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Role of Protein Kinase C in Melanocortin-3 Receptor Endocytosis

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G protein-coupled receptors (GPCRs) are a superfamily of cell-surface proteins that are characterized by seven transmembrane alpha helices and that function through trimeric G proteins [1]. The molecular functioning of GPCRs is determined in part through selective activation of specific trimeric G proteins that consist of alpha and beta-gamma subunits [2]. The alpha subunits activate the canonical signaling pathways as follows: $G_i\alpha$ which is inhibitory to adenylyl cyclase; $G_s\alpha$ that activates the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA) pathway; $G_q\alpha$, which is linked to the phospholipase C-beta/protein kinase C pathway (PLC/PKC); $G_{12/13}\alpha$, which activates small G-proteins [2]. This diversity is overlayed by another complexity arising from a multitude of subfamilies of the G proteins in the individual families [2]. As a consequence of activation, GPCRs are desensitized through phosphorylation accompanied by endocytosis [3]. Upon endocytosis, the receptor can be recycled back to the membrane or degraded [4]. In effect, the trafficking of receptors is an interesting cell biology problem that is amenable to study with fluorescence microscopy.

Melanocortin receptors (MCRs) belong to the rhodopsin family of GPCRS and are highly conserved in vertebrates, reflecting their important physiological functions of regulating adrenal gland development and function (MC2R), melanocyte function (MC1R), central nervous system control of energy homeostasis and cardiovascular function (MC3R and MC4R), and regulation of metabolism in peripheral tissues and glandular function (MC5R) [5]. Whereas all MCRs couple to $G_s\alpha$, they have additionally been shown to regulate other pathways, perhaps in a context-specific manner. Furthermore, they exhibit varying tissue distribution patterns that do not entirely mirror their well-established functions [6]. MC3R is intricately involved in regulating energy metabolism through the hypothalamus, yet it is expressed in the cerebral cortex, hippocampus, retina, whole eye, pituitary gland, and bone marrow [6]. This distribution suggests other undiscovered functions of MC3R.

In addition to characterizing tissue distribution patterns through molecular and histochemical techniques, the molecular functioning of MC3R and other MCRs has been studied by different research groups [5]. Current understanding confirms the initial reports that all MCRs stimulate cAMP production but that signaling to other pathways, such the extracellular kinase (ERK) and PLC/PKC pathways has been reported [5]. GPCR signaling to noncanonical pathways is attributed to receptor phosphorylation and interactions with β-arrestins that occur concomitantly with endocytosis [4]. It is also known that different ligands can activate alternative signaling pathways through the same receptor and this biased signaling has been attributed to differential ligand-mediated allosteric effects. This is has led to a search for molecules that activate alternative pathways as way to minimize side effects. Receptor desensitization is another factor requiring better clarification given that it can lead to sequestration in recycling endosomes or degradation through lysosomes for unclear reasons [7].

The goal was to investigate the role of specific downstream kinases on MC3R endocytosis.

A CAD neuronal cell line expressing rat MC3R tagged with green fluorescent protein (GFP) has been previously described [8]. The cells were counterstained with DRAQ5. Kinase inhibitors targeting PI3K (wortmannin), protein kinase B (PKB) (triciribine) and protein kinase C (PKC) (bisindolylmaleimide 1, BIM-1), were used to interrogate the cellular distribution of MC3R. Kinase inhibitors were purchased from commercial suppliers and solubilized as recommended. All compounds were used at a final concentration of 1 µM. Images were collected with a Leica Stellaris 5 confocal microscope system.

Motif analysis with Expasy Prosite [9] revealed the presence of PKC phosphorylation motifs in the second intracellular loop and in the carboxyl-terminal helix of MC3R. G protein coupling preferences were predicted with the program PRECOG [10]. MC3R shows a high probability of coupling to $G_q\alpha$, ($G_{14}\alpha$), which is upstream of the PLC/PKC pathway (Table 1). The cell imaging data show, as expected, membrane localization of MC3R and nuclear localization of DRAQ5 (Figure 1A). The cellular distribution of MC3R was unaffected by treatment with wortmannin and triciribine (not shown). Conversely, treatment with the BIM-1 leads to an enhanced internalization and depletion of MC3R (Figures 1B and C).

BIMs are synthetic inhibitors of PKC that are designed based on staurosporine structure, which is an antibiotic isolated from *Streptomyces staurosporeus* [11]. They have been used to localize PKC in cells as conjugates of fluorescein [12]. The results presented in this paper show the depletion of MC3R in BIM-1 treated cells, suggesting enhanced degradation. While GPCRs activate intracellular Ser/Thr kinases, they are in turn regulated by specific members of this family of kinases, including GPCR kinases (GRKs) and PKC [13]. This leads to receptor desensitization through internalization and lysosomal degradation or intracellular sequestration. The presence of PKC phosphorylation motifs in MC3R intracellular regions in conjunction with G protein coupling prediction data suggest a feedback inhibition loop whereby activation of the PLC/PKC pathway by $G_q\alpha$ is followed by MC3R phosphorylation. This is consistent with previous studies demonstrating the colocalization of PKC and MC3R in CAD cells [8]. However, the BIM-1 treatment data does not support this interpretation. Still, others have reported that PKC activation diverts endocytosed ErbB3 from the degradative pathway leading to intracellular accumulation while PKC inhibition promotes degradation [14]. Thus, further work is needed to delineate the apparent functional interaction between MC3R and PKC.

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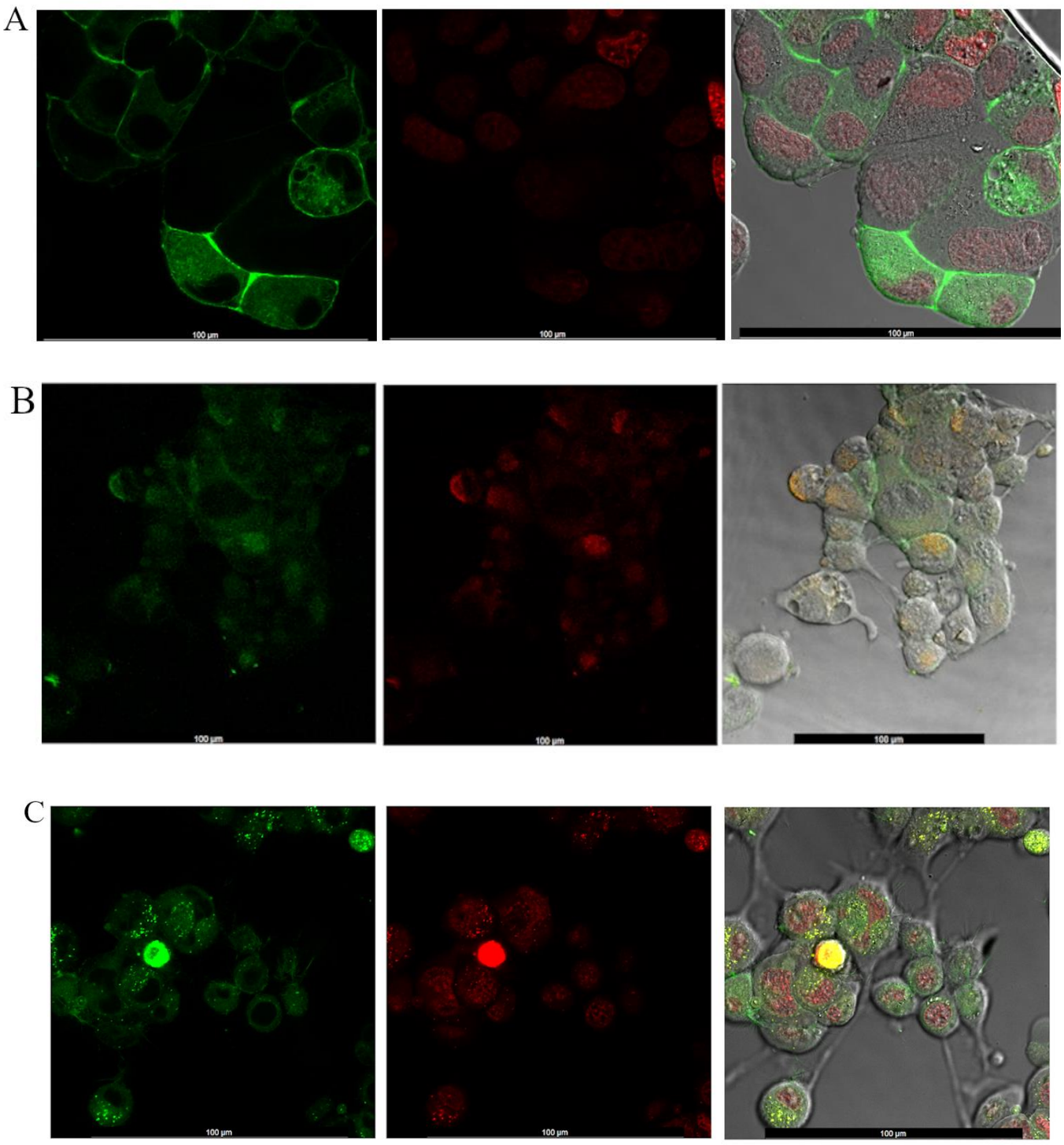

Figure 1. PKC inhibitor modifies the distribution of MC3R in CAD cells. (A) MC3R-expressing cells were counter-stained with DRAQ5 (nuclear stain), (B) the cells were treated with BIM-1, (C) the cells were treated with BIM-1 and stained with DRAQ5.

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Table 1. Prediction of MC3R coupling to G proteins. The probability of coupling is shown, with a probability of ≥ 0.7 shown in green. The table shows that a value higher than 0.4 predicted coupling.

MC3R	GNAI3	GNAI1	GNAZ	GNAO1	GNA12	GNA13	GNAQ	GNA14	GNA15	GNAS	GNAL
Rat	**0.607**	**0.36**	**0.594**	**0.342**	**0.645**	**0.726**	**0.872**	**1**	**0.004**	**0.233**	**0.105**
Fish	**0.6**	**0.258**	**0.405**	**0.171**	**0.797**	**0.427**	**0.252**	**1**	**0.771**	**0.775**	**0.537**
Human	**0.591**	**0.375**	**0.594**	**0.333**	**0.6**	**0.799**	**0.84**	**1**	**0.004**	**0.329**	**0.69**

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