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ROLE OF m1 RECEPTOR-G PROTEIN COUPLING IN CELL PROLIFERATION IN THE PROSTATE

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Summary

The prostate gland from several animal species contains variable levels of muscarinic subtypes, but only the human prostate expresses significant levels of the m1 subtype. We studied muscarinic receptor activity in human benign prostatic hypertrophy (BPH) as well as several cell lines derived from prostate cancer. The BPH we studied expresses approximately 75% of the m1 receptor and undetectable levels of the other receptor subtypes whereas PC3 cells express only the m3 receptor subtype. DU145 and LnCaP cells express approximately equal levels of m1 and m3 receptor subtypes. Only the PC3 cells responded to carbachol with an increase in turnover of polyphosphoinositides, and none of the cell lines responded with effects on cAMP metabolism. Co-precipitation of receptors with heterotrimeric guanine nucleotide-binding regulatory proteins demonstrated interactions of the m1 receptors with Gi, Gq and G16 in BPH tissue and of the m1 and m3 receptors with Gi, Gq and G12 in PC3 and DU145 cells. Mitogen activated protein kinase (ERK) activity was seen in response to carbachol in PC3 and DU145 but not LnCaP cells. Finally, carbachol promoted cell proliferation in all three cell lines. Thus, there appears to be no consistent relationship between ERK activity, cell proliferation, and the subtype mediating the proliferative response, amongst these prostate cancer cell lines.

Keywords

muscarinic receptor; prostate; G protein coupling; cell proliferation

Hammer and colleagues (1) were the first to use the drug pirenzepine to suggest that muscarinic receptors could be subdivided into at least two categories, then called M1 and M2. The M1 receptor was purified and subsequently cloned from mammalian cerebral cortex, a tissue rich in this pharmacologically defined receptor subtype (2). It is now well established that five receptor subtypes with differing protein sequences are expressed in brain. The m1 receptor protein (as defined by molecular sequence) is abundant in brain, constituting roughly 30–40% of the total muscarinic receptors present in several brain regions, yet is present in a mixture with other receptor subtypes. The pharmacological and biochemical analysis of this receptor subtype has thus focussed on the use of cell lines transfected with plasmids encoding and expressing the cloned m1 receptor DNA. We recently showed, using surgically-acquired samples of human benign prostate hypertrophy (BPH) and using an immunoprecipitation assay to measure expressed protein levels, that BPH expresses significant levels of the m1 receptor protein, constituting almost all of the measurable levels of muscarinic receptor in this gland (3). The role of the cholinergic innervation of this gland has surprisingly not been well-characterized. We therefore chose this tissue as a model in which to study the m1 muscarinic receptor in a prototypical

physiological environment to relate the distribution, biochemistry and ultimately the physiological role of the m1 receptor in regulating prostate function.

Methods

The methods used for immunoprecipitation of receptors and complexes of receptor and guanine nucleotide binding (G) protein have been described (3). The assays for phosphoinositide (PI) and cAMP metabolism were performed using whole-cell assays as described (4). Activity of mitogen activated protein kinase (ERK) was measured using a solid phase immunoassay (5) with myelin basic protein as a substrate for phosphorylation. Jun kinase (JUNK) was measured as described (5). [³H]Thymidine uptake into TCA-precipitable material was determined using conventional methods. Anti-G protein antibodies used in these studies were a generous gift of Dr. David Manning, University of Pennsylvania as described previously (3). PC3 and DU145 cells were a gift from Dr. Chung Lee, Northwestern University, and LNCaP cells were obtained from the ATCC collection.

Results

We have published that muscarinic receptors, measured using [³H]QNB binding, are present in prostate tissues from several species, at levels of 30–50 fmol/mg protein. Levels of the m1–m5 subtypes were different between species, and depended on the anatomical region (e.g., ventral, dorsal, lateral) investigated, which differs across species. To compare animal models to relevant human disease states, we measured levels of muscarinic receptor subtypes in BPH using samples derived from patients following transurethral radical prostatectomy (TURP) procedures (3). We found that in these samples, low to undetectable levels of muscarinic receptor subtypes of the m2–m5 class were found, but that roughly 75–80% of the detectable receptors were of the m1 subtype, based on an immunoprecipitation assay using subtype selective antibodies. We have performed extensive studies to determine that those receptors that are refractory to precipitation are likely proteolysed, and cannot be measured in this assay (unpublished data).

We have since oriented our efforts to find a model system to study prostate function, using cell lines derived from normal and malignant prostate tissues. There are three widely used cell lines in which the cell biology has been extensively studied. The PC3, DU145 and LNCaP cell lines were derived from human prostate cancer cells. Unlike human prostate cancer, the PC3 and DU145 cell lines do not respond to androgen, whereas the LNCaP cells do. All of these cells have changed phenotype with passage, and therefore are not necessarily the ideal model with which to study prostate function and metastasis. However, they have given us systems to develop assays to measure parameters that would likely lead to fruitful examination of prostate function and the transformation into a malignant phenotype.

The PC3 cells express almost exclusively the m3 subtype of receptor. The DU145 and LNCaP cells express roughly equal numbers of m1 and m3 receptors. Both the m1 and m3 receptors have been expressed in cells not normally known to contain these receptors, and have been shown under these conditions to actively promote metabolism of polyphosphoinositides (PI), and not to be active in the metabolism of cAMP. We used a whole-cell assay of PI and cAMP metabolism (4) to investigate this in the prostate-derived cell lines, expressing relatively low levels of receptor that are comparable to those observed *in vivo*. In PC3, but not DU145 or LNCaP cells, carbachol promoted an increase in PI turnover that was reversed by atropine. This effect was insensitive to pertussis toxin, although all pertussis toxin substrates were inactivated. There was no effect of carbachol on cAMP metabolism in any of the cell lines. Therefore, and contrary to our expectations, the

signaling of the m1 and m3 receptors was quite different in these cells, in particular regarding PI turnover.

We have developed an assay to measure interactions of receptors with G proteins. This relies on precipitation of the agonist-activated receptor with anti-receptor antibodies, followed by identification of G proteins that co-precipitate using western blotting. In BPH, this was shown to result in co-precipitation of Gi, Gq/11, and G16 alpha subunits with the m1 muscarinic receptor (3). In PC3 and DU145 cells, the alpha subunit of G16 was not present, and in the presence of carbachol, precipitation of the m1 (DU145) or the m3 (PC3 and DU145) receptors resulted in co-precipitation of Gi, Gq, and G12 alpha subunits (submitted, Mol. Pharmacol). These findings were interesting, considering that expression of dominant positive ("gain of function") alpha subunits of G12 in NIH 3T3 fibroblasts induces cell proliferation and transformation, whereas expression of a dominant positive G16 construct inhibits these same effects. Some data similar to these were presented at the meeting and are reported *vide infra* (Gutkind *et al.*). This led us to pursue the effect of carbachol on two parameters associated with cellular proliferation, namely, the jun (JUNK) and mitogen activated (ERK) kinase pathways.

In PC3 and DU145 cells, basal levels of ERK were elevated by incubation with carbachol, while basal levels of JUNK were unaffected. LNCaP cells do not exhibit either basal or stimulated levels of ERK. In all three cell lines, carbachol caused an increase in cellular proliferation. This occurred over a 2–4 hour time period in PC3 and DU145 cells in the presence, but not the absence of fetal calf serum. This was not inhibited by pertussis toxin under conditions where the pertussis toxin substrates were fully inactivated. These results have been compiled in a submitted manuscript.

We used two cell lines derived from normal human prostate, obtained from the NIH (1532 and 1535), to compare with cancer-derived prostate cells using the above assays. Our results are preliminary, but suggest the following. These cells express low levels of the m1 and m3 subtypes of receptor (30–50 fmol/mg protein), yet neither cell line responds to carbachol with alterations in PI metabolism. We have shown consistently activation by carbachol of JUNK but not ERK in these cells. Cell division was not changed in the presence of carbachol, measured using thymidine uptake.

Discussion

Our data show that the human prostate gland is unlike that of other animal species in that it expresses the m1 muscarinic receptor exclusively in TURP samples, and a mixture of the m1 and m3 receptors in cell lines derived from normal and cancerous tissue. The levels of receptor in all cell types studied are virtually identical, ranging from 30–50 fmol/mg protein. These two receptor subtypes are thought to mediate PI turnover, yet only one cell line of five tested was shown to generate a PI response in the presence of carbachol. We measured this using a whole cell assay in the presence of lithium, and feel it unlikely (although possible) that our assay conditions prohibit measurement of this response. We have confirmed our ability to measure PI turnover in cells expressing cloned receptors (m1 and m3) in addition to the PC3 cells, and it is therefore not clear why this response was not seen in the other cell lines. We are currently using other measures of PI turnover that measure transient metabolite production to clarify this issue. As predicted from work using cells expressing recombinant receptors (6), no cAMP response has been detected in any of the cell lines used for these studies.

Both m1 and m3 receptors were found to co-precipitate with alpha subunits of Gi, Gq/11, and G12 in the cancer-derived cell lines, whereas in the TURP specimens we have published

co-precipitation of the m1 receptor with Gi, Gq/11 and G16 under the same conditions. This is interesting in light of findings using transfected NIH 3T3 cells, where dominant positive G12 constructs potently induced cell proliferation and transformation while similar G16 constructs inhibited cell proliferation (see 7 for review). By nature, the TURP specimens are not amenable to study in the cell proliferation assay, so the relationship of m1 receptor coupling to G16 in this tissue compared to G12 in cancer-derived cells is unclear. Our laboratory is attempting to derive cell lines directly from BPH specimens in primary culture to address some of these issues. We also wish to make the point that almost all previous studies on signaling pathways used by these receptors and G proteins have used cells transfected with cDNAs encoding cloned muscarinic receptor subtypes. By definition, these cells do not normally express muscarinic receptors, nor do they express significant levels of the respective G protein to be studied, and hence are used to reduce background “noise” that would potentially confound any results obtained after transfection with either receptor, G protein, or both. On the other hand, the cellular milieu in which these signal transduction events are studied cannot be considered in any sense normal in terms, particularly, of the expression level of receptor. The studies we present here rely on clonal cell lines, that likewise do not have phenotypes totally characteristic of normal, or for that matter, cancerous, prostate cells. Additionally, transfection with *predicted* GTPase-deficient, “gain of function” G protein mutants has not specifically been shown to render these proteins constitutively active in all reported usages.

While carbachol could induce elevation of ERK activity in PC3 and DU145 cells, though not LNCaP cells, all three cell lines were shown to respond to carbachol by an increase in cell number. In PC3 and DU145 cells (LNCaP were not studied) there was no effect of carbachol on JUNK activity. We point out that we do not measure levels of the respective kinase proteins in these assays, but rather the activity of the protein representing the phosphorylated and hence active form. In the cells derived from normal prostate, JUNK but not ERK activity was elevated in response to carbachol. The cancer-derived PC3, DU145, and LNCaP, and not the normal-derived 1532 and 1535 cell lines respond to carbachol by an increase in cell proliferation. This clouds the issue of the relationship between ERK, JUNK and cell proliferation in response to muscarinic receptor activation, at least in prostate cells in culture. Since all cell lines studied express identical levels of the same muscarinic receptor subtypes (m1 and m3), yet do not respond with the same second messenger response to receptor activation, we are unable at this time to pinpoint, even in a general way, the events that lead from receptor activation, to G protein coupling, and ultimately to cellular proliferation.

In summary, we are presenting what we think is a view of the m1 (and m3) muscarinic receptor proteins that varies greatly with published work regarding second messengers and predicted responses that have used transfected cell lines for these determinations. We have shown that these receptors couple as predicted to PI turnover in one cell line, yet not in another four that express the same level of receptor endogenously. We have shown dramatic effects of muscarinic receptor activation on cell division in cancer-derived cells, that is not seen in cells derived from normal prostate. However, the relationship between receptor activation and those signaling pathways thought to mediate cell division and transformation is, if anything, more complex than would be predicted based on assays using cells over expressing cloned receptors. We hope, through genetic approaches, to begin to unravel this web of nuance as our studies proceed.

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Figure 1. Hypothetical mechanisms for activation of ERK and JUNK through muscarinic receptors: comparison to mitogenic receptor signaling

TKR, tyrosine kinase receptor; STR, seven transmembrane receptor; bFGF, basic fibroblast growth factor, an example of a mitogen; m1, PI-linked muscarinic receptor; m2, cAMP-inhibitory muscarinic receptor; ras, “small” mitogenic G protein; G16, Go, Gq, G12, PLC/PI-linked G proteins; Gi, cAMP-linked G protein. Other abbreviations are described in the text.

TABLE I
Precipitation of complexes of receptor and G protein from prostate cells

Specific anti-muscarinic receptor antibodies were used to precipitate complexes of agonist-bound receptor and associated G protein from BPH, PC3 and DU145 cells. G proteins were identified by western blotting as described (3).

<u>Tissues:</u>	<u>Subtype:</u>	<u>G_i</u>	<u>G_o</u>	<u>G_{q/11}</u>	<u>G₁₂</u>	<u>G₁₃</u>	<u>G₁₆</u>	<u>G_z</u>
BPH	m1	++	--	++	--	--	++	--
PC3	m3	++	--	++	++	++	--	--
DU145	m1	++	--	++	++	++	--	--
	m3	++	--	++	++	++	--	--