Choline and acetylcholine: what a difference an acetate makes!

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Choline is a water soluble B-group vitamin, which humans must consume through their diet to remain healthy. Meat, eggs and yeast extract are great sources of choline, an essential component of cell membranes and also the precursor of the neurotransmitter, acetylcholine (ACh). College students are traditionally introduced to synaptic physiology by study of the neuromuscular junction: a synapse where ACh is the neurotransmitter. One of the beauties of biology is quickly appreciated: the 'signal', (the ACh) is terminated by the enzyme ACh esterase which hydrolyses ACh to choline and acetate. The beauty of this system is partly efficiency, because the choline and the acetate are rapidly (within seconds) recycled. Further thought raises a question about this piece of Physiology which Bruhova and Auerbach explore with deep understanding: if ACh activates the receptor, why does not choline? The two are chemically similar and since ACh transiently reaches a concentration of over a mM in the synaptic cleft, then the choline concentration, will transiently be high too. In fact choline *can* activate the receptor, but weakly and it binds with much lower affinity.

So why are the interactions between the receptor and ACh or choline different? Insight into this question could be gained from study of crystal structures e.g. of the snail ACh binding protein (Celie *et al*., 2004) with/without ligand bound. However, crystal structures are static, showing a frozen picture of the receptor, and give no information about the speed of receptor activation, receptor open probability, or the energetics of the protein conformational changes. These functional measurements are key to understanding the physiology. Bruhova and Auerbach answer this question using a combination of sophisticated molecular tools and clever analysis of single-channel patch-clamp electrophysiological recordings. Single channel recordings, almost uniquely in biology, allow the observation, in real time, of the activity of a single protein molecule. This exquisite resolution provides the means to infer the affinity (binding free energy) of the receptor (with channel open or closed) for ACh or choline. Binding of ACh to the receptor and channel opening can be described by the mechanism:

$$A + R \underset{k_{-1}}{\overset{2k_{+1}}{\rightleftharpoons}} AR + A \underset{2k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} A_2R \underset{\alpha}{\overset{\beta}{\rightleftharpoons}} A_2R^* \qquad (1)$$

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where A represents ACh, R the receptor, and A_2R^* is the open channel conformation of the doubly liganded receptor. In adult mouse acetylcholine receptors (AChRs) the two transmitter binding sites are approximately equivalent and independent (Nayak et al., 2016) and so the agonist dissociation equilibrium constant, $K_D = k_{-1}/k_{+1} = k_{-2}/k_{+2}$. However, this mechanism (1) is not the full story. One of the keys to the Bruhova and Auerbach analysis is the observation by Meyer Jackson of rare, unliganded AChR channel openings (Jackson, 1984). These happen, in the absence of ACh, when a receptor (R) spontaneously flips into the open channel configuration (R*). Although the importance of these was largely overlooked at the time, thermodynamic principles tell us that these events must happen (Monod *et al.,* 1965; Karlin, 1967). The value of this observation has been elegantly developed in recent years by Auerbach and colleagues to give a more complete understanding of the energetics of AChR activation. In wild-type mouse muscle AChR, unliganded openings are extremely rare (happening about once every 15 minutes!). Bruhova and Auerbach use receptors with previously characterised combinations of 'background mutations' that raise the probability of unliganded openings to measurable levels. These mutations do not affect the binding site (Nayak et al., 2012; 2016) and so they allow the effects of a binding site mutation to be measured.

To understand the difference between ACh and choline binding, the channel gating constants, $E = \beta/\alpha$, (E_0, E_1, E_2 for unliganded, monoliganded and doubly liganded receptors) were estimated from the channel open probability, P_o ($E = P_o/(1- P_o)$) in absence of agonist and at very high agonist concentrations. K_D is calculated from E_0 and E_2 using a convenient shortcut: $K_D = (E_2/ E_0)^{0.5}$. For example, $E_{2(ACh)} = 25$ and $E_{0(WT)} = 7.4\text{x}10^{-7}$, so $K_D = 172$ μM, and the agonist binding energy is $0.59\ln K_D$ = -5.11 kcal/mol . For choline $E_{2(Cho)} = 0.046$ and $E_{0(WT)} = 7.4\text{x}10^{-7}$, so the binding energy = -3.25 kcal/mol (K_D= 4 mM) and the difference in binding energy between ACh and choline of 1.86 kcal/mol corresponds to a 23-fold difference in affinity. Using energy changes as a way to describe agonist affinity is not common in Physiology, but as Bruhova and Auerbach point out, is no more complicated than using a Richter scale to describe earthquakes!

Bruhova and Auerbach use previously characterised mutations at the alpha subunit transmitter-binding site to explore the contribution of individual amino acid side chains to the overall binding energy of ACh and choline. Focussing on the aromatic rings of αY190, αW149 and αY198, each provides ~50% less binding energy for Cho compared to ACh. Considering the analogy of a weather vane on a clock tower, ACh is predicted (based on the structure of the snail ACh binding protein) to orientate itself in a North-Easterly direction (consider 'North' as pointing towards the extracellular space) with the acetyl part of ACh forming an H-bond that helps to optimally orientate the agonist. The mutation analysis of the binding site predicts the quaternary ammonium interacts preferentially with the aromatic rings of αY190, αW149 and αY198, with αY190 being deprotonated by K145. In contrast, because choline lacks the acetate moiety, it's optimal position is in a Westerly orientation with its OH group able to H-bond with αW149 and the quaternary ammonium group interacting only weakly with αY190 and αY198. So the difference between ACh and choline turns out to be determined by the optimal position of the ligand in the binding pocket and is not just a matter of which way the wind is blowing when the agonist binds.

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