Sounds Physiological

Shedding light on the action potential: An Interview with Brian Salzberg

Transcript of a conversation between Elizabeth M. Adler and Brian M Salzberg[1,2]

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Transcript condensed and edited by EMA

EMA: I'm Liz Adler, Executive Editor of *The Journal of General Physiology*, and I'm in San Francisco for the 58th Annual Meeting of the Biophysical Society, speaking with Brian Salzberg of the University of Pennsylvania. Brian, thanks so much for agreeing to speak with me.

BMS: Hi, Liz. It's a pleasure. Glad to be here.

EMA: Nowadays, using optical methods—or I might say dynamic optical methods to distinguish them from microscopes—to investigate physiological functions is fairly common. But when I first met you, back in 1978, it wasn't very common at all. And you were mostly using optical methods to look at changes in membrane potential.

BMS: It's been really extraordinary, seeing the growth of functional optical methods, you know, dynamic optical methods. Because when we started looking for voltage-sensitive molecules, essentially molecular voltmeters, back in 1971, it was unheard of. And for the first decade or so, there were very, very few optical methods around. But it's taken off remarkably in the last 20–25 years.

EMA: So how does it work [laughs]?

BMS: Well, to this day, we don't *really* know [*editor's note*: electro-chromism is a good part of it; see Loew et al., 1985]. We know better for calcium indicators, because there it's a simple matter of calcium chelation by a fluorophore.

But in the case of voltage indicators, the molecule of interest binds to the cell membrane—the good ones don't cross the cell membrane—and they obviously experience part of the membrane electric field. And it's a representative part, because the good voltage-sensitive dyes, the fast ones, respond linearly over a range of probably about plus or minus 200 mV from rest. So they're very linear, and they're very, very fast. In squid axon, at least, with a very fast voltage clamp, we've measured them to respond in less than 2 µs at room temperature … those experiments that Pancho Bezanilla and Ana Lia Obaid and I did many years ago [*editor's note*: see Salzberg et al., 1993].

Now, of course, there's a whole new generation of voltage sensors. There are voltage-sensitive fluorescent proteins, which have the advantage that they are genetically targetable, so we can have particular cell types express voltage sensors. They're not *nearly* as good yet as voltage-sensitive dyes, in the sense that they're neither linear nor fast.

EMA: Are those also targeted to the plasma membrane?

BMS: Yes, they are. They sense membrane field. And the way most of them seem to work is they're attached by some sort of linker to the S4 voltage sensor. So they typically now use derivatives of the voltage-sensitive phosphatase. So, that's the most common type. They're attached to the S4, and they move with voltage. But probably because . . . of course, we're not sure of this . . . but probably because they're rather large fluorophores, they slow down the voltage sensor, and they don't respond as fast as the gates do, for example.

So rise times of these fluorescent proteins are typically, oh, in the best case, maybe 3 or 4 ms instead of less than 2 µs. So they're three orders of magnitude slower, and they're not linear.

EMA: What are some of the advantages of using dyes rather than electrodes?

BMS: Well, photons are gentle; it's not sticking a telephone pole in through the plasma membrane, or attaching a patch pipette to the plasma membrane. And, of course, you can also record from many cells simultaneously, or many parts of a cell. So, as long as you can optically dissect the image of a preparation, you can record from as many places in the preparation as you have pixels.

EMA: You alluded earlier to calcium dyes.

BMS: Mmm-hmm.

EMA: That's, of course, exploded.

BMS: Right.

EMA: I guess you were involved with the development of some of the early. . .

BMS: Let's see. In 1974, Joel Brown and Larry Pinto got word of the fact that there were metallochromic ion indicators, in particular calcium indicators, that were used by inorganic chemists. And we had the idea that we could use these in biological systems to measure calcium changes. And so Joel Brown and Larry Pinto and Larry Cohen and I, and Paul De Weer and Bill Ross, got together and did a series of experiments using voltage-clamped squid axons, which had been stained with arsenazo III, and we measured fairly large calcium changes in squid axons due to calcium permeation through voltage-sensitive sodium channels. We could block the calcium signal with tetrodotoxin, which is a *very* specific blocker of sodium channels in squid axon.

We saw very big calcium changes, and we repeated those experiments and did more experiments in the summer of 1975, and then we published a paper in the *Biophysical Journal*, so next year will be 40 years since we published that paper [*editor's note*: see Brown et al., 1975]. Of course, within I guess it was 2 or 3 years, Roger Tsien had the idea of using fluorescent calcium chelators to measure calcium, and what followed was first quin2 and then fura-2, and then a whole raft of new calcium indicators [*editor's note*: see Tsien, 1980, 1982a,b; Grynkiewicz et al., 1985]. And they quickly supplanted arsenazo. Arsenazo has very particular uses still, but they are overshadowed completely by Roger's dyes.

And now, of course, in the last decade, we have genetically targetable calcium indicators. So, there are things like the GCaMP series [*editor's note*: see Ackerboom et al., 2012].

EMA: What about other ions?

BMS: Well, there are now fluorescent indicators at least for potassium, sodium, [zinc, and] hydrogen, you know, pH indicators. There have been pH indicators for a long time, of course.

EMA: Litmus [laughs].

BMS: Right. Of course, there are now optical indicators for larger molecules, like cAMP and things like that. It's just extraordinary, the degree to which optical methods have taken off in the last couple of decades.

I remember in 1971 I was a postdoc in Larry Cohen's lab, and we bought a vibration isolation table, and—as far as I know—that was the first vibration isolation table that was used in any kind of biological experiments.

EMA: I guess Larry started out looking at birefringence even before?

BMS: Yeah, Larry did his PhD in zoology, and then did a postdoc with Richard Keynes in England. And it was in Babraham, an outpost of Cambridge University, that he worked together with Richard Keynes and Bertil Hille on birefringence and light-scattering [*editor's note*: see Cohen et al., 1970]. So those were the first—or among the first—intrinsic optical changes that were detected.

They were complicated. For light-scattering, for example, there were voltage-dependent components and there were current-dependent components, and it was difficult to sort them all out. It was only, really, in about 1971, when I joined Larry's lab, that we began doing voltage-clamp experiments on single squid axons, and looking for *extrinsic* indicators.

We were looking for molecules that showed voltage sensitivity, and we really worked pretty much in the dark, literally and figuratively. But we started looking at fluorescent molecules. And I remember we got hold of the Dyers and Colourists Index, and looked at all the fluorescent dyes that people had been using for mainly staining textiles.

We were trying the oddest things. I mean, aside from all the obvious coloring agents, we started looking at saffron, for example. Saffron, it turns out, is fluorescent, but shows no voltage sensitivity, unfortunately.

EMA: Uh-huh.

BMS: Chlorophyll. Chlorophyll is interesting, because chlorophyll does have a small amount of voltage sensitivity. But it turns out to be extremely phototoxic.

All green plants obviously express chlorophyll—but it's phototoxic, and plants protect themselves from chlorophyll's phototoxicity using carotenoids, like β-carotene. So you find these carotenoids associated with chlorophyll, and they protect the plants from the singlet oxygen, which is produced by chlorophyll.

The first *real* voltage-sensitive dye was merocyanine-1 or merocyanine-540, which Larry and Vicencio Davila and I found in 1972. Merocyanine-1—or merocyanine-540—turns out to be *extremely* phototoxic, and the mechanism seems to be production of singlet oxygen. And so we had the idea that maybe we could protect nerve membranes from phototoxicity by using carotenoids, as plants do, so we started trying to get β-carotene to membranes.

I remember some experiments that Ana Lia Obaid and I did trying to protect *Aplysia* neurons from phototoxicity, and we tried to get β-carotene into *Aplysia* neurons.

EMA: Uh-huh.

BMS: You know, *Aplysia* really don't like to eat carrots, so we had to put it in artificially, and it's extremely hydrophobic, so it's not easy to get into a bath. It turns out that bovine serum albumin has a hydrophobic pocket, and we thought we could use that as a carrier for β-carotene. So we delivered β-carotene to membranes using BSA, and lo and behold, the phototoxicity was gone. But we discovered very quickly that what it was doing actually was pulling the dye out of the membrane. . .

EMA: ***Oh.***

BMS: It was protecting the cell by taking away the voltage-sensitive dye. So it's a very good way, it turns out, of destaining membranes, but it doesn't work as a photo-protective agent.

In any case, we started working on looking for voltage-sensitive dyes [in] about 1971. The first experiments, obviously, we worked on squid giant axon because of the ability to voltage clamp it, and we worked on that in Woods Hole. But, during the rest of the year, Larry spent his time writing grants and working on papers, and Vicencio Davila and I had to devise a way of doing optical experiments in the winter. So we started using walking legs of lobster.

We must have screened about 150 dyes before we found merocyanine-540. And that was kind of serendipitous. Larry and I wrote to Eastman-Kodak, because we had heard that Eastman-Kodak had developed a large number of merocyanine dyes, and merocyanine dyes were used by film manufacturers as photosensitizers. Because, you know, in film, the silver halide crystals, which are the light sensors, are not sensitive in the red. They are only sensitive to relatively high energy blue photons. So—even for black-and-white film—what the film manufacturers had discovered was they could use photosensitizers to transfer energy from the red-sensitive pigments to the silver halide crystals, and therefore push the sensitivity of the film into the red.

EMA: Uh-huh.

BMS: They had made something like 35,000 merocyanine-type molecules, and they just kept it in their vaults, and they didn't release them. Larry and I wrote a letter to Eastman-Kodak and asked them if they could possibly send us some merocyanine samples.

And we said, well, we wanted them to have a certain degree of water-solubility, and we hoped that they would be biologically compatible and nontoxic, and they sent us four dyes, called merocyanine-1, -2, -3, and -4. And they did give us the structures, which was surprising.

Vicencio Davila and I looked at the structures, and for no particular reason we hit on one of them, and said, "Well, that looks interesting," and we tried that. And that was at least 10 times better than any of the 150 dyes that we'd tried. It was a dramatic change. We thought the chances that we'd hit on the best one on the first try was small, so we screened the other three, and the other three were complete duds.

It gave a very, very big signal, and it was a very fast signal.

I still remember the thrill of looking at the oscilloscope. Our pulse protocol was a 50-mV hyperpolarizing pulse followed by a 50-mV depolarizing pulse from rest. And there were no tops or bottoms on the optical signal, so we had to turn the oscilloscope gain down, and there were still no tops and bottoms. I think we turned the gain down three times before we saw the top and bottom of the optical signal, and it was an incredible thrill.

EMA: That must have been tremendously exciting.

BMS: It was. I can still feel the goosebumps.

EMA: So you looked at squid axons and lobster walking legs, and I remember you doing experiments with *Xenopus* posterior pituitary.

BMS: That's right. We started out with *Xenopus* posterior pituitary, because there are, oh, something like 40 million nerve terminals, which you can activate more or less synchronously. But we actually quickly switched to mouse, so we started looking at optical signals from mouse pituitary, or [more precisely] mouse posterior pituitary [*editor's note*: the neurohypophysis]. And we found that the wavelength dependence of the dye, when applied to the mouse posterior pituitary, was a bit anomalous. And we very quickly realized that what was happening was there was a large admixture of—or contamination by—light-scattering, and it had nothing to do with the dye. So, it turns out there was a large optical signal from the mouse pituitary in the absence of any extrinsic dye. So that light-scattering signal then occupied us for the next decade, trying to figure out what it was due to.

EMA: So what was it due to?

BMS: It seems to be due [in part] to the actual fusion of the dense core vesicles. We can actually detect the fusion of the vesicles, and the signal is complicated: It has at least three components. A very fast component, which is related to the membrane voltage [and/or current] itself, so [that signals] the arrival of the action potential in the terminals. And that's similar to the kind of voltage [and current] dependence that Larry had seen in the intrinsic optical signals from the nerve; it seems to be related either to the action potential or the action currents, or probably both. You know, the action currents slightly precede the action potential, and the optical signal is intermediate, so it rises a little bit later than the action currents and a little bit earlier than the action potential. So there's that component, which we called the E-wave, because it was "E" for "excitation."

EMA: Uh-huh.

BMS: And then there was a slow component in the opposite direction, which we called the S-wave, for "slow," but also we had it in the back of our mind that it might be related to secretion. So it turns out the S-wave *is* related to secretion, and then there are slower components still, which seem to be related to reequilibration of the terminals. That's a long story, but skip forward about two decades, and we decided that, since light-scattering changes are often related to volume changes, we wanted to see whether the light-scattering changes that we were seeing in nerve terminals might be related to volume changes in the terminals themselves. So we bit the bullet, and we put together an atomic force microscope, which is the most direct way of measuring distance changes.

We didn't try and scan the preparation. We didn't try and image the preparation. All we did is use a bare cantilever, with no tip, and we laid it on top of the posterior pituitary gland, and then we stimulated the axons. And sure enough, the cantilever of the atomic force microscope jumped.

It jumped fast, and it jumped big.

And it was *so* big that we were sure it was an artifact. We were stimulating with, you know, 100 V applied extracellularly. So we thought, "Well, it's clearly some kind of artifact, but it's a *nice* artifact..." So, we just put on a couple of hundred nanomolar TTX, and it went away: It was related to the action potential.

Well, to make a long story short, it turns out that the posterior pituitary—because of its anatomy—when the cantilever was sitting on top of the posterior pituitary, it's sitting on top of a stack of about

100 terminals deep, and, assuming the terminals all act in series, then a 10-nm jump in the height of the preparation actually corresponds to about a 1-Å jump in the diameter of a single terminal.

So we were actually detecting, with very large signal-to-noise, a 1-Å change in diameter. And if you do a quick, back-of-the-envelope calculation on a 5-µm nerve terminal, if you calculate how many sodium ions enter a 5-µm terminal during the action potential, it's on the order of 50 million, and an approximately equal number of potassium ions leave during the repolarization phase of the action potential. And then you ask yourself how many water molecules would have to accompany each sodium ion in order to give rise to the volume change that we see. And turns out it's only two or three.

EMA: ***Really*? Huh.**

BMS: It's quite remarkable. So you begin to picture sort of a cartoon of a not fully dehydrated sodium ion—but a *partially* dehydrated sodium ion—which has lost most of its hydration shell, but still carries with it perhaps two water molecules, one in front and one behind, and that gives rise to an increase in volume, which will have the time course of the upstroke of the action potential, and then, similarly, a potassium ion may be accompanied by two or three water molecules going out, and that gives rise to the repolarization phase of the action potential and the downstroke of what we call the mechanical spike.

EMA: That's really amazing.

BMS: It's really quite remarkable you can detect this change in volume. Now, we're not 100% sure that we're not seeing something else. I've woven this quite nice tale about water entry [but] it's difficult to test.

It could be that we're seeing a pure electromechanical effect of the action potential. We've done some experiments, though, which suggest that it really *is* water entry. So, if you make the bath hypertonic, say, by 20% with sucrose, then what will happen is water will leave the terminals, and you'll get a slightly shriveled membrane. And you might expect, naively, that a shriveled membrane would give less of a mechanical spike than a membrane under tension. And sure enough, that's what we see. We got better at doing the experiments, so we used the voltage-sensitive dye so we could measure the action potential at the same time that we measured the diameter, and it turns out that the action potential is completely unaffected by the hypertonic solution, but the mechanical spike is reduced by about 30%. Then we did the opposite experiment, where you make it hypotonic, and sure enough, if you stretch the terminal membrane, you get a larger mechanical spike, but the action potential was unaffected.

So that also suggests that it really is water movement that is causing the mechanical spike. The other thing that you can do is, you can reduce the amount of sodium that enters during the action potential by replacing sodium with an impermeant cation like choline. And it turns out that you can replace a lot of the sodium with choline and still not affect the action potential significantly. You slow it a little bit and you make it a little bit smaller, but the amount of water that should enter with sodium would be greatly decreased, and that's what we see. We see a decreased size in what we call the mechanical spike, and no *big* change in the action potential.

And the other thing that we did with this beautiful preparation of the posterior pituitary—and this is a funny, kind of serendipitous story as well. We thought it might be nice to measure secretion with millisecond time resolution. So we made a mouse where we expressed a fluorescent moiety, a derivative of EGFP, which is called SynaptopHluorin.

EMA: Uh-huh.

BMS: And the idea is that SynaptopHluorin's fluorescence is highly quenched when it sees a low pH environment.

EMA: Uh-huh.

BMS: So if it sits in the vesicle membrane, it sees the vesicle pH, which is about 5.5. The idea was that every time a vesicle fuses, it would then be exposed to bath pH, which is 7.2–7.3, and we should see a flash of fluorescence.

So we made the mouse, and we did the experiments, and sure enough, we saw an increase in fluorescence at about the wavelength which we expected, about 470 nm. The only thing that was very disappointing to us was the increase in fluorescence was slow, and our whole idea was to make a millisecond response to secretion. We did the experiment a bunch of times; we were convinced that the fluorescence change was real—it was fairly large—but it was slow.

And then we did the really *damning* control experiment. We did the exact same experiment on a wild-type mouse, and got exactly the same result. So it had *absolutely* nothing to do with the expression of SynaptopHluorin. It was an intrinsic fluorescence change. And why there would be an intrinsic fluorescence change which was *loosely* coupled to the action potential—we gave it a train of maybe 25 action potentials, and we got a gradual increase in fluorescence.

So I'm very fortunate—I *was* very fortunate—in being one building away from Britton Chance, and I went and talked to Brit, and I said, "Well, what is it about nerve terminals that might change their intrinsic fluorescence at about 470 nm?" He said, "Oh, it's probably flavoproteins; it's probably FAD, maybe." I said, "Well, that's interesting." He said, "Well, why don't you look for fluorescence changes from NADH? That should be at about 350 nm, and it should be in the *opposite* direction because NADH, when it's oxidized to NAD, should *decrease* its fluorescence, whereas $FADH_2$, when it's oxidized to FAD, should *increase* in fluorescence." So we went and did the experiment at 350 nm, and sure enough, there was a decrease in fluorescence. So it looked like it was a change in oxidative phosphorylation in the mitochondria, which were responding to the action potential. . .

EMA: To a *train* of action potentials.

BMS: To a train of action potentials. So it turns out that in nerve terminals at least, the mitochondria seemed to be *counting* the action potentials, and that makes a lot of sense, because you need ATP in order to get fusion of the dense core vesicles. So the mitochondria have to know to make more ATP when there have been a lot of action potentials.

So what's the signal during an action potential for increased activity to the mitochondria, since they're not directly coupled?

And the obvious guess was calcium. So there is *some* calcium entry during the action potentials, and of course, mitochondria are very sensitive to intracellular calcium concentration. So we did the obvious thing. We blocked the calcium entry, and we blocked about 50% of the intrinsic fluorescence change.

So it was about half-gone [but *only* half-gone], and we *assured* ourselves that there was no rise in internal calcium by using calcium indicator dyes inside, and there was absolutely *no* change in calcium, and yet we only blocked 50% of the intrinsic signal. So there had to be something else that was going on.

We realized that these are relatively small terminals, and it doesn't take *too* many action potentials to upset the ionic gradients. So we've got sodium entry and potassium exit and, after a relatively small train of action potentials, a Na^+/K^+ pump has to turn on.

EMA: Mmm-hmm.

BMS: So what happens when the Na^+/K^+ pump turns on? You're hydrolyzing ATP into ADP. So you change the ADP/ATP ratio, and that's something which mitochondria are very sensitive to *also*, because they actually know that there's less ATP, so they make more. So we did the obvious thing again: We put on [the alkaloid] ouabain to block the pump. And sure enough, ouabain plus calcium blockers [or zero calcium] eliminated the intrinsic fluorescence change completely.

So the mitochondria *tally* the action potentials in nerve terminals where they're close to the membrane but not directly coupled to the membrane. So that was fascinating also.

EMA: Oh, that's *totally* fascinating.

BMS: So it's amazing. You know, you get led in strange directions from odd observations. We found the light scattering by looking for a wavelength dependence that was wrong, and we found the intrinsic fluorescence change by looking for secretion, using a mouse that didn't work.

EMA: That's pretty amazing. Do you have any other particularly interesting stories or findings or a favorite experiment that you did, or . . . ?

BMS: Well, you know, we've been using voltage-sensitive dyes for 40 years now—and I've been working especially with Ana Lia Obaid for the last 30 years or so—and we've done a number of interesting preparations.

Recently, Ana Lia took a sabbatical in Amita Sehgal's lab working on *Drosophila*. She came back to my lab, and we decided that we would look for voltage changes in the neuromuscular junction of *Drosophila*. And we thought, "Well, these are likely to be very, very small. We are looking at individual boutons, or small collections of boutons." And, to our surprise, at least certain voltage-sensitive dyes, like the di-4-ANEPPDHQ we've been using, gave relatively large signals. So we can actually see voltage changes in individual boutons. So that's exciting.

EMA: That's pretty amazing, that sort of resolution.

BMS: Yeah. So that leaves open the possibility of looking at genetic variations, and how they relate to spontaneous activity in nerve terminals, and lots of interesting things.

EMA: Well, thank you. I learned all sorts of stuff that I didn't know.

BMS: It's been a pleasure.

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