Characterizing the G-protein activities of the membrane Androgen Receptor, ZIP9

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Androgens mediate their physiological functions via the classical nuclear receptor, AR. However, recent studies have identified a new plasma membrane (and non-genomic) androgen receptor- the Zn(II) channel, ZIP9. ZIP9 is ubiquitously expressed and localised to gonadal tissue. Recent studies have suggested the role of zinc transporters in ovarian follicle growth, with several ZRT- and Irt-like Protein (ZIP) family members shown to be expressed in a stage-specific manner during follicle development, highlighted potential physiological importance of ZIP9 in modulating ovarian functions. The binding of androgens to ZIP9 has been proposed to activate "non-classical" testosterone signalling pathways, via coupling to G proteins. However, how androgens and zinc interplay to modulate G protein activation remains unclear. This study therefore aimed to determine how androgens and zinc regulate ZIP9-dependent G protein coupling. To determine the effect of ZIP9 and its interaction with ligands and G proteins, HEK293 cells over-expressing ZIP9 were utilised to assess ZIP9-dependent Gs and Gi coupling via assessment of cAMP production using a live kinetic cAMP reporter- GloSensor. To assess Gi coupling, HEK293 cells expressing ZIP9 were pre-treated with 10 μM forskolin for 10 minutes to elevate cAMP prior to ligand treatment, whilst Gs coupling was detected with the addition of ligand alone. Cells were stimulated with 100 nM testosterone alone, 20 μM zinc alone or the combination treatment of testosterone and zinc and cAMP production was monitored for up to 60 minutes. Experiments were conducted with a minimum of n=3 in triplicate measurements. Treatment of HEK293 cells expressing ZIP9 with either testosterone or zinc alone, or in combination had no effect on cAMP production, suggesting that ZIP9 ligands didn't include coupling of ZIP9 to Gs. To rule out that transiently transfected ZIP9 was expressed at a protein level with the plasmid concentrations transfected, Western blot analysis of transfected cell lysates was carried out and showed that increasing Zip9 plasmid concentration increased the protein expression of ZIP9, supporting that ZIP9 was expressed and didn't couple of Gs. Next, we investigated if ZIP9 was coupling to Gi. Interestingly, in forskolin pre-treated conditions basal cAMP was modulated by the amount of ZIP9 plasmid expressed, with increasing inhibition of forskolin-induced cAMP production corresponding with increasing ZIP9 expression. This suggests that ZIP9 constitutively couples to Gi. Next, ligand treatment was assessed. At a high Zip9 expression, treatment with either testosterone, zinc or a combination of both showed no significant change in cAMP production. Interestingly, at low ZIP9 expression, single treatments with zinc or testosterone abrogated basal ZIP9-Gi coupling. However, combined testosterone and zinc treatment enhanced cAMP production, suggesting a potential switch to Gs coupling, dependent on Zip9 expression level and ligand. To understand the potential physiological implications of this dual ZIP9-Gs/Gi coupling, isolated mouse granulosa cells were treated with either 20 μM of zinc and 100 nM of testosterone, or co-treatment 24-hour exposure on Zip9 expression. Whilst single treatments of either testosterone or zinc alone did not affect Zip9 expression, co-treatment downregulated Zip9 expression. The G protein coupling of ZIP9 in granulosa cells is currently underway to explore the mechanism underpinning this. These data indicate that ZIP9 displays dual Gs/Gi coupling that is dependent on its expression level and ligand concentrations. This may have important implications in the regulation of reproductive disorders with high androgens including PCOS, with the ovarian roles important next steps to explore.