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Topical Review

Potassium Channels, Proliferation and G1 Progression

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Introduction

Potassium channels are the most ubiquitous and diverse family of plasma membrane ion channels, and this is reflected in a large variety of essential roles they perform in different cells. Voltage-gated K channels modulate the excitability of excitable cells, and K channels gated by intracellular ligands, such as calcium or ATP, provide a functional link between the physiological properties of the plasma membrane and the activity of intracellular metabolic pathways. There is now substantial evidence that drugs which block K channels also inhibit the proliferation of many types of cells, but the cellular mechanism(s) by which the level of K channel activity might be related to proliferation remains unclear. A particularly intriguing possibility is that the opening, or activation, of K channels might be required for the passage of cells through a specific stage in their cell cycle; this would provide a fundamental link between physiological and biochemical signaling pathways which regulate progression through the cell cycle (Fig. 1). The role of K channels in mitogenesis and proliferation has been previously reviewed [20,24,60]. The focus of the present review is the hypothesis that the activation of K channels is required for cells to progress through the G1 phase of the cell cycle. We will examine first the evidence supporting this hypothesis, and then we will discuss the processes or events within G1 phase that are most likely to require the activation of K channels. Identification of these critical events is important because their dependence on the activation of ion channels in the plasma membrane would represent a novel type of regulatory checkpoint, compared to other checkpoints previously identified within the G1 phase of the cell cycle [66].

Key words: Potassium channels — Cell cycle — G1 phase — Proliferation

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Is the Activation of K Channels Required for Proliferation?

Potassium channel antagonists inhibit the proliferation of many types of cells, ranging from quiescent lymphocytes stimulated by mitogens to rapidly cycling tumor cells (Table 1, Fig. 2*A*). The rank order of potency of K channel antagonists for inhibiting proliferation and K currents in the same population of cells are generally similar, and this relationship has frequently led investigators to conclude that the activation of K channels might be required for proliferation. Although the inhibition of proliferation by K channel antagonists has been observed in many populations of cells (Table 1), very few studies have rigorously demonstrated that the inhibition of proliferation by these agents resulted from the specific interactions of these agents with K channels and not other cellular processes required for proliferation. Without resolution of this issue, it is very difficult to identify the mechanisms whereby the activity of K channels is required for proliferation.

We would like to examine the concentration-dependent inhibition of proliferation and K currents, because this relationship has provided the basis for most conclusions that the inhibition of K channels was responsible for inhibition of proliferation. In most studies in which the inhibition of proliferation and K currents were directly compared [e.g., 4,12,13,18,70,98], both the IC_{50} (the concentration required for 50% inhibition) and the

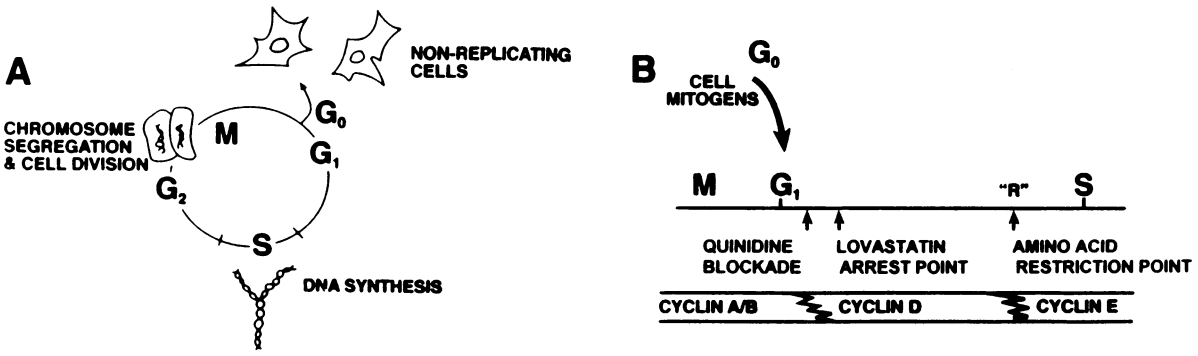

Fig. 1. Proliferating cells must carry out an ordered series of biochemical steps for DNA synthesis and, subsequently, mitosis to occur. This process has been summarized in a *cell cycle* model, in which G1 and G2 phases are preparatory to S (DNA synthesis) and M (mitosis) phases, respectively. Nonproliferating cells are in a resting stage, G0. Polypeptide growth factors stimulate cell proliferation by moving cells from G0 into G1 and also by stimulating progression through G1. Progression through G1 phase requires passage through several control points [*reviewed in* 66]. (*A*) At the microscopic level, S-phase and M-phase cells exhibit distinctive morphological properties. S phase cells are identified autoradiographically by the presence of grains of newly-incorporated [^{3}H]-thymidine into the replicating DNA. M-phase cells exhibit condensed chromosome structures in different stages of segregation to the two new daughter cells. (*B*) At the biochemical level, events during G1 phase are extremely important to cell proliferation. Degradation of cyclins A and B permit entry of cells into G1 phase. Cyclin D levels control the rate of progression through early G1 phase via phosphorylation of the retinoblastoma (Rb) protein. Phosphorylation of Rb acts as a trigger for activation of the transcription of genes required for DNA synthesis. Cyclin E activity spans the G1/S transition but its role is not well defined [54].

steepness of the dose-dependence were higher for inhibition of proliferation than for inhibition of K currents (Table 1, Fig. 2*A*). The extent to which the inhibition of K currents and proliferation differ is not the same for all K channel antagonists, and this has been noted by several investigators [12,70,82]. Identification of the sources of these differences might provide clues for determining whether the antagonists inhibit proliferation by interacting with targets other than K channels.

Several factors could increase the IC_{50} for proliferation, relative to the IC_{50} for K currents. Serum is typically added to the culture medium used during proliferation assays, but not to the physiological salt solutions used for recording K currents. Price et al. [70] identified two mechanisms whereby serum affected the inhibition of K currents and proliferation in peripheral blood mononuclear cells (PBCMs) by the peptide toxin charybdotoxin (ChTx), quinine, 4-aminopyridine (4-AP) and tetraethylammonium (TEA). Serum reduced both the inhibition of K currents and proliferation by quinine and 4-AP, suggesting binding of these drugs to the serum. In contrast, serum did not affect the inhibition of K currents by ChTx, but completely eliminated the inhibition of proliferation by ChTx. Freedman et al. [27] suggested that serum might overcome the inhibition of proliferation by ChTx through the release of unidentified, serum-bound factors that stimulate proliferation. In support of this conclusion, Freedman et al. [27] observed that serum decreased the ability of ChTx to inhibit the release of interleukin-2 (IL-2), and inhibition of the release of IL-2 is critical to the inhibition of proliferation by ChTx. We observed that serum also reduced the potency of glibenclamide for inhibiting the proliferation of MCF-7 human breast cancer cells [105], perhaps due to the binding of glibenclamide to serum proteins. The ability of an antagonist to inhibit proliferation might also be reduced if it is metabolized during the treatment period, thereby decreasing its effective concentration. For K channel antagonists that produce a voltage-dependent block, the relative sensitivity of K currents and proliferation might be different if the inhibition of K currents is measured at potentials that are different from the resting membrane potential of proliferating cells [18,24]. The extent to which proliferation is apparently inhibited by K channel antagonists might also depend on the technique employed to measure proliferation. The incorporation of [^{3}H]-thymidine ([^{3}H]-tdr) into DNA has been used most frequently, but these data can differ markedly from direct measurement of proliferation by cell counts [91]. A specific concern is that inhibition of the cellular uptake of [^{3}H]-tdr by a test drug can artefactually appear as an inhibition of proliferation [1]. Finally, a cell might express more than one type of K channel whose activation could permit proliferation. If an antagonist selectively inhibited one population of K channels but not the other, the second population of K channels might permit proliferation, perhaps at a reduced rate, to continue. The combined influence of these factors can obviously complicate any comparison of the dose-dependent inhibition of K currents and proliferation. We describe below an additional factor, the presence of spare K channels, which might also contribute to differences in the dose-dependent inhibition of K currents and proliferation.

The dose-dependent inhibition of K currents in the studies listed in Table 1 was generally very well fitted by a single-site blocking model, as might be expected for

Table 1. Effects of K channel blocking drugs on K current and proliferation

		Inhibition (IC_{50})					
Cell type	Drug	K current	S	Proliferation	S	P/K	References
Human T-lymphocyte	TEA	8 mM	0	13 mM	10	1.63	18
	4-AP	0.19 mM	0	2.1 mM	10	11.05	18
	Quinine	14 μM	0	33 μM	10	2.36	18
	Verapamil	6 μM	0	24 μM	10	4.00	12
	Diltiazem	60 μM	0	114 μM	10	1.90	12
	Retinoic acid	10–50 μM	10	10–50 μM	10		86
Human PBMC	TEA	8.0 mM	0	8.3 mM	0	1.04	70
	4-AP	0.32 mM	10	3.8 mM	10	11.88	70
	4-AP	0.25 mM	0	0.9 mM	0	3.60	70
	Quinine	30 μM	10	82 μM	10	2.73	70
	Quinine	22 μM	0	39.5 μM	0	1.79	70
	ChTx	0.3 nM	0	0.5 nM	0	1.67	70
L2 lymphocyte	Quinine	<50 μM	0				43
Murine B Lymphocyte	Quinine	12.4 μM	0	13.7 μM	10	1.10	4
	TEA	11.9 mM	0	6.9 mM	10	0.58	5
	4-AP	0.1 mM	0	3.8 mM	10	38.00	4
	Verapamil	5 μM	0	6.9 μM	10	1.38	4
Nb2 lymphoma	TEA	2.5 mM	0	5.5 mM	1	2.20	98
	Quinidine	4.2 μM	0	8.9 μM	1	2.12	98
	4-AP	90 μM	0	756 μM	1	8.40	98
	Glibenclamide			25–200 μM			39
Human brown Fat	Verapamil	17 μM	0	17 μM	5	1.00	65
	TEA	1.6 mM	0	2.0 mM	5	1.25	65
Human melanoma	TEA	2–4 mM	0	<20 mM	12		58
MCF-7 Human Breast cancer	TEA			5.8 mM	5		105
	4-AP			1.6 mM	5		105
	Quinidine			25 μM	5		105
	Glibenclamide			50 μM	0		105
	Linogliride			770 μM	0		105
Rat Schwann (Newborn)	Tetrabutylammonium			140 μM	5		103
	Tetrapentylammonium			15 μM	5		103
	Tetrahexylammonium			0.5 μM	5		103
Rat Schwann (Adult)	Quinine	30 μM	0	40 μM	?	1.33	13
	TEA	0.2 mM	0	1.5 mM	?	7.5	13
	4-AP	400 μM	0	200 μM	?	.5	13
Rat glial cells	TEA			<20 mM	?		71
	TEA			>5 mM	?		64
	4-AP			<1 mM	?		64
NG 108-15 Neuroblastoma	Tamoxifen	2.5 μM	0	2 μM	5	0.80	78,79
	4-AP	0.5–1.0 mM	0	0.5—1.0 mM	5		79
	TEA	>1 mM	0	>1 mM	5		79
NCI-H69 Small-cell lung Cancer	4-AP	4 mM	0	<0.1 mM	10		63
HTB-9 human Bladder cancer	TEA			1–20 mM	5		85
	Quinine			1–100 μM	5		

Drug concentrations are given as IC_{50} values or a range of concentrations that include the IC_{50}. The ratio P/K is the ratio of the IC_{50} values for proliferation and K currents. The column labeled S indicates the percentage of serum present during the measurement.

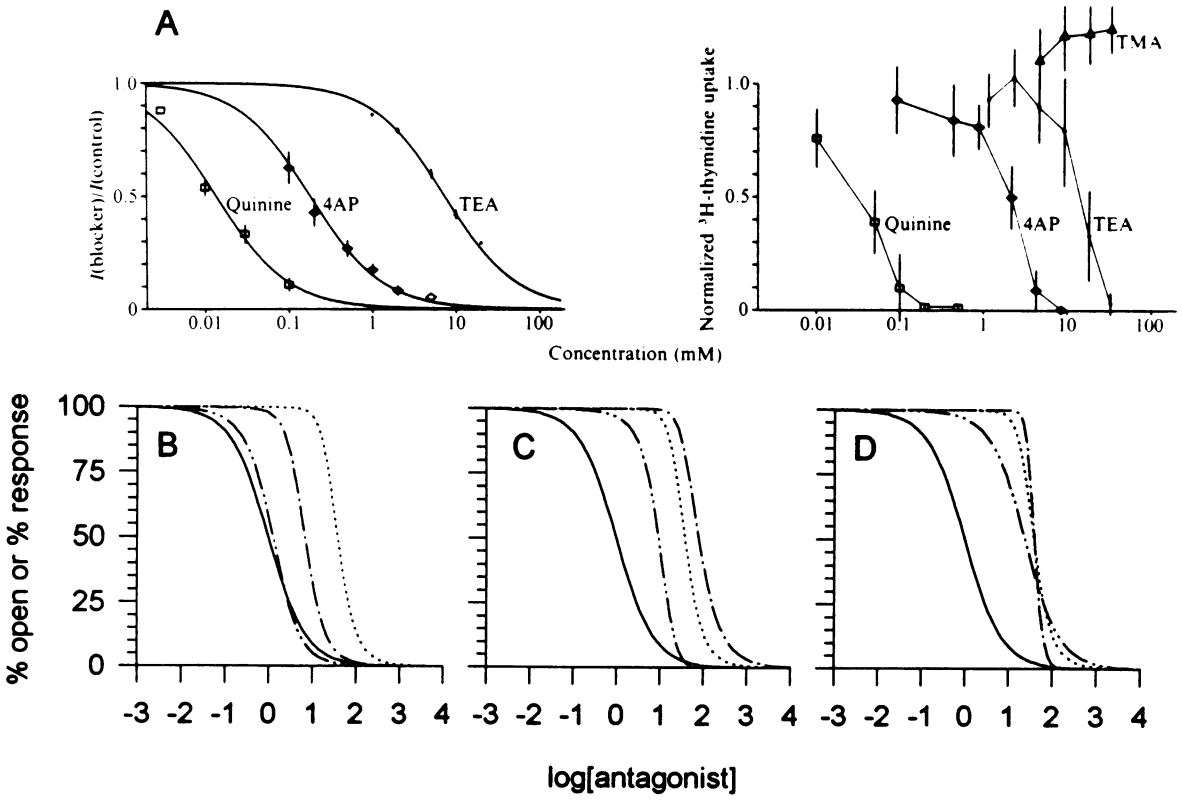

Fig. 2. (*A*) Dose-dependent inhibition of K currents (left panel) and mitogenesis (right panel) by quinine, 4-AP and TEA in T lymphocytes. Also shown at right are data for tetramethylammonium (TMA), which did not appreciably block K currents [*figure from ref* 18]. (*B–D*) Inhibition of K currents and proliferation predicted by the hyperpolarization-threshold model (*described in the text*). In each panel, the solid curve is the percentage of open K channels as a function of antagonist concentration (*c*) (Eq. 1; $IC_{50} = 1$; same curve in each panel). The dotted curve in each panel is the percentage of proliferation (*P*, % response) *vs c*, given $V = -25$ mV, $Ek = -85$ mV, $Gm = 5$, $\Delta V_t = -20$ mV and $\sigma = 10$ mV. For each concentration, *Gk* was calculated as $F_o \cdot 100$ (the absolute values of *Gk* and *Gm* are arbitrary, only the relative values affect the position of the curves). (*B*) The shift in IC_{50} for *P vs. c* is proportional to the ratio of *Gk* to *Gm*. Increasing *Gm* to 25 (–·–) or 100 (–··–) decreased the rightward shift in the IC_{50} for *P vs. c*, with little change in the slope of *P vs c*. (*C*) The shift in IC_{50} and, to a lesser extent, the slope of *P vs. c* is sensitive to ΔV_t. When ΔV_t was decreased to −10 mV (– ·–), the IC_{50} was increased, and when ΔV_t was increased to −40 mV (–··–), the IC_{50} was decreased. (*D*) The slope of *P vs. c* is inversely related to σ, increasing when σ was decreased to 5 mV (–·–) and decreasing when σ was increased to 40 mV (–··–).

open-pore K channel blockers such as TEA and 4-AP. Therefore, the IC_{50} for inhibition of K currents probably reflects the true equilibrium dissociation constant (*Kd*) for the binding of these drugs to the K channels, and the fraction of open channels (F_o) can be calculated as a function of the concentration (*c*) of antagonist according to

$$F_o = \frac{1}{1 + c/Kd} \tag{1}$$

The opening of K channels increases the membrane conductance, *Gm*, by an additive factor, *Gk*, which is proportional to *Fo*. This increment of K conductance produces a K current, *Ik*, that will move the membrane potential, *V*, towards the K equilibrium potential, *Ek*. Ohm's Law gives:

$$Ik = Gk \cdot (Ek - V), \tag{2}$$

and the change in membrane potential, ΔV, is therefore related to the total conductance, *Gm* + *Gk*, by the following function:

$$\Delta V = \frac{Ik}{Gm + Gk} = \frac{Gk \cdot (Ek - V)}{Gm + Gk} = \frac{Gk}{Gm + Gk} \cdot (Ek - V) \tag{3}$$

According to Eq. 3, ΔV is a nonlinear, saturating function of *Gk*, with a half-maximal hyperpolarization occurring when *Gk* is equal to *Gm*.

We assume in our model that progression through a control point in *G*1 requires a signal corresponding to a ΔV greater than some threshold value, ΔV_t, and, if ΔV is larger than ΔV_t, the cell will progress through the control

point in an all-or-none manner.[1] We also assume that, in a large population of cells, ΔV_t is distributed among different cells as a Gaussian function with variance σ^2 [6], and the fraction of cells that reach threshold for a given value of ΔV can be calculated by integrating this Gaussian distribution from 0 to ΔV. Therefore, the percentage (P) of cells that progress through the control point, as a function of ΔV, is:

$$P(\Delta V) = 100 \cdot \int_0^{\Delta V} \frac{1}{\sqrt{2\pi\sigma}} exp\left(-.5\left(\frac{V - \Delta V_t}{\sigma}\right)^2\right) dV \quad (4)$$

Proliferation should be proportional to P to the extent that the rate of proliferation is limited by the rate of passage through this control point.

The dose-response relations predicted by the hyperpolarization-threshold model described above (Fig. 2*B–D*) are consistent with three features of the dose-response relations reported for the inhibition of K currents and proliferation. First, the inhibition of K currents is based on a simple, single-site blocking model, which is evident in published dose-response curves. Second, it predicts an increase in the IC_{50} for proliferation relative to K current inhibition, which can be explained most easily in the context of spare receptor theory [81,89]. According to spare receptor theory, the concentration of an *agonist* required to elicit 50% of the maximal response (EC_{50}) will be less than the *Kd* of the agonist for its receptor if fewer than 100% of the receptors need to be occupied to produce a maximal effect. Alternatively, the concentration of an *antagonist* required to inhibit a response by 50% will be greater than the *Kd* of the antagonist for its receptor if fewer than 100% of the receptors need to be occupied to produce a maximal effect. As described in Eq. 3, the hyperpolarization produced by activation of K channels is a hyperbolic, saturating function of *Gk*, with 50% of the maximal hyperpolarization produced when *Gk* is equal to *Gm*. For a given value of ΔV_t, the smaller the value of *Gm*, the smaller the value of *Gk* that is required to produce a hyperpolarization greater than ΔV_t. If *Gm* is very small, then the number of K channels that must open to produce a value of *Gk* sufficiently large to hyperpolarize by ΔV_t might represent only a small fraction of the total K channels available. The channels not required to open represent spare channels, analogous to spare receptors. If the reserve of K channels is large, then it might be necessary to inhibit a very large fraction of K channels to prevent an increase in *Gk* that will hyperpolarize the membrane potential by ΔV_t. The IC_{50} for inhibition of proliferation will then be *increased* relative to the *Kd*, in proportion to the size of the channel reserve. In fact, Sabath et al. [82] estimated that at least 95% of K channels in T lymphocytes must be blocked to inhibit proliferation. The third feature of our model is the increased steepness of the dose-dependent inhibition of proliferation, compared to inhibition of K currents, which is indicative of a threshold phenomenon [6]. We incorporated a simple, all-or-none threshold into our model, in which the steepness of the dose-response curve for proliferation is inversely proportional to σ, the width of the Gaussian distribution of threshold values.

We presented this hyperpolarization-threshold model to illustrate the potential influence of spare K channels using a model that incorporates a plausible mechanistic linkage between the activation of K channels and proliferation. However, we simplified the model for clarity at the expense of leaving out factors, such as voltage-dependent block or voltage-dependent gating, that would be essential for this model to be fully generalizable. These factors could be incorporated into more complicated versions of this model. It is important to recognize that the influence of spare channels is not limited to the specific model we presented, because spare channels would have a similar effect in any model in which the activation of less than half of the K channels can produce half of the maximal proliferation.

Our hyperpolarization-threshold model demonstrates that it is impossible to test, for a single antagonist, the hypothesis that inhibition of K channel currents underlies the inhibition of proliferation by simply determining if the IC_{50} values for proliferation and K channel currents are significantly different, because significantly-different IC_{50} values could occur if either: (i) inhibition of proliferation resulted from interaction of the antagonist with a target other than K channels or (ii) the presence of spare channels increased the IC_{50}, even when the inhibition of K channel currents was solely responsible for inhibiting proliferation. Therefore, similar IC_{50} values for K channel currents and proliferation are consistent with the hypothesis of a common target, but different IC_{50} values cannot be used to reject the hypothesis. However, we might distinguish between these two alternatives by comparing, for *two or more* K channel antagonists, the ratio of the IC_{50} values for inhibition of K channel currents and proliferation. If all of the antagonists inhibit proliferation solely by inhibiting a single population of K channels, then the ratio of the IC_{50} values for inhibition of K channel currents and proliferation should be the same for all of the antagonists. Different ratios among the antagonists would suggest that the antagonists interact with different cellular targets or that

[1] Note that ΔV_t is a change in membrane potential, the smallest hyperpolarization constituting a signal sufficient to permit passage through *G*1. An analogous model could be developed in which the critical threshold is a fixed value of the membrane potential that must be exceeded for passage through *G*1, but a fixed-threshold model has different implications for cell cycle regulatory signals, which are discussed later.

they are differentially influenced by other factors, such as serum, metabolism or voltage-dependent inhibition. Our prediction of a similar ratio of IC_{50} values for a series of antagonists is in contrast to the predictions of spare receptor theory for a series of agonists, in which each agonist can reveal a different fraction of spare receptors and, therefore, produce a different shift in the EC_{50}, depending on their individual intrinsic activities.

Our final point regarding spare channels is that they might be functionally important in regulating the passage of cells through a control point in the cell cycle. If K channels must open before a cell can pass through a control point, then endogenous signaling pathways (or externally applied drugs) might slow or prevent passage through the control point by decreasing the probability of the K channels being open. However, if the K channels that regulate passage through the control point are overexpressed such that only a very small fraction of the expressed channels must open to increase *Gk* to a level sufficiently high relative to *Gm*, then the ability of either endogenous pathways or inhibitory drugs to regulate passage through the control point will be diminished without requiring any concomitant change in the activity of the signaling pathway. Therefore, a loss of proliferative control could result simply from overexpression of a population of regulatory K channels.

Is the Activation of K Channels Required for Progression through G1?

If inhibition of proliferation by K channel antagonists can be demonstrated, can we conclude that the activation of K channels is actually required at a specific stage in the cell cycle? Although it is certainly true that arrest at a specific stage in the cell cycle will inhibit proliferation, proliferation might also be inhibited by a nonspecific slowing of the entire cell cycle, without arrest at any specific stage. A general slowing of the cell cycle need not imply a nonspecific action of a drug, and, in fact, it might represent the very selective interaction of a drug with a receptor/enzyme whose activity influences the cell's behavior throughout the cell cycle (e.g., energy production). Inhibition of proliferation by K channel antagonists, therefore, provides necessary, but not sufficient, evidence that activation of K channels is required for passage through a specific stage in the cell cycle.

The application of techniques that resolve, in greater detail, the passage of cells through the cell cycle must be used to test the hypothesis that K channel antagonists arrest at a specific stage in the cell cycle. Arrest in either G0/G1, S or G2/M regions of the cell cycle can be assayed using flow cytometry to assess DNA content and calculate the cell-cycle distribution, in which case a selective, cell-cycle arrest will lead to an accumulation of cells in a specific region. Although flow cytometry can provide strong evidence for the arrest of cells in G1 phase, further resolution of the temporal position of the site of arrest requires examination of the progression of phenotypic changes that occur during passage through G1 phase, such as changes in the level or activity of cyclin proteins, RNA synthesis or cell volume. Table 2 provides a listing of G1 events that have been used as indices to assess the arrest of cells in G1 phase by K channel antagonists.

Table 2. Indices used to assess arrest in G1 by K Channel Antagonists

Index	References
Cell-cycle distribution (flow cytometry)	43, 105
G1-specific protein expression:	
IL-1	12,27,30,48,70
IL-2 receptor	12,27,30
Cyclin D1	97
Transferrin receptor	4
Other proteins	4,82
Increased RNA synthesis	4,23
Increased cell size	4
Cytosolic Ca signals	30,48

The role of K channels in G1 progression has been studied most thoroughly during the activation of quiescent T lymphocytes or PBMCs by mitogens, such as phytohemagluttinin (PHA). Activation by PHA is dependent on extracellular Ca and involves the movement of lymphocytes from G0 to G1, the expression of cell surface receptors for the cytokine interleukin-2 (IL-2) and the production and release of IL-2. The binding of the released IL-2 to the IL-2 receptors initiates a second signal required for progression from G1 to S phase, and the stimulation by IL-2 is not dependent on extracellular Ca.

The strongest evidence linking the inhibition of K currents, proliferation and G1 progression is inhibition of the mitogen-stimulated proliferation of human T cells and PBMCs by high-affinity peptide toxins [27,48,70]. These toxins include charybdotoxin (ChTx), which inhibits large-conductance Ca-activated K channels (K_{Ca}) and inactivating voltage-gated K channels [28], and margatoxin (MgTx), which specifically inhibits Kv1.3 inactivating voltage-gated K channels [29]. Both ChTx and MgTx inhibited the Ca-dependent stimulation of human T lymphocytes by mitogens, reducing both the increase in cytosolic Ca [48] and the production of IL-2 [27,48,70]. Although ChTx can inhibit both voltage-gated and Ca-activated K channels, ChTx binds with high affinity to a single site on human T lymphocytes [23]. The similar inhibitory action of MgTx [48], a selective inhibitor of Kv1.3 channels [29], provides additional evidence that inhibition of voltage-gated Kv1.3 channels underlies the inhibitory effects of ChTx.

The Kv1.3 channels are the most abundant voltage-

gated K channel in T lymphocytes, and were originally designated as *n* type channels (*reviewed in* 47). Inhibition of the PHA-P-stimulated release of IL-2 from PBMCs by ChTx was accompanied by a 50% suppression of the transient increase in IL-2 mRNA levels typically observed after stimulation [27]. The transient stimulation of c-myc mRNA expression by PHA-P was also reduced by ChTx, but to a lesser extent than IL-2 mRNA, whereas constitutively-expressed Class I MHC HLA-I mRNA was unaffected by ChTx [27]. The inhibition of the expression of IL-2 mRNA and the release of IL-2 was very consistent among cell donors and nearly complete at high ChTx concentrations, whereas inhibition of proliferation by ChTx was highly variable among cell donors, with a maximum of less than 60% inhibition [27]. Although inhibition of Kv1.3 channels prevented IL-2 production, the inhibition of proliferation by ChTx could be overcome by treatment with IL-2, indicating that the IL-2 receptor-effector systems were intact in the presence of ChTx [48,70]. A variable level of baseline IL-2 production might therefore underlie the variable efficacy of ChTx in inhibiting proliferation [27]. These observations suggested that ChTx primarily blocked the induction of IL-2 expression, which is required for progression through G1 phase. The site of arrest by ChTx was located in early G1 phase, because ChTx was most effective when added to the culture medium very early (<4 hr) after stimulation by mitogens [48,70]. Thus, a link between K channel activity and G1 progression in T lymphocytes involves the early G1 production of an essential progression factor, IL-2.

Amigorena et al. [4] reported that TEA, 4-AP, quinine and verapamil inhibited the Ca-dependent activation of murine B lymphocytes by the mitogen lipopolysaccharide (LPS). The cells appeared to be arrested in mid-G1 phase, based on the appearance of early G1 markers, including the expression of Ia and FcγRII proteins, early RNA synthesis and cell enlargement, but the absence of a late-G1 marker, the expression of transferrin receptors. The stimulation of B lymphocytes by LPS was not inhibited by ChTx [8], which indicated that the activation of Kv1.3 channels was not required for the LPS-stimulated proliferation of murine B lymphocytes.

The proliferation of T lymphocytes can also be stimulated by IL-2 via a Ca-independent pathway. In the murine noncytolytic T lymphocyte clone, L2, unstimulated cells were predominately in G1 phase, rather than G0 phase, and IL-2 stimulated progression through the cell cycle [43]. Concurrent addition of quinine and IL-2 inhibited the IL-2-stimulated proliferation of L2 cells [43], and the addition of quinine 2 hr, but not 6 hr, after stimulation by IL-2 blocked the synthesis of a set of proteins expressed in late G1, whose level was correlated with cell proliferation [82]. Quinine also inhibited K currents in L2 cells [43], but it is not known if the quinine-sensitive event that occurs 2–6 hr after stimulation by IL-2 was related to inhibition of these K currents. It appears that Kv1.3 channels, which are required for the Ca-dependent, mitogen-stimulated proliferation of human T lymphocytes (*see above*), are not required for the Ca-independent stimulation of human T lymphocytes by IL-2, because the stimulation of human T lymphocytes by IL-2 was not inhibited by ChTx [48].

The arrest of cells other than lymphocytes in G1 phase by K channel antagonists has been characterized in greatest detail in MCF-7 human breast cancer cells. We reported that TEA, 4-AP, quinidine, glibenclamide and linogliride were equally effective in inhibiting the proliferation of MCF-7 human breast cancer cells, but only a subset of these antagonists, including glibenclamide and linogliride, inhibitors of ATP-sensitive K channels (K_{ATP}) [25,31,77], and quinidine, which also inhibits K_{ATP} currents [31], arrested the cell cycle in G0/G1, as measured by flow cytometry [105]. The inability of TEA and 4-AP to arrest the cell cycle, even though they were very effective inhibitors of proliferation in MCF-7 cells [105], is a good example of why a mechanism of cell-cycle arrest should not be inferred from the inhibition of proliferation. The G0/G1 arrest in MCF-7 cells by these agents was reversible. The cells entered S phase 12–18 hr after washout of drug, and a peak in the fraction of cells in S phase was observed 24 hr after washout. Similar washout kinetics were observed in MCF-7 cells arrested with lovastatin, an agent which is known to arrest MCF-7 cells in early G1 [41]. We recently began to examine the expression of cell cycle regulatory proteins to identify the site of arrest by these drugs. We compared the expression of cyclin D1 protein by cells arrested in G1 with quinidine and lovastatin. Quinidine-arrested cells exhibited reduced cyclin D1 levels relative to unsynchronized, growing cells. However, in lovastatin-arrested cells, cyclin D1 levels were similar to the unsynchronized, growing cells [97]. These data indicate that the site of arrest by quinidine is very early in G1, slightly preceding the lovastatin arrest site (Fig. 1).

Which K Channels are Required for Progression through G1 Phase?

Identification of the specific K channels which are required for progression through G1 phase is a high priority, because it will enable a more direct study of the relationship between the activation of these K channels and the activity of cell-cycle signaling pathways.

Voltage-gated K Channels

At present, only the activation of voltage-gated Kv1.3 channels in T lymphocytes has been definitively linked

to progression through G1 phase, as described above. The strength of this conclusion rests on the use of ChTx and MgTx to examine the role of Kv1.3 channels. These peptide toxins are unlikely to enter cells, and their action should be limited to the block of specific K channels in the plasma membrane. This specificity was confirmed in human T lymphocytes by the high-affinity binding of ^{125}I-ChTx to a single class of sites, which represented the predominant voltage-gated K channels in these cells [23]. Inhibition of Kv1.3 channels might also be partially responsible for inhibition of the mitogen-stimulated proliferation of T cells by TEA, 4-AP and quinine [12,18], although these antagonists most likely interact with other targets as well (*discussed below*). Voltage-gated K channels have also been suggested to be required for the proliferation of human melanoma cells [58] and Nb-2 lymphoma cells [98].

Ligand-gated K Channels

ATP-sensitive K channels (K_{ATP}) might be involved in regulating G1 progression in MCF-7 cells. We identified three components of the macroscopic current-voltage relations in MCF-7 cells, only one of which, an ATP-sensitive linear outward current, displayed the proper pharmacological sensitivity to be the target of the agents that produced G1 arrest [42]. This current exhibits run-down during whole-cell recording, which has slowed our efforts to characterize the dose dependence of its inhibition by G1-arresting agents. Recently, we identified a small-conductance (8.5pS) K_{ATP} channel in MCF-7 cells which might underlie the macroscopic linear outward current [42]. In contrast to lymphocytes, ChTx and MgTx did not arrest MCF-7 cells in G1 [105], and we have not observed any K currents that resemble Kv1.3 currents. There is only one voltage-dependent current in MCF-7 cells, a TEA-sensitive, outwardly rectifying current that is activated at very positive potentials [42], but TEA does not arrest the cell cycle of MCF-7 cells. A functional role of K_{ATP} channels has also been proposed for the proliferation of hair follicle cells [10] and the maturation of oocytes [101], and the proliferation of Nb-2 cells was also inhibited by glibenclamide [39]. There are also reports that agents that activate K_{ATP} channels, including diazoxide, minoxidil and cromakalim, can inhibit proliferation [44,45,56,61]. Diazoxide and cromakalim also inhibited the agonist-induced mobilization of intracellular Ca in these cells [44,45]. However, the inhibition by cromakalim was only partially reversed by glibenclamide [45], which suggests that the inhibition of proliferation by cromakalim was not mediated solely by activation of K_{ATP} channels.

Ca-activated K channels (K_{Ca}) might also play a role in regulating progression through G1 phase. As described below, mitogenic stimulation of proliferation often leads to an increased level of activity of K_{Ca} channels, which can include the expression of new K channel proteins. In fibroblasts, inhibition by ChTx of the K_{Ca} channels expressed after mitogenic stimulation also inhibited proliferation [37].

Nonspecific Actions of K Channel Antagonists

The studies discussed above and listed in Table 1 suggest that several different types (and possibly combinations) of voltage- and ligand-gated K channels might be required for the progression of different populations of cells thorough G1 phase, and this is not surprising, considering the very different repertoires of K channels expressed by different types of cells. However, with the exception of the peptide toxins, the K channel antagonists used in these studies are not highly selective, and their ability to arrest in G1 phase or inhibit proliferation might be due to interactions with intracellular targets other than K channels.

The limited number of K channels for which high-affinity antagonists are available, combined with the fact that many relevant investigations were performed prior to the biochemical isolation and purification of these toxins, has resulted in a large number of published studies employing a repertoire of organic K channel antagonists that have much lower affinity and selectivity for K channels. Examples of frequently used antagonists are TEA, 4-AP, verapamil, quinine and quinidine (Table 1). Unlike the peptide toxins, these K channel antagonists can enter cells (verapamil [17]; TEA [C. Deutsch, *personal communication*]), and this greatly increases the possibility of nonspecific inhibitory actions. Although interpretation of the effects of these low-affinity antagonists is problematic, they are very effective inhibitors of K currents and proliferation, and this has contributed to their popularity as tools for studying the role of K channels in proliferation.

It is very likely that the inhibition of proliferation and/or arrest in G1 phase by TEA and 4-AP might not be due simply to inhibition of K currents. In human T lymphocytes, 4-AP and TEA completely inhibited proliferation [18], whereas ChTx, which completely inhibited the Kv1.3 currents, inhibited the proliferation of human T cells by a maximum of 50–60% [27]. Furthermore, although 4-AP blocked IL-2 production without affecting IL-2 receptor expression, the inhibition of proliferation by 4-AP could not be overcome by exogenous IL-2 [12], as was the case for inhibition by ChTx [70]. These observations suggest that the more complete arrest in G1 by TEA and 4-AP probably involved interactions at other targets, in addition to Kv1.3 channels. This surmise is consistent with the repeated observation that the ratio of the IC_{50} values for proliferation and K currents are much greater for 4-AP than for TEA and other K channel an-

tagonists (Table 1). Although the disparity in IC_{50} values is partly due to the presence of serum during the measurement of proliferation, the ratio of IC_{50} values under serum-free conditions (Table 1) is still larger than expected if 4-AP inhibits proliferation only by blocking the same K channels inhibited by the other antagonists. There could be many cellular targets for TEA and 4-AP, other than K channels. For example, Schell et al. [84] concluded that TEA and 4-AP inhibited the mitogen-stimulated activation of lymphocytes by blocking the uptake of thymidine, amino acids and other essential metabolites, rather than by blocking K channels. Pappas et al. [64] reported that 4-AP, which is a weak base, inhibited the proliferation of cultured astrocytes concomitant with an alkalization of the intracellular pH that was probably sufficient to account for the inhibition of proliferation. We observed that TEA and 4-AP effectively inhibited the proliferation of MCF-7 cells without affecting cell cycle progression [105], suggesting that these agents can also slow the cell cycle, without arresting at a specific stage in the cell cycle. All of these observations underscore the great caution that should be exercised in attributing the effects of TEA and 4-AP on proliferation solely to inhibition of K channels.

The high concentration of selective K channel antagonists sometimes required to inhibit proliferation is also a concern. The concentration of glibenclamide required to inhibit the proliferation of MCF-7 and Nb-2 cells was several orders of magnitude higher than the concentration required to inhibit most K_{ATP} channels [31]. At this high concentration, glibenclamide has been reported to inhibit voltage-gated K currents in neuroblastoma cells [74], cAMP-activated Cl currents in heart [92] and CFTR Cl currents in an inner medullary collecting duct cell line [93]. We do not know if the low potency of glibenclamide in inhibiting proliferation resulted from its interactions with targets other than K_{ATP} channels, a lower sensitivity of K_{ATP} channels in these cells, or if it simply resulted from the presence of spare K_{ATP} channels, as described in our model. The resting membrane potential of MCF-7 cells is insensitive to substitution of extracellular Cl [104], indicating that the G1 arrest by glibenclamide is probably not due to inhibition of Cl currents. The putative target of glibenclamide is the sulfonylurea receptor (SUR), which is a member of the superfamily of ABC (ATP-binding cassette) proteins [38], among which is the CFTR. Further investigation will be required to determine if inhibition of the proliferation of MCF-7 and Nb-2 cells by glibenclamide specifically involves K_{ATP} channels or, perhaps, other proteins that might be regulated by the SUR or other ABC proteins [36].

In summary, there is very strong evidence linking the inhibition by ChTx of the mitogen-stimulated proliferation of T lymphocytes to the inhibition of a specific K channel, Kv1.3, and this is associated with a decreased production of IL-2 in early G1 phase. In MCF-7 cells, inhibitors of K_{ATP} channels arrest the cell cycle in early G1 phase, and a small-conductance K_{ATP} channel has been identified that might represent the target of these drugs, but further investigation will be required to demonstrate that inhibition of this channel is responsible for the G1 arrest. Potassium channel antagonists also arrest mitogen-stimulated murine B lymphocytes and IL-2-stimulated murine L2 lymphocytes in mid-G1 phase, but the identity of a K channel target of these drugs that might be responsible for the arrest has not been confirmed. For cells in which no high-affinity peptide toxins have been identified that arrest in G1 phase, molecular approaches, such as the heterologous expression of K channels or the antisense knockout of endogenous K channels, might be employed to manipulate the activity of K channels in these cells and test hypotheses that specific types of K channels must be activated for progression through G1 phase.

How Might the Activation of K Channels Control Progression through G1 Phase?

During G1 phase, cells prepare for entry into S phase by performing many essential tasks, such as the uptake of metabolic substrates, the synthesis of RNA and proteins, and the processing of cell-cycle regulatory signals [*reviewed* in 66,100]. The activation of K channels might be required for successful completion of one or more of these tasks. Identification of the specific tasks which are directly dependent on the activation of K channels is difficult. Because of the sequential nature of many of the events that occur during G1 phase, arrest of progression through G1 phase at a specific site by K channel antagonists might block multiple downstream events, even if they are not directly dependent on the activation of K channels. Therefore, the conclusion that any G1 event, absent following arrest by a K channel antagonist, is dependent on the activation of K channels requires very specific tests of that dependence. We review below several processes that might be required for progression through G1 phase and which might require the activation of K channels.

Hyperpolarization of the Membrane Potential

The Membrane Potentials of Cycling vs. Noncycling Cells

Comparison of the membrane potentials of many types of cells has revealed a strong relationship between membrane potential and mitotic activity: (i) terminally differentiated cells in G0 phase are very hyperpolarized, (ii)

cycling cells that do not enter G0 phase (e.g., tumor cells) are very depolarized, and (iii) quiescent cells that require stimulation by mitogens to enter the cell cycle from a resting state in G0 (e.g., lymphocytes) are relatively hyperpolarized [7]. The cycling cells and mitogen-stimulated quiescent cells can be further distinguished by different patterns of changes in membrane potential during their passage through G1 phase. Some cycling cells undergo a large hyperpolarization between early G1 phase and S phase. For example, the resting membrane potentials of Chinese Hamster V79, Neuro-2A neuroblastoma and MCF-7 cells synchronized in early to middle G1 phase were typically depolarized (−5 to −25 mV), and the membrane potential hyperpolarized by 15–30 mV during progression through G1 and entry into S phase [9,83,104]. We termed the site at which MCF-7 cells were arrested in early G1 by K channel antagonists the ''D control point,'' because the cells were very depolarized (−9 mV) at this site of arrest [104]. Hyperpolarization during G1 has not been reported in all cycling cells, and some cycling cells might depolarize during progression from G1 to S phase [5]. The prevalence of hyperpolarization *vs.* depolarization during the passage of different types of cycling cells through G1 has not been systematically studied, but hyperpolarization has been reported more frequently in the literature.

In contrast to the cycling cells that do not enter G0 phase, the membrane potentials of quiescent, unstimulated lymphocytes in G0 are much more negative (−50 to −70 mV) [32], similar to the membrane potentials of differentiated cells [7]. The entry of these cells into G1 phase following stimulation by mitogens is probably accompanied by a small hyperpolarization [32]. It is important to emphasize that mitogen-stimulated lymphocytes do not appear to pass through a depolarized state similar to the D control point in MCF-7 cells during their progression from G0 through G1 phase. The stimulation of cells other than lymphocytes by mitogens can produce either hyperpolarization or depolarization, depending on the pre-stimulus membrane potential [13,52,103]. Although K channel antagonists appear to block both the progression of cycling cells through G1 and the mitogen-stimulated movement of quiescent cells into and through G1, the cellular signals and G1 events associated with the two processes might be very different, and we should be cautious in generalizing, between the two groups, any putative mechanisms by which the activation of K channels might be required for progression through G1 phase.

Is Hyperpolarization Required for G1 Progression?

Inhibition of the proliferation and/or G1 progression of both cycling and mitogen-stimulated cells by K channel antagonists has led to the hypothesis that hyperpolarization of the membrane potential by K channels is required for the progression of both groups of cells through G1 phase. Several investigators have attempted to test this hypothesis by determining if proliferation and/or specific cell cycle-related parameters are sensitive to depolarization of the membrane potential by an increase in the extracellular K concentration (K_o). Freedman et al. [27] demonstrated that depolarization of PHA-P-stimulated PBMCs to the same membrane potential observed during treatment with ChTx (35 mM K_o, −44 mV) mimicked the inhibitory action of ChTx, including inhibition of proliferation, reduction of IL-2 mRNA levels and inhibition of IL-2 release, and they concluded that the inhibitory action of ChTx might be accounted for by its depolarization of the membrane potential. Freedman et al. [27] also demonstrated that larger increases in K_o produced a dose-dependent inhibition of proliferation, IL-2 mRNA expression and IL-2 release that reached nearly complete inhibition at 100 mM K_o (≈−10 mV). Gelfand et al. [30] observed a similar inhibition of IL-2 release and proliferation of PHA-stimulated human T lymphocytes following complete substitution of extracellular Na by K, and the mitogen-stimulated proliferation of Schwann cells was also inhibited by high (>30 mM) K_o [103]. The evidence that mitogenic stimulation can be inhibited by depolarization with high K_o suggests that the membrane potential must be maintained at a sufficiently hyperpolarized level to permit the entry of mitogen-stimulated cells into the cell cycle. In cycling cells, a critical role of membrane hyperpolarization during G1 progression has been supported by the observation that high K_o reversibly arrested Chinese Hamster V79 cells in early G1 phase [88] and BHK 21 cells in middle G1 phase [62]. The proliferation of astrocytes [11], human astrocytoma [44] and human neuroblastoma cells [44] was also inhibited by high K_o. Collectively, these studies appear to provide strong support for a conclusion that membrane hyperpolarization is required for the proliferation of both mitogen-stimulated and cycling cells.

In each of these studies, K_o was increased by equimolar substitution for extracellular Na, thereby maintaining a constant tonicity. Unfortunately, the concomitant reduction in Na makes it difficult to separate the effects of the depolarization by high K_o from the possible effects of a decreased Na gradient, including the inhibition of Na-dependent membrane transport processes required for progression through G1 phase (*discussed below*). Furthermore, proliferation was assayed in most of these studies by measuring the incorporation of [^{3}H]-tdr into DNA, yet the uptake of [^{3}H]-tdr in some cells is Na dependent [94], and reduction of the Na gradient could therefore artefactually affect this assay so that inhibition of cell proliferation might be overestimated. This latter concern is supported by the observation in astrocytes that DNA synthesis, measured by [^{3}H]-

tdr incorporation, was considerably more sensitive to substitution of extracellular K for Na than was proliferation measured directly by cell counts [11]. The possibility that substitution of extracellular K for Na artefactually appeared to inhibit proliferation might also be supported by the observations that relatively small increases in K_o made without substitution for Na did not inhibit the proliferation of astrocytes (25 mM K_o) [64] or PHA-stimulated lymphocytes (50 mM K_o) [15]. Wilson and Chiu [103] reported that complete substitution of extracellular K for Na (116 mM) in the absence of mitogen nearly completely inhibited the proliferation of Schwann cells but substitution of choline for Na had virtually no effect, indicating that the Na gradient was not important when cells were proliferating slowly in the absence of mitogen. However, in the presence of mitogen, substitution of choline for Na produced substantial inhibition, although less than the complete inhibition produced by substitution of K for Na. It appears, therefore, that the effect of the loss of the Na gradient might be dependent on the growth conditions, producing a much greater inhibitory effect when cells are rapidly proliferating.

Although the available evidence does not permit an unambiguous separation of the effects of depolarization by elevated K_o from the effects of a decreased electrochemical gradient for Na on G1 progression, a critical role of depolarization is still supported by two points. First, depolarization by elevated K_o arrested the cell cycle of some cells in early G1 phase, which might be prior to the critical Na-dependent uptake of substrates, such as amino acids, which is more prominent in middle to late G1 phase [66]. Second, it seems unlikely that some early G1 events, such as the mitogen-stimulated increase in intracellular Ca or the expression of IL-2 message, would be inhibited simply by a decrease in the electrochemical gradient for Na. We believe that sufficient evidence exists to allow us to surmise that membrane potential does not simply passively follow the cell cycle, as argued by some investigators [83]. Rather, a hyperpolarization of the membrane potential might be required for the progression of cycling cells through G1 phase and maintenance of a sufficiently hyperpolarized potential might be required for the entry of mitogen-stimulated cells into the cell cycle. In both groups of cells, the activation of K channels would be required for progression through G1 phase. Clearly, however, further investigation will be required to test more rigorously the requirement for membrane hyperpolarization during G1 progression.

Hyperpolarization-dependent Processes During G1 Progression

If hyperpolarization is required for progression through G1 phase, which events in G1 are dependent on hyperpolarization of the membrane potential? The two processes that have received the most attention are the regulation of Ca influx and Na-dependent membrane transport. An increase in cytosolic Ca activity due to the combined release of Ca from intracellular stores and the influx of extracellular Ca is generally required at two stages during progression through G1 phase, the G0/G1 transition and the G1/S transition [*reviewed in* 100]. It is important to note that most proliferating cells lack voltage-gated Ca channels, and Ca influx is stimulated by hyperpolarization of the membrane potential, which increases the driving force for the entry of Ca through poorly identified pathways.

Mitogenic stimulation of quiescent cells increases the cytosolic Ca activity via the inositol 1,4,5-trisphosphate (IP_3) -mediated release of intracellular Ca, which may be followed, to a variable extent, by a prolonged influx of extracellular Ca [52, *reviewed in* 100]. The time course of these Ca signals is highly variable among different types of cells, and even in single cells responding to different mitogens, with signals ranging from repetitive spikes to oscillations to a single, prolonged rise in Ca activity. Potassium channels might influence the shape of these Ca signals by at least two mechanisms. First, the agonist-induced generation of IP_3 has been shown in HL-60 cells to be inhibited by depolarization of the membrane potential by gramicidin [69]. Therefore, a membrane potential hyperpolarized by active K channels might be required for the IP_3-mediated release of intracellular Ca. This role for K channels might be important for many studies discussed in this review, because some of the agents/treatments frequently used to test the role of K channels in the cell cycle, including TEA, 4-AP and depolarization by high K_o, have been shown to inhibit the agonist-induced release of intracellular Ca without affecting the basal Ca activity in unstimulated cells [44]. A second mechanism by which K channels might influence mitogenic Ca signals is their ability to provide or maintain a sufficient driving force for Ca entry. This might be an especially important role for K_{Ca} channels, because positive feedback provided by the activation of K_{Ca} channels by cytosolic Ca might enhance or prolong Ca influx following mitogenic stimulation [68].

Lin et al. [48] observed in human T lymphocytes that ChTx inhibited the rise in cytosolic Ca activity that normally follows stimulation by mitogens, indicating that activation of Kv1.3 channels was required. Depolarization by high K_o also inhibited the Ca signal in T lymphocytes elicited by mitogenic stimulation [30,48], and the inhibitory effects of ChTx and depolarization by high K_o were not additive [48], suggesting a common mechanism of action. However, the functional relationship between depolarization by high K_o, inhibition of the mitogenic Ca signal and proliferation remains unclear. Gel-

fand et al. [30] observed that treatment with the Ca ionophore ionomycin overcame much of the inhibition of proliferation by depolarization, and Freedman et al. [27] observed that increasing the extracellular Ca increased the IC_{50} for inhibition of proliferation by high K_o, but Freedman et al. [27] also reported that treatment with ionomycin could not overcome the inhibition of proliferation by either high K_o or ChTx, which would be expected if the inhibition of a Ca signal was solely responsible for inhibition of proliferation. Although inhibition of a mitogenic Ca signal by ChTx might provide a plausible explanation for inhibition of the production of IL-2, further investigation will be required to fully define the relationship between membrane potential, mitogenic Ca signals and proliferation.

Calcium signals are also required for the progression of cycling cells through G1 phase, but the role of K channels in these Ca signals is not as well studied. Nilius et al. [60] observed in melanoma cells a sigmoidal relationship between the cytosolic Ca activity and the membrane potential, with Ca activity increasing with hyperpolarization. This relationship was centered near the resting potential, indicating that changes in the membrane potential near the resting potential, due to the opening or closing of K channels, could produce large changes in the cytosolic Ca activity. Nilius et al. [60] also observed that when a melanoma cell voltage-clamped at 0 mV was released by switching to current clamp, the membrane potential rapidly recovered to a resting potential near −50 mV. This increased the driving force for Ca and elicited a slow increase in cytosolic Ca which accelerated with further hyperpolarization produced by activation of K_{Ca} channels, ultimately resulting in a large, sustained Ca signal. The elicitation of a sustained Ca signal by a large hyperpolarization might be very relevant to the progression of cycling cells through a Ca-dependent step in G1 phase [59,60]. We suggest that in MCF-7 cells an initial hyperpolarization produced by the opening of K_{ATP} channels could trigger a Ca influx that would be sustained by the subsequent activation of K_{Ca} channels. Activation of the K_{ATP} channels would only need to be transient and could be tightly linked to other cell-cycle signals, such as a brief fall in the ATP/ADP ratio. The accuracy of this model, and its generalizability to other types of cycling cells, remains to be determined.

The Na-dependent transport of metabolic substrates and ions across the plasma membrane is also required for the G1 to S transition, and hyperpolarization during progression through G1 can increase the rate of Na-dependent transport by increasing the electrochemical gradient for Na [55,95,96]. Potassium channel antagonists might arrest in G1 by slowing these Na-dependent transport processes.

Fixed-threshold vs. Hyperpolarizing-transition Models

To summarize the relationship between membrane potential and the cellular processes that might control G1 progression, we would like to distinguish between two possible requirements for passage through a G1 control point. In the first model, the membrane potential must simply be hyperpolarized relative to a fixed threshold potential to permit passage through the control point. In the second model, a hyperpolarization must occur that produces a *change* in membrane potential larger than a threshold value, regardless of the final value of the membrane potential. There is some overlap between these two models, since a large hyperpolarization of the membrane potential might satisfy both requirements, but there are also important differences.

In the fixed-threshold model, the membrane potential provides a permissive function, rather than producing a true, discrete signal. This places minimal demands on synchronization between the activity of K channels and other cell cycle regulatory events, and, in an extreme case, we might expect that the hyperpolarization produced by chronically activated K channels could actually facilitate passage through G1. The fixed threshold model also places minimal demands on the type of K channel that could serve a regulatory function at the control point since its activity does not need to be precisely controlled by other cell cycle signaling pathways. In fact, chronic exposure to a K ionophore, such as valinomycin, might be expected to substitute for K channel activity. G1 events that are simply dependent on the membrane potential as a source of energy would be especially likely to underlie a fixed-threshold model for regulation. The Na-dependent uptake of metabolic substrates, such as amino acids [96], would be a good example.

In the hyperpolarizing transition model, on the other hand, it is critical that a hyperpolarization of the membrane potential by synchronized with other cell-cycle regulatory processes at a G1 control point, and this would limit the types of K channels that might serve this regulatory function since their activity would have to be appropriately controlled by other cell-cycle regulatory signals. Furthermore, chronic hyperpolarization of the membrane potential might actually inhibit progression through the control point, since it would reduce the size of any hyperpolarization that could be produced by activation of K channels. The G1 events most likely to require a hyperpolarizing transition would include processes that inactivate or reset following prolonged stimulation and, therefore, would respond only to a change in membrane potential. Also, many Ca signals tend to be transient, which not only prevents cellular toxicity due to a prolonged rise in cytosolic Ca activity, but also allows a sequential coordination between Ca signals and other

signaling pathways. If hyperpolarization stimulates Ca entry (*as described above*), then it might also be necessary that a cell be depolarized at specific points in the cell cycle (e.g., early G1) to prevent inappropriate Ca entry and Ca signals. This might lead to the requirement that the membrane potential hyperpolarize only at the specific point during G1 at which the triggering of a Ca signal would be appropriate.

It seems likely that hyperpolarization beyond a fixed threshold might be a very common requirement, because of the importance of a hyperpolarized membrane potential for a large number of energy-dependent processes (e.g., Na-dependent substrate uptake) necessary for passage through G1 phase and entry into S phase. On the other hand, a requirement for a hyperpolarizing transition in the membrane potential might be more variable, perhaps being most important in cycling cells. The notion of a discrete hyperpolarization synchronized with other cell cycle signaling pathways has not received as much attention as the fixed threshold model, perhaps because of the more common experimental focus on the role of K channels in mitogenic stimulation, which is often associated with small hyperpolarizations of quiescent cells that are already very hyperpolarized, rather than the progression of cycling cells through G1 phase.

The relative importance of the fixed threshold *vs.* hyperpolarizing transition requirements during passage through G1 phase could influence the sensitivity of G1 progression and/or proliferation to various manipulations. For example, if the transient activation of a population of K channels produces a hyperpolarization that is required for progression, then a selective channel antagonist might inhibit progression through G1 by blocking these channels. However, because the regulatory channels are only transiently activated during the cell cycle, acute application of an antagonist of these K channels would depolarize these cells only if they were at the point in the cell cycle at which these K channels were activated, and there would be no effect at other positions in the cell cycle. This might account for the observations, in some cells, that the acute application of K channel antagonists which inhibited proliferation did not depolarize the membrane potential [64,79,80]. A transient activation of regulatory K channels is supported by our observation that acute application of the K_{ATP} antagonist glibenclamide to unsynchronized MCF-7 cells depolarized very few cells (1 in 20, *unpublished observation*), yet in whole-cell recordings in which ATP was not present in the recording pipette, a much larger fraction of cells exhibited glibenclamide-sensitive K currents [42]. This would be consistent with only a transient activation of K_{ATP} channels during the cell cycle.

It might be possible to determine the relative importance of the fixed threshold *vs.* hyperpolarizing transition requirements during passage through G1 phase by manipulating the membrane potential during the cell cycle independently of the endogenous K channel activity. Daniele and Holian [15] reported that the K ionophore valinomycin inhibited the PHA-stimulated proliferation of lymphocytes. The inhibition was prevented by increasing K_o, which indicated that valinomycin was acting at the plasma membrane, and these results suggested that chronic hyperpolarization by valinomycin inhibited the proliferation of lymphocytes. However, these investigators did not report the actual effect of valinomycin on membrane potential, which might have been small since the resting membrane potential of quiescent lymphocytes is rather hyperpolarized, and the inhibitory effects of valinomycin might have resulted from other effects on membrane transport, as acknowledged by the authors. Although ionophores such as valinomycin are attractive because of their simplicity, their effects can be difficult to interpret, and more rigorous tests might be performed by manipulating the expression of heterologously expressed K channels.

Regulation of Cell Volume

Some cells swell and then undergo a regulatory volume decrease (RVD) when they are placed in a hypotonic medium. The RVD requires the efflux of water and solutes, and it is produced by the simultaneous opening of K and C1 channels. In lymphocytes, RVD was inhibited by antagonists, such as charybdotoxin, that block Kv1.3 channels [33], and a specific link between Kv1.3 channels and RVD in lymphocytes was demonstrated when the heterologous expression of Kv1.3 channels conferred the capacity for RVD onto a T lymphocyte cell line that does not normally volume regulate [21]. Although RVD is a response to a nonphysiological stimulation, an analogous process might occur during growth under isotonic conditions. Rouzaire-Dubois and Dubois [24,79] invoked a volume regulatory process in their model for the regulation of mitogenesis by K channels. In their model, an increase in cell volume due to the influx of Na is prevented by the simultaneous efflux of K, and this maintains intracellular solutes, such as Na, at a concentration high enough to promote DNA synthesis. These investigators also demonstrated that valinomycin could partially restore the proliferation of neuroblastoma cells which had been inhibited by quercetin, an inhibitor of voltage-dependent K currents in these cells [80]. Neither quercetin nor valinomycin affected the membrane potential, which suggested that the coupling between K channel or ionophore activity and proliferation might be due to an electroneutral function, such as volume regulation. The volume-regulation model developed by these investigators might be applicable to some cell populations; however, there currently is no evidence directly linking

volume regulation and proliferation in lymphocytes, and MCF-7 cells do not exhibit RVD [3]. Further investigation will be required to determine the extent to which volume regulation by K channels might play a role in regulating G1 progression.

Regulation of the Expression of Cell Cycle Regulatory Proteins

At the biochemical level, cell cycle progression is controlled by a family of protein kinases that are stimulated by regulatory proteins called cyclins. Cyclin-dependent protein kinases are constitutively expressed in proliferating cells, whereas the cyclin regulatory subunits are expressed in a cell-cycle specific fashion. To understand fully how the activation of K channels might regulate progression through G1 phase of the cell cycle, it will be necessary to determine if the activation of K channels can regulate the activity of certain cyclin-dependent protein kinases. The cyclin-dependent kinases which initiate the passage of mammalian cells through G1 [*reviewed in* 54] are activated by the binding of the D family of cyclin proteins, cyclins D1, D2 or D3. The level of cyclin D1 is rate-limiting for the passage of several mammalian cell types through G1, including human breast cancer cells and macrophages; however, in normal human T lymphocytes and granulocytes, G1 progression appears to be regulated by cyclins D2 and D3 [2, 40,49,53,57,72,76].

The finding that cyclin D1 expression is critical for transit through early G1 phase in human breast cancer cells suggests that the temporal relationship between the hyperpolarization of the membrane potential at the D control point and cyclin D1 expression might be important. We observed that cyclin D1 protein levels were depressed in MCF-7 breast cancer cells arrested in early G1 by quinidine [97]. Quinidine-arrested cells also exhibited reduced levels of cyclin D1 mRNA [97], and cyclin D1 protein levels are controlled by regulated expression of cyclin D1 mRNA. Further investigation should help to define the position of the D control point relative to the expression of cell-cycle regulatory proteins in early G1 phase and to determine if the activation of K channels influences the expression of these signalling proteins.

Do Cell-cycle Signals Regulate the Activity of K Channels?

We have focused on the evidence that the activation of K channels might be required for progression through G1 phase, and we now raise the question of whether cell-cycle signals might regulate the activity of K channels. Regulation might occur either by controlling the level of K channel expression or by modulating the activity of existing K channels. Increased K channel activity could rapidly and simultaneously influence many signaling or homeostatic processes via changes in membrane potential, Ca signals or cell volume, thereby functioning as a switch or checkpoint controlling progression through G1 phase.

Mitogens frequently increase the level of activity of K channels concomitant with the stimulation of resting cells in G0 phase to enter G1 phase and proliferate. Examples of mitogen-stimulated K channel activity include EGF-stimulated NIH-3T3 cells [52], EGFR-T17 fibroblasts [50] and human A431 carcinoma cells [68], IL-2- and phorbol ester-stimulated T lymphocytes [22,43], PHA-stimulated T lymphocytes [34], concanavalin-A stimulated murine T cells [19], LPS- and anti-IgM-stimulated murine B cells [67], mitogen-stimulated Schwann cells [103] and EGF- and insulin-stimulated mouse mammary epithelial cells [26]. The increase in K channel activity following mitogenic stimulation can involve the expression of new K channels. This has been demonstrated most convincingly in human T lymphocytes, in which mitogenic stimulation produced a 2.5–3-fold increase in the number of ^{125}I-ChTx binding sites, compared to unstimulated cells [23]; this increase probably reflected the expression of new Kv1.3 channels. There is also evidence for the expression of new Ca-dependent K channels (K_{CA}) in murine B lymphocytes after treatment with anti-IgM antibodies [67]. The appearance of these K_{Ca} channels required gene transcription and protein translation, indicating the expression of new K channel proteins [67].

The signaling pathways that link the activation of receptors by mitogens to the expression of K channels have not been clearly identified. Huang and Rane [37] reported that the Ras/Raf pathways were probably involved in the increased appearance of ChTx-sensitive K_{Ca} channels in fibroblasts following mitogenic stimulation. The increased activity of these K_{Ca} channels appeared to be required for the stimulation of proliferation by the mitogens, because combined treatment with mitogen and ChTx reduced the stimulation of proliferation produced by the mitogen alone. Transfection of the fibroblasts with $p21^{ras}$ also increased the appearance of these K_{Ca} channels, and the increase appeared to involve the activation of Raf serine/threonine kinases. These investigators concluded that the increased appearance of the K_{Ca} channels was due to induction of the expression of new K_{Ca} channels, rather than an increased activation of quiescent channels. These studies support a role for increased gene expression in the elevated activity of K channels following mitogenic stimulation, and it is interesting to note than an increase in the expression of K_{Ca} channels, which might play an especially important role in the early, mitogenic Ca signals, is most common.

As described above, the mitogen-stimulated entry of

quiescent cells into the cell cycle is usually accompanied by a small hyperpolarization of the membrane potential, although this varies among different populations. This is markedly different from the large hyperpolarization during the progression through G1 of some cycling cells that do not enter G0, and an important, unanswered question is whether this hyperpolarization is also the result of an increased expression of new K channels or if there is an increased activity of existing K channels. The membrane potential of MCF-7 cells undergoes a large hyperpolarization during progression through G1 phase, and we observed smaller macroscopic K currents in MCF-7 cells arrested in early G1 phase by lovastatin, compared to unsynchronized cells [*unpublished observations*]. The smaller K currents are probably responsible for the more depolarized membrane potentials of MCF-7 cells arrested in early G1 phase [104], but the basis for the different level of K currents in the arrested versus unsynchronized cells is not known. A higher incidence of K_{Ca} channels has been observed in cell-attached patches from MCF-7 cells during log-phase growth, compared to plateau-phase growth [99]. The higher incidence of K_{Ca} channels in log-phase cells could be due to an increased level of expression of these channels during phases of the cell cycle that are more prevalent during log-phase growth, but the higher incidence might also have resulted simply from the shift of the cell cycle distribution during log-phase growth into phases which exhibit a higher cytosolic Ca activity, resulting in a higher open probability. The level of activity of many types of K channels, especially ligand-gated K channels, can be rapidly altered by changes in the open probability, without requiring a change in the level of expression of the channels. For example, we have surmised that the activation of K_{ATP} channels is required for the passage of MCF-7 cells through G1 phase [42,105]. K_{ATP} channels are highly regulated by cytosolic factors, including ligand binding and phosphorylation [25,31], and these properties provide many potential connections with other signaling pathways. For example, these channels could be activated by a fall in the cytosolic ATP/ADP ratio similar to the fall observed during the early G1 phase of Ehrlich ascites tumor cells [87]. Finally, the activation of protein kinases is a mechanism common to many signaling pathways that stimulate movement from G0 into G1 (e.g., growth factor receptors) and G1 signaling pathways, such as the cyclin-dependent kinases. Therefore, phosphorylation might also regulate the activation of K channels and passage through a control point in G1 phase.

It is important to recognize that the pattern of K channel regulation is likely to be very different for different types of K channels and/or cell populations. For example, a decrease in the level of activity of inwardly rectifying K channels has been observed during the G1/S transition in mouse embryos [16], HeLa cells [90] and neuroblastoma cells [5], perhaps in association with a depolarization of these cells during progression from G1 phase into S phase.

Can Overexpression of K Channels Cause a Loss of Regulation of G1 Progression?

We predicted above that overexpression of K channels could increase the fraction of spare K channels and reduce the ability of endogenous signaling pathways that normally regulate the activation of K channels to control passage through G1 phase. This loss of regulation would not require any change in intracellular signaling, only an increased expression of K channels. There is evidence of a link between oncogenic transformation and K channel expression. Transfection of the AtT20 cell line with the EJ-*ras* oncogene increased the transcription of Kv3.1 mRNA and altered the pattern of K channel expression [35]. Oncogenic transformation of cells by *ras* [73], Rous sarcoma virus [75] or SV-40 virus [91] also increased K channel activity. In contrast, the decreased proliferation following the differentiation of ML-1 human myeloid leukemia cells by TPA [51] or the differentiation of HL-60 cells by retinoic acid [102] was associated with decreased K channel activity. The likelihood that overexpression of K channels will stimulate proliferation would depend on two factors. The first factor is the degree to which the activation of K channels is rate-limiting in progression through G1 phase, and cells that are either terminally differentiated (e.g., neurons) or already cycling at a maximal rate would not be affected. A second factor is the type of K channel that is overexpressed. If only a fixed-threshold requirement is important, then the overexpression of many types of K channels might hyperpolarize the membrane potential and facilitate G1 progression. Alternatively, if a synchronized hyperpolarizing transition is required, then overexpression of only the specific type of K channel that serves this regulatory function would facilitate progression, and the overexpression of other, chronically active K channels might be inhibitory to G1 progression.

Conclusions

Elucidation of the role(s) of K channels in G1 progression is a challenging endeavor, due to the small set of experimental tools available to dissect a complex system of signaling pathways. We conclude from a selected body of experiments that the activation of K channels is, indeed, required for progression through G1 phase. This has been demonstrated most rigorously for the voltage-gated Kv1.3 channels in T lymphocytes, and there are also several lines of evidence supporting a requirement for K_{ATP} channels in the progression of MCF-7 cells

through early G1 phase. Inhibition of the proliferation of a large number of types of cells by TEA, and other low-affinity K channel antagonists, suggests a very broad role of K channel activity in proliferation. However, critical tests of the effects of these agents on the cell-cycle distribution and specific indices of G1 progression will be required to demonstrate that the inhibition of proliferation is caused by a specific effect within the G1 phase of the cell cycle. Many important details regarding the mechanisms of K channel involvement in G1 progression remain unresolved. With the exception of Kv1.3 channels in T lymphocytes, the types of K channels, the temporal position within G1 at which they must be active and the G1 events which require their activation have not been clearly identified. In the future, investigations employing high-affinity K channel antagonists, such as the peptide toxins, and molecular biological techniques that manipulate the expression of specific types of K channels will lead to a clearer understanding of the relationship between K channels, proliferation and G1 progression.

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