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Are any of the GDX are heterodimers or are you working on the assumption they are homodimers?

Randy Stockbridge: The ones that I mostly spoke about, are homodimers. So, you may remember in the sequence similarity network I showed, there are three major nodes in the GDX cluster. The biggest node were homodimers and then the other two smaller nodes were an inward facing and an outward facing subunit of a heterodimer. We have biochemically characterized two of the heterodimers, very surface level characterization, showing that they transport guanidinium and that they behave as a heterodimer that you need both subunits there.

Did you see major differences in current trace kinetics across the different substrates, e.g. faster decay of the signals? Could you identify substrates which electronically bind to the transporter, but are not transported?

RS: I'll answer the binding question first, I think just with the positively charged substrates, if we were to get a binding event but not a transport event, we would see a positive current rather than a negative current. You really need two protons going the opposite direction in order to get that negative current – that's a nice thing about this system that makes us feel a little more confident that we're looking at transport and not binding.

And then the next part of that question, yeah, these do all look sort of like transport-ish kinetics to us, it is not the very sharp current that people have seen for example with ion-binding to an ion channel. For sure we see differences in the kinetics, I think that would be expected that these various substrates are transported at different rates and that that decay that represents the rate at which this charge is capacitively charging liposome, that is different from substrate to substrate. We have not analyzed that in detail at this point yet, although that may be the next frontier for us.

Can you go over washing to change internal solution? Can you change any internal solution?

Katherine Henzler-Wildman: We've done it with changing the internal proton concentration and guanidinium concentration and chloride concentration for ClC, I didn't show that data but we haven't done it with bulky EMER substrates so there is a limit to the number of experiments we've done so far!

RS: You can see the exchange with pH's in my experience and I really haven't tried it with much else, rather easier to equilibrate.

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Could you explain again why a tightly coupled transport in ClC means there is no change in signal when you change the anion concentration? Thermodynamic considerations would say that higher anion gradient would still change the integrated current.

KHW: Yeah, we're not changing it hugely, we're only changing it a few fold. What we are looking for is a reversal condition and then here where we are running not at a reversal condition, so we actually get a current as we change that gradient... The proton is driving chloride transport but there is no chloride gradient, so we are running it under a very particular condition. What is important to remember is that we are starting with equal chloride on both sides and a constant proton gradient and then we're running it with either 5 millimolar or 10 millimolar, 30 millimolar chloride on both sides so there is no chloride gradient. In the absence of a gradient of chloride, if it is tightly coupled you should not see a change in that integrated current because the only thing driving the transport is the proton gradient.

How does 'overuse' of a sensor manifest? Noisier data, or something else?

RS: I guess I'm not sure what overuse would mean, I mean at some point we just get it very noisy and we start to see rapid degradation of the currents we're seeing… so when we do these experiments what we always do is a positive control perfusion at the beginning where we perfuse with guanidinium, we say okay we see a nice current there and then we perfuse with our test substrate and say its dimethylguanidine, usually we see a smaller current and then we go back to the original guanidinium and perfuse with that and say okay was the first guanidinium current the same size as our third trace, our second guanidinium collection. If that is not happening, and that happens eventually with these sensors then we just throw out that last little bit of data and go back to this positive control throughout the course of using one of these sensors.

KWH: We also see that the data gets very noisy, you can run a lot of experiments on one sensor but eventually it does start to degrade, and the data starts to get noisier, and we also tend to run the same experiment. So, we run our initial experiment periodically to check we are still getting consistent data.

Maybe I missed it but for the SSM assay with the substrate that has a lipid tail, did you add this to the liposomes or to the activating buffer?

RS: The octoguanidinium, the one with the tail, we didn't, it's a detergent and it destabilizes the liposomes, and it doesn't work so we haven't tried that with transport. With the other substrates, we can go diphenol, tetramethol, tetrapropammonim, all of these we just add to the perfusion buffer and there is a point eventually with higher concentrations of these more hydrophobic things, where you start to see with the empty liposome control kind of

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an intolerable amount of non-specific interaction so because these are positively charged substrates this manifests a positive current in the empty liposome control. There is a point that is related to the hydrophobicity and the concentration, where we cannot really do the experiments and trust it. But I feel we are going pretty hydrophobic and still getting reasonable results and still not NOT getting this high background current and it's still an easy control to do it with the empty liposomes and check that.

What is the ideal control experiment? Empty liposomes or proteoliposomes with non-functional transporter

KWH: From our experiments, they both seem to work really well. One of the things we think about, goes back to one of the earlier questions; is there say an additional binding site where you can get binding and not transport that you could potentially get from a non-functioning mutant like this (minute 54) E13QDGX, so we worry about getting some current due to binding that is not due to transport in that situation. Obviously, we are not empirically seeing that here, but I think in that scenario if you are worried about that then an empty liposome actually makes a better control system.

RS: It depends on what you are controlling for, if it is just the substrate partition into the membrane then yes empty liposome is fine for that. As with everything, I think it is worth trying everything! There is no answer as to what the perfect control is. If you have something that is locked or you know doesn't bind substrate that might be a fine control to tease out whether you're seeing binding currents or not, it depends on the system.

Will this assay work to determine the stoichiometry of any transporter? What are the limits of the assay?

KWH: I'm going to interpret this as transport rates. Obviously the faster your transport is, then the higher your current so the easier it is to read it out with SSM-electrophysiology. Faster transporters are better but with that said, we've gone as slow…we've done some assays with EMRE where we know from the NMR experiments what the alternative access rates are, so that sets an upper limit for the rate of transport and we've gone as slow as 0.5 per second and still detected reversable transport currents but I would say you probably can't go a lot slower than that.