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Listening in to the cell: experiments in bioelectricity

About me – escaping the Higgs Boson

In his office one day at Imperial College, Abdus Salam looked over his copy of Pakistan News and said 'Well what are you doing after your PhD'? As a very naïve 23 year old I said that I hadn't thought about it. Two weeks later I found myself a postdoc the Institute for Theoretical Physics in Trieste. Salam, the first muslim to win the Nobel Prize for Physics for his work on supersymmetry, had set up the Institute to enable researchers in developing countries to keep in contact with new developments. Although on a short contract, it was an exciting time as it was realised that the Higgs Boson was a renormalizable (read 'respectable') theory and although I was caught up in some of the technical problems I realised pretty soon that I was not cut out to be a deep physics thinker – I just did not know enough. I had undertaken a degree, and then a PhD, in theoretical physics as it seemed difficult and challenging. But it was really a flight from what I had spent most of schooldays doing, electronics and small engineering projects.

An introduction to biophysics
In the 1970s the Nuffield Foundation had a scheme to try to recruit physicists to biology. There are now many programmes which encourage this, but at the time it was an enlightened approach. Believing that biology could use some more mathematics, a year on I attended lectures on genetics, and neurobiology, subjects full of words I did not understand. At some point during the year I encountered the Hodgkin-Huxley model of the nerve action potential, and was astonished how very arbitrary the model seemed to be. It did not really have the feel of a physics model. I do not know whether this is initial experience of many physicists; the defining equations seemed to be pulled out of thin air and it is not clear why any of the exponential curves used to fit the data should be there at all. No conclusion seemed to be derived from first principles and the whole style seemed to be very different from the linear thinking of the physical sciences.

At some point during the year I retreated to Trieste for conference on mathematical biology. During a talk about a very elaborate chemical scheme to explain neural conduction, a member of the audience got up and started shouting that there was absolutely no evidence for the theory: 'You cannot get the electrode through the myelin', he said. I thought it was wonderful that in biology people expressed such vehement views; physicists were far more restrained. This was my introduction to Paul Fatt and his then wife Gertrude Falk. As a PhD student with Bernard Katz Paul in the early 1950s Paul had discovered miniature synaptic potentials. In London I took their biophysics course – six non-stop laboratory weeks of recording from the neuromuscular junction – hard work, but the best introduction to electrophysiology.

The retina and the single photon signal

As a result I became a member in mid 1970s of the UCL Biophysics Department, one of the major centres of physiological biophysics founded by AVHIll and then run by Bernard Katz. Paul and Gertrude wanted to understand the origin of the b-wave of the electroretinogram (the electrical signal elicited by light which can be recoded simply by putting an electrode on the cornea), and to explain how it could be such a sensitive marker of excitation. Much of my equipment had to be built as the technology for recording membrane potentials reliably from small cells was just getting going. Inspired by the work of Alan Hodgkin, Denis Baylor and Trevor Lamb at Cambridge who had been recording from photoreceptors, the project needed very fine microelectrodes – with resistances over 200 megohms. We used a pulling device which required so much force to load the spring that it was too much for Gertrude, a handy tip if you wish to discourage your supervisor from looking over your shoulder in the lab. I built a lot of the high impedance electronics, the manipulators and light stimulator to record from cells and within a year we were beginning to record from bipolar cells in the isolated retina of dogfish. Everything had to be done in the dark to make sure that we were only getting signals from the rod photoreceptors when light was projected onto the retina surface. The room also had to be quite cool, or else the tissue just died.

The surprise was that most of the bipolar cells depolarised when illuminated with a short very dim light flash. This remained a puzzle for the next decade, well after I left the lab, for it was known then that in the dark photoreceptors released neurotransmitter continuously and light suppressed this release. Since most known transmitters opened receptor channels there was a paradox, as the response seemed to be behaving in a 'non-classical' way. (Actually there are also bipolar cells which hyperpolarise, following the neurotransmitter release, and much more understandable).

The second surprise was that the responses fluctuated. Since the 1940s it had been known that a rod photoreceptor could respond to the absorption of a single photon. Could the bipolar cells also be collecting information from single photon hits? I tried for a while to build an analog device to measure the fluctuations, but eventually resigned myself to learning how to program an enormous computer that Katz and Ricardo MIledi had been using to analysis noise fluctuations of the acetylcholine receptor. The PDP8 computer – made by the now deceased Digital Equipment Corporation (DEC) was about the size of a large kitchen dresser and had to be programmed in very low level code. It was a question of booking the computers for short periods between Katz and Miledi often in the middle of the night and analysing our data. The result was evidence that bipolar cells could detect a single photon absorption, the fluctuation arising from the statistics of the quantal nature of light.
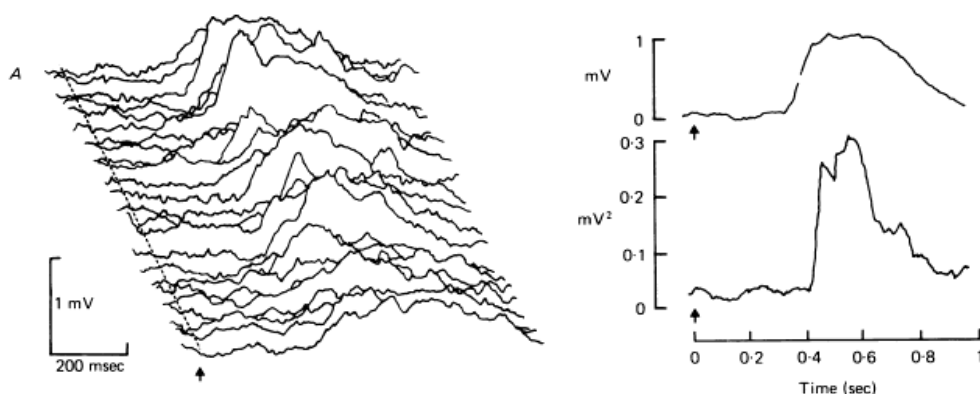
Figure Fluctuations in response to dim light flashes of a rod bipolar cells. The means and ensemble variance allows an estimate of the responses to a single photon event. From (Ashmore and Falk, 1980)

The noisy retina.

A letter arrived one dank February day from David Copenhagen, newly appointed as an Assistant Professor at UCSF, inviting me to join his lab. This would be my third postdoc. With visions of a sunny California, we arrived there the following November and it proceeded to rain for the next six months. We again had to set up a lab form scratch although by this time some of the equipment was commercially available. The computers had begun to shrink in size too. With a few thousand lines of code 'Baslab' (BASic LABoratory programme) brought from London and that Ian Parker had developed at UCL, we started to record analyse membrane electrical signals from bipolar cells, both depolarizing and hyperpolarizing. But this time we used from turtles, so although the room remained dark and one would emerge blinking into the light, it did not have to be quite so cold.

We looked at fluctuations the bipolar cell membrane potential due to the random arrival of transmitter molecules. Although the fluctuating rate itself was modulated by fluctuating light, a mathematical complication, there was a clear difference between the synaptic noise of depolarizing and hyperpolarizing bipolar cells. We could not quite identify the receptor mechanisms but it served as descriptive indicator that distinct receptor systems were being by the two cell types (Ashmore and Copenhagen, 1980). I felt a little guilty that David had employed me on his grant to look at electrical coupling between photoreceptors, but the lab had been subverted into a synaptic biophysics laboratory.

Hearing from the bottom up

Everyone says that so much of science discovery is serendipity. I did not think it true but reluctantly I now think it is. Ian Russell had just made the first intracellular recordings from hair cells in a mammalian cochlea and was visiting UCSF in San Francisco where I was then a postdoc (my third) as the best intracellular microelectrode puller was being developed there. It was the machine which eventually formed the cornerstone of Sutter Instruments. Ian and I knew each other a little from my my time as a Nuffield fellow and so I was invited to take up his position as a temporary lecturer at Sussex University while he enjoyed research leave. Weary of sitting in dark rooms, I thought this a great opportunity to learn about hearing.

A few laboratories with experience of intracellular recording in the retina were beginning to look past the eye and ask how the sensory hair cells of the cochlea could be working. All the technology for recording electrical signals was there, it was just a question of using it correctly. Robert Fettiplace and Andrew Crawford at Cambridge had started to record electrical signals from turtle hair cells (Crawford and Fettiplace, 1981) and Jim Hudspeth and David Corey at Cal Tech had shown how to measure electrical signals from frog hair cells (Corey and Hudspeth, 1979).

After building the microelectrode recording equipment yet again (although to begin with, for lack of funds, some of the items were scavenged from the Brighton rubbish dump), it was possible to record from hair cells in the frog. These cells are quite robust and can be dissected out as an epithelium to be pinned down under microscope objective. The Medical Research Council surprisingly awarded me an equipment grant with an impressively short turn-around time, I suspect that it was realised that Sussex was a centre of excellence. The problem was that nobody

understood then how the mammalian cochlea could be mechanically tuned to produce the high frequency selectivity in the auditory nerve fibres.   Ian and I thought at the time that frog hair cells would be a good model system, technically approachable, to explore basic cochlear principles. Although the recordings were possible and I showed that frog hair cells could underpin a particular type of auditory tuning mechanism it dawned on us that this system was not the way to understand how mammals hear, as the frequency tuning of the hair cells was relatively low frequency. Different, high frequency, mechanisms operate.
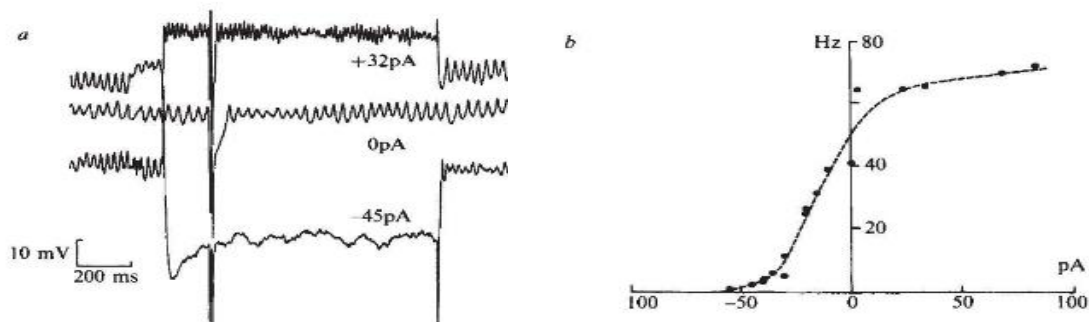
Figure. Spontaneous membrane potential oscillations in a frog saccular hair cell recorded with an intracellular electrode. The oscillation frequency is a function of potential determined by injected current. The frequency does not exceed 80Hz. From (Ashmore, 1983)

Outer hair cells as actuators

At the beginning of the 1980s a number of separate results merged to change the way in which the the cochlea was understood. Although it was known that cochlear auditory nerve fibres were highly frequency selective (so that each fibre responded to only a single pure tone), the mechanism was unknown. The recording by Russell and Peter Sellick of electrical signals from inner hair cells, the presynaptic source of auditory nerve fibres, showed that the selectivity was a property of the cochlea rather than some subsequent neural enhancement. The issue is that the mammalian cochlea is a coiled fluid filled coiled tube and the effects of viscosity around any structure should be mechanically dissipative.

We now know that a subpopulation of cochlear cells outer hair cells (OHCs) behave like ultrafast actuators that can undo the viscous dissipative damping that would be expected of a structure, the basilar membrane, surrounded by fluid. The direct evidence came from a group in Geneva who showed that a subpopulation of cochlear hair cells, the outer hair cells (OHCs), changed length when their membrane potential changed. Many cells can be stimulated to change shape on electrical stimulation (muscle cells for example) but this phenomenon was different, the OHC mechanism was robust and potentially provided a missing link to understand how power could be fed into the basilar membrane motion.

Unlike the retina, cell biology and cell biophysics was quite late coming to hearing. Much of the impetus had come from Swedish groups centred around the Karolinska Institute. Primarily ENT clinicians, they were the first to start looking at the elegant inner ear structures using electron

microscopy. Ian Russell, Jim Hudspeth and Bill Brownell had all spent time in Ake Flock's laboratory in Stockholm. Flock realised the significance of the outer hair cell population and had rediscovered a way of microdissecting individual hair cells that had first been described at the beginning of c.20 by Hensen. This was the key. Ian and I visited Stockholm with a whole suitcases of electrical recording equipment and managed to push a microelectrode into an isolated outer hair cell. It had no membrane potential to speak of, but we occasionally found that the cell seemed to jump apart when impaled. We thought not much of that. Bill Brownell in conjunction with a group in Geneva, with better equipment and the willingness to look at isolated cells (anathema to a retina biophysicist when everything was done in the dark) spotted that the cells moved. In hindsight, the recordings from OHCS made back at Sussex showed that the cells moved on current injection (usually off the end of the microelctrode) but the significance escaped us.

Patch clamp recording hair cells.

The first successful recording of the flickering of a single ion channel, the acetylcholine receptor, by Neher and Sakmann in 1976 was an eye opener for biophysics. The influential paper, describing the main methods of patch clamp recording, came later. Although single channel recording remains one of the highest resolution single molecule methods in biology, it is probably whole cell recording, where the measured current flows through all the channels in a cell, that has yielded the most information. Whole cell recording allows currents to be measured with a bandwidth 10 kHz, much better than with a sharp microelectrode. Beyond that limit one has to make many corrections.

In Bristol, Rob Meech and I started to record from guinea pig OHCs. We reported single potassium channels from isolated OHCs (Ashmore and Meech, 1986), but it was the advent of good commercial patch clamp amplifiers in the mid 1980s which opened up the possibilities for measuring ionic currents using whole cell recording. At the same much of the software for controlling the amplifier and analysing the data had improved considerably, so it was no longer necessary, quite, to write an application for every new experiment. Despite the limited bandwidth for membrane potential control, I demonstrated that the patch clamp could extended Brownell's observations to show that OHCs could change length fast enough to be involved in acoustic processes.
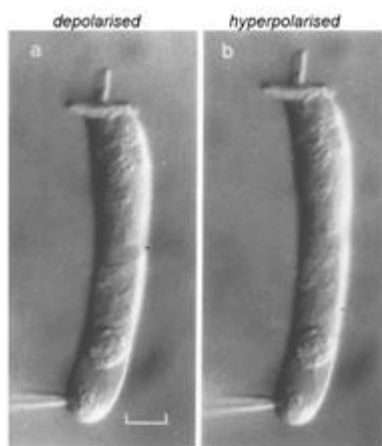
Figure: One of the longer OHCs from the guinea pig cochlea with a recording pipette at the base, depolarizing or hyperpolarizing cells. The sensory hair bundle is at the apex of the cell. The length change of maximally 4% is maintained for the duration of the stimulus allowing this photograph to be taken (Holley and Ashmore, 1988)

Ionic currents in outer hair cells

OHCs are long cylindrical cells and can be over 70 μm long in some cases, particularly when dissected from the apical end of the cochlea which detects low sound frequencies. The trick that we discovered, following Flock's protocol to isolate cells, was to patch the cells at the base where the membrane is more fluid. The lateral sides of the cells were hard to patch well as the surface was densely packed with protein. From the start we had worked with guinea pig OHCs: these were mature adult cells and at the time the guinea pig was a main animal of choice for auditory physiology. Much of what we know of hair cell biophysics has come from carried out on early stage mouse inner ears as the tissue survives well and mouse genetics is well developed. One problem though is that developmental changes in the mouse are often not complete and some features of early stage cells are not representative of the final cochlea.

One question was how the electrical properties of the cells varied from one end of the cochlea to the other. To our surprise not all hair cells expressed the same currents and to the same extent. The OHC properties varied along the cochlea coil. Long apical cells expressed a potassium current, $I_{K(ca)}$, whereas the shorter OHCs expressed a surprising novel current which we called I_{Kn} as it activated a negative potential, -80 mV, below the cell resting potential (Housley and Ashmore, 1992). I_{Kn} we now know is the current KCNQ4 (alternatively Kv7.4) in the same KCNQ family as those implicated a variety of clinical symptoms ranging from epilepsy to cardiac problems.
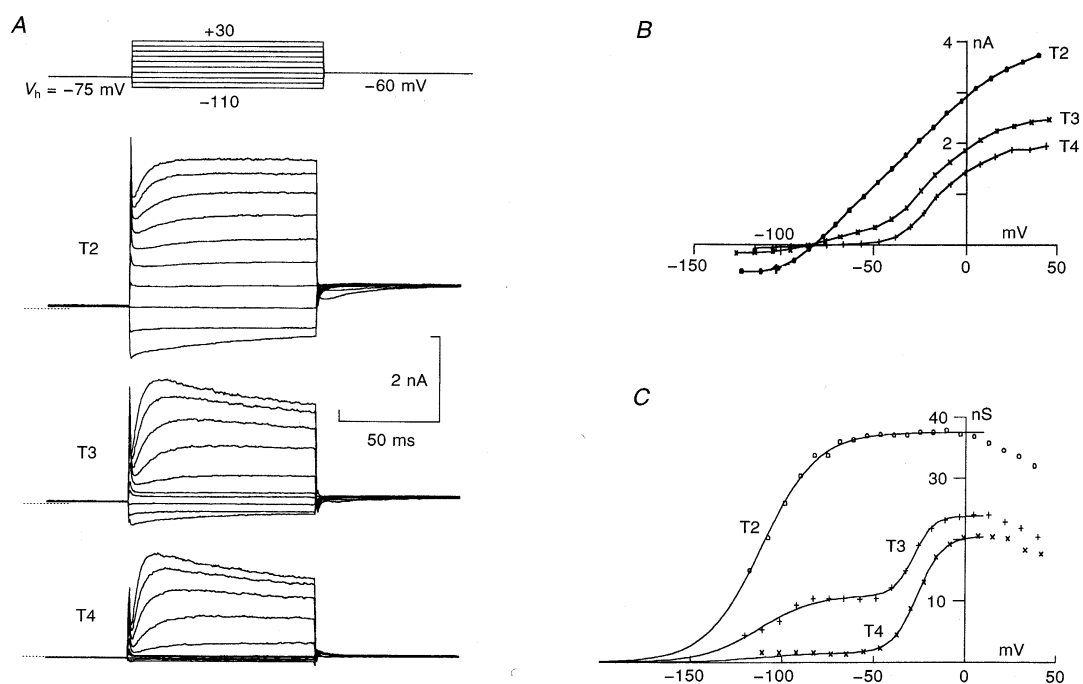

Figure: OHC currents measured with a whole cell patch clamp when stepped from a holding potential of -75 mV, for various turns of the guinea pig cochlea, Turn 4 being the most apical (low frequency end. The initial de-activating current evident in Turn 2 is I_{Kn} (KCNQ4). Shorter cells form the cochlear base have larger currents and larger conductances. From (Mammano and Ashmore, 1996).

Gating charges and the molecular basis of OHC motility

The molecular 'motor' which makes OHCs move was eventually identified in 2000 by a group at NorthWestern University at Evanston using a subtraction DNA library for hair cells (Zheng et al., 2000). They called the molecule 'prestin' as it made the hair cell move 'presto'. The name prestin stuck even though it was soon found that the molecule was a member number A5 of a family SLC26 of anion-bicarbonate transporters. How could a transport protein could act to distort cell membranes?

We had found a hint of this a decade earlier. In order to record ionic currents with a patch clamp it is necessary to minimize current that flows to charge the membrane capacitance. The curiosity about OHCs was that this capacitance compensation was very tricky and had to be redone at each holding potential. I ran down the corridor (I rarely do that) to show Meech when I realised that on the screen was evidence of an underlying gating charge movement, usually evidence of a molecular reorganization. Since capacitance $C = dQ/dV$, the anomalous capacitance was due to a moving charge in addition to the charge required for the membrane capacitance itself. This so called 'nonlinear capacitance' became an electrical fingerprint for the identification of prestin. In fact the Northwestern experiment measured a NLC in a transfected cell as a marker for prestin.

There are so many copies of prestin per OHC that Jonathan Gale and I managed to measure the gating charge in small patches of OHC membrane. All along the side of an OHC there was a small gating charge movement, matching the movement of the membrane. Even though small, the gating current was sufficiently stable and rapid to be able to pull it out of the noise with averaging the data a few thousand times.
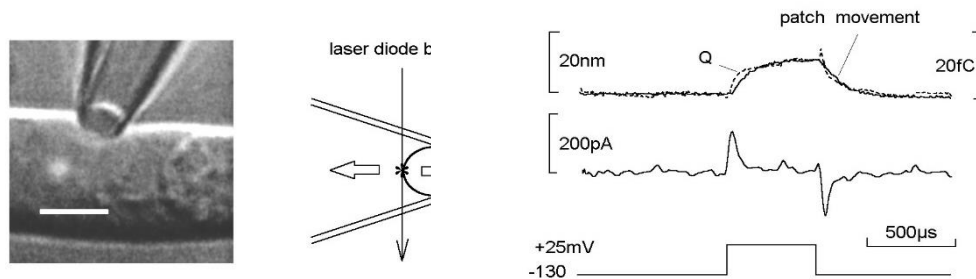

Figure: The gating charge from a small patch of lateral OHC membrane. By measuring the local patch displacement with a laser beam, the movement tracks the total charge, Q, moved. From (Gale and Ashmore, 1997).

The only point we missed was that the charge was carried by chloride *inside* the cell. But that needed the molecular identity of prestin to be known. Prestin/SLC26A5 was eventually expressed and analysed in CHO cells by Dominik Oliver and his colleagues then at Freiburg. They showed that the charge movement could largely be explained if intracellular chloride ions were shuttled though the cytoplasmic vestibule of the molecule but only about 0.7 of the way though the membrane leaving the transport cycle incomplete. This would look like a gating charge movement. The hypothesis was (and still is to a large extent) that as chloride moves into the electric field the molecule expands, probably less than 4% in the plane of the membrane. As a result of the close packing of prestin the OHC the cell elongates.

We still do not know the precise structure of prestin. There are a number of studies which show that prestin/SLC26A5 is, under some conditions, a low efficiency transporter as are other members of the SLC26 family but it is clearly a chloride/bicarbonate antiporter. The other surprising finding is that SLC26A5 homologs are found in all hair cells, vertebrate and invertebrate, but not as at the same density as in mammalian OHCs. The conclusion, although speculative, is that prestin provides pH regulation in sensory hair cells. It is just that mammalian OHCs have found an additional role for the protein.

Cochlear synapses and confocal imaging

Although it became routine to record the ionic currents from single cells the site of the current flow, possibly at multiple sites, and the link to the internal signalling pathways becomes important. The use of calcium sensitive dyes and single cell imaging has thus been added to the electrical measurement. In neuroscience, calcium signalling at the neuronal synapses has become a major interest. Very often the calcium currents themselves, dependent on a few tens of single channels are quite small. In the cochlea, the inner hair cells form multiple synaptic contacts, not all equivalent with the auditory nerve became the next step of exploration of the laboratory, preferably exploiting the techniques to work with adult hair cell systems (Griesinger et al., 2005).

We were lucky enough to have almost exclusive access to a multiphoton confocal microscope. Adding patch clamp recording to the system became a technical problem to synchronise the two systems with submillisecond accuracy. But this system then allowed us to look at multiple sites of calcium entry could be imaged discretely and at the same time once the cell was stimulated.

Recording electrical signals in cancer

Many of the techniques for recording signals from cells can be moved to other systems once the tricks to work with new tissues are developed. Five years ago a chance conversation with Aamir Ahmed from Kings College, who had been working on a prostate cancer cells, imaging calcium but not the electrical signals, turned into another project. He had shown that Wnt peptides initiated a burst of calcium in the cell and subsequent cell proliferation. Wnts are familiar from developmental biology, where gradients of the protein determine cell division and organisation, but the idea that some Wnts could trigger tumours, particularly in prostate and breast tissues, was intriguing.

Aamir had collaborated with a company Sophion in Denmark that made high throughput multiwell patch clamp machines. These are designed primarily not as discovery but as drug screening stands. He wanted to see whether a 'classical' patch clamp – a single micropipette - gave the same answers. We began a slow collaboration – slow because there was about one experiment every fortnight – but discovered how to patch the cells and image the calcium signals at the same time. The technical problem with the Wnts is that the receptor sensitivity is in the nanomolar range. Thus any leakage of the peptide before we were ready meant a premature response and a new dish of cells. Nevertheless, we found that a short pulse of Wnt hyperpolarised the cells and this could be traced to a small rise in a calcium activated channel. The bioelectric signal was thus a very sensitive and fast indicator of Wnt activity, but at a single cell level. The interesting issue in trying to publish this essentially interdisciplinary work was that it fell between a rock and hard place: cancer biology is seen as a molecular biology problem and, at the other end, a clinical issue and very often neither set of reviewers quite grasped the biophysics. A certain amount of mutual education was called for.
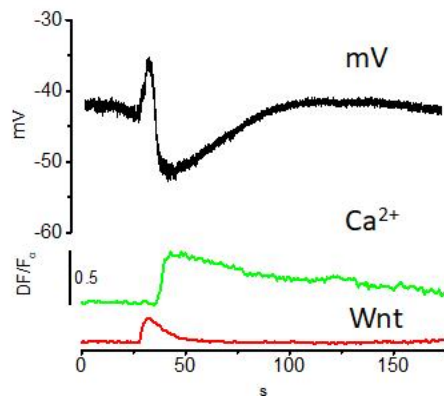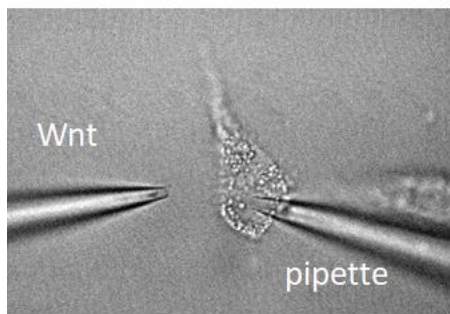
Figure: The effect of Wnt peptide on a PC3 cancer cell: a biphasic response. Adding a calcium indicator (OGB5N) to the pipette solution allows intracellular calcium to be measured at the same time as the cell potential, while adding a neutral dye (Alexa647) to the Wnt pipette monitor the peptide dose and application. From (Ashmore et al., 2019).

What can we learn from bioelectricity?

I have tried to give a sampler of a few topics which can be accessed by measuring the bioelectrical signals from the cell membrane. The techniques have become almost standard in neurobiology although some of them take a while to acquire and more importantly to interpret. There are limiting technical issues in the trying to measure ultrafast events, very obvious in hearing where the biological signals extend to 100 kHz, yet many of the technologies (the patch clamp for example) has a recording bandwidth of 20kHz at most. An outstanding basic question, for example, is how fast does an ion channel open? At the moment we can only guess using models, extrapolation or coarse grained molecular dynamics.

There is little doubt that recording electrical signals from single cells requires a certain degree of dedication, but such hard won data can provide fundamental insights into biological processes on which industrial and medical advances may well depend. Understanding the dynamics and modulation of the electrical potentials across a functioning cell membrane is an essential complement to the progress of appreciating the structural, molecular, cellular and systems properties of living systems.

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