

REVIEW ARTICLE

A personal historic perspective on the role of chloride in skeletal and cardiac muscle*

Otto F. Hutter

Physiology, University of Glasgow, Glasgow, United Kingdom of Great Britain and Northern Ireland



Otto Hutter, born in Vienna, reached England by Kindertransport in 1938. He began to study physiology at the Wellcome Physiological Research Laboratories. Graduating from University College London, he stayed on as Sharpey Scholar and lecturer until 1960, interrupted by two years in Stephen Kuffler's Laboratory in Baltimore. He was then appointed to the Scientific Staff at the National Laboratories for Medical Research, and in 1970 to the Regius Chair of Physiology at the University of Glasgow. His work on neuromuscular and ganglionic transmission, skeletal and cardiac muscle is published mainly in *Journal of Physiology*. In retirement he now lives in Bournemouth.

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Correspondence

Otto F. Hutter, Physiology, University of Glasgow, G12 8QQ, Glasgow, United Kingdom of Great Britain and Northern Ireland.

E-mail: OFHutter@aol.com

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Introduction

The best philosophers illumine our thoughts and guide our actions. Since Karl Popper proved that scientific knowledge advances through conjectures and refutations (Popper 1963), that insight has become the recurrent theme of numerous retrospective scientific narratives. I also have already borrowed this leitmotiv in a different context on a previous occasion (Hutter, 2006). Here, I turn to how our knowledge of the role of chloride in muscle has grown

Abstract

During the early decades of the last century, skeletal muscle was held to be impermeable to chloride ions. This theory, based on shaky grounds, was famously falsified by Boyle and Conway in 1941. Two decades later and onwards, the larger part of the resting conductance of skeletal muscle was found to be due to chloride ions, sensitive to the chemical environment, and to be time-and-voltage dependent. So, much of the groundwork for the physiological role of chloride ions in skeletal muscle was laid before the game-changing discovery of chloride channels. The early history of the role of chloride in cardiac muscle, and work on the relative permeability to foreign anions of different muscles are also here covered from a personal perspective.

because that subject illustrates *par excellence* how scientific knowledge advances through the falsification of theories. Also, the role of chloride in muscles is a topic to which I happen to have made contributions way back in the 1950s and 1960s. And I have watched later developments with ever increasing fascination. So this will be a personal, a subjective account by an old hand in the game.

I started to study physiology in 1942, when at age 18 I took a war-time job as a laboratory technician at the Wellcome Physiological Research Laboratories. Quite

apart from the rich laboratory experience I gained there, I also received a wonderful continuing education. Many of the war-time scientific staff at the laboratories had been University Lecturers, and they were only too willing to guide me in my studies as an external student.

The text books from which I studied were inevitably of pre-war vintage. As regards the role of ions in muscle, the latest authoritative account was an article in *Physiological Reviews* by Fenn (1936). In it he concludes:

“This review has been presented chiefly from the point of view of the theory that the muscle membrane is impermeable to all common ions except the potassium and the hydrogen ion. This theory explains satisfactorily most of the facts”. What were the grounds which convinced a good scientist like Wallace Fenn that skeletal muscle fibers were not permeable to chloride ions?

Three, at the time seemingly valid findings, underpinned that conclusion: First, way back, Höber (1905) showed that the resting potential of a sartorius muscle – as measured between one external electrode on an injured tibial end of the muscle, and another on its intact pelvic end – that the resting potential so measured was not altered when extracellular chloride was replaced by sulphate or sucrose. If chloride were permeable, he argued, then a reversal of the chloride concentration gradient should have caused a depolarization. As we shall see later, that argument was correct, but the experimental set-up was inadequate.

Secondly, one can point to work on model membranes done by Michaelis (1925). He showed that the ionic permeability of collodion membranes depended on the charge of molecules admixed into the collodion film. When a collodion membrane was doped so as to become negatively charged, it repelled chloride but admitted potassium. This was an influential finding, and Michaelis model seemed persuasively applicable to the skeletal muscle membrane (Mond and Amson 1928).

Finally, Fenn et al. (1934) concluded, on basis of histological measurements, that the extracellular spaces between muscle fibers can accommodate all the chloride within an anatomical muscle, implying that the muscle fibers themselves are free of chloride.

But, all this proved to be a pack of cards that was blown over by the great Irish physiologist E J Conway, born in Co Tipperary in 1897. As a schoolboy, he carried off all the prizes. And after qualifying in medicine, he joined the Physiology Department in Dublin. At first, he engaged in research on renal physiology. Then, in 1937, he and his colleagues showed that muscle was permeable to lactate. That was the first demonstration that an anion could cross the muscle membrane, but it could be waved away by supposing that the permeating species was not the lactate anion, but rather undissociated lactic acid.

Then, in 1939, in a letter to *Nature*, Conway & Boyle put forward a new model for the muscle membrane in the following words: “The membrane considered, is one of special type with cation *and* anion pores, permeable to potassium but not to the larger sodium ions, and permeable at the same time to small anions of the type of chloride . . .”

Here (omitted) is a pictorial representation of that model. The pores in the membrane are shown large enough to admit the relatively small K and Cl ions. But the membrane pores are too small for the internal macromolecular anions and for the more heavily hydrated external Na ions; and for present purposes, it does not matter whether Na is really impermeable or only effectively so. This model is sometimes called a Double Donnan Equilibrium, after another great Irish scientist F G Donnan.

Now as is well known, according to the rules of the Donnan Equilibrium (Donnan, 1911) the permeable ions will distribute themselves between two compartments so that the concentration gradients of the permeant cations and anions are identical, as there can be only one membrane potential; or to put it another way for the present case, so that the product of the outside concentrations $[K_o] \times [Cl_o]$ is equal to the product of the inside concentrations $[K_i] \times [Cl_i]$. So, following in Donnan's footsteps, Boyle and Conway argued that because the inside concentration of potassium is so high, the inside concentration of chloride may well be vanishingly low.

Two major papers from Conway's laboratory followed. In the first, Boyle et al. (1941) arrived at a lower estimate for the extracellular space, than that previously estimated by Fenn et al. (1934). This allowed for a finite but low intracellular Cl^- concentration in accordance with a Donnan equilibrium. In the second, famous paper, Boyle and Conway (1941) demonstrated – in a variety of elegant ways mostly involving measurements of muscle volume changes – the ability of chloride to enter muscle.

As to the mechanism by which both potassium and chloride ions may cross the membrane, Boyle and Conway (1941) surmised: “The similarity of anion and cation diameters for diffusion through the membrane suggests the view that the same molecular pore exists for both and that this is probably not charged within the membrane”. Essentially, this was still the “pore-sieve” model of ion permeation first put forward by Ostwald at the end of the 19th century. So, as regards the mechanism of ion permeation, no great conceptual advance was made during the first part of the 20th century. But importantly, Conway overturned an ill-founded dogma which had held sway for too long.

Interestingly, Conway and Boyle's 1939 letter to *Nature* appeared in the same issue as Hodgkin & Huxley's

famous letter in which they first demonstrated the overshoot of the squid nerve action potential. In fact, these two epoch defining letters immediately followed one another. But as it happened, in the immediate post-war years, it was the role of Na and K in the generation of the action potential which claimed center stage, while the role of Cl in excitable cells remained a backwater for some more years.

It was into that backwater that I chanced to put my paddle to give it a bit of a stir. It happened this way. In the summer of 1955, when I had returned to University College London after two fruitful years in Stephen Kuffler's Laboratory in Baltimore, I was allotted a young Pakistani medical graduate named S M Padsha as my first PhD student. Looking for a promising problem, I seized upon work then just done at UCL by Hill and McPherson (1954). They had shown that replacement of chloride by nitrate prolonged the active state of muscle. Moreover, the speedy onset of that effect pointed to an action of nitrate on the surface membrane.

So, we examined what nitrate might do to the electrical properties of the muscle membrane. We soon found that replacement of chloride by nitrate doubled the muscle membrane resistance. And we were just in time to demonstrate this effect at the March 1956 meeting of the Physiological Society at UCL under the title: "Effect of nitrate on the electrotonic potential of skeletal muscle". In those grand old days, the Demonstrations were regarded as the most important part of the meeting, a practice that has since sadly gone overboard.

The most obvious interpretation of our finding was that chloride normally contributes at least one-half of the muscle membrane conductance. But, this met with some skepticism because to that point in time no one had shown that substitution of chloride by a less permeable anion had a major effect on the membrane potential.

Now, Alan Hodgkin always attended the UCL meeting, so he must have been aware of our demonstration, but I cannot remember whether he made any comment. At all events, he obviously savvied that if the muscle membrane were indeed really highly permeable to chloride ions, as our demonstration suggested, then external chloride would need to be removed so rapidly that the internal concentration of chloride cannot fall at the same time. And at the next UCL Meeting, in March 1957, Hodgkin & Horowitz described experiments on single muscle fibers that had been loaded with chloride by equilibration in a solution of high KCl product. If such a chloride rich fiber is then suddenly exposed to an external solution low in chloride so as to reverse the chloride concentration gradient effectively— as Höber never could do — the membrane potential turns inside positive by as much as +70 mV, showing that the membrane potential can be dominated

by chloride. At the same meeting, I introduced S M Padsha (1957) to communicate the effects of an extended series of foreign anions on muscle membrane resistance.

The full papers from the two teams were published in 1949: ours appeared in volume 146. In it we concluded: "...that chloride ions normally contribute the major share of the membrane conductance", the justification being, that chloride replacement by iodide — the most effective anion we tested — caused as much as a 2.4-fold increase in membrane resistance, equivalent to a fall in membrane conductance to 0.4 of its normal value.

The paper from the Cambridge team appeared in volume 148. Much of this informative paper dealt with the rectifying property of the potassium conductance. This meant that the relative contributions of the potassium and chloride conductances varied according to the experimental conditions. For muscle equilibrated in Ringer's solution, Hodgkin and Horowicz (1959) found the transport number for chloride to be 0.6, whereas that for potassium to be about 0.3. Gratifyingly, Hodgkin gave space to acknowledge and discuss our similar results; but inevitably our paper, though original and informative, remained overshadowed by the greater refinement of Hodgkin & Horowicz's use of single muscle fibers and by Hodgkin's consummate analysis of the results the single fibers yielded.

Anyhow, my first PhD student Padsha duly gained his PhD at the hands of AV Hill. My next PhD student was a conspicuously talented medical student, who had taken the intercalated BSc Physiology course and who had acquired a taste for more physiology. His name was Denis Noble. Denis joined me in September 1958. By then Harris (1958), also at UCL, had shown that the efflux of isotopic Chloride³⁶ from frog muscle was slowed by nitrate and iodide. This interaction between the ions of the lyotropic series prompted us to study also the effect of replacing chloride with a noninterfering impermeable anion.

At this point, Paul Fatt came up with the suggestion that methylsulphate might do the trick, and this proved to be the case. So, Denis and I set about to compare the membrane conductance of muscle in Ringer's solution with that of muscle made chloride-free by use of methylsulphate. Our paper appeared in April 1960. Our main conclusions were:

- (1) Chloride normally contributes 68% to the resting membrane conductance, in confirmation with our earlier results with iodide.
- (2) The potassium conductance — that is the conductance left after the total removal of chloride — rectifies inwardly.
- (3) The chloride conductance — as derived by subtracting the chloride conductance from the total membrane conductance — showed outward rectification in accordance with the Constant Field Theory.

All these findings agreed with the conclusions at which Hodgkin & Horowicz had arrived at by different experimental approach. So, by the beginning of the 1960s the magnitude of the muscle chloride conductance was firmly established. But the contemporary view remained that chloride was passively distributed (Adrian 1961) and that chloride ions crossed the membrane by electro-diffusion through an aqueous channel subject to a linear potential drop. As will become apparent, this was still a somewhat simplistic picture.

Meanwhile, Denis Noble and I continued our collaboration by examining also the role of chloride in cardiac muscle. In the course of that work, about which more later, I was offered an appointment to the scientific staff at the National Institute for Medical Research. Much, much later I learned that I owed that appointment to a recommendation by Alan Hodgkin.

In moving to Mill Hill, I was allowed to bring with me a new postgraduate student from the 1961 crop of Physiology graduates. I chose a Miss Anne Brooks, soon to become Mrs Anne Warner. I chose her mainly because of her role in the students' Operatic Society. Not as a singer, I hasten to add, rather as stage lightening technician. I argued that a girl who can handle a lot of switches probably had the makings of good electrophysiologist. And so it eventually turned out.

Once installed at Mill Hill, we set about to study the effects of pH on the chloride conductance of skeletal muscle. Remembering the work of Michaelis (1925), I expected that protonation of a membrane would make it more permeable to anions. So, imagine my surprise when we found exactly the opposite: acidification of the external solution greatly reduced the chloride conductance, whereas raising the pH increased it to about twice its normal value. Eventually, we published a series of three papers (Hutter and Warner 1967a,b,c).

The first described the surprising pH sensitivity of the chloride conductance. The second paper nailed down this effect by showing that the efflux of Cl^{36} was similarly altered by pH changes. And in the third paper, we showed that low concentrations of the metal cations Cu^{2+} or Zn^{2+} or UO^{2+} also greatly reduce the chloride conductance.

In retrospect, we should have divided the first paper because it contained a separate new finding. We found that when a muscle is left in an acid solution for some time chloride accumulates in the fiber. This pointed to the existence of an inward chloride transport system which is normally masked by the high chloride conductance. This was in fact a correctly interpreted early demonstration of the existence of active chloride transport in muscle of which more later.

Two further papers followed before I and Anne Warner left the National Institute to go our separate ways. In the

first, Hutter and Warner (1972), we studied the current-voltage relationship of the chloride conductance at different pH. As before, the relationship at near-neutral pH resembled the predictions of constant field theory. But in acid and alkaline solutions, it departed from the predictions of the constant field theory in different ways. Moreover, we found indications that the chloride conductance was time dependent in different ways depending on the external pH. These indications were then confirmed by Anne Warner in a nice last paper (Warner 1972) in which she employed the three electrode voltage clamp technique invented by Adrian et al. (1970). Whether bringing up children in a family, or young colleagues in the lab, you have to give them the opportunity eventually to do their own thing.

So, here is what we knew about chloride and skeletal muscle by the end of the 1960s. (1) Under normal conditions, chloride accounts for the larger part of the resting membrane conductance (Hutter and Padsha 1956, 1959; Hodgkin and Horowicz 1957, 1959; Hutter and Noble 1960) (2) The chloride conductance is labile, that is, sensitive to pH and metallic cations (Hutter and Warner 1967a,b,c) (3) The full range of behavior of the chloride conductance is more complex than the predictions of the Constant Field Theory (Hutter and Warner 1972; Warner 1972) (4) Under conditions of low chloride permeability, inward transport can raise the intracellular concentration of chloride to above the level expected for a purely passive distribution (Hutter and Warner 1967a) (5) The rank order of anion permeability in skeletal muscle at normal pH is $\text{Cl} > \text{Br} > \text{NO}_3 > \text{I} > \text{SCN}$ (Hutter and Padsha 1959), but at pH 5.0 NO_3 is more permeable than Cl (Hutter and Warner 1967d).

Now before dealing with later work on skeletal muscle, let me introduce some work on cardiac and crustacean muscle. When I came back to UCL, I brought with me some experience in cardiac electrophysiology thanks to a collaboration with Wolfgang Trautwein (Hutter and Trautwein 1956). So, once we had rounded off our work on skeletal muscle, Denis and I turned to explore the role of chloride in cardiac muscle. We used Purkinje fibers from sheep or dog hearts which mostly showed spontaneous rhythmic activity. Here are the main findings of our paper (Hutter and Noble 1959, 1961). Edward Carmeliet (1961) from Silvio Weidmann's laboratory reached essentially the same conclusions independently in his paper.

(1) Replacement of chloride by methylsulphate produced only a small increase in membrane resistance, indicating that the contribution of chloride to the total membrane conductance is much smaller in cardiac than it is in skeletal muscle. (2) In spontaneously beating preparations, replacement of chloride by methylsulphate eventually slows the rhythm. This suggests that E_{Cl} is

normally positive to the maximum diastolic potential, as would be expected if chloride was passively distributed according to the mean membrane potential over time. (3) Replacement of chloride by I or NO₃, causes hyperpolarization and arrest or slowing of the rhythm. (4) On basis of the above measurements of bi-ionic potentials and of measurements of membrane resistance, the relative permeability of anions was found to be: I > NO₃ > Br > Cl.

So, the differences between skeletal and cardiac muscle were quite striking. In skeletal muscle, the combination of a high chloride conductance with a chloride equilibrium potential normally near the resting potential serves to stabilize the resting potential. However, in spontaneously beating cardiac muscle fibers, the more positive value of the chloride equilibrium potential means that chloride current facilitates pacemaker activity, although the chloride conductance is relatively low. Most surprising to me of all was that the rank order of foreign anion permeability in cardiac muscle was the reverse of that in skeletal muscle.

I took up this theme again when Walmor Carlos deMello from Puerto Rico came to Mill Hill as a visiting worker and introduced us to crustacean muscle. Table 1 summarizes how we found the relative anion permeabilities to differ with muscle type and pH.

Taking first the now familiar situation in skeletal muscle, the chloride permeability is greater in alkaline and neutral solution than in acid solution. And when the chloride permeability is high, bromide, nitrate, and iodide are progressively less permeant than chloride. But at pH 5, when the chloride permeability is low, the foreign anions – in particular iodide – are more permeable than is chloride (Hutter and Warner 1967d). In crustacean muscle by contrast, the chloride permeability is higher in acid solution than in alkaline solution. And throughout the pH range, nitrate is more permeable than is chloride.

Now at first sight, all this was perplexing. But it falls into place within the Field Strength theory developed by Eisenman (1961) to account for ionic selectivity. According to Eisenman, the relative tendency of an ion to enter into association with a channel site depends on the balance of two energies: the strength of the interaction of an ion with its hydration shell on the one hand, and on the

other the strength of its interaction with the membrane site. If one then supposes that field strength of a membrane site with anion affinity can be increased by protonation, and that intrinsic field strength of such sites varies with species and muscle type, then such results can be rationalized. The difference between the anion selectivity of skeletal and cardiac muscle can similarly be accommodated.

Moving on now into the 1970s and 80s, the invention of chloride-sensitive microelectrodes by J L Walker greatly advanced our knowledge of active chloride transport in the hands of several teams. As regards skeletal muscle, Bolton and Vaughan-Jones (1977) confirmed the existence of active inward transport of chloride that is normally shunted by passive chloride movements. And later on Aickin et al. (1989) showed that a NaKCC co-transport system was responsible for the inward transport of chloride.

As regards cardiac muscle, Richard Vaughan-Jones (1979, 1986) found that even resting Purkinje fibers contained more chloride than would be expected if chloride were passively distributed as result of the operation of a chloride-bicarbonate exchange mechanism. According to a more recent reviews (Vaughan-Jones 2009; Alvarez-Leefman 2012), an electrogenic chloride/bicarbonate transporter also serves as a mechanism for uphill chloride accumulation in cardiac cells, and this is further complemented by an NKCC transporter (Clemo et al. 1992) as in skeletal muscle and smooth muscle. In beating cardiac fibers, these mechanisms presumably work in addition to the effect the relatively low mean membrane potential has on the chloride distribution. Be that as it may, in cardiac muscle the chloride conductance – because of its relatively low value – normally continues to play second fiddle to the cations; though under pathological conditions, the potentially destabilizing effect of the chloride distribution may become arrhythmogenic (Huang and Duan 2011).

Moving now into the 1980s, two new techniques took the field. The first was the incorporation of membrane vesicles bearing ion channels into planar phospholipid bilayer systems. The second newcomer was the patch-clamp technique. In the hands of Christopher Miller and his colleagues (White and Miller 1979; Miller and White 1984), these two approaches led to the discovery of a novel chloride channel in the electric organ of *Torpedo marmorata*. Thomas Jentsch and his colleagues then succeeded in cloning the electroplax chloride channel and this opened the way to the discovery of a whole family of chloride channels. Thus, Pusch et al (1991) identified and characterized the major skeletal muscle chloride channel which they called CLC-1. It turned out that the single channel conductance of CLC-1 is very low, only just 1pS. And this explains why attempts to find a convincing

Table 1. Relative anion permeabilities.

Frog skeletal muscle	Crustacean muscle
pH 9.8 Cl > Br > NO ₃ ≥ I <div style="text-align: center;"> v v v </div>	pH 8.5 Cl < NO ₃ <div style="text-align: center;"> ^ ^ </div>
pH 5.0 Cl ≤ Br ≤ NO ₃ < I	pH 4.5 Cl < NO ₃

Source: *An Application of Field Strength Theory* OF Hutter et al. (1969).

chloride channel through patch clamping of intact muscle fibers proved largely fruitless during the 1980s.

Now, it is neither possible nor necessary for me to describe how much the application of Molecular Biology has advanced our knowledge of anion permeability. A year ago, a special issue of *JPhysiol* celebrated 25 years of wonderfully productive research into chloride channels. Nevertheless, let me single out one remarkable finding that is especially cogent in present context. According to Fahlke et al. (1997), the pore properties of a recombinant human CLC-1 channel are dramatically altered by substitution of Gly230 by glutamic acid (G230E). Although the anion selectivity of the wild-type channel accords with our original findings on intact muscle fibers with chloride as the most permeable anion, the G230E variant exhibits the reverse selectivity series with chloride as the least permeable anion of the lyotropic series. Moreover, the cation to anion permeability ratio of the mutant channel is much greater than that of the wild-type channel, suggesting that the anion conductance of fibers expressing the mutant channel would be abnormally low for skeletal muscle. So effectively, that single substitution of glycine by glutamate would result in a skeletal muscle fiber that mimics the properties of cardiac muscle as regards to anion permeability. Could then a naturally occurring glutamic acid residue at a critical site be the cause of the observed differences between the anion conductances of skeletal and cardiac muscle?

The broad picture, as far as I can see it, is that great advances have been made regarding the molecular architecture of chloride channels, and in regard to their equally novel gating mechanism. As regards ionic selectivity, however, the principles formulated now 60 years ago by George Eisenman have continued to find application. If I have one small quibble, then it is that in reworking the properties of chloride channels in expression systems, earlier relevant work has often been overlooked, as if it belonged to a closed dark age before the dawn of molecular biology.

Finally, while much of the modern work on chloride channels has been done by use of in vitro expression systems, our knowledge of the role of chloride in the functioning of skeletal muscle has also taken a new turn. From the beginning it was obvious that the high chloride conductance of skeletal muscle exerted a stabilizing influence on its excitability. And this function was driven home by Adrian and Bryant's (1974) demonstration that a low chloride conductance was the cause of the involuntary repetitive discharge characteristic of myotonia. But, in the last decade or so, an astonishing new twist has been given to the physiological role of chloride in controlling muscle excitability. The Danish team led by Thomas Pedersen has shown that during normal muscle action potential activity, multiple cellular signals control

the chloride conductance. With the onset of activity the chloride channels are inhibited, which serves to preserve excitability in the face of potassium accumulation within the transverse tubules. But during prolonged activity that inhibition is lifted and the chloride conductance starts to rise. This late effect reduces the excitability of metabolically exhausted muscle fibers and so protects them from suffering damage.

All this and more is covered in the splendid, recent review by Pedersen et al. (2016). I found reading that admirably lucid review more enthralling than the most exciting thriller. And I hope that in good time you also will experience that exquisite pleasure which is reserved for those whose laboratory days are long behind them, but who can still take delight in how physiology is flourishing.

Conflict of Interest

None declared.

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