Developing an Assay to Determine the Signaling Pathway of GPCRs

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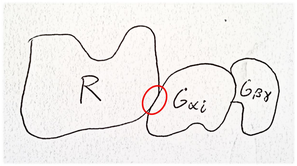
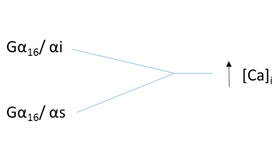
Abstract

The G alpha 16 subunit is from the Gq protein family, but has been shown to be promiscuous (it can sometimes bind to Gi or Gs signaling receptors as well). Due to their promiscuity and intrinsic Gq behavior, they have been used to engineer chimeras that couple to specific receptors. By replacing varying lengths of their c-terminal ends with those of other G proteins' which couple to other pathways (e.g. Gi or Gs), chimeras can be constructed for further experimentation [1]. In this project the c-terminus 11-aa of human G alpha 16 was replaced with those of other proteins to construct G alpha 16 chimeras. These G alpha 16 chimeras can be particularly useful as they can couple to a receptor that couples to Gi or Gs for example while at the same time exhibiting Gq behavior (G alpha q signals PLC → hydrolysis of PIP2 to DAG and IP3 → IP3 mediated release of calcium from the endoplasmic reticulum → activates calcium-activated chloride channels). The activation of these chloride channels can be qualitatively seen by the "chloride spikes" which are ordinarily detected in Gq signaling using the Two-Electrode Voltage Clamp (TEVC). The hydrolysis of PIP2 from the membrane also results in channel current inhibition. This current inhibition can also be seen on channel recordings using the TEVC.

As previously mentioned, Gq signaling results in the increase of intracellular calcium after its release from the endoplasmic reticulum. This increase of intracellular calcium can also be measured in cells. Using calcium dyes, and a potentially working chimera we now have the tools to develop a high throughput assay in cells. This high throughput assay could be very beneficial as a system to screen receptors and drugs.

Background

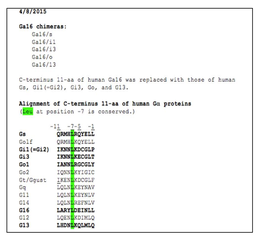




G protein-coupled receptors (GPCRs) have seven transmembrane helices, a C terminus located on the cytoplasmic side, and an N terminus on the extracellular side. GPCRs are involved in initiating many different cellular transduction mechanisms in our cells, and many present day drugs on the market (~50%) target GPCRs [3].

There is a critical interface between the receptor and the alpha subunit of the G protein. There are defined regions on G alpha subunit that interact with the receptor which couples to these proteins. Varying lengths of the c-terminal end of the alpha subunit are important in this interface.

Given powerful Ca^{2+}-binding fluorescent probes it is easier to measure $G\alpha_{16}$ activity by analyzing PLC stimulation than studying Gi adenylyl cyclase inhibition. This is one of the reasons why the use of G alpha 16 proteins is beneficial [2].



Methods

***Xenopus* oocytes injected with cRNAs -** the oocytes are injected with different cRNAs dependent on the experiment. After approximately two days, the cRNA's have translated into protein and are at optimal expression levels (depending on the cRNA's injected).

Two-Electrode Voltage Clamp (TEVC) used for ion channel recording – The GeneClamp500 amplifier (Axon Instruments) is used to perform the TEVC recordings. The voltage protocol used is a voltage ramp from -80 mV to + 80 mV **[3]** .

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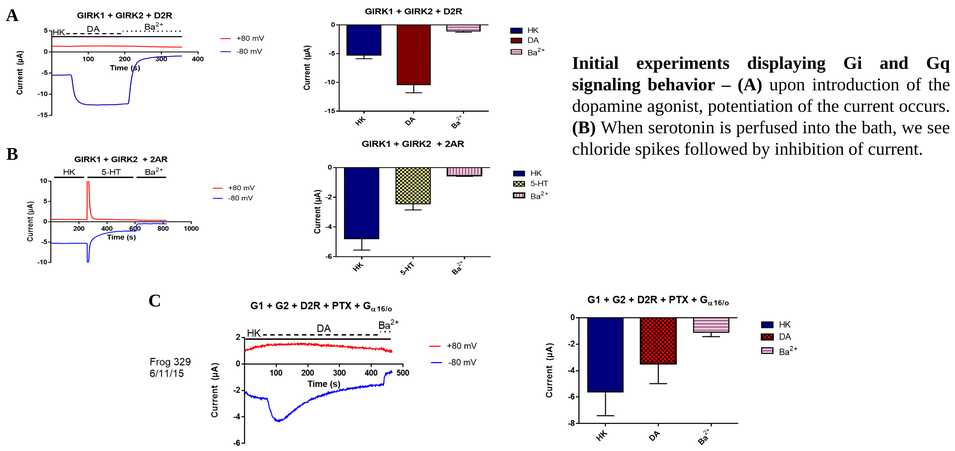
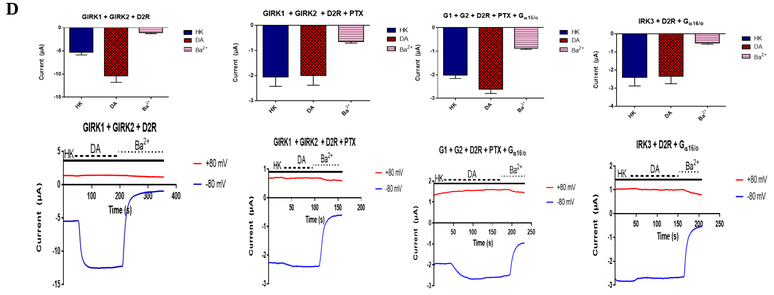
Results

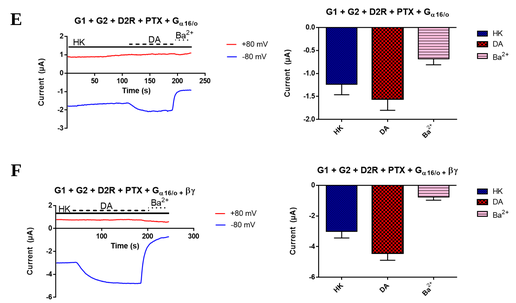


Initial experiments displaying Gi and Gq signaling behavior – (A) upon introduction of the dopamine agonist, potentiation of the current occurs. **(B)** When serotonin is perfused into the bath, we see chloride spikes followed by inhibition of current.

First experiments using the $G_{\alpha16/o}$ chimera – Before running this experiment our initial hypothesis was to see Gq behavior (chloride spikes followed by inhibition of current) due to the chimera. **(C)** The chloride spikes did not occur, instead we saw potentiation (presumably Gβγ-induced) followed by steady inhibition of the current (inhibition did not always occur). PTX was used to block Gi signaling.



Experiments with G protein insensitive channel IRK3 – (D) Recordings from this experiment provide us with an example of the PTX Gi signaling block, potentiation of current from the chimera (no inhibition), and no significant effect upon using the G protein insensitive channel IRK3.



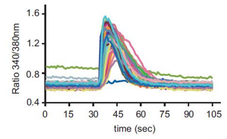
Forming a heterotrimer to keep the chimera initially inactive – (E) shows another $G_{\alpha16}$ recording this time with no inhibition. **(F)** The heterotrimer group displays gradual potentiation upon agonist introduction. The heterotrimer group showed larger potentiation, most likely due to the greater amount of βγ further activating the channel.

Future Directions

G alpha 16 wt - The next experiment with oocytes will be using the G alpha 16 wt and expressing it with GIRK 1/2 and the muscarinic M2 or the β2 adrenergic receptors that have been reported to couple to $G\alpha_{16}$ [4]. Upon introduction of the agonists (ACh or Iso) we hope to see a Gq response (Chloride spikes followed by inhibition of current). This experiment will serve as a positive control for the $G\alpha_{16}$ in the *Xenopus* oocyte system.

After analyzing the results from the G alpha 16 wt experiments, two major possibilities exist:
1 – the wild type works, in which case the problem lies within the chimera.
2 – the wild type does not work, in which case a different G protein or chimera may have to be used.

High throughput Assay in Cells - If eventually we obtain results showing Gq behavior from a working chimera that can couple Gi, Gs, and Gq signaling receptors, then it would be convincing to see this Gq response in cells using calcium dyes (e.g. Fura-2) to see rises in intracellular calcium.



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Conclusion

The first results using the $G_{\alpha16/o}$ chimera did not provide us with desirable results of the chimeras function. Although we did see inhibition of current in some recordings, we did not see any chloride spikes. One hypothesis made from these outcomes was that the chimera induced too much basal Gq activity resulting in prior PLC stimulation and PIP2 hydrolysis before agonist introduction. This may have stifled the agonist induced PIP2 hydrolysis potentially causing insufficient IP3 mediated calcium release from the endoplasmic reticulum. Two ideas stemmed from this result, either using a G protein insensitive channel (ex: IRK3) or injecting βγ to form a heterotrimer (αβγ) preventing basal Gq activity. Neither experiments proved promising. This resulted in the questioning of the chimeras function, leading us to our next plan of testing the wild type $G_{\alpha16}$ to see whether or not it can provide us with a legitimate Gq response. After obtaining the wild type results we can hopefully find where the problem lies and move on. Either way, the chimera will most likely require reengineering to render it full suitable to initiate a confident Gq response. After seeing such response, we are optimistic that this system will function properly in cells as a high throughput assay that can identify the signaling pathway of receptors and screen drugs.

References

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[2] "G Protein Chimera User Manual." *OpenWetWare RSS.* N.p., n.d. Web. 19 July 2015.
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