Basic Medical Research Award

Ion channels: From idea to reality

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Pores in the early days

BERTIL HILLE

Today it seems self-evident that neurons make their electrical signals by opening gates of water-filled pores (ion channels) in the plasma membrane. But it was not always so. Remarkably, pore theory in biology is easily traced as far back as the 1840s when von Brücke and the circle of 'biophysicists' Helmholtz, Ludwig, DuBois Reymond, Fick and others invoked them as a new hypothesis to explain osmosis[1]. The principal property of pores as conceived then was that the channels (kanäle) would pass water and other small particles ranging in size up to the rigid pore diameter. The pore hypothesis remained prominent in many textbooks of biology and physiology from then on, but except for osmosis, tools for making biological tests were lacking. In this century, Michaelis was a strong proponent, discussing proposed effects of hydration and of pore charge on "capillary canals" in apple skin and other membranes.

In 1952, Hodgkin and Huxley published their quantitative description of action potential propagation in the squid giant axon[2], for which they received a Nobel prize in 1963. Although they described beautifully the changes of membrane Na^+ and K^+ permeability after changes in membrane potential, they concluded that their study did not favor any particular mechanisms for the permeability. Subsequently, Hodgkin and Keynes[3] made subtle isotopic K^+ flux-ratio experiments in Cambridge. In a stroke of genius, they said their unexpected results would be explainable if "ions cross the membrane along a chain of negative charges or through narrow tubes or channels... in which they are constrained to move in single file [with] several ions in the channel at any moment."[4]

In the next decade, Clay Armstrong and I began our independent research. In our first papers we brought a clear list of 'molecular' assumptions to the table (Fig. 1). They included the following ideas: ions are passing through aqueous pores that we called channels, ion channels are proteins, the channels for Na^+ and K^+ are different, they have swinging gates that open and close them, we can study their architecture by using electric currents to measure gating, permeation and block, and channel blockers are molecules that enter the pores and physically plug them.

Our starting agenda could have been entirely wrong, and there were plenty of skeptics, but it was essentially what we set out to show. Although this does not sound like open-minded scientific inquiry, I certainly picked problems to study because of their potential to show that these ideas were right! From 1965 to 1973, such ideas were debated annually at meetings of the Biophysical Society. There, prominent scientists would routinely rise to request that anyone who chose to use the word "channel" avow first that it bears absolutely no mechanistic implications! It is probably fair to say that most people thought that discussions about molecular mechanisms were premature.

In 1969, when I had drafted a summary review of these ideas[4], Kenneth Cole, the dean of American biophysics wrote to me: "I'm ... worried you may be pushing some of your channel arguments pretty far." In 1978, Wolfgang Schwarz and I wrote an article[5] showing that many features of permeation, selectivity, and block of K^+ channels can be explained by extending the long-pore theory of Hodgkin and Keynes. This manuscript had a hard time in review. Even then, there was a widespread presumption that inward rectifier K^+ channels could be carriers and not pores. Three of an eventual five reviewers asserted that it was "uncritical salesmanship" to write a paper on the subject "potassium channels as multi-ion, single-file pores."

A serious difficulty in formulating the early structural hypotheses about ion channels as molecular pores was that not one structure was known (until 1998) and therefore our biophysical methods of inference were untested and uncalibrated. For example, biologists had long accepted that the permeating particle had to be the *hydrated* ion, whereas crystallographers and physical chemists were just beginning to explain the selectivity of ionophores like valinomycin on the basis of direct interactions with the *unhydrated* ions. Which was right for channels? When I had to explain by a pore theory how the Na channel could be permeable to Na^+, K^+, amino-ammonium (hydrazinium), guanidinium, but not methylammonium ions, after months of hard thinking I felt required to invoke direct interactions between channel residues and the ion[6]. I could not see how details of the ion inside could be detected through a

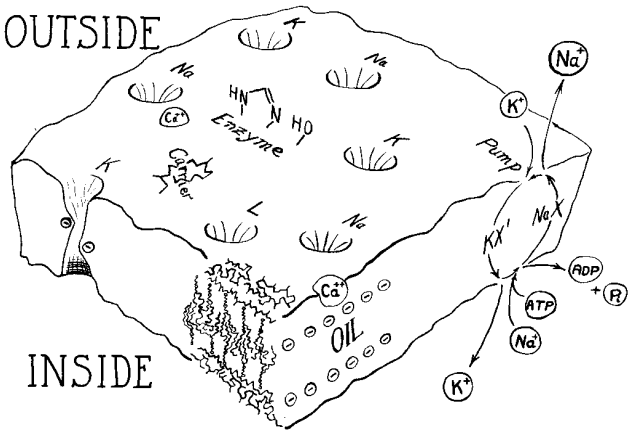


Fig. 1 My 1967 thesis drawing of an excitable membrane showing three separate types of ion channels Na, K and leak (L), as well as the Na-K ATPase pump, a "carrier," a serine protease modeled after acetylcholinesterase, and lipids.

full fuzzy coat of water molecules. Clay and I reached similar conclusions about K^+ channels[7,8]. Twenty years later, Rod MacKinnon's crystal structure of the KcsA channel confirmed our guesses in a most pleasing way[9].

When did cells evolve this style of pore? Classical electrophysiological experiments had shown regenerative action potentials in Paramecium, green algae and higher plants. This suggested that there might be homologous voltage-gated channels throughout the eukaryotes, an idea that was well borne out when cloning of the genes began. From their sequences, we now suppose that K^+ channels arose first and then spawned voltage-gated Ca^{2+} channels early in eukaryotic history by tandem duplications. The voltage-gated Na^+ channels may have arisen only with animal axons, making our nervous systems possible. Altogether we know so far of about 100 homologous genes for K^+, Na^+ and Ca^{2+} channels in mammals and nearly as many in *Caenorhabditis elegans*. Nevertheless, it was a considerable surprise when a gene in *Escherichia coli* was also reported to be homologous to animal K^+ channels[10]! The possibility that this occurred by some kind of reverse gene transfer was dispelled when additional K^+ channel genes were found by most of the archaeal and bacterial genome projects. One of these channels was the subject of the initial MacKinnon K^+ channel crystal structure[9]. I hope we can learn soon what these proto-channels do—are they used for signaling or do they transport K^+ ions for osmotic adjustments?

Thus, the pore theory, originally conceived for osmosis and ultrafiltration, received its definitive proof in biology by application to ion channels of excitable membranes. Happily, the theory has seen full closure during the last 12 years with the additional discovery of the family of aquaporins[11]. These are the real water pores of eukaryotic and prokaryotic cells. Remarkably, they have an aqueous path that passes water without being permeable to the common ions.

Early views of channels and gates

CLAY M. ARMSTRONG

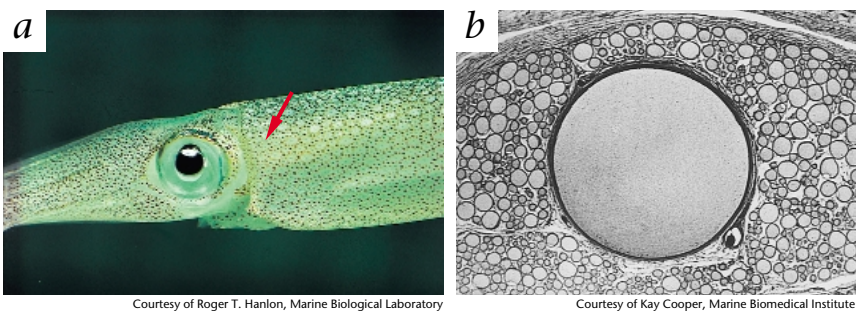
Ion channels are involved in every thought, every perception, every movement, every heartbeat. They developed early in evolution, probably in the service of basic cellular tasks like energy production and osmotic stabilization of cells, and evolved to underlie the elaborate electrical system that provides rapid perception and control.

Surprisingly, as Bert Hille notes, the existence of ion channels was controversial until about 20 years ago. The behavior of what we now recognize as channels was brilliantly described in squid axons (Fig. 1) by Hodgkin and Huxley in 1952 (ref. 2). I was fascinated by their work when I encountered it in medical school, and coming to understand it was the main element of my scientific education. In brief, they said that unknown structures, possibly carriers, allow ions to pass through the membrane. The structures are selective, some for Na^+ and others for K^+ ions. They can be activated and deactivated within a fraction of a millisecond by changing membrane voltage.

My chance to work on these questions came with an appointment in the laboratory of K.S. Cole, a pioneer of cellular electrical studies. K^+ conductance with the property of inward rectification (current flows inward more easily than outward, a property now found in many cells) had been described, and invoked to explain some properties of heart action potentials. Leonard Binstock and I showed that squid axon K^+ conductance could be made to inwardly rectify by putting tetraethylammonium (TEA^+) ion in the axoplasm, suggesting a simple mechanism for the phenomenon based on channel block. A rough measure of the K^+ flux through each conducting unit could be derived from the kinetics of TEA^+ block, about one K^+ ion per microsecond, which seemed too fast for a carrier mechanism. It was about this time that I met Bert Hille, and we began to share ideas about channels.

A useful variant of TEA^+ was $C9^+$ (nonyltriethylammonium ion) which has seven methylene groups added to one of the ethyl groups of TEA^+, forming an ion with a charged head and a hydrophobic tail (Fig. 2*a*). Study of this substance gave a detailed picture of what seemed clearly to be a transmembrane channel[12]. It had a narrow, selective region near the outside of the membrane, a wider vestibule further in, and a voltage-operated gate at the inner end. A useful clue regarding the inactivation mechanism of normal Na^+ channels was that $C9^+$ diffused into the channels slowly, so they conducted for a time before being blocked or 'inactivated' (Fig. 2*a*, right). All of this gave a very tangible picture of a channel with a gate, as did the incisive studies of Bert Hille on Na^+ conductance[6].

The problem was that many or most workers were convinced that K^+ conductance resulted from a carrier, like the K^+-selective ionophore valinomycin. It seemed easy to make a carrier selective, by simply endowing it with a high affinity for one ion species. Making a channel selective was more difficult, for if the ion stuck too tightly it would block rather than permeate. A reasonable solution (Fig. 2*b*) seemed to be one in which a K^+ ion (r = 1.33Å) in the pore is so snugly invested with carbonyl oxygens that it cannot tell that it is not in water[7]. A rigid K^+ pore, however, cannot close down around a Na^+ion (0.95Å), which does not bind snugly in the pore and thus has a much higher energy than in water. The result is that K^+ enters the pore easily, whereas Na^+ faces an energy barrier and does not. This agreed well with the ideas Bert Hille had advanced regarding dehydration of Na^+ ions in Na^+ chan-



Courtesy of Roger T. Hanlon, Marine Biological Laboratory

Courtesy of Kay Cooper, Marine Biomedical Institute

Fig. 1 ***a***, The squid *Goligo pealei*. The giant axon, which begins near the arrow and runs to the right in the mantle, is shown in cross-section in the micrograph in (***b***). The axon is about 0.5 mm in diameter, and is surrounded by smaller axons. Its large size makes it a high-speed conductor of action potentials.

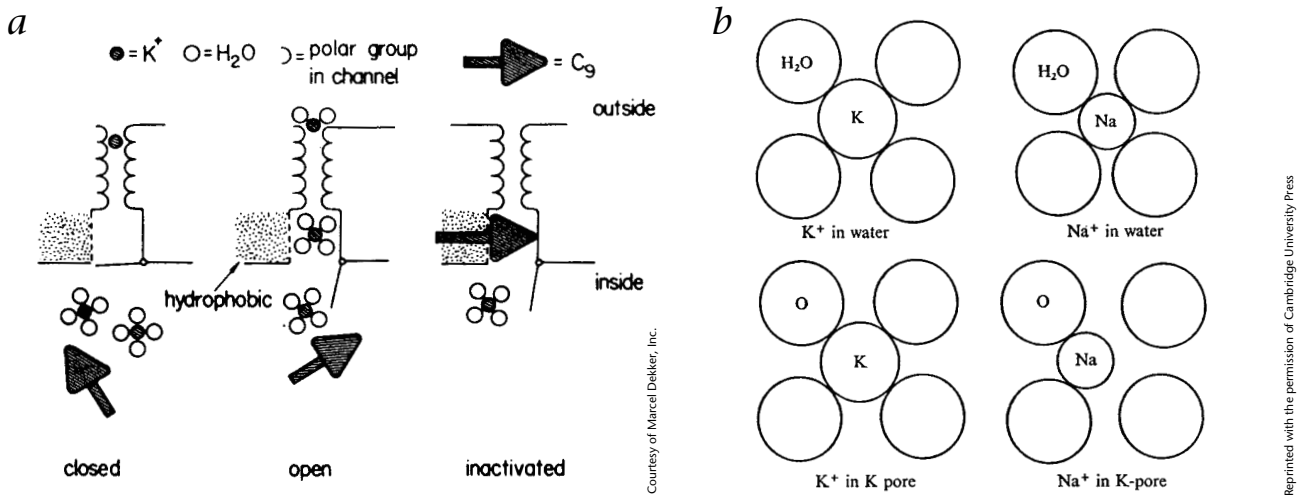


Fig. 2 Early ideas of channel architecture and selectivity (1971–1972). ***a***, Nonyltriethylammonium ion ($C9^+$) was used to explore K^+ channel structure. In the resting state (left) the gate is closed, and $C9^+$ is unlikely to be in the channel. When the gate is opened by depolarization the channel conducts K^+ ions for a time (middle), and $C9^+$ then diffuses into the pore (right). The charged group of $C9^+$ is about the size of a hydrated K^+ ion. K^+ dehydrates to pass through the narrow outer part of the channel, but $C9^+$, with covalently linked ethyl arms, cannot follow, and thus blocks or 'inactivates' the channel. ***b***, Selectivity of the outer part of the channel. K^+ is closely bound to oxygen atoms that bear a partial negative charge either in water (oxygens of water molecules) or in the channel (carbonyl oxygens of the channel wall). Na^+, on the other hand, is closely invested by oxygens in water, but not in the channel, which is too large: only two of the four oxygens form a good complex with Na^+, making its energy much higher than in water. The result is that K^+ enters the channel easily, whereas Na^+ does not.

nels[6], and his measurements of the size of K^+ channels[8]. The final proof of channels was the single channel measurements of Neher, Sakmann, and colleagues.

These pictures of pores and selectivity seemed as good as we were likely to get without detailed molecular structures, so it was time to study gating. Fortuitously, it transpired that pronase, used to facilitate internal perfusion, removed inactivation of the Na^+ channel without appreciably altering activation. By analogy with the effect of $C9^+$ on K^+ channels, the effect of pronase suggested a blocking particle (the ball) tethered by an enzymatically cleavable chain to the inner end of each Na^+ channel[13]. A 'ball and chain' mechanism was strongly supported by experiments on the effects of inactivation on gating current, the small current associated with the conformational changes that open and close the activation gate of the Na^+ channel[14]. Gating current further suggested a physical model in which a helix with repeated positive charges moves relative to a matching helix with repeated negative charges, thus driving the conformational changes of gating[15]. This turned out to be half right, and the hypothetical positive helix, corresponding to the S4 helix, has been found in all voltage-dependent channels.

At this point the channel story acquired new tools, cloning and crystallization, and new investigators. With amazing speed, the ball and chain mechanism was shown to fit the inactivation mechanism of *Shaker*B K^+ channels[16]. MacKinnon, Miller and Yellen began mutagenesis experiments that soon identified the pore region of K^+ channels. Having exhausted mutational analysis, MacKinnon transformed himself into a crystallographer and gave us pictures of a non-voltage-gated channel from bacteria[9]. This channel has two transmembrane helices and almost certainly constitutes the 'pore module' of all K^+ channels. Pleasingly, the general structure resembled the old ideas (Fig. 2).

Where next? Perhaps the main question is how the 'gating module' of four transmembrane helices opens and closes a gate at the inner end of the pore module, a question on which biophysical-mutational experiments are already in hand from the Yellen lab[17]. Crystallization of a voltage-gated K^+ channel has not yet been achieved.

From the medical point of view, the story is just beginning. Considering their ubiquity, it seems safe to predict that ion channel mutations will be found to be involved in many diseases.

Potassium channel's secret

RODERICK MACKINNON

The evolution of the lipid cell membrane solved one problem and created another. It enabled compartmentalization of life's essential ingredients, but it made it almost impossible for charged atoms, the ions, to move into and out of cells. The electric field around an ion causes it to be repelled away from the oil-like membrane of cells. The repulsion, known as the dielectric barrier, is so great that nature had to fashion specific mechanisms to get ions across the membrane. For rapid, selective transmembrane ion flow—the kind underlying electrical signaling in the nervous system and many other cellular processes—membrane-spanning proteins called ion channels were nature's solution.

I began studying ion channels long after it was established that they are protein pores in the cell membrane. In 1986, I abandoned plans to practice medicine and began postdoctoral studies with Christopher Miller, who provided an inspi-

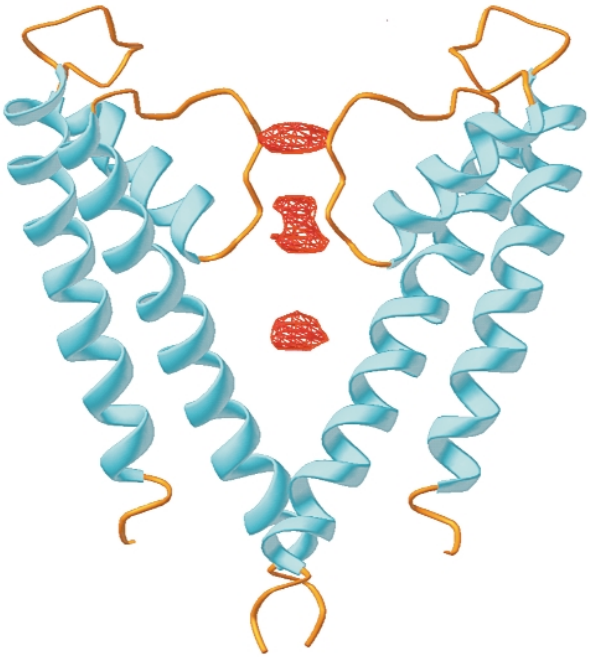


Fig. 1 Three ions in the potassium channel. Two subunits of the potassium channel are shown as ribbons (blue and gold). Electron density (red mesh) shows three ions within the ion pathway: two are located in the selectivity filter (top) and one, in a cavity at the membrane center (bottom). The subunits are oriented with the extracellular solution on top.

rational beginning in science. The experiments of Clay Armstrong and Bertil Hille describing the gated pores of sodium and potassium channels were already classics. Genes encoding sodium and calcium channels and the acetylcholine activated channel had been cloned, and potassium channel clones were on the horizon. The fundamental questions had reached a new level. What is the structure and chemistry behind the operation of an ion channel?

I began by asking how a small scorpion toxin inhibited a potassium channel. Using electrical measurements, I deduced that the toxin occludes the channel's ion pathway rather than interfering with gating of the channel[18]. That humble conclusion set the course that I still follow today—one aimed at understanding how a potassium channel so precisely selects the potassium ion over sodium, and conducts it near the diffusion limit.

The first potassium channel gene was cloned in 1987 from fruit fly: the *Shaker* gene[19]. The gene gave us the amino-acid sequence, but little information about the arrangement of amino acids in the channel. The scorpion toxin allowed me to identify which of the amino acids form the ion pathway, the first step toward understanding the channel's three-dimensional structure. I began this work in Christopher Miller's laboratory and then continued in my own laboratory, in 1989, at Harvard Medical School. Over the next several years, my laboratory reached a number of important conclusions about the architecture of a potassium channel using site-directed mutagenesis, electrical measurements, and simple reasoning. We showed that the channel contains four identical subunits arranged in a symmetric ring around a central pore[20]. We also defined a special amino acid stretch known as the pore loop. Our experiments revealed that four pore loops, one from each subunit, meet near the channel's central axis to form the narrowest point along the ion pathway. Gary Yellen and I exploited the same potassium channel blocker used decades earlier by Armstrong and Hille, tetraethylammonium, to show that our pore loop conclusion must be true. Finally, my laboratory demonstrated that a stretch of eight amino acids within the pore loop is responsible for potassium selectivity[21]. We called these amino acids the potassium channel signature sequence.

The signature sequence has been used as a key to find potassium channels even in bacteria. The obvious implication is that nature used the same structural scaffold to form the selectivity filter in all potassium channels. This intriguing conclusion inspired me to want to directly visualize a potassium channel in a way that mutagenesis experiments could not accomplish. I therefore set out to learn X-ray crystallography, spending long hours purifying and attempting to crystallize a few soluble proteins, learning a new trade while gathering advice from experienced colleagues. A complete change of environment, from Harvard to Rockefeller University, removed any temptation to fall back on mutagenesis experiments, and helped me to focus intensely on the new effort. It was frightening! From my laboratory at Harvard only one beginning postdoctoral scientist, Declan Doyle, and my wife Alice (a chemist who felt sorry for me) joined the effort to crystallize a potassium channel.

Two very good things happened before despair set in. First, the *Streptomyces lividans* potassium channel (KcsA) was described[22], and second, my laboratory grew quickly into a still small but very enthusiastic group of talented scientists, dedicated to solving ion channel structures. The KcsA channel has only two membrane-spanning segments per subunit and closely resembles the *Shaker* channel in its amino acid sequence. The ever-valuable scorpion toxin confirmed that the KcsA channel had to be closely related in structure to its eukaryotic counterparts[23]. Through much hard work and determination, crystals were obtained that allowed us to solve the

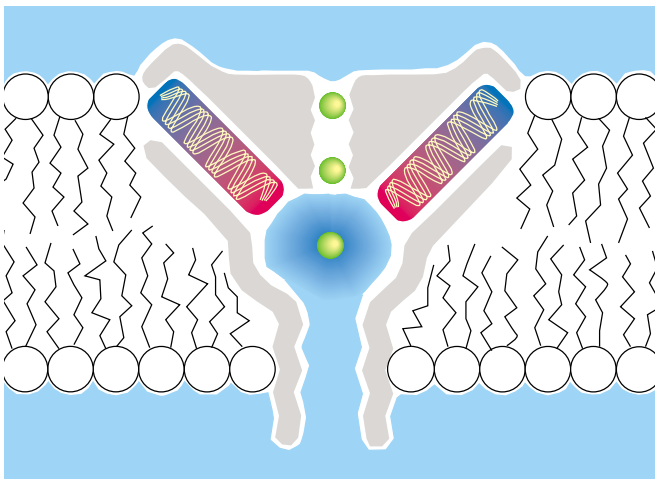


Fig. 2 Potassium channel demonstrates nature's mechanism of lowering the dielectric barrier. The channel contains a water-filled cavity (pale blue) in its ion pathway, halfway through the cell membrane (white). Alpha helices direct their cation-attractive negative ends (red) toward the cavity, stabilizing the positively charged potassium ion (green sphere). Without the cavity and helices, a potassium ion would be unable to cross the membrane center. Reprinted with permission from *Science* **280**, 75 (1998).

structure at a resolution of 3.2Å (ref. 9). Years of pharmacology and ion permeability studies were suddenly understandable at a new and deeper level. Two aspects of the potassium channel structure impressed me the most. The first was the presence of three ions in a queue within the pore, a result predicted more than 40 years earlier in a most elegant manner by Alan Hodgkin and Richard Keynes[3] (Fig. 1). The second aspect was nature's solution to the fundamental issue raised in the opening paragraph, overcoming the dielectric barrier to ion flow across the cell membrane. It was immediately apparent that the potassium channel has a very special design that includes a water-filled cavity to raise the dielectric constant at the membrane center, and oriented α-helices to provide favorable electrostatic interactions (Fig. 2). The solution is elegant in its simplicity.

Many questions still remain unanswered. I suspect that ions in the pore interact with each other through the structure of the protein. But higher resolution data that more accurately define the chemistry of the selectivity filter, and perhaps protein dynamical studies, will be needed to test this idea. The ability to actually look at ion channel structures has already begun to stimulate altogether new directions in ion channel research. Our recent findings of a β subunit oxidoreductase enzyme on voltage-dependent potassium channels[24] and of a PAS 'sensory' domain on the HERG potassium channel[25] have completely revised my view of potassium channels and their roles in cell biology. These discoveries keep me fascinated and wondering what is next to come.

1. Hille, B. in *Ionic Channels of Excitable Membranes* (Sinauer Associates, Sunderland, Massachusetts, 1992).
2. Hodgkin, A.L. & Huxley, A.F. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–544 (1952).
3. Hodkgin, A.L. & Keynes, R.D. The potassium permeability of a giant nerve fibre. *J. Physiol.* **128**, 61–88 (1955).
4. Hille B. Ionic channels in nerve membranes. *Prog. Biophys. Mol. Biol.* **21**, 1–32 (1970).
5. Hille, B. & Schwarz, W. Potassium channels as multi-ion single-file pores. *J. Gen. Physiol.* **72**, 409–442 (1978).
6. Hille, B. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* **58**, 599–619 (1971).
7. Bezanilla, F. & Armstrong, C.M. Negative conductance caused by the entry of sodium and cesium ions into the potassium channels of squid axons. *J. Gen. Physiol.* **60**, 588 (1972).
8. Hille, B. . Potassium channels in myelinated nerve. Selective permeability to small cations. *J. Gen. Physiol.* **61**, 669–686 (1973).
9. Doyle, D.A. *et al.* The structure of the potassium channel: molecular basis of K^+ conduction and selectivity. *Science* **280**, 69–77 (1998).
10. Milkman, R. An *Escherichia coli* homologue of eukaryotic potassium channel proteins. *Proc. Natl. Acad. Sci. USA* **91**, 3510–3514 (1994).
11. Heymann, J.B., Agre, P. & Engel, A. Progress on the structure and function of aquaporin 1. *J. Struct. Biol.* **121**, 191–206 (1998).
12. Armstrong, C.M. Interaction of tetraethylammonium ion derivatives with the potassium channels of giant axons. *J. Gen. Physiol.* **59**, 413 (1971).
13. Armstrong, C.M., Bezanilla, F.M. & Rojas, F. Destruction of sodium conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* **62**, 375–391 (1973).
14. Armstrong, C.M. & Bezanilla, F. Inactivation of the sodium channel. II. Gating current experiments. *J. Gen. Physiol.* **70**, 567–590 (1977).
15. Armstrong, C.M. Sodium channels and gating currents. *Physiol. Rev.* **61**, 645–683 (1981).
16. Hoshi, T, Zagotta, W.N. & Aldrich, R.W. Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science* **250**, 533–538 (1990).
17. Liu, Y., Holmgren, M. Jurman, M.E. & Yellen, G. Gated access to the pore of a voltage-dependent K^+ channel. *Neuron* **19**, 175–184 (1997).
18. MacKinnon, R. & Miller, C. Mechanism of charybdotoxin block of the high-conductance, Ca^{2+}-activated K^+ channel. *J. Gen. Physiol.* **91**, 335–349 (1988).
19. Tempel, B.L., Papazian, D.M., Schwarz, T.L., Jan, Y.N. & Jan, L.Y. Sequence of a probable potassium channel component encoded at *Shaker* locus of *Drosophila*. *Science* **237**, 770–775 (1987).
20. MacKinnon, R. Determination of the subunit stoichiometry of a voltage-activated potassium channel. *Nature* **350**, 232–235 (1991).
21. Heginbotham, L., Abramson, T. & MacKinnon, R. A functional connection between the pores of distantly related ion channels as revealed by mutant K^+ channels. *Science* **258**, 1152–1155 (1992).
22. Schrempf, H. *et al.* A prokaryotic potassium ion channel with two predicted transmembrane segments from *Streptomyces lividans*. *EMBO J.* **14**, 5170–5178 (1995).
23. MacKinnon, R., Cohen, S.L., Kuo, A., Lee, A. & Chait, B.T. Structural conservation in prokaryotic and eukaryotic potassium channels. *Science* **280**, 106–109 (1998).
24. Gulbis, J.M., Mann, S. & MacKinnon, R. Structure of a voltage-dependent K^+ channel β subunit. *Cell* **97**, 943–952 (1999).
25. Morais Cabral, J.H., Lee, A., Cohen, S.L., Chait, B.T., Li, M. & MacKinnon, R. Crystal structure and functional analysis of the HERG potassium channel N terminus: A eukaryotic PAS domain. *Cell* **95**, 649–655 (1998).

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