haemagglutinin membrane glycoprotein of influenza virus at 3 Å resolution. *Nature (Lond.)*. 289:366–373.

Yellen, G., M. Jurman, T. Abramson, and R. MacKinnon. 1991. Mutations affecting internal TEA blockade identify the probable pore-forming region of a K^+ channel. *Science (Wash. DC)*. 251:939–942.

Yokoyama, S., K. Imoto, T. Kawamura, H. Higashida, N. Iwabe, T. Miyata, and S. Numa. 1989. Potassium channels from NG-108-15 neuroblastoma-glioma hybrid cells; primary structure and functional expression from cDNAs. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 259:37–42.

Yool, A. J., and T. L. Schwarz. 1991. Alteration of ionic selectivity of a K^+ channel by mutation of the H5 region. *Nature (Lond.)*. 349:700–704.

DISCUSSION

Session Chairman: Ron Kaback *Scribes:* Juan M. Pascual and Mingyao Liu

THADDEUS BARGIELLO: Have you examined the behavior of the reciprocal exchange chimera where the Kv3.1 channel has a Kv2.1 pore?

GLENN KIRSCH: No, we have not done that.

BARGIELLO: My concern is essentially to be able to distinguish interactions between amino acids within the pore region domain from interactions outside the domain, and the reverse chimera can give you a lot of information about it.

KIRSCH: Yes, I agree.

ALFREDO VILLARROEL: You have found a very interesting change when you mutate this L374V. I think that what is missing in your compensation experiment is to put a Leu at either position 368 or 369.

KIRSCH: At 368 we changed Val into Thr without affecting conductance. That satisfied our curiosity about the point reversion and we did not go any further with that. I guess your point is that we should find out what Leu does.

VILLARROEL: I would like to know why you changed Val to Thr at position 368.

KIRSCH: Thr is the host residue at this position, and we wanted to identify amino acid residues that specify functional differences between the host and the donor channels. We have not yet done the experiment you are asking about.

RON KABACK: Have you investigated Pro mutations in that region?

KIRSCH: We have not mutated the Pro. They are highly conserved. Our model predicts a serious disruption of the pore, but we should test this.

ROLF JOHO: Rod MacKinnon has changed one of these prolines to an alanine. They got no functional channels.

BOB GUY: In one of your earlier papers on wild-type channels you reported that internal TEA binds with the same affinity when either Val or Ile is in position 369, as long as you have Val in position 374. Now you have mutant in which a Val is present at both 369 and 374 but the TEA sensitivity is much lower than that of the two wild-type channels. Do you have any experimental evidence that these residues are involved in TEA binding?

KIRSCH: We have measured sensitivity to internal TEA in several wild-type and mutant K channels. We have found that the identity of residues at position 369 and 374 makes a difference. In the chimera, which has Val and Leu, we found low internal TEA sensitivity. When we individually revert these residues back to Kv 2.1, we find no change in internal TEA block. When we revert both of them simultaneously to Kv2.1 we restore the internal TEA sensitivity. We think that these residues are involved in specifying internal TEA binding, but we don't think they are the only residues involved.

The sequence of the wild-type Kv1.6 channel has Val in positions 374 and 369; and it also has high internal TEA sensitivity. This seems contradictory, because when we make the chimera L374V mutation, which is the same at 369 and 374 as Kv1.6 we get a different TEA sensitivity. I think their are two explanations. One reason is that we have not identified all of the determinants of the internal TEA binding site. This site is located in the internal mouth of the channel, whereas the amino acid segment we are dealing with contains mostly the external mouth, the central part, and a small part of the internal mouth. Apart from positions 374 and 369 the internal mouths of CHM and Kv2.1 are identical, whereas that of Kv1.6 may be quite different. A second reason is that we also have to consider that TEA is a open channel blocker, so its access to the binding site will depend on the gating kinetics. Mutations which decrease probability of opening will reduce TEA block. The chimera mutant L374V has low open probability and very short open times.

GUY: You are focusing only on residues that are naturally mutated in the sequence, and not working on the residues that never change normally. The potential problem is that you might be concentrating on the functionally less important residues. The fact that your mutations have effects does not necessarily mean that these are the most important residues.

KIRSCH: I agree, but wild-type K channels differ in their K/Rb conductances and position 374 may be important in specifying this difference. It is clear that we have to mutate highly conserved residues. It is also true that many of these mutants will yield nonexpressing channels. The problem is that the channel consists of four identical subunits, so every time we introduce a mutation, we are replicating it four times in the channel. There are two strategies to avoid this problem: the most direct one is to attach the subunits together with artificial linkers and make mutations in only one subunit. Alternatively, we could try rescue experiments, coexpressing wild-type and mutant subunits, and then try to distinguish the different hybrids at the single channel level.

DAVID BUSATH: Changing L374 to Val causes an increase in Rb conductance and decrease in K conductance, with little change in the selectivity ratio of Rb and K measured from bi-ionic reversal potentials. For a single ion channel, this would suggest that the affinity of a low energy site in the permeation path has increased for Rb and decreased for K. However, it is hard to imagine a binding site lined by hydrophobic side chains. (The data of Villarroel and Sakmann present the same dilemma, but at least with their data, you can invoke the Thr hydroxyl dipoles as possible cation "binders" in the wild-type). What

kinds of mechanisms could there be for your results? Are there functional groups in the neighborhood of L374 that could bind cations?

KIRSCH: When we change Leu into Val we get decreased K conductance. Suppose we are changing the binding site, so that K binds more tightly and Rb less tightly, this would be accomplished by increasing the field strength of the binding site. Rb, which is a larger ion, does not interact as strongly with the binding site and has a higher conductance. If this were the case, we would predict that CS block would be less effective, and that is what we have found. External Cs block of inward K current in the chimera is strong compared with Kv2.1; the IC50 is about seven times lower in the chimera. Therefore, we can use Cs as a probe for localizing the binding site. Cs block is strongly voltage-dependent, increasing steeply at more negative potentials, indicating a binding site deep in the electrical field of the membrane. The only pore mutation that affects Cs block is L374V which reduced Cs affinity ~20-fold. However, for Rb currents, Cs block is reduced only ~3-fold, suggesting that Rb competes less effectively than K at the binding site controlled by position 374.

BUSATH: You mean this a weak binding site for Rb?

KIRSCH: Yes, it is a weaker binding site for Rb than for K when Val is present. The question is why should repacing Leu and Val increase the field strength of a binding site. When we made a number of different side chain substitutions at position 374 we could not see a consistent pattern of changes in K and Rb conductance. For large positively or negatively charged or large aromatic rings, we do not get functional channels. The largest side chain that allows a functional channel is Leu, which in fact has the largest K conductance, whereas the Val gives the lowest K conductance, suggesting a reciprocal relationship between side chain size and conductance. The problem is that Ile has a similar size to that of Leu, but its conductance is close to that of Val. So size does not seem to account for these differences. It might have something to do with the geometry of these residues.

OLAF ANDERSEN: Have you determined the selectivity of Q382K?

KIRSCH: That is a mutation on the external mouth of the pore. We have not concentrated much on this because its effect on external TEA block and current-voltage rectification were consistent with the localized changes in electric field and cation concentrations that might be expected when you place a positively charged residue in the external mouth of the pore.

ANDERSEN: Generally it is of interest that Q382K can either increase (Chimera L374V + Q382K) or decrease (Chimera L374V + V369I + Q382K) the Rb over K conductance. Doesn't that mean that we should be willing to abandon the notation of a localized selectivity filter?

KIRSCH: Yes, our results are consistent with selectivity being distributed among several sites within the pore. Thus, the L374V mutation altered Rb^+/K^+ selectivity without affecting Na^+/K^+ selectivity. The other point is, how do we know that our results are not simply an artifact arising from multiple occupancy of interacting sites?

ANDERSEN: I would not call it an artifact in any case. Multiple occupancy might be the mechanism by which the G_{Rb^+}/G_{K^+} ratio is modulated.

KIRSCH: That is not exactly the case. Our data were obtained under two different conditions, in one case we had Na^+, which is an impermeant substitute on the outside and K^+ or Rb^+ on the inside; in the other case either K^+ or Rb^+ was present on both sides. Now we compare the conductances that we get in those conditions and this would correspond to filling or not filling an external site with a permeant ion. You can see that we have multi-ion effects, because the outward conductance for K^+ currents that were measured with Na^+ outside was always less than the outward K^+ conductance with K^+ outside. This can be explained if the dwell time of ions in the pore is reduced by multiple occupancy. But this effect does not explain the mutation-induced change in the G_{Rb^+}/G_{K^+} ratios because external Rb^+ can substitute for K^+ in boosting outward conductance so that the conductance ratios are the same in the presence or absence of multi-ion effects. This holds true for both Chimera and L374V; the conductance ratios are not effected by occupancy of an external binding site.

ANDERSEN: Returning to the question of the binding side, Leu vs Val and Ile. There is a major difference between Leu and the other two side chains. The former branches at the gamma carbon while the latter two have beta branches. This is reflected in different channel dynamics, that can be interpreted to reflect different side chain-backbone interactions. The backbone would be expected to be more flexible in the case of the gamma-branched amino acids and changes in backbone flexibility will alter the effective field of the carbonyl group with respect to solvating the permeating ions.

KIRSCH: Yes, we agree. We do not think that the side chain of L374 is a binding site, but rather it could influence a nearby binding site by distorting the packing of the chains. In fact there is a conserved Gly in 375. Since Gly does not have a side chain it may form a cavity and that would favor cation binding to nearby carbonyl oxygens.

ROGER KOEPPE: With regard to the backbone, your data at 374 and 369 suggest that fluctuations of the peptide backbone will be important. I want to point out that fluctuations in the backbone are an important feature from dynamic modeling of the gramicidin channel. Are these backbone effects occurring within one subunit or between different subunits that cross-talk? Have you done any experiments that can support any of these hypotheses?

KIRSCH: Yes, we have. We think there is a functional interaction between 369 and 374, because the single mutations L374V or V369I have similar effects on gating; both mutations shorten channel open times from ~40 to 5 ms. However, when the two mutations are made simultaneously, the mean open time increases to ~15 ms (the same as in Kv2.1). This suggests a functional interaction, but it does not distinguish intra- versus intersubunit interactions. However, when we performed coinjection experiments with subunits of L374V plus subunits with V369I we were surprised to find that in addition to homotetramers (distinguished by their conductances) that had short open times, all of the heterotetramers had long open times, indicative of intersubunit interactions.

BUSATH: How well does Rb displace Cs in the Leu version of chimera?

KIRSCH: Much better than K^+. In the chimera the blocking potency of Cs^+ is reduced by a factor of three for Rb^+ current compared with K^+ current.

BUSATH: So all your data can be rationalized in terms of a single binding site that can be mutated to reverse its selectivity for Rb^+ versus K^+. The site has a higher affinity for Rb^+ when Leu is present and higher affinity for K^+ when valine is present.

KIRSCH: Yes, that is our conclusion. From the voltage dependence of the Cs block we estimate that the site lies 80% of the way through the membrane electric field from the extracellular side.