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DISCUSSION

Session Chairman: Alan Finkelstein *Scribes:* Han-qing Xie and Marc J. Glucksman

OLAF ANDERSEN: What is the basis for the asymmetry of the Cx26 gating curve for the symmetrical channel? That is, why does the inside-out voltage-dependent process produce asymmetry?

JOSHUA RUBIN: There are two independent processes interacting to produce the asymmetry. The steady-state curves reflect the presence of a fast $V_{i\text{-}o}$ dependence and a slower V_j dependence.

Asymmetry is a result of a process that is dependent on the membrane potential. One oocyte is kept at a fixed potential while the other oocyte is stepped to various potentials. The current flow across the membrane is measured and indicates that there is a fast change in conductance as a result of changes in the membrane potential. Depolarization of the cell causes a fast increase in conductance and hyperpolarization causes a fast decrease in conductance. On top of this there are slower changes in conductance arising from the V_j dependence.

ANDREW HARRIS: If you include all of the data from stepping one cell then stepping the other and plot against to V_j, would you get a symmetrically shaped curve?

RUBIN: Yes.

ALAN FINKELSTEIN: Let me clarify some terms: V_j refers to the potential difference between the two cells. If for example there is a 20-mV difference between cell 1 and 2, the absolute voltage does not matter.

There is another voltage dependent factor $V_{i\text{-}o}$, the potential difference between the cell and the external medium. There is no potential difference between the cells, and you change the potential difference between the inside and outside of the cells. In some gap junctions the change in coupling between the cells is a consequence of this transmembrane potential.

ANDERSEN: Getting back to the issue of hemichannels. One cannot "tear" the gap junctions apart, we are told that they can dissociate by incubation in hypertonic solutions, which may be analogous to the two different ways you can open a zippered jacket.

(*a*) If you pull in a direction perpendicular to the zipper, you will tear the fabric.

(*b*) If you unzip from the end you will have two hemizippers.

DAVID SPRAY: We looked for that "zipper" for a long time. Many people would agree that there is no stoichiometric result in hemichannels in gap junction preparations. $V_{i\text{-}o}$ dependence is an uncommon property found only in some vertebrate connexins. So what is being measured? Is it the field across one or another extracellular loops?

RUBIN: We have exchanged both extracellular loops and have not changed the $V_{i\text{-}o}$ voltage dependence. The ES mutant also has inside-out voltage dependence. The extracellular loops may have a role in $V_{i\text{-}o}$ dependence but they certainly cannot by themselves confer this kind of voltage dependence.

JOE MINDELL: If you normalize out the fast V_{io} in the symmetric Cx26 junction, is the slow process becoming symmetrical?

JOSHUA RUBIN: Yes.

GERHARD DAHL: In connexins, there is no equivalent to the S4 segment of other voltage-gated ion channels. In fact, all connexins known today have the same set of a few charged amino acids in their transmembrane segments, while voltage sensitivity of various connexons differs considerably. Do you dare to speculate where the voltage gate could be located?

RUBIN: In our studies we are trying to identify regions of the molecule involved in voltage gating. There are biophysical data generated by Andrew Harris and David Spray that suggests that a component of the voltage sensor may reside along the channel lining sequences. They studied the time course of changes in conductance in

amphibian blastomeres when they flipped the polarity of V_j from +100 to −100 mV. It appeared that the open hemichannels could not close until the closed hemichannels first opened, suggesting that with one gate closed the other hemichannel voltage sensor could not see the change in the V_j field. Another interpretation could be allosteric interaction preventing the open gate from closing so there is no double closure.

We replaced all the predicted pore lining sequences of Cx32 with the sequences from Cx26 and observed no change in the gating charge.

Recently, we have observed the behavior of a mutant in the first extracellular loop, ES mutants (Fig. 2). As Cx26 comes out of the first transmembrane domain, it has a Lys, Glu at the border; all the other connexins have Glu, Ser. We changed these amino acids and now we see a large increase in the gating charge. This suggests that we are in a region of the molecule involved in voltage sensation; no other charges have done this.

HARVEY POLLARD: This discussion about the voltage sensor could be broadened to include more than a few of the residues. Indeed, the global ensemble of charges does result in a dipole, which can be acted on by a voltage pulse. A reorientation of the protein could ensue. This could explain how in the absence of a discrete charge site, voltage dependence can still be found.

RUBIN: We approached the voltage sensor as if there were discrete functional domains. We have exchanged various regions and there was no difference. There are 1.8 equivalent gating charges for Cx32 and 3.8 for Cx26, this means that only a fraction of charges move with each subunit. The mutant Cx32 closes for relative negativity. Since Cx26 closes for relative positivity, it would seem that somewhere there must be localized charges conferring polarity.

POLLARD: If the structural model were correct and you looked at the distribution of charges in the membrane, would there be any asymmetry of charge? Could a vector of some sort be modified by the mutant.

RUBIN: The transmembrane charges for Cx26/Cx32 are conserved.

FINKELSTEIN: Can you tell us how much of Fig. 2 is based on data?

RUBIN: The original evidence is hydropathy plots. Additional evidence of topology is from membrane protection studies and antibody binding studies. These have been done with Cx32 and Cx43 and these two connexins represent two branches of the connexin family tree.

FINKELSTEIN: What about evidence for α-helix structure within the membrane?

RUBIN: There was x-ray diffraction data that suggested the presence of a β-sheet, but that may have been due to the tilt of the α-helices.

B. VEERAPANDIAN: What x-ray structures are you referring to?

RUBIN: Those of Makowski et al. from two-dimensional (2-D) crystals. Current 2-D x-ray diffraction data has a resolution of 18 A.

HARRIS: For the mechanism of contingent gating proposed by David Spray and myself, it is not necessary for the voltage sensor charges to be inside the pore but only for the field across those charges to be affected by the conductance state of the pore. My question is, what other aspects of the channel behavior were altered in these mutants?

RUBIN: In homotypic junctions, those composed of identical hemichannels, the only changes we observed were shifts in V_o. These occur with replacement of the first extracellular loop or the cytoplasmic loop. The second loop shows no change in voltage dependence of the chimaera.

HARRIS: More specifically, in mutants that change voltage sensors, were there also changes in kinetics?

RUBIN: The Cx32*26 KE mutant has a greatly increased gating charge but there were obvious changes in the kinetics.

DAHL: Does mutation of the extracellular loops, including domain swapping, affect the efficiency of channel formation? How do the macroscopic junctional conductances compare between wild-type connexins and mutants?

RUBIN: Not for the most part. The ES mutant does not appear to make homotypic junctions but will make heterotypic ones with Cx26 or Cx32.

MARC GLUCKSMAN: I believe there are more than five connexins that have been cloned. Are there significant homologies beyond the first loop that may play the "other" role in changing the conformation of the channel.

RUBIN: There are regions of tremendous conservation. The two extracellular loops are conserved between all vertebrate Cxs as well as the transmembrane domains.

The amino terminus diverges as well as the cytoplasmic domain and carboxyl terminus.

GLUCKSMAN: Are there any features of the models of Nigel Unwin (with electron microscopy), or of Lee Makowski (combining EM and x-ray diffraction) to support or to conflict with the heterotypic constructs, that would work at the resolution of those structural models?

RUBIN: Unwin predicted a clockwise rotation in the hemichannel as part of the gating mechanism. It occurred to us that asymmetry in slow V_j dependence could be explained by steric interactions.

GLUCKSMAN: Have you tried any single mutations instead of the double mutation of Lys, Glu to Glu, Ser in Cx26?

RUBIN: Not yet.

MARCO COLOMBINI: I have a comment about the location of the sensor. Any portion of the protein that moves out of the field, whether neutral or not, once you introduce a charge, will act as a sensor. Maybe the protein moves and you have voltage dependence, or maybe somewhere a charge moves.

RUBIN: That is right. These charges could be serving as a probe for regions of the molecule that are moving.

MICHAEL GREEN: Why is the gating charge so small (is it possible that there are charges of opposite sign, or charges of the same sign moving in opposite directions)?

RUBIN: That is possible. There are positive and negative charges conserved in different transmembrane domains. We made mutants that have not been characterized that may address whether multiple ion movements yield a small change in net charge distribution.

GREEN: Is this (above) connected to the effect of changing two

charges by changing two amino acids on Cx26 in the chimera with Cx32 (at the end of your paper)?

RUBIN: The opposite occurs too.

GREEN: Can these charges be titrated?

RUBIN: We never tried. Shifts in voltage dependence as a result of a change in the pH have been observed.

THADDEUS BARGIELLO: In many of the chimeras we have made for examining the cytoplasmic loop, we have simultaneously changed the position and number of charges in the molecule without changing the gating charge.

RUBIN: That brings up a good point. The first extracellular loop exchange involves changes in five charged positions but doesn't produce any change in the gating charge. Mutation of two charges within the loop creates a large change in gating charge. Perhaps the whole domain transfer preserved intradomain interactions and these same interactions were disrupted by the two amino acid mutations. This may give us a clue as to the nature of the intradomain interactions that are important in structure and function.

GLUCKSMAN: You mention at several points in your paper that not all of the properties of rectification could be accounted for in the heterotypic junction, could you speculate what other than the first loop may be responsible for rectification?

RUBIN: In Cx32 and Cx26 there are only slow V_j changes when the Cx26 side is relatively positive. The slow changes have Boltzmann parameters consistent with Cx26 closure. Cx38 has been demonstrated to close for relative positivity so we thought Cx32 was inactivated in this junction. Recent results indicate that Cx32 may close for relative negativity and so the asymmetry in slow V_j may arise from simultaneous closure of Cx32 and Cx26 hemichannel slow V_j gates.

DAHL: Could you comment on the effects of the environment on the voltage gate? When Cx32 is expressed in transfected cells, the voltage gating appears different compared with hepatocytes and oocytes.

RUBIN: The gating charge is identical in all cases, the V_o has shifted. V_o in oocyte is 55 mV, in hepatocyte 40 mV, and in transfected cells 25 mV. The G_{min} may also change.

Oocytes may influence Cx38 gating, which is different in oocytes and blastomeres. If there is an environmental change, it can shift the ΔG_{chem}.

VALERIE HU: My question concerns the irreversibility of gap junction formation. How do you explain the decrease in gap junction permeability along the progression of the cell cycle towards G2/M phase? We have observed this and permeability may be metabolically regulated.

DAHL: Turnover of gap junction protein is very fast with a $t_{1/2}$ of 2–3 h in hepatocytes.

HU: So uncoupling observed as cells go through mitosis is degradation without resynthesis at that time.

DAHL: Cells have sufficient time for a cycle of synthesis and degradation.

HU: Once gap junction channels form irreversibly, are they subject to degradation?

RUBIN: Degradation could occur on the whole channel. In cells, double membrane structures have been observed. You may be degrading full channels and not hemichannels.