^- channel is actually more permeant to NO_3^- and will conduct malate anions to a lesser extent as well (Keller et al., 1989; Hedrich et al., 1990). This permeability sequence is characteristic of several anion channels derived from vacuolar membranes (*cf.* Hedrich, Flugge & Fernandez, 1986; Pope & Leigh, 1988; Pope et al., 1990; Tyerman & Findlay, 1989). Most striking, however, is the voltage dependence of the channel. Current activation was found on (positive-going) depolarization and appeared fixed to a relatively narrow voltage span; the current-voltage characteristic thus showed a distinct region of negative conductance of about -100 to -50 mV (Keller et al., 1989; Hedrich et al., 1990). Furthermore, once activated in depolarizing voltage steps, the current was seen to inactivate with a half-time of 10–12 sec (Hedrich et al., 1990). These are characteristics prerequisite for spontaneous electrical activity, such as action potentials, and may contribute to the voltage transitions recently recorded from intact guard cells following ABA stimulation (Thiel, MacRobbie & Blatt, 1991*a,b*; *see ABA potentiates K^+ Efflux . . .* below).

For the moment, there is no evidence for Cl^- channel activity modulated by G-proteins (Hedrich et al., 1990). Both GTP and ATP were equally effective in channel preconditioning; comparable channel activities were found also with the nonhydrolyzable analog ATP-γ-S. It will be interesting to see whether a similar basal level of control is exerted on other channels in the guard cell plasma membrane. *A priori* the consequences are difficult to predict, as nucleotide binding or phosphorylation need not enhance channel activities (*cf.* Cook & Hales, 1984; Ashford et al., 1988; also the review by Ashcroft, 1988). ATP appears to have the reverse effect on a K^+ channel in *Nitellopsis* (Katsuhara et al., 1990); this report is preliminary, but clearly shows a reduction in open channel probability with 1 mM of either ATP or AMP independent of Mg^{2+}. Also reported is an ATP-activated K^+ channel in *Saccharomyces* (Ramirez et al., 1989) which, by contrast, requires Mg^{2+} and ATP rather than the nonhydrolyzable analog. [Arguments for coupling between this K^+ channel and the yeast H^+-ATPase, however, are not compelling; note the similar Mg^{2+}-ATP requirement exhibited by the squid axon delayed rectifier and ATP sensitivities of other neuronal tissues (*cf.* Bezanilla et al., 1986; Ashford et al., 1988; Ashcroft, 1988).]

Ca^{2+} Channel Characteristics are Uncertain

Direct evidence for boni fide Ca^{2+} channels in guard cells remains elusive, although there is considerable indirect evidence of their existence, both in the plasma membrane and tonoplast. A rise in cytoplasmic free Ca^{2+} concentration is known to be evoked by abscisic acid (ABA), a phytohormone and water stress signal in plants which leads guard cells to lose osmotica and stomata to close (McAinsh et al., 1990). Extracellular Ca^{2+} acts synergistically with ABA in stomatal movements (DeSilva, Hetherington & Mansfield, 1985; Atkinson et al., 1989), although the associated Ca^{2+} fluxes across the plasma membrane are probably very small (MacRobbie, 1989). A parallel Ca^{2+} flux across the to-

noplast is likely also. Calcium channels and fluxes activated by inositol 1,4,5-trisphosphate (IP_3) are known in tonoplast-enriched vesicles from *Avena* (Schumaker & Sze, 1987), and the tonoplast of *Beta* (Alexandre & Lassalles, 1990; Alexandre, Lassalles & Kado, 1990; Brosnan & Sanders, 1990) and *Acer* (Ranjeva, Carrasco & Boudet, 1988; Canut et al., 1989); Ca^{2+} release from intracellular stores in guard cells is inferred from recent observations that the cytoplasmic Ca^{2+} signal—and consequent changes in K^+ channel and other (anion? *see also* Lunevsky et al., 1983; Shiina & Tazawa, 1988; Thiel et al., 1990) currents (above)—can be induced also following the release of caged IP_3 and in the presence of La^{3+} outside (Blatt et al., 1990*a*; Gilroy et al., 1990).

Schroeder and Hagiwara (1990) have attempted to identify the Ca^{2+} conductance at the plasma membrane, using simultaneous voltage-clamp measurements and Ca^{2+}-dependent dye fluorescence to distinguish this from Ca^{2+} release across the tonoplast of *Vicia* guard cell protoplasts. Their results highlight a voltage dependence to the ABA-evoked Ca^{2+} signal which coincides roughly with a variable current; the data—notably the dependence of cytoplasmic free Ca^{2+} concentration on membrane voltage—are consistent with ABA eliciting a Ca^{2+} flux across the plasma membrane. Nonetheless, the evidence Schroeder and Hagiwara offer for a Ca^{2+}-permeable, but nonselective channel is not convincing.

A fundamental flaw in this study is that ABA activates currents, both through outward-rectifying K^+ channels (Blatt, 1990; Thiel et al., 1991*b*) and through Cl^- (anion) channels (Schroeder & Hagiwara, 1989; Hedrich et al., 1990; Thiel et al., 1991*b*), the former (Blatt et al., 1990*a*) and possibly the latter (see *ABA potentiates K^+ Efflux . . .* below) independent of changes in cytoplasmic free Ca^{2+} concentration. Contributions from these currents were not accounted for in the analysis, either by eliminating them using nonpermeant ions or channel blockers, by comparing currents after varying the thermodynamic driving force(s) on the several ionic species, or directly by identifying single, Ca^{2+} channels. As such, the "whole-protoplast" records do not differentiate between a Ca^{2+} current, on the one hand, and a sum of K^+, Cl^- and other non-Ca^{2+} currents, on the other. For the moment, details of any Ca^{2+} channel(s), their characteristics and control must await a more rigorous analysis. This information will be essential also for assessing the immediate contribution of plasma membrane Ca^{2+} flux to changes in cytoplasmic free Ca^{2+} and transport control, and for determining its role in triggering Ca^{2+} release from intracellular stores (*cf.* Berridge & Irvine, 1989; Petersen & Wakui, 1990).

Mechanosensitive Channels: Stretch Activation or Poise?

There have been several reports of ion channels, most weakly to moderately anion selective, which activate (Falke et al., 1987, 1988; Gustin et al., 1988) or deactivate (Morris & Sigurdson, 1989) on stretching the plasma membrane of plant and fungal protoplasts. Hedrich and Schroeder (1989; *see also* Schroeder & Hedrich, 1989) alluded to stretch-activated channels in the guard cell plasma membrane, and a recent abstract (Cosgrove & Hedrich, 1990) reports the presence of three channel types, each selective for Cl^-, K^+ or Ca^{2+} in this case. The possibility of a mechanosensitive Ca^{2+} channel is particularly noteworthy and could represent a significant link in the transduction chain leading to stomatal closure (*see also* Okazaki & Tazawa, 1990). Nonetheless, for most circumstances in plant cells it is difficult to envisage how primary control of channel activities could be mediated by shearing forces in the same manner as in animal cells with any degree of homeostatic efficiency. Hydrostatic pressures on the plant plasma membrane are commonly maintained in a steady state for long periods of time—in guard cells, frequently for hours—suggesting, rather, that mechanosensitivity is more likely to be important in a "preconditioning" step, poising ion channels for activity.

In large measure, research continues to focus on mechanosensitive ion channels as entities discrete from other channels in the plant plasma membrane, thus following the precedents of work with animal cells (*cf.* Olesen, Clapham & Davies, 1988; Moody & Bosma 1989; also the review by Morris, 1990). Guharay and Sachs (1984) originally proposed that cytoskeletal elements linked to channel proteins could transmit the shearing forces for channel activation; Edwards and Pickard (1987) later adapted this model to include the cell wall, speculating that contacts between the wall and membrane could provide the necessary anchorage for sensing spatial displacement within the plane of the plant plasma membrane. However, many ion channels could exhibit some degree of sensitivity to membrane "stretch" (*cf.* Kourie & Findlay, 1990); recent work (Martinac, Adler & Kung, 1990) indicates that forces within the lipid bilayer itself may be sufficient to modulate channel gating. So it is pertinent to ask whether membrane shear could affect the gating of channels, such as those already characterized principally by their ionic and voltage dependencies.

H^+ ATPase Activity is Voltage Dependent

The membrane potentials of plants and fungi often lie well negative of all dominant diffusion regimes and exhibit a marked sensitivity to vanadate and metabolic antagonists (*see* Sanders, 1988, 1990), attesting to the activity of ATP-dependent, electrogenic transport. Hence, controls on channel activity must be aligned with primary ion transport. Kinetic features of the H^+-ATPase and its contribution to the membrane electrical properties in *Vicia* guard cells have been studied in some detail (Blatt, 1987*a,b,* 1988*b*; Thiel et al., 1991*a*) and show many characteristics comparable to the primary electrogenic pumps of *Neurospora* (*cf.* Sanders, 1988; Sanders & Slayman, 1989) and *Chara* (Blatt, Beilby & Tester, 1990*b*). Steady-state current-voltage profiles for the pump (Blatt 1987*b*, 1988*b*) indicated an apparent coupling stoichiometry of 1 charge (H^+) transported per ATP hydrolyzed and a predicted equilibrium potential near -360 mV at pH_o 7.4. Reaction kinetic analyses showed membrane charge transit to be intrinsically rate limiting, thus accounting for a broad region of voltage dependence, which extended well into the physiological voltage range.

Pump currents at 0 mV—near the current maximum at saturating positive voltages—have been estimated in the range of 2–20 μA cm^{-2} (Blatt, 1987*b*, 1988*b*; Thiel et al., 1991*a*); taking the voltage dependence of the pump into account, currents at the free-running membrane potential may be as little as 20–50% of this figure. Even so, pump current and conductance can dominate the membrane electrical characteristics of the guard cells; this was most evident when the external K^+ concentration was held below 1 mM, a manoeuvre eliminating current through the K^+ inward rectifier which otherwise masks the principal conductance of the pump (above; also Blatt, 1987*b*; Thiel et al., 1991*a*). Under these conditions, membrane voltages frequently exceeded -250 mV and could be shown to be maintained by the pump against a low background, or "leak" conductance comprising a sum of the remaining transport processes operative across the membrane.

Integrating Channel Gating for Transport Control

Ion channels are fundamentally passive devices in the membrane, providing for ion fluxes which are energetically "downhill" but, nevertheless, intrinsically rapid. Channel activities, thus, anticipate close and often multiple controls in coordinating ion fluxes within the needs of cellular physiology. For stomatal guard cells, as for other plant cells, channel function must be integrated also within a framework for transport which can adapt to appreciable fluctuations in the extracellular ionic environment (*cf.* Pitman, 1988; Sanders & Slayman, 1989). An important manifestation of this requirement is that membrane voltage commonly predominates in the primary ion gradient and energy charge on the membrane. Animal cells, by contrast, maintain a primary electrochemical potential for Na^+, which is dominated typically by the chemical gradient across the plasma membrane; cellular homeostasis, in this case, depends critically on the Na^+ concentration on either side of the membrane being held within relatively narrow limits. Classic examples of channel function in animals, too, reflect the comparatively stable ionic environment of these cells (*cf.* Hodgkin et al., 1952; also discussions in Hille, 1984). The habit of plant cells thus demands that we consider questions of channel physiology together with those of the ionic environment and of membrane energization.

Defining the ionic millieu for many higher-plant cells, and especially for guard cells, presents a singular challenge because of uncertainties about the surrounding microenvironment of the plant tissue (*cf.* Maier-Maercker, 1983; Blatt, 1985). Nonetheless, stomatal function persists over a wide range of conditions in the laboratory (*cf.* Fisher & Hsiao, 1968; Raschke, 1979; Willmer, 1984; Blatt, 1987*a*; MacRobbie, 1988*a,b*) and is likely to do so also in the intact leaf (*see* Pitman, 1988; also Atkinson et al., 1989). The immediate questions, thus, need focus as much on understanding how the characteristics of channel behavior may accommodate and utilize variations in the environment about the guard cell, as on how channels may contribute to the relevant fluxes under a single set of ionic conditions.

A complementary plasticity may be anticipated also in the integration of channel activity with membrane energization. Voltage cues for channel gating are of basic importance in a membrane so energetically dominated by transmembrane voltage. The requirement, in this case, is to coordinate channel activity with the physiological needs (ion uptake and loss for osmotic processes such as stomatal movements) and prevailing driving force(s) for ion flux. At the same time, the cell must maintain a balance between the energetic requirements for channel activity—its voltage dependence and current drain—and the demands of other cellular homeostatic functions. Thus, for integrating channel activity, voltage control alone will be insufficient in most cases. Circumstances are easily found in which the need for controlling cytoplasmic concentrations of the permeant species within acceptable limits would

otherwise entail excessive and prolonged swings in membrane energy charge (voltage) or futile and potentially damaging transport cycling.

Models for Stomatal Function

All evidence points to a dynamic mosaic of controls on transport which may be brought to bear in any one circumstance. Models invoking "static" transport control—in which the activities of one or a few processes dictate the ion flux through other transporters solely on a basis of the inherent kinetic properties (e.g. voltage dependence) of the latter—are clearly inadequate to account for guard cell membrane behavior; instead, control of ion flux is achieved through directed adjustments in the activities of several channels in concert, a strategy well able to accommodate the demands for osmotic solute flux under a variety of environmental circumstances. The schematics in Fig. 2 summarize our current understanding of the transitions between modes of net solute uptake of net solute loss and are divided between two distinct scenarios, accounting for guard cell behavior with supra- and with submillimolar concentrations of K^+ outside.

Abscisic Acid Potentiates K^+ Efflux and Stomatal Closing

To date, best characterized is guard cell response to abscisic acid (ABA), a plant water-stress signal which results in stomatal closure (*see* Harris et al., 1988; also MacRobbie, 1988*a* for review). Its action is thought to be effected by ABA binding at an exterior site on the plasma membrane (Hartung, 1983). The ion fluxes evoked by the phytohormone evolve through concerted modulations of at least five different transport pathways. In millimolar K^+_o and acid pH_o (Fig. 2*A,B*), net K^+ (and, to an extent, presumably anion) uptake is balanced by ATP-coupled H^+ extrusion via the pump operating against an otherwise low background conductance (Thiel et al., 1991*a*). Abscisic acid triggers a cascade of events as follows (numbers cross-reference the figure): (a) activation of an inward-directed current (*1*) which depolarizes (positive-going) the membrane; (b) a rise in cytoplasmic free Ca^{2+} concentration (*5, 6*) which (c) inactivates the K^+ inward rectifier (*3*) and (d) further activates inward-directed currents (*1*), including (*7*) a Ca^{2+}-dependent and voltage-gated anion current; and (e) activation of the K^+ outward rectifier (*4*). As illustrated, ABA does not alter the H^+ pump characteristics (*8*), but this remains to be established. The first three events (a–c), and possibly the fourth (d), are complete within a time frame of seconds. Activation of the K^+ outward rectifier is slower with a halftime of approx. 1 min.

At present, less detail can be brought to bear on the situation with submillimolar K^+_o and alkaline pH_o, but it is reasonable to expect a somewhat different pattern will emerge (Fig. 2*C*). Under these conditions, the K^+ inward rectifier is silent (Blatt et al., 1990*a*; Blatt, 1991*a*); instead, charge movement balancing the H^+ pump is dominated by a sum of other currents across the membrane, plausibly including high-affinity $H^+ - K^+$ cotransport analogous to that found in *Neurospora* (Rodriguez-Navarro et al., 1986; Blatt et al., 1987). There is evidence for net K^+ uptake via an energy-coupling mechanism capable of operating, in 0.1 mM K^+_o($E_{K+} \cong -180$ mV), at voltages between -100 and -150 mV (Clint & Blatt, 1989). As such, voltage transitions across E_{K+} need not be critical to a passage between modes for net solute uptake and for its loss. Both activation of the K^+ outward rectifier and sufficient driving force for net K^+ efflux are realized at membrane voltages near -150 mV in this case (Blatt, 1990). What is essential for the transition to stomatal closing is that the distribution of currents be shifted from the H^+ pump to the K^+ outward rectifier and anion currents. In 0.1 mM K^+_o and alkaline pH_o (Fig. 2*C*), ABA is known to activate an inward-directed current (*1*) and the outward-rectifier (*4*; Blatt, 1990); what response the pump (*8*) shows, if any, remains unknown.

Activation of Inward-Directed Currents

To realize a net K^+ (and anion) loss, it is essential that the K^+ outward rectifier be engaged and, hence, that the membrane be situated at a voltage positive of E_{K+}. In supramillimolar K^+_o, especially, this can entail membrane depolarization resulting in stable voltages typically near -70 mV in 10 mM K^+_o (Blatt, 1988*a*, 1990; Blatt & Clint, 1989; Thiel et al., 1991*a,b*). There is some question about the nature of the current activated for depolarization. It is tempting to speculate that one component of the current could be carried by Ca^{2+} (*cf.* Schroeder & Hagiwara, 1990), thus providing the necessary driving force for depolarization and, at the same time, possibly evoking Ca^{2+} release from intracellular stores (*cf.* Berridge & Irvine, 1989; Valdeolmillos et al., 1989; Petersen & Wakui, 1990). One difficulty with this proposal is that the initial K^+ ($^{86}Rb^+$) efflux transient in ABA (<1 min), which

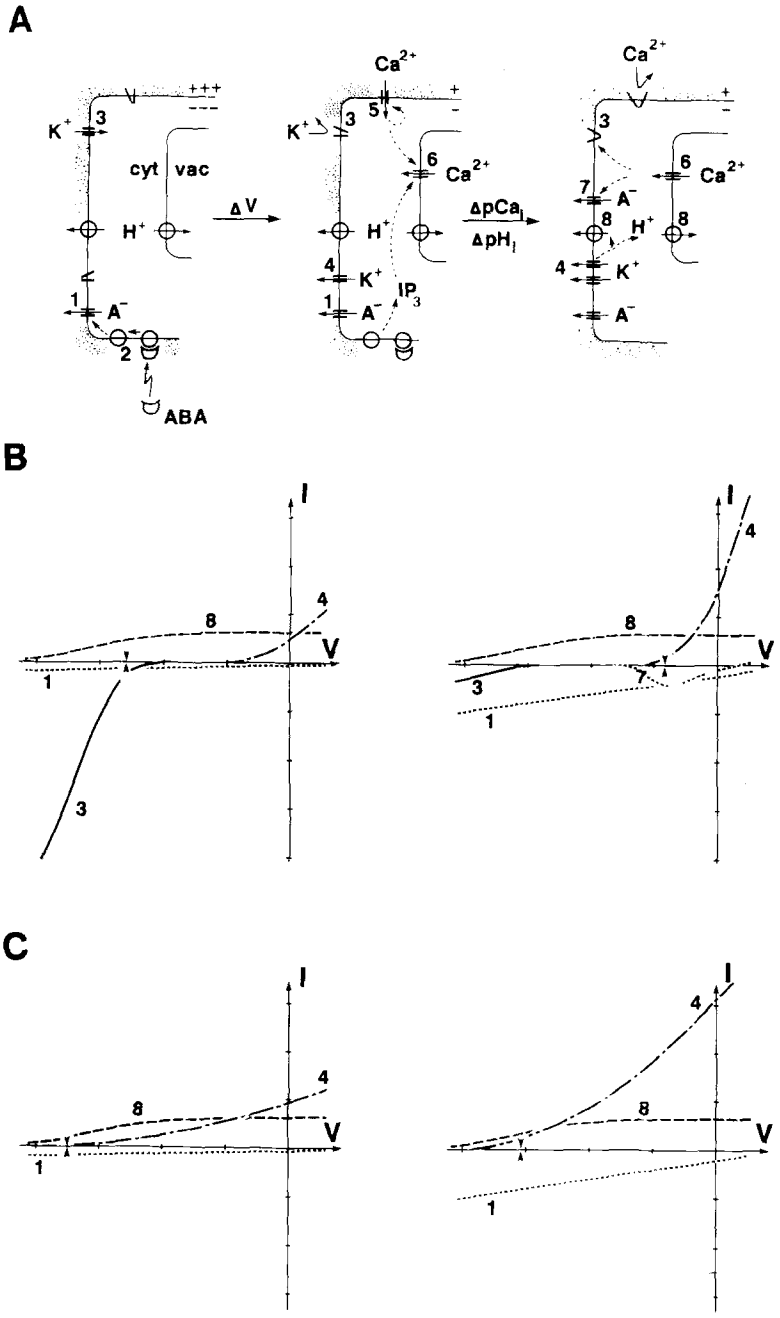

Fig. 2. Abscisic acid-evoked stomatal closure (additional details in the text). (*A*) Abscisic acid signal cascade is shown with ion fluxes (arrows, solid lines) and control links (arrows, dashed lines), channels (|↑|, activated; |\, deactivated; /\, inactivated) and H^+ pumps (○). The cascade is seen to begin (frame, left) with activation of an inward current, possibly carried by anion efflux (*1*) and plausibly mediated by a G-protein coupled to the ABA receptor (*2; see* Hartung, 1983; Allan et al., 1989). In millimolar K^+_o, membrane depolarization (frame, center) brings the voltage out of range for the K^+ inward rectifier (*3*) and into range for the K^+ outward rectifier (*4*), and activates Ca^{2+} influx (*5*). One or both Ca^{2+} influx and inositol trisphosphate (IP_3) trigger Ca^{2+} release from the vacuole (*6*) to inactivate (frame, right) the K^+ inward rectifier (*3*) and to activate a Ca^{2+}- and voltage-dependent anion current (*7*); the Ca^{2+} signal is proposed (*see* Tsien et al., 1988; Petersen & Wakui, 1990) to inactivate Ca^{2+} influx across the plasma membrane (*5*) either directly or otherwise (Schroeder & Hagiwara, 1990). Potentiation of the K^+ outward rectifier (*4*) may result from a rise in cytoplasmic pH (*8*; Blatt, 1991*a*). (*B* and *C*) Summary of the steady-state characteristics of component pump and channel currents detailed so far in millimolar (*B*) and submillimolar (*C*) K^+_o. Curves illustrate the situation before (left) and shortly after (right) exposure to abscisic acid. Identifying numbering cross-references to the cartoons in *A*; the double carats mark the free-running membrane potential.

probably reflects membrane depolarization, appears independent of external Ca^{2+}; only a second efflux transient, evident after 1–2 min and extending over a 10–20 min period, is Ca^{2+} sensitive (MacRobbie, 1990). Similar considerations may apply to light-evoked flux transients (Brindley, 1990). There are also other reasons which make primacy and dominance of a Ca^{2+} flux across the plasma membrane suspect (below).

Cytoplasmic Free Ca^{2+} Concentration

Free Ca^{2+} concentrations in the guard cell cytoplasm have been reported to rise from about 100–200 nM to values in excess of 1 μM on exposures to ABA (McAinsh et al., 1990; Schroeder & Hagiwara, 1990; Fricker et al., 1991). These concentrations are suffucient to effect the gating of anion and other inward-directed currents (Hedrich et al., 1990; Blatt et al.,

1990*a*) as well as inactivating the K^+ inward rectifier (Schroeder & Hagiwara, 1990; Blatt et al., 1990*a*). All the same, attaching a physiological interpretation to the observations has not been straightforward. Notably, a clear correlation between the Ca^{2+} signal and stomatal closure is lacking. In approx. 40–60% only of stomata has closure correlated with a rise in free Ca^{2+} concentration (McAinsh et al., 1990; Fricker et al., 1991); also, there is some disparity in the time course for the ABA response, with the Ca^{2+} signal first evident from minutes (McAinsh et al., 1990; Fricker et al., 1991) down to a few seconds (Schroeder & Hagiwara, 1990; Gilroy et al., 1991; Fricker et al., 1991) into exposures.

One plausible explanation lies in technical difficulties inherent to fluorescent measurements of Ca^{2+} in plant cells. As Fricker et al. (1991) have pointed out, the bulk of the cytoplasm in guard cells and much of the endomembrane system is located around the nucleus; most of the plasma membrane bounds a very thin layer of cytoplasm. In short, the cytoplasmic regions in which Ca^{2+} signals are likely to be most important for transport control are also the regions in which they are most difficult to resolve.

Knowing the primary source of Ca^{2+} for release in the cytoplasm would go a long way toward addressing questions of Ca^{2+} localization and, hence, of timing. For the moment, plausible candidates include inositiol trisphosphate-mediated release from intracellular stores (Blatt et al., 1990*a*; Gilroy et al., 1990) and Ca^{2+} influx across the plasma membrane (DeSilva et al., 1985; Schroeder & Hagiwara, 1990). Both are likely to be important, plausibly coupled for a high gain in Ca^{2+} release (*cf.* Berridge & Irvine, 1989), and together could offer considerable scope for cooperativity (e.g. Kasai & Augustine, 1990). The effect of a local all-or-none response, in this case, might account for the temporal variability and poor correlative frequencies observed.

Alternatively, it is possible that the role for Ca^{2+} is secondary, as a means for adaptive control of transport capacity, rather than a primary signal in the initial transition for net solute loss. An analogous role for Ca^{2+} is found in adaptive signal processing of visual photoreceptors (*cf.* Stryer, 1986; Nakatani & Yau, 1988; Kawamura & Murakami, 1991), and there is some reason to suspect this situation could occur in guard cells as well. For one, considerable variability in basal levels of cytoplasmic free Ca^{2+} concentration have been reported from guard cells (*cf.* Fricker et al., 1991; Gilroy et al., 1991), although the key questions, its correlation to the ABA response and membrane transport characteristics, remain unanswered. Second, gating of the K^+ outward rectifier which mediates guard cell K^+ loss is singularly *in*sensitive to cytoplasmic free Ca^{2+} concentration (Hosoi et al., 1988; Schroeder & Hagiwara, 1989; Blatt et al., 1990*a*; contrast with Ketchum & Poole, 1991!); rather, the most pronounced effect of cytoplasmic Ca^{2+} is seen in gating of the K^+ inward rectifier. This response may be seen as a "preventative" measure (Blatt et al., 1990*a*) but, since depolarization would normally bring the membrane out of range for K^+ influx through the inward rectifier, it is difficult to see how its modulation contributes to the transition itself. [It is arguable, too, whether Ca^{2+}-dependent activation of inward-directed currents (Schroeder & Hagiwara, 1989; Blatt et al., 1990*a*; Hedrich et al., 1990) contributes to any initial depolarization (below).]

Finally, a third indication that Ca^{2+} release may not be primary to signalling draws on the otherwise paradoxical juxtaposition of flux (MacRobbie, 1990) and combined Ca^{2+} and electrical measurements (Schroeder & Hagiwara, 1990) early on in ABA treatments. One key distinction between these cytoplasmic Ca^{2+} and K^+ efflux measurements is that membrane voltage was brought under experimental control in the former; this is not practical in flux measurements, and the initial efflux transient MacRobbie observed (above) most likely reflected membrane depolarization (Thiel et al., 1991*a*,*b*). In question, then, is the voltage dependence of the inferred Ca^{2+} flux across the plasma membrane (*Ca^{2+} Channels*, above). From MacRobbie's data we may suspect that initial depolarization can be achieved by activating an inward-directed current of ions other than Ca^{2+} (Fig. 2*B*), and the implication is that the Ca^{2+} current may be voltage gated; on exposure to ABA it, too, could be activated, but only following depolarizations sufficient to open the Ca^{2+} channels (e.g., Brown, Lee & Powell, 1981; *see* review by Tsien et al., 1988). Such a scheme would still bestow a voltage dependence on the cytoplasmic Ca^{2+} signal consistent with Schroeder and Hagiwara's data by simple virtue of the reduced driving force for Ca^{2+} influx with positive-going voltages. It might also account for some of the variability observed in the Ca^{2+} signal; in short, a rise in cytoplasmic free Ca^{2+} concentration might be evoked only under more pronounced depolarization. Decisive studies combining electrical and fluorescent techniques are now essential to resolving these issues.

Ca^{2+}-Activated Cl^- (Anion) Channel

Activation of this current by Ca^{2+} reveals a conductance with a voltage dependence sufficient for regenerative depolarization and may account for a negative slope conductance evident in whole-cell *I-V*

relations (Thiel et al., 1991*a,b*). All the same, other ABA-evoked anion currents (Fig. 2*B*) must be anticipated for two reasons, in addition to those outlined above. (1) Activation appears restricted to a narrow voltage span positive of −100 mV (Hedrich et al., 1990); as such, the current is not a prime candidate for any early depolarizations from more negative voltages (*see* Thiel et al., 1991*a*). There are also no indications for it as a component of the ABA-activated inward current in submillimolar K^+_o conditions (Blatt, 1990). (2) The current has been found to inactivate with halftimes about 10–12 sec (Hedrich et al., 1990); it is therefore unlikely to be capable of sustaining the solute flux necessary for stomatal closure, although the crucial issue of any voltage dependence for inactivation remains to be examined. The current could contribute to oscillatory phenomena which, together with its Ca^{2+}-dependence, might be important in "fine-tuning" for solute loss under some conditions.

K^+ Outward Rectifier

Parallel flux and current measurements have yielded strong evidence that the outward rectifier is the dominant pathway for K^+ efflux during stomatal closing (Cling & Blatt, 1989). Extracellular K^+, itself, modulates the gating of the outward rectifier (*Two Classes of K^+ Channels . . .* above, and Fig. 1), and this level of inherent kinetic control may be seen to ensure that channel activity is restricted to a voltage range for net K^+ efflux, irrespective of the prevailing electrochemical equilibrium for K^+. Thus, with a rise in inward-directed (anion?) current in ABA, charge balance is maintained largely by the K^+ outward rectifier and holds the free-running potential close and positive to E_{K+}.

In addition, ABA activates this current without a measurable change in its voltage dependence or gating kinetics, suggesting that the phytohormone either recruits channels to an active pool or affects single-channel conductance (Blatt, 1990). The response has been observed both in sub- and supramillimolar K^+_o concentrations and in acid and alkaline pH_o (Blatt, 1990; Thiel et al., 1991*b*). Activation occurs with halftimes near 60 sec (Blatt, 1990; Thiel et al., 1991*b*), thus suggesting its role as contributory, but not primal to early stages of solute efflux stimulation by ABA (MacRobbie, 1990). Significantly, the activity of the K^+ outward rectifier is apparently independent of cytoplasmic free Ca^{2+} concentration (Schroeder & Hagiwara, 1989; Blatt et al., 1990*a*), although its rise in ABA roughly coincides with ABA-evoked changes in cytoplasmic free Ca^{2+} concentrations (above).

Control of the K^+ current amplitude could be achieved through phosphorylation of the channel, a possibility raised by recent studies with okadaic acid and complementary protein phosphatase antagonists (G. Thiel and M. Blatt, *in preparation*). There are indications also of a link to pH. A voltage-independent overshoot in the current has been observed to follow channel block with TEA and was ascribed to cytoplasmic alkalinization and its recovery (Blatt & Clint, 1989) imposed by an impurity in TEA, triethylamine (Zucker, 1981). Cytoplasmic acid loads have the opposite effect, selectively reducing or eliminating the K^+ outward rectifier but, again, in a largely voltage-independent manner (Blatt, 1991*a*). Finally, ABA has been reported to induce cytoplasmic alkalinizations together with a rise in free Ca^{2+} concentration in *Zea* coleoptile tissues (Gehring, Irving & Parish, 1990). The question now is whether the K^+ outward rectifier response can be linked to a similar course of events in the guard cells and, equally important, whether the origin of any ABA-evoked pH_i signal can be ascertained.

Is H^+ Pump Activation Prerequisite for Stomatal Opening?

A number of factors promote K^+ uptake and stomatal opening, notably blue light (Sharkey & Raschke, 1981; Iino, Ogawa & Zeiger, 1985), low pCO_2 (*see* Raschke, 1987), and a variety of fungal toxins including fusicoccin (Marre, 1979), as well as an endogenous circadian rhythm (*cf.* Holmes & Klein, 1986; Gorton et al., 1989). Primary H^+ extrusion by the pump is clearly essential to generating a driving force K^+ entry. In turn, cation influx may be mediated, in supramillimolar K^+_o and neutral or acid pH_o, by the K^+ inward rectifier (Thiel et al., 1991*a,b*) or, in submillimolar K^+_o, by an energy-coupling process (Clint & Blatt, 1989). Virtually no information can be brought to bear on anion uptake, although thermodynamic considerations leave little doubt of the requirement for H^+ coupling (*cf.* Sanders & Hansen, 1981; MacRobbie, 1988*a*; Sanders, Hopgood & Jennings, 1989) or other energetic input. These mechanistic considerations aside, positive evidence is sparce that bears on the control of transport and its integration for ion uptake and stomatal opening; there is much scope for future research here.

Membrane Potential and the H^+-ATPase

Past notions for guard cell control of stomatal opening have centered on modulations of the H^+ pump and, hence, of membrane voltage to control the driv-

ing force for K^+ influx (*cf.* Raschke, 1979, 1987; Zeiger, 1983). Blue light, especially, has been reported to hyperpolarize the plasma membrane (Schroeder, 1988) and acidify the bathing of guard cell protoplasts (Shimazaki, Ino & Zeiger, 1986) with a time course comparable to that for stomatal opening (Iino et al., 1985). Both blue (Assmann, Simoncini & Schroeder, 1985; Schroeder, 1988), and photosynthetically active red light (Serrano, et al. 1988) are known to alter membrane current of protoplasts under voltage clamp and, in each case, a requirement for ATP in the patch pipette was demonstrated. Analogous responses to fusicoccin are known as well (Schroeder 1988; Serrano, Zeiger & Hagiwara, 1988). The idea, then, is that increasing pump output might drive the membrane sufficiently negative to activate the K^+ inward rectifier (*see* Schroeder, 1988).

The evidence for control specifically of the H^+-ATPase by light remains ambiguous all the same. One problem is that pH measurements necessarily reflect the balance of H^+ fluxes between the cell and bathing medium. For a H^+-coupling membrane, this means that in the bulk pH it is not possible to distinguish, among others, between a true rise in H^+ output by the pump, on the one hand, and a decline in H^+ return via H^+-coupled transport, on the other. As Blatt and Clint (1989) point out, a change in net H^+ flux (pH) indicates only a redistribution of the current fraction carried by protonic and nonprotonic ion movement across the membrane. Equally inconclusive are the steady-state currents which were measured at a single potential negative of all dominant diffusion regimes. The underlying presumption, in each case, was that a net positive (outward-directed) current could arise at these voltages only if energy-dependent ion pumping were stimulated. The alternative of a reduced membrane conductance(s) for current return against a *constant* background of ATP-dependent pump current, was not considered.

More detailed analyses have been carried out for *Vicia* guard cells in fusicoccin (Blatt, 1988*b*; Blatt & Clint, 1989; Clint & Blatt, 1989). In this case, *I-V* characteristics do reveal a limited response of the pump during prolonged exposures to the toxin; at the free-running membrane potential pump current rose by approx. 10% over 50 min. Most pronounced, however, were marked and rapid reductions (two- to three-fold, $t_{1/2}$ = 3–6 min) in background conductance (Blatt, 1988*b*) and inactivation of the K^+ outward rectifier (Blatt & Clint, 1989; Clint & Blatt, 1989). These studies also yielded evidence, both from tracer flux and voltage-clamp analyses, for an energy-coupling K^+ uptake mechanism which was activated by fusicoccin (Clint & Blatt, 1989). [No information was forthcoming on the K^+ inward rectifier—measurements were conducted in submillimolar (0.1 mM) K^+_o and at pH_o 7.4—and this point will need addressing in future work.] In short, rather than supporting notions of simple pump control for K^+ uptake, the data conform to a pattern of concerted transport modulation complementary with that of guard cell response to ABA (Fig. 2).

It must be stressed that there is considerable scope for variation in the output of the guard cell H^+ pump. So far, the evidence has highlighted seasonal adjustments (*cf.* Blatt, 1988*a*, 1990), but it does carry implications for pump modulation in the short term. Thiel et al. (1991*a*,*b*) have documented two, well-defined "states" in *Vicia* guard cells, analogous to the "pump" and "K^+" states of the *Chara* plasmamembrane (*see* Beilby, 1986*b*, 1990). The first of these "states" was dominated by a high pump activity, yielding membrane voltages (-237 ± 11 mV in 0.1 mM K^+_o) which were situated well negative of E_{K+}; pump current in 10–30 mM K^+_o was demonstrably balanced by a K^+ influx through the inward rectifier. In the second, the membrane was dominated by a diffusional conductance, with membrane voltages (-143 ± 4 mV in 0.1 mM K^+_o) close to and positive of E_{K+}. Most striking were observations of spontaneous and reversible transitions between the two "states"; all indications are that the same alterations may be elicited by abscisic acid as a preface to K^+ loss and stomatal closure (Thiel et al., 1991*b*). Both the spontaneous and evoked transitions resulted from large variations in background conductance, including a voltage-dependent conductance similar to that which Hedrich et al. (1990) described; so, for the moment, it is not clear whether the kinetic characteristic of the pump may also be modulated.

Integration of the K^+ Inward Rectifier

Gating of this current is well accommodated to the requirements of an H^+-coupling membrane and offers two final insights into guard cell behavior and channel integration with the pump. One otherwise puzzling aspect of the voltage dependence for gating of the current is its insensitivity to the prevailing K^+ concentration outside the cell (Schroeder, 1988; Blatt, 1991*a*); the characteristic is juxtaposed in the same membrane with the K^+ outward rectifier, for which gating follows E_{K+} as K^+_o varies (Blatt, 1988*b*, and above). The effect, for the inward rectifier, is to enforce a minimum polarization of the membrane for K^+ uptake—at pH_o 6.1, to voltages greater (inside negative) than (−)120–140 mV (Schroeder, 1988; Thiel et al., 1991*a*)—*irrespective* of E_{K+} and the prevailing K^+_o concentration. As a result, the K^+ conductance cannot overwhelm the membrane be-

tween E_{K+} and this "gating voltage"; given a moderate background conductance to the membrane, voltages in excess of (−)150 mV may be achieved in 10 mM K_o^+ (Thiel et al., 1991*a*) with relatively little expenditure of energy by the pump. In short, gating of the inward rectifier ensures that K^+ uptake cannot short circuit other energetic demands on membrane voltage.

This strategy is effective so long as the current activates at voltages negative to E_{K+}—practically speaking, for $[K^+]_o > 1$ mM—but it does pose a serious problem for the situation with submillimolar K_o^+. In this case, the inward rectifier could present a pathway for net K^+ efflux over a wide voltage range [from −120 to −200 mV, for example, were the current to activate near −120 mV in 0.1 mM K_o^+ ($E_{K+} \cong -200$ mV; *cf.* Blatt, 1988*a*)]. In fact, the circumstance does not arise, because the K^+ inward rectifier appears inactivated in submillimolar K_o^+ (Blatt et al., 1990*a*; Blatt, 1991*a*; *compare* Ohmori, 1978) and, under these conditions, alternative mechanisms for K^+ uptake (*see* Clint & Blatt, 1989) are likely to be important.

The second feature of the K^+ inward rectifier is that its gating anticipates, and compensates for, a pH_o-dependence liable of the H^+-ATPase. The steady-state *I-V* relations in Fig. 3 were calculated from kinetic parameters for the H^+-ATPase of *Vicia* guard cells (Blatt, 1987*b*, 1988*b*) and based on the pH_o dependence of the kinetically similar H^+-ATPase of *Chara* (Blatt et al., 1990*b*); the principal effect expected of alkaline-going pH_o is that the *I-V* characteristic of the pump shifts to the left (negative-going) along the voltage axis. Gating of the inward rectifier tracks the H^+-ATPase over the pH_o range from 5.5 to 7.5 (Blatt, 1991*a*) so that, with increasing alkaline pH_o, the current activates at increasingly negative voltages (Fig. 3). All other factors being equal, similar rates of K^+ uptake might be realized with a similar pump output at each pH_o, but at increasingly negative free-running potentials reflecting the predominance of membrane voltage in the primary ion (H^+) electrochemical gradient with neutral and alkaline pH_o. So, the effect may be seen to offset K^+ uptake with pump output, again ensuring that the inward rectifier cannot short circuit other energetic demands on membrane voltage, in this case irrespective of the prevailing extracellular H^+ concentration.

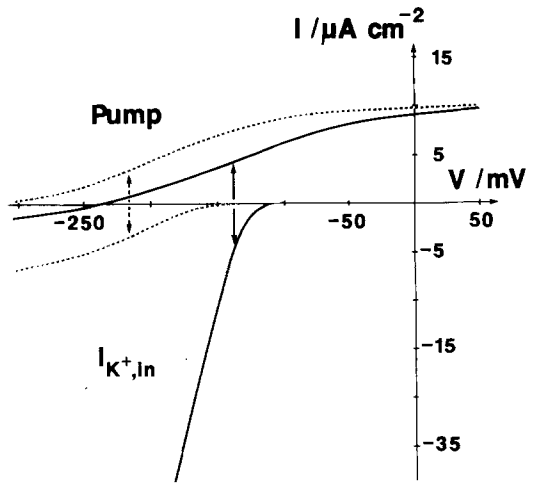

Fig. 3. Kinetic coordination of H^+-ATPase and K^+ inward rectifier. Pump and channel currents at pH_o 5.5 (——) and 7.4 (·····). Arrows mirrored on the voltage axis mark the voltages at which the paired currents are equal in magnitude. Note the roughly equivalent current magnitudes predicted when currents balance in each case, despite a ca. −90 mV shift to the alkaline pH. Representative K^+ currents were taken from one *Vicia* guard cell bathed in 10 mM KCl (*see* Blatt, 1991*a*). H^+ pump currents were calculated from reaction kinetic analyses carried out at pH_o 7.4 (Blatt, 1987*b*, 1988*b*) and incorporate a pH_o dependence based on comparable results of parametric fittings for the *Chara* H^+-ATPase (Blatt et al., 1990*b*). The parameters (Class I, 2-state model) and corresponding pump equilibrium potential (E_P) were

	k_{io}^o	k_{oi}^o	K_{io}	K_{oi}	E_P
pH_o7.4	500	0.04	0.1	10	−359
pH_o5.5	200	0.04	3.3	10	−243

Concluding Remarks

The last decade has witnessed a remarkable development in our understanding of the ion transport characteristics of the guard cell plasma membrane; the most elementary questions of the ionic basis of the membrane potential have been superceded by particular descriptions of primary electrogenic transport and ion channels, including multiple pathways for K^+ flux across the plasma membrane. Critical to the development have been advances in the application of intracellular and patch electrode methods and of quantitative radiotracer flux analysis. This detail, as well as the unique physiology of guard cells, establishes the stomatal system as a singularly attractive transport model for higher-plant cells.

Research on stomatal guard cells, to date, has focused principally on transitions between the open and closed stoma and, especially, on the action of abscisic acid. It is primarily through this work that many of the dominant transport and control processes have been identified and can now be pieced together. To what extent these patterns of transport and control may extrapolate to phenomenologically related responses—notably to light and CO_2—remains to be seen. We may bear in mind, too, that guard cells also exist quasi-stably in the open and closed stoma; the characteristics of these two condi-

tions are likely to prove equally important to an understanding of transport control in guard cells. Thus, while current models for stomatal function are clearly illustrative, it must be questioned whether they are, as yet, wholly representative.

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