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COMMENTARY

ARACHIDONIC ACID AND ITS METABOLITES IN THE REGULATION OF G-PROTEIN GATED K^+ CHANNELS IN ATRIAL MYOCYTES

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In 1985, Pfaffinger *et al.* [1] and Breitwieser and Szabo [2] showed that the muscarinic gated-K^+ channel, $I_{K.ACh}$, in cardiac myocytes was dependent on a pertussis toxin-sensitive G protein. This work was followed by an elegant paper which clearly demonstrated the GTP dependence of gating at the single-channel level [3]. Soon thereafter, our groups (Clapham and Neer), then at Harvard Medical School, and Brown and Birnbaumer's groups at Baylor College of Medicine, began using the inside-out patch as an assay for the ability of purified G protein subunits to activate the K^+ channel. In this commentary I will present current theories on the mechanisms of signal transduction in this system.

G protein linked receptors comprise a class of proteins with analogous structure and sequence. In particular, all receptors in this family are theoretically made up of seven transmembrane spanning segments. The G proteins in these systems are heterotrimers consisting of α, β, and γ subunits. In the inactive form, the α subunit binds GDP, and is functionally associated with the β and γ subunits. When agonist binds the receptor, GTP replaces GDP on the α subunit. This association changes the affinity of α for $\beta\gamma$ and the subunits split into α-GTP and $\beta\gamma$ subunits. The α subunit, through its intrinsic hydrolytic activity cleaves GTP and returns to the GDP bound state which reassociates with $\beta\gamma$ and the receptor. Current knowledge on effector activation by G protein linked receptors has been based on the rhodopsin/transducin/cGMP phosphodiesterase system in the retina and the β receptor/G_s/adenylyl cyclase system [4–6]. In both of these systems, it is the α subunit of the activated G protein which binds the effector (adenylyl cyclase or cGMP phosphodiesterase) leading to stimulation of the enzyme. The $\beta\gamma$ subunit was thought to serve as an anchor for the α subunits or as a means by which excess α activity could be attenuated. Recent experiments with yeast have clearly demonstrated an effector function for the $\beta\gamma$ subunit [7]. Our experiments on the cardiac channel, $I_{K.ACh}$, have also suggested an effector role for the $\beta\gamma$ subunit in a mammalian system [8]. Since β and γ are not separated except under denaturing conditions, their separate functions are not known; γ may serve simply as a membrane anchor for the β subunit or it may have an independent function.

Molecular cloning has revealed fourteen α, four β, and three γ subunits. There are four splice variants of α_s, the subunit which stimulates adenylyl cyclase; three α_i's ($\alpha_{i1,2,3}$), the subunit which was originally defined as inhibitory for cyclase; two transducins (α_T) which are specific for the retinal system; α_{olf}, which is homologous to α_s and stimulates cyclase in the olfactory system; and several α subunits of unknown function—among these are α_o which is abundant in brain and α_Z which is found in platelets and neural tissue. Four β subunits ($\beta_{1,2,3,4}$) have been sequenced from brain. Of the three γ subunits, one is specific for the retinal system, and one which was found in brain has homology to *ras* (an oncogenic α subunit-like protein [9]). There are at least twelve $\beta\gamma$ combinations and fourteen α subunits to provide specificity of signal transduction pathways for over seventy receptors.

What are the roles of the G protein subunits in signal transduction? α_s and α_T are activators of adenylyl cyclase and cGMP phosphodiesterase respectively. α_i was thought to inhibit cyclase directly; however, since relatively high concentrations of purified α_i were necessary to inhibit cyclase, it was proposed that activation of G_i led to the release of $\beta\gamma$ subunits which bound α_s. This hypothesis presumes that $\beta\gamma$ binds the activated α or facilitates removal of GTP to form α-GDP. Although the role of $\beta\gamma$ as an attenuator of α function is the most commonly accepted view, there is still controversy concerning the mechanism of inhibition of adenylyl cyclase.

Yet another controversial G protein-linked effector system is that of the acetylcholine-activated inwardly rectifying atrial potassium channel, $I_{K.ACh}$. By direct application of GTP to the intracellular surface of inside-out patches of atrial membranes, Kurachi *et al.* [3] demonstrated the GTP-dependence of this channel. Direct application of individual purified subunits yielded contradictory results—our laboratory [8] found that $\beta\gamma$ (0.2 to 1 nM) activated the K^+ channel, whereas Codina *et al.* [10] found that α_{40} (called α_K and α_{i-3}; 1 pM) activated the channel. Later, we found that both α_i and α_o (10 pM) could activate the channel [11]. Since lower concentrations of α activated the channel, Brown, Birnbaumer, and colleagues argued that α was the physiologic activator of the muscarinic receptor. Furthermore, an antibody which blocked the function of frog transducin α blocked muscarinic activation

of $I_{K.ACh}$. Finally, purified recombinant α subunits (nanomolar concentrations) also activated the channel. In summary, current evidence supports a role for channel activation by α subunits although there appears to be no preference among the various pertussis toxin sensitive α subunits. To date there is no convincing evidence for or against *direct* gating by the α subunits.

$\beta\gamma$ *activation of* $I_{K.ACh}$

Is the $\beta\gamma$ activation first described by our laboratory [8] mere artifact? Our preparations of $\beta\gamma$ contained less than 0.01% α subunits (0.1 pM α in 1 nM $\beta\gamma$ subunits), so $\beta\gamma$ activation could not be attributed to α contamination. Initial controls showed that boiled $\beta\gamma$ and buffer (including 3-[(3-cholamidopropyl) - dimethylammonio - 1 - propanesulfonate (CHAPS), the detergent used to suspend the hydrophobic $\beta\gamma$) failed to activate the channel. In contrast, Kirsch *et al.* [12] reported that CHAPS alone activated $I_{K.ACh}$. This report is puzzling since experiments by both Kurachi's ($N > 35$) [13] and our ($N > 50$) laboratory have failed to reveal activation by CHAPS even at concentrations which are three times higher (552 μM) than those used to suspend the $\beta\gamma$. A preparation of transducin $\beta\gamma$, a similar protein, also failed to activate the channel even when suspended in CHAPS [11]. Furthermore, preincubation with a 2-fold excess of α_{41}-GDP or a 4-fold excess of α_{39}-GDP prevented $\beta\gamma$-dependent activation, presumably by binding free $\beta\gamma$. Finally, addition of α_{41}-GDP to patches preactivated by $\beta\gamma$ turned off $I_{K.ACh}$, consistent with our α preincubation experiments. We are therefore convinced that $\beta\gamma$ activation is not simply an artifact.

Since application of α-GTPγS followed by $\beta\gamma$ (and vice versa) did not increase channel activity, we concluded that both α and $\beta\gamma$ were activating the same population of channels. Why would the cell use two messengers to modulate one population of channels? An explanation for the puzzle was provided by Kurachi and coworkers [14] who showed that $I_{K.ACh}$ was activated by arachidonic acid. Since Jelsema and Axelrod [15] had shown previously that transducin $\beta\gamma$ activated phospholipase A_2 (PLA_2) in retinal rods, it was logical to test the hypothesis that $\beta\gamma$, acting through PLA_2, generated arachidonic acid and its metabolites which, in turn, activated the channel. Although specific pharmacologic inhibitors of PLA_2 were absent, Bar-Sagi *et al.* [16] had reported an antibody that blocked the generation of lysophospholipids in fibroblasts by inhibiting the function of porcine pancreatic PLA_2. This antibody has since been shown to effectively block PLA_2 in other systems as well.

Block of $\beta\gamma$ (2 nM) activation by the anti-PLA_2, antibody of Bar-Sagi and coworkers provided the strongest evidence for $\beta\gamma$ activation of $I_{K.ACh}$ via PLA_2 [17]. Boiled antibody and control preimmune serum did not block $\beta\gamma$-induced $I_{K.ACh}$ activation. We then tested various metabolites of arachidonic acid [17]. Indomethacin, a specific inhibitor of cyclooxygenase, had no effect. Nordihydroguaretic acid (NDGA), a lipoxygenase inhibitor, blocked $\beta\gamma$-dependent channel activation. The 5-lipoxygenase inhibitors (including baicalein and AA861 [14]) blocked arachidonic acid activation. 5-Hydroperoxyeicosatetraenoic acid (5-HPETE), 12-hydroxyeicosatetraenoic acid (12-HETE), and leukotrienes (LT) B_4 and C_4 all activated the channel. Kurachi *et al.* [14] found that 5-HPETE and LTA_4 and LTC_4 stimulated channel activity.

To summarize, our conclusion is that $\beta\gamma$ activates PLA_2 to produce arachidonic acid. Arachidonic acid and/or its metabolic products then activate $I_{K.ACh}$. The fact that NDGA and antibody to PLA_2 did not block muscarinic-induced GTP-dependent activation [16] suggests that α may be the mediator of muscarinic-dependent activity while $\beta\gamma$ plays a different role, perhaps linked to another receptor. However, there are several points that require further experiments. First, $\beta\gamma$ produces full activation (50- to 100-fold over basal levels) of the channel, whereas arachidonic acid, 5-HPETE, 12-HETE and leukotrienes produce 10- to 20-fold increases in channel activity. Thus, products of PLA_2 metabolism do not fully reproduce $\beta\gamma$ activation. Perhaps $\beta\gamma$ application generates several short-lived metabolites closer to the site of action.

What is the final mechanism of action by which the lipoxygenase pathway produces activation? There are five possibilities. First, each of the compounds, 5-HPETE, 12-HETE, LTA_4, LTB_4, and LTC_4 may directly activate the channel independent of one another. This seems unlikely given their structural diversity and lack of activation by closely related compounds. Second, these lipophilic compounds may have a nonspecific action in destabilizing the G protein subunits. The freed α subunit would then somehow activate $I_{K.ACh}$. However, even if the α subunit was liberated, no GTP would be available for binding and activation of α. Furthermore, addition of GTP to leukotriene- or arachidonic acid-activated patches did not increase channel activity as would be expected for a destabilization scheme. Third, the receptor, G protein, and channel may form a macromolecular complex which is disturbed by addition of the compounds. Again, this description is flawed by the specificity of action of only certain compounds. A fourth possibility is that the leukotrienes generated cross the patch membrane and bind a leukotriene receptor, finally releasing an active α. The absence of GTP on the intracellular surface of the patch argues against this possibility. Leukotrienes activated $I_{K.ACh}$ even in pertussis toxin treated cells; if a G protein is indeed linked to the leukotriene receptor, it must be pertussis toxin insensitive. Finally, a yet unidentified product may be generated which specifically activates the channel. Sorting out the possibilities will require specific antagonists to leukotrienes and eventually reconstitution of the entire system.

Regardless of the details of the cardiac muscarinic receptor activated K^+ channel, two larger questions are raised by this work. First, does $\beta\gamma$ serve as the activator for PLA_2 in all the systems in which G proteins are thought to interact with PLA_2 (e.g. chemotaxis in neutrophils, mitogenesis in NIH3T3 fibroblasts, exocytosis in mast cells; reviewed by Bourne [18] and Burch [19]). Second, do G proteins produce two "second messengers", one ($\beta\gamma$) which migrates in the membrane to reach very hydrophobic

regions of effectors and a second (α) which interfaces the membrane to more loosely bound or cytoskeletal components of transmembrane proteins? Since *ras* has a region of significant homology to γ, and both *ras* and $\beta\gamma$ activate PLA_2, perhaps the γ subunit is the physiologic stimulator of PLA_2, whether or not γ remains tethered to β. This hypothesis also raises the possibility that other enzymes such as phospholipase C may be activated by $\beta\gamma$. Testing this hypothesis will require more specific methods than simple reconstitution. Deletion of genetic products, either by mutagenesis in yeast or use of antisense techniques in mammalian cells, may provide the necessary specificity to delineate the pathways.

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