IONIC CHANNELS IN EXCITABLE MEMBRANES

CURRENT PROBLEMS AND BIOPHYSICAL APPROACHES

BERTIL HILLE, *Department of Physiology and Biophysics, University of Washington School of Medicine, SJ-40, Seattle, Washington 98195 U.S.A.*

ABSTRACT Ionic channels are gated aqueous pores whose conformational changes are driven by the electric field in the membrane. Gating may be studied by three electrical methods: ionic current transients, ionic current fluctuations, and "gating current," and probably occurs through a series of conformational changes in the channel leading to an all-or-nothing opening of the pore. When the potential is held constant, the gating steps come to equilibrium rather than reaching an energy-dissipating, cyclic steady state. The kinetic models now in use eventually need to be changed to correct disagreements with several recent studies. Diffusion of ions through open channels is very fast but involves many interactions of ions, pore, and solvent that lead to ionic selectivity, saturation, block, and flux coupling. Our description of the ionic fluxes can be improved by abandoning continuum models in favor of more structured ones. Problems to be solved include determining how many ions occupy a channel at once and what kind of energy barriers they must cross in traversing the membrane. Ultimately we will need to know the chemical structure of the whole system to understand how it functions.

My purpose in this paper is to review the major problems posed in studies of ionic channels today and to outline a variety of promising approaches for the next 5–10 years. This paper is an expanded version of an earlier one (Hille, 1977*a*) and focuses on channels with a voltage-dependent permeability mechanism.

Electrical excitation of axon and muscle membranes involves transient permeability increases to Na^+, K^+, and Ca^{++} ions. These voltage-dependent ionic permeabilities can be modified selectively and independently by applied drugs and chemical agents, and there remains no further doubt that axon and muscle membranes contain at least three separate ionic pathways, called Na, K, and Ca channels, plus in muscles a variety of "inward rectifiers." Indeed, it is now usually assumed that a new channel type is required whenever a new permeability change with different kinetics and different ionic selectivity is discovered. On this basis there may be at least six types of electrically excitable ionic channels in cardiac Purkinje fibers (McAllister et al., 1975). One of the major research goals today is to elucidate the molecular mechanisms of operation of these channels. Recent progress has been reviewed by many authors (e.g., Almers, 1978; Armstrong, 1975*a*,*b*; Lüttgau and Glitsch, 1976; Hille, 1970, 1975*a*, 1976, 1977*b*; Taylor, 1974; Ulbricht, 1974, 1977). The hypothesis that the basic mechanisms

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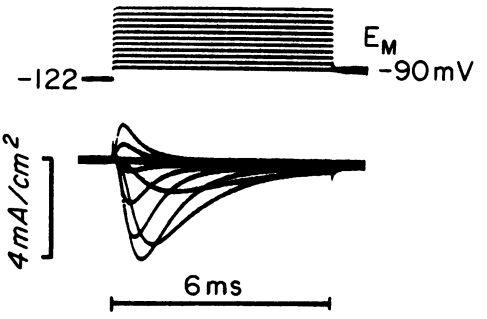

FIGURE 1 Ionic currents in Na channels elicited by step depolarizations of the membrane of a frog semitendinosus muscle fiber at 5°C. At each depolarization, currents carried by Na^+ ions grow and decline as the gating processes activate and then inactivate Na channels. A downward deflection of the current trace indicates net inward flow of charge. Potassium, leak, and capacity currents have been subtracted pharmacologically or electronically from the records. (From Hille and Campbell, 1976).

in all channels are similar will probably be important in guiding our experiments and interpretations. Thus far the known features of the three major channel types are so similar that one could easily imagine that they were developed by divergent evolution from a single ancestral device.

Two essential properties of excitable ionic channels, permeability to ions and responsiveness to stimuli, are demonstrated in Fig. 1 by the appearance of large sodium ion currents that change rapidly after a step depolarization applied to a muscle membrane. The approximate number per unit area and the permeability of single Na and K channels have been determined by measuring the uptake of the specific toxins tetrodotoxin and saxitoxin and by analysis of ionic current fluctuations (Almers and Levinson, 1975; Begenisich and Stevens, 1975; Conti and Wanke, 1975; Conti et al., 1976*a*,*b*; Ritchie et al., 1976; Ritchie and Rogart, 1977, Sigworth, 1977). In the 5×10^{-4} cm^2 of muscle membrane used for the experiment of Fig. 1 there are roughly 2×10^7 Na channels (Almers and Levinson, 1975), opening and closing to produce the sodium ion currents measured. Under physiological conditions, single open channels have an electrical conductance of 1–10 pS and can select and pass a permeant ion roughly every 200 ns. Because of this prodigious throughput rate, Na and K channels are now accepted to be pores, as is shown diagrammatically in Fig. 2. The open pore provides an aqueous pathway for ions to cross the membrane.

GATING

In any functional excitable system, at least one of the channel types present must change its conformation in response to a physiological "stimulus" such as an applied electric field, a chemical transmitter, or some other agent. The conformational changes are usually called gating, since they gate the movement of permeant ions by opening and closing the pore. In Na and K channels the conformational changes are driven by the electric field within the membrane. Experiments with internally applied blocking

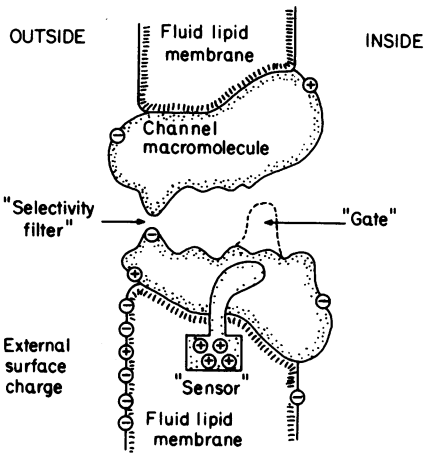

FIGURE 2 Schematic diagram of an ionic channel like the Na or K channel. The channel is a protein macromolecule forming a pore through the lipid-bilayer membrane. The pore has a narrow selectivity filter near the outside and a gate near the inside. A voltage sensor extending into the lipid moves under the influence of the intramembrane field and controls the opening of the gate. Surface charges and the ionic double layer associated with them modify the field within the membrane. The drawing is fanciful and the dimensions and shapes of the components are not known. (From Hille, 1977*a*).

ions suggest that the actual gate that controls ion flow is near the inner end of the pore, as in Fig. 2, although the process of gating can be altered by venoms, enzymes, and chemical modifying agents applied either externally or internally (Armstrong, 1975*a*,*b*; Hille, 1976). The conformational changes occur stochastically on a time scale of 30 μs to 10 ms, with kinetics that can now be studied by three different techniques: ionic current transients, ionic current fluctuations, and "gating current."

Hodgkin and Huxley's (1952*b*) classical analysis of sodium current transients showed, for example, that Na channels "activate" with a delay, as if by a multistep process, and then "inactivate" with a time-course close to first order (see Fig. 1). They proposed a model equivalent to saying that during a depolarization three "*m* gating particles" in each Na channel must undergo independent but identical transitions to activate, and at the same time one "*h* gating particle" undergoes a single transition to inactivate the channel. The experimental method they used is very powerful and needs to be repeated in full in a more modern setting, as there remains much more to be found this way. At the same time it is limited by being able to detect only those transitions of the gating system that change the conductance of the channel. Any other transitions may only be inferred by fitting kinetic models. The newer method of fluctuation analysis has the same limitation, but emphasizes other kinetic features than the transient analysis does, as well as providing a measure of single-channel conductance (Conti and Wanke, 1975; Neher and Stevens, 1977). Fluctuation studies are just beginning to appear, and none have had the resolution needed to say how many steps there are in gating or how many gates there are in a channel. Such information may begin to appear soon. The newest method, gating current, gives important clues on

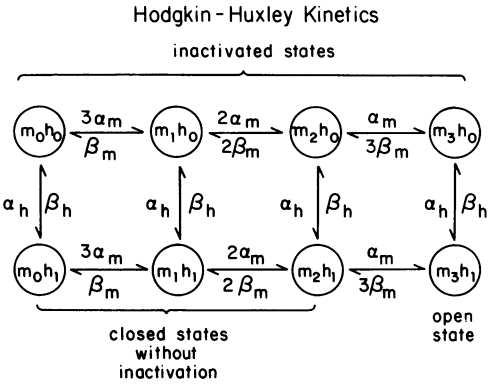

FIGURE 3 A kinetic representation of gating transitions of Na channels, equivalent to the Hodgkin and Huxley (1952*b*) kinetic model. If there are three *m* "gates" or "particles" and one *h* gate, then the subscripts indicate how many gates are in the correct position to make a conducting channel. Only the state m_3h actually conducts. The α's and β's are rate constants given by Hodgkin and Huxley. Modern experiments show that this diagram needs revision at least in details.

each conformational change that results in a change in the overall charge distribution of the channel macromolecule. In these experiments the ionic currents are eliminated as much as possible and then changes of electric field are imposed to elicit intramembrane charge movements presumed to arise as charged or dipolar components of the channel macromolecules are rotated or moved by the field (Armstrong and Bezanilla, 1974; Keynes and Rojas, 1974; reviewed by Almers, 1978). The major component of current seen this way seems to relate to steps underlying activation of Na channels, and from the behavior of this component a kinetic interdependence of activation and inactivation has been demonstrated (Armstrong and Bezanilla, 1977).

In most kinetic schemes of gating, the Na and K channels undergo transitions among numerous conformational states. For example, the Hodgkin-Huxley (1952*b*) model is formally equivalent to having eight distinct conformations of Na channels (Fig. 3) and five of K channels, with only one of these states able to conduct ions. Some schemes postulate that several of the conformations are conducting states. In any case, most workers operate on the hypothesis that gating appears smooth and graded only because the number of channels flipping stochastically in an all-or-nothing manner from conducting to nonconducting states is very large. If channels have more than one open state, all the significant states have about the same single-channel conductance and ionic selectivity. These ideas are qualitatively supported by the apparent constancy of the reversal potential for current and the apparent constancy of single-channel conductance determined by fluctuation analysis while the population of Na channels is making transitions from closed to open to inactivated states (Chandler and Meves, 1965; Hille, 1971; Conti et al., 1976*a*,*b*; Sigworth, 1977). In the last analysis, of course, it is not possible for two different open states of a channel to be absolutely identical.

The question of the energy source for gating transitions of a channel is an important one, as it limits the range of kinetic models permissible. 15 years of experience with internal and external perfusion of squid giant axons has demonstrated convincingly that sources of high-energy compounds and indeed all solutes other than simple salts are quite unnecessary for channels to be opened and closed a million times. For example an axon perfused continuously with 500 mM K_2SO_4 through the inside at 18°C can fire more than 4×10^5 action potentials (Baker et al., 1962). However since at some potentials channels become open and at others they become closed in a fully repeatable manner, there is entropy production and free energy consumption during every gating cycle. The underlying energy source is coupled to the electric field in such a way that the equilibrium direction of the gating transitions can be reversed simply by changing the field.

How does the field act on the gating mechanism? Processes depending on the square of the field, like membrane thickness changes arising from electrostrictive pressure, may be ruled out because the equilibria of the major gating steps change dramatically over the membrane potential range from −75 to −20 mV, without complementary transitions occurring in the ranges from 0 to +200 mV or −75 to −200 mV. Hence the vector direction of the field is as essential as the magnitude. This still leaves two major possible mechanisms: The field may act on diffusible ions which then act on components of the membrane, or it may act on components of the membrane directly. Much evidence has accumulated against mechanisms requiring an indirect action of the field mediated by any anion or by Na^+, K^+, H^+, or Ca^{++} ions. Axons function nearly normally with any of at least 13 different anions outside or inside (Koppenhöfer, 1965; Tasaki et al., 1965). The activation and inactivation of Na channels measured by the time-course of ionic currents (Fig. 1) is nearly normal if all the external Na^+ is replaced by K^+ or by any of at least 30 different other cations (Hille, 1971) or if the internal K^+ is replaced by Na^+ or by several other cations (Chandler and Meves, 1965, 1970) or if the external pH is changed in the range pH 6–10. The kinetics and equilibria of gating are the same whether the net current in the channel flows inward or outward.

The only common ion with an effect on gating is external Ca^{++}, changes of which act in many ways like a change in membrane field (Frankenhaeuser and Hodgkin, 1957). This so-called voltage shift with Ca^{++} ions has spawned at least 25 different gating models involving an electric field-dependent binding and unbinding of Ca^{++} to some controlling structure of ionic channels. However, further, more detailed investigations on K channels (Mozhayeva and Naumov, 1972 *a*,*b*,*c*) and Na channels (see references in Hille et al., 1975) have shown that the shifts may be accounted for completely by a static binding and screening of fixed negative surface charges near the channel (see Fig. 2). An excess of Ca^{++} ions near the nerve surface sets up a local potential felt in part by the "voltage sensor" of the channel. Furthermore excitability is maintained in the presence of Ca^{++} chelating agents that reduce the free calcium to less than 10^{-8} M (Armstrong et al., 1972). Hence dynamic models with Ca^{++} acting directly as the sensor probably have no place in explaining normal activation and inactivation of

channels. Nevertheless, both metabolism and ions including Ca^{++} do have other important modulatory effects on channels.

We are left with the conclusion that the electric field acts on components attached to or within the membrane, and the electric work done on them is the energy injected into the gating process. For compactness these components may be called "sensors" or "voltage sensors." They have approximately the same significance as the "gating particles" introduced by Hodgkin and Huxley (1952*b*). The location of the field-sensing components of the channel is unknown, but one might expect them to be immersed in a fluid region of the insulating dielectric of the membrane rather than in the very polar, ion-permeable part (see Fig. 2). In accord with the transient time-course of gating current, a time-invariant field cannot continue to do work on sensors indefinitely. Unlike the number of dissolved ions, the number of these sensors associated with a channel is probably small. Once their distribution reaches its new average equilibrium value, it can only fluctuate reversibly without absorbing more energy from the field. The crucial point is that the gating system comes to a true *equilibrium* rather than to a continuously cycling steady state under constant conditions, and therefore any kinetic scheme such as the representation of Fig. 3 must satisfy the thermodynamic properties of an equilibrium system, namely microscopic reversibility and detailed balances. No step may be irreversible, and, in order not to create a perpetual motion machine, each cycle in the diagram (there are six in Fig. 3) must have the product of all rate constants taken in the clockwise direction equal to the product in the counterclockwise direction. Models disobeying this criterion (Moore and Cox, 1976) and models with negative rate constants (Goldman, 1976) are wrong in detail, although it may be relatively easy to bring them into compliance by adjusting a few constants or adding an arrow without altering some of their novel kinetic features.

By now a fair body of evidence has accumulated that the m^3h kinetics of Hodgkin and Huxley (1952*b*) with kinetically independent activation and inactivation processes and with first-order transitions of m and h parameters simply do not fit the details of gating in Na channels (see review by Goldman, 1976; Bezanilla and Armstrong, 1977; Armstrong and Bezanilla, 1977). What should be the goals of improved models? It will be more important now to seek a physically realistic model than to maintain the symmetry and simplicity useful for automatic computation of membrane responses. There probably will still be features remaining of activation and inactivation and there may not be many more steps than the eight of Fig. 3, but now inactivation steps will depend somewhat on the status of activation as in the proposal of Armstrong and Bezanilla (1977). Partial models that imitate only half the facts should be abandoned. The essence is to keep within the framework allowed by all the many acceptable experiments, the existing molecules, and the laws of nature. In addition, since no authors except Hodgkin and Huxley (1952*b*) and Chandler and Meves (1970) have looked at more than a few isolated aspects of gating, new models will need to go hand in hand with new comprehensive measurements done with the best techniques available to measure the macroscopic kinetics, the fluctuations of gating, and gating currents.

It is definitely too early to begin to build models based narrowly on the properties of

specific amino acid side chains or phospholipid head groups, because we have no knowledge of the chemistry of ionic channels. On the other hand, study of model compounds of known structure will probably be essential to give a clearer idea of practical molecular mechanisms of gating and the rules that apply. We need to know more about the kinetics of conformational changes in proteins in general, and specifically there should be more study of the effect of intense electric fields. What kind of conformational changes or subunit interactions can be driven by oriented electric fields, and can they be steeply voltage dependent and very quick? Do such changes have multi-step kinetics as in nerve? What might be the influence of the relatively high membrane viscosity and the low dielectric constant? These kinds of studies might be done theoretically by molecular dynamics simulation, a powerful new method involving direct calculation of the equations of motion for all the atoms of molecules as large as proteins (McCammon et al., 1977). Or they may be done experimentally on oriented protein or peptide films and crystals or on lipid bilayer membrane systems doped with peptides or proteins. Many small molecules can form gated ionic pores in bilayer systems (see reviews: Mueller and Rudin, 1969; Haydon and Hladky, 1972; McLaughlin and Eisenberg, 1975; Ehrenstein and Lecar, 1977). These systems offer great promise because they imitate phenomena in nerve closely and are completely defined. Although not understood in any molecular detail yet, the chemical requirements for gating can be investigated directly by systematic chemical synthesis and modification of the pore-forming molecules and lipids. Two major factors for pore formation have been identified, namely aggregation of between two and nine of the peptide molecules and a match between membrane thickness and pore length. In some years similar experiments should be possible with ionic channel components isolated from excitable membranes. It is conceivable that the high-order kinetics needed to describe the activation of Na and K channels in nerve actually reflect underlying diffusion and aggregation steps within the membrane. There is, however, no evidence that the rate of activation is systematically faster in membranes with a higher density of sites as might be expected from that theory.

PERMEABILITY

The open pore provides an aqueous pathway for ions to hop over a sequence of energy barriers, pausing for various times at binding sites on the way (Läuger, 1973; Hille, 1975*a*). Like enzymes, channels have the job of catalyzing a specific "reaction" selectively, increasing the rate of ion movement many orders of magnitude above that possible without the catalyst. The highest energy barrier serves as the rate-limiting "selectivity filter." The filter appears so narrow that ions must become at least partially dehydrated. It will pass several alkali metal cations, NH_4^+, and, in Na channels, a few organic cations, but no molecules with diameters as large as 6 Å (Hille, 1975*a*). The dehydration steps are catalysed by compensatory dipoles or negative charges in the filter which keep the height of the net energy barrier below 8 kcal/mol (Hille, 1975*b*). In order to have the highest possible conductance of an open channel, the narrow part of the pore is probably also only a few angstroms long and the rest of the pore may be

much wider. In both Na^+ and K^+ channels, this filter seems to lie near the external mouth of the pore, as represented in Fig. 2. The difficulties of describing ion flow in such a pore are related to the lack of any general theory for the movements of atomic particles through an atomic pore filled with an atomic fluid.

The traditional descriptions of ionic fluxes in excitable membranes use almost interchangeably forms based on Ohm's law with a Nernst-potential battery (Hodgkin and Huxley, 1952*b*) and forms based on integrated versions of the Nernst-Planck electrodiffusion equations (Goldman, 1943; Hodgkin and Katz, 1949). At first the flux equations were applied to the membrane as a whole, but now, where at all possible, they should be applied separately to each type of ionic channel. As the phenomena needing explanation have become more complex, investigators have tried to use the Nernst-Planck equations by integrating them for systems with dielectric constants, standard chemical potentials, or diffusion coefficients varying with distance, and for membranes with fixed charge throughout the volume or at one or both surfaces (see review by Adrian, 1969). However, we now realize that certain phenomena simply cannot be explained by free diffusion, so further theoretical work with the Nernst-Planck equations is probably of limited interest in the study of ionic channels.

Phenomena not explainable by free diffusion include flux coupling, flux saturation, voltage-dependent block by permeant and impermeant ions and concentration-dependent selectivity (see reviews of Armstrong, 1975*a*,*b*; French and Adelman, 1976; Hille, 1975*a*). In systems with free diffusion, the ratio of outward and inward tracer fluxes of an ion is equal to the ratio of the internal and external electrochemical activities of the ion (Ussing, 1949). And in systems where ions move independently, the net flux at a fixed membrane potential and internal concentration is a linear function of the outside concentration, and the tracer efflux is independent of the external concentration, (Hodgkin and Huxley, 1952*a*). These several criteria are not satisfied by Na channels or K channels, and in order to explain the deviations it has become necessary to develop models where the movements of individual ions are negatively or positively correlated with the presence of flux of other ions in the channel.

One such model envisions a relatively narrow pore with ions moving ("hopping") stepwise between weak binding sites arranged in linear sequence across the membrane, and the fluxes are calculated by solving a system of first-order rate equations representing transitions of the channel among the various allowed states of occupancy (Hodgkin and Keynes, 1955; Heckmann, 1972, Läuger, 1973; Markin and Chizmadjev, 1974; Hille, 1975*b*). The essential feature is that the occupancy of channels is limited by simple competition for a limited number of sites and/or by mutual repulsions. It is possible to fit many observations this way. In many cases there may be more than one ion in the channel at a time and then the repulsive (or attractive) interactions must be included. Additionally the narrow part of the pore might contain a column of ions and water molecules in a row which could be constrained to move more as a whole than as independent particles. The present models do not take explicit account of such single-file or "slip" interactions between ions and water molecules; indeed the pore is simply represented as a series of energy barriers and energy wells with-

out any clear physical picture of what structural features give rise to them. In the future methods will be needed to relate the chemical structure and possible motions of the pore wall to the energy profiles for movements of solutes and solvent, as well as to calculate the potential gradients set up by the applied external field in the quite inhomogeneous channel-membrane-electrolyte system. As with gating models, new modeling of the fluxes in specific ion channels will need to be realistic and based on extensive new experiments. Ideally the experiments should include unidirectional tracer-flux measurements as well as electrical current-voltage measurements uncontaminated by either gating or local ionic accumulations. The use of small blocking ions that are driven in and out of the channel by the electric field continues to seem one of the most informative ways to probe the barriers and wells of the channel (Armstrong, 1975*a*,*b*; Hille, 1975*a*; Woodhull, 1973).

The two most promising methods for determining empirical rules relating permeability to pore structure are direct experimental measurement on pores of defined structure and molecular dynamic simulation with a computer. Molecular dynamics studies are only in their infancy and much will certainly be done in the future (Levitt, 1973; Subramanian and Levitt, 1976). Experimental measurements on the once promising mechanical holes formed in materials like mica have now been eclipsed in interest by those on pores formed in lipid bilayers by relatively small molecules like gramicidin A and analogues (Haydon and Hladky, 1972; Apell et al., 1977). However, a remaining weakness of the known pore-forming small molecules is that, although they have a high turnover number for ions, none has the high ionic selectivity of physiological Na, K, or Ca channels. Perhaps the pores they form are slightly larger and more flexible, but with less intense internal (local) electric fields than the natural channels. It will be a challenge to synthetic chemists to "improve" upon this first generation of model compounds. Another source of information is diffusion into protein crystals of precisely known crystallographic structure (Bishop and Richards, 1968), but perhaps most available crystals have aqueous interstices too large to be good models of selective ionic channels.

CHEMISTRY AND MOLECULAR BIOLOGY

Virtually all our understanding of channels has come from the combination of electrical experiment and theoretical analysis discussed so far. However, such traditional biophysical studies discover and describe a physiological function but cannot give a full explanation in isolation. They must be complemented by a direct chemical approach and by the powerful techniques of molecular and developmental biology, genetics, and immunology so we can learn the full chemical structure of the channel. A productive indirect approach has been to modify the function of channels *in situ* with chemical reagents and to infer from the reported chemical specificity of the reagent used what types of chemical groups are important in the channel. For example, internal application of some proteolytic enzymes eliminates inactivation gating in Na channels (Armstrong et al., 1973) and external application of the carboxylmethylating Meerwein's reagent prevents the binding of the specific Na channel toxin tetrodotoxin

(Reed and Raftery, 1976). This kind of work produces new forms of the channels that have properties preferable to those of native channels for certain experiments, and also give modest clues to the chemistry of the channel, but still is no substitute for the ultimate chemical analysis. Within a few years some natural pores will be isolated and their primary chemical structures will be completely determined. Very probably one channel will resolve into several molecular subunits and reconstitution experiments and immunological methods will be needed to determine which parts are essential for specific aspects of function. Genetics can also help count the number of parts and their function by determining, for example, the number of complementation groups involved in an Na channel. Indeed, there are already reports of single-gene modifications of both Na channels and K channels in *Drosophila* as well as of Ca channels in *Paramecium* (Pak and Pinto, 1976; Schein et al., 1976), and there are many reports of channels that might represent incomplete, or differently combined, or modified parts of the standard "adult" channels in starfish eggs (Hagiwara et al., 1975), tunicate eggs (Okamoto et al., 1976), embryonic or denervated skeletal muscle (Kidokoro, 1975; Redfern and Thesleff, 1971), and other systems.

Progress in understanding ionic channels in the past 10 years has been very rapid, and we can anticipate that the resulting improved definition of the problem sets the stage for even more dramatic advances in the next 10 years.

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