Receptor-mediated activation of phospholipase A<sub>2</sub> and arachidonic acid release in signal transduction

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Introduction

Neurotransmitters, hormones, cytokines, growth factors and other ligands transmit their specific messages by binding to receptor molecules on the cell surface. The ligand-bound receptors activate effector systems such as adenylate cyclase, cyclic nucleotide phosphodiesterase, ion channels, and phospholipases via membrane-associated transducing systems to generate second messengers. Receptors activate effector systems via signal transducing elements called GTP-binding proteins (G-proteins) which are heterotrimers composed of α-, β- and γ-subunits. When a receptor binds a ligand, the G-protein disassociates into the α- and βγ-subunits [1]. The dissociation of subunits is caused by the exchange of GDP for GTP which is bound to the α-subunit. Specific α-subunits are associated with the inhibition of adenylate cyclase and activation of cyclic GMP phosphodiesterase, voltage-sensitive ion channels, adenylate cyclase and phospholipase C. The α-subunits have endogenous GTase activity which hydrolyses the bound GTP to GDP, resulting in the reassociation of the α- and βγ-subunits and subsequent termination of the signal [1].

Phospholipase A<sub>2</sub> as an effector enzyme can mediate receptor-induced arachidonic acid release regulated by an unknown G-protein [2]. Phospholipase A<sub>2</sub> hydrolyses arachidonic acid-containing phospholipids, such as phosphatidylcholine, phosphatidylinositol, phosphatidic acid, and phosphatidylethanolamine. The arachidonic acid is further metabolized by cyclo-oxygenase to prostaglandins E<sub>2</sub> and thromboxanes; by lipoxygenase to leukotrienes and hydroxyeicosatetraenoic acids (HETEs), and by epoxygenases to epoxides. Metabolites of arachidonic acid have many important biological actions [3, 4]. Our interest in phospholipase A<sub>2</sub> as a receptor-mediated effector enzyme stemmed from the initial observation that the chemotactic peptide, fMetLeuPhe, liberated free arachidonic acid from neutrophils prelabeled with <sup>14</sup>C-arachidonic acid [5]. A direct association between the amount of arachidonic acid released by fMetLeuPhe and the extent of chemotaxis was also demonstrated [6].

Several pathways exist for the receptor-mediated generation of arachidonic acid. The most direct involves coupling of the receptor protein with phospholipase A<sub>2</sub> activation through a G-protein of unknown character [6]. Indirect routes have been demonstrated involving the release of arachidonic acid from diacylglycerol by diglyceride lipase [7]. Diacylglycerol can be generated from the action of phospholipase C on several phospholipids such as phosphatidylcholine and phosphatidylserine. Alternatively, arachidonic acid can be released from phosphatidic acid by diglyceride lipase. Phosphatidic acid can be produced from the hydrolysis of phospholipids by phospholipase D [8]. In many studies, receptor-mediated release of arachidonic acid was found to be calcium dependent, while phospholipase C and D are partially or insensitive to calcium [9, 10].

Noradrenaline via α<sub>1</sub>-adrenergic receptors liberate arachidonic acid by phospholipase A<sub>2</sub>

Experiments in our laboratory indicated that α<sub>1</sub>-receptor-mediated release of arachidonic acid occurred by the activation of phospholipase A<sub>2</sub>, independent of phosphatidylinositol-specific phospholipase C. An opportunity to examine the role of G-proteins and phospholipase A<sub>2</sub> in the receptor-mediated release of arachidonic acid was made possible by the availability of a rat thyroid cell line, FRTL5, in which thyrotropin (TSH) induced the formation of α<sub>1</sub>-adrenergic receptors [11]. In these cells, noradrenaline stimulated the release of arachidonic acid and inositol phosphates [12], and increased DNA synthesis [13]. The major metabolic product formed from arachidonic acid was prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). When FRTL5 cells were starved of TSH, noradrenaline increased the incorporation of <sup>3</sup>H-thymidine into DNA. It was later determined that PGE<sub>2</sub> induced the eicosanoid-stimulated incorporation of <sup>3</sup>H-thymidine into DNA [13]. This provided an example where stimulation of an adrenergic receptor on the cell surface could induce mitogenesis via a cascade of intracellular signals. Guanosine 5'-[γ-thio]triphosphate (GTP[S]) a nonhydrolysable analogue of GTP which persistently activates G-proteins, stimulated inositol phosphate formation and arachidonic acid release when introduced into permeabilized FRTL5 cells [12]. These findings suggested that arachidonic acid was liberated from a G-protein linked to phospholipase A<sub>2</sub>, or through an alternative pathway involving phospholipase C or phospholipase D as previously mentioned. The addition of pertussis toxin, a compound that inhibits phospholipase C, but not phospholipase A<sub>2</sub>, blocked the stimulation of inositol phosphate formation induced by GTP[S], but not the release of arachidonic acid. These experiments provided evidence that the GTP[S]-stimulated release of arachidonic acid was due to the activation of phospholipase A<sub>2</sub>. Further evidence for G-protein-coupled phospholipase A<sub>2</sub> activation was obtained with the use of pertussis toxin, which prevents the disassociation of the α- and βγ-subunits of the inhibitory G-protein [1]. The toxin inhibited noradrenaline- and GTP[S]-induced release of arachidonic acid in FRTL5 cells, but had no effect on inositol phosphate accumulation. Direct evidence for a receptor-mediated and G-protein-linked activation of phospholipase A<sub>2</sub> was obtained using FRTL5 cell membranes and <sup>3</sup>H-arachidonoylphosphatidylcholine as a substrate [12]. GTP[S] stimulated the release of <sup>3</sup>H-arachidonic acid, and the further addition of noradrenaline increased the release of <sup>3</sup>H-arachidonic acid. From these studies, it appears that the α<sub>1</sub>-adrenergic receptors in FRTL5 cells are linked to two G-proteins, one which activates phospholipase

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A2, and the other which activates phospholipase C. The G-protein associated with phospholipase A2 activation is pertussis toxin sensitive, while phospholipase C is linked to a pertussis toxin-insensitive G-protein. It is not clear whether a single \( \alpha \)-adrenergic receptor is coupled to both phospholipases or if subpopulations of \( \alpha \)-adrenergic receptors exist, each linked to a separate G-protein and effector enzyme.

The role of \( \alpha \)-adrenergic receptor-mediated activation of phospholipase A2 and release of arachidonic acid was also studied in the brain [14]. In primary cultures of spinal cord neurons, noradrenaline stimulated the release of arachidonic acid as well as the turnover of inositol phosphates via \( \alpha \)-adrenergic receptors. Archidonic acid was found to be released from neurons, while inositol phosphates were released from both neurons and glia cells. Treatment of spinal cord cultures with phorbol esters, which activates protein kinase C, inhibited turnover of inositol phosphates, but increased the noradrenaline-stimulated release of arachidonic acid. Pertussis toxin inhibited inositol phosphate release from arachidonic acid in cells, but not inositol phosphates. Blocking the release of intracellular calcium reduced the production of inositol phosphates, but had little effect on the release of arachidonic acid. These findings indicated that occupation of \( \alpha \)-adrenergic receptors activated the release of arachidonic acid from neurons in primary cultures of spinal cord cells, possibly via phospholipase A2 and independent of the activation of phosphatidylinositol-specific phospholipase C. Similarly, \( \alpha \)-adrenergic receptor-mediated release of arachidonic acid via phospholipase A2 was also observed in MDCK kidney cells [15].

**Bradykinin induces the release of arachidonic acid by the activation of phospholipase A2**

The peptide bradykinin induces the release of arachidonic acid and subsequent prostaglandin synthesis, and also increases inositol phosphate formation [16]. It is recognized that bradykinin generates inositol phosphates by activating phospholipase C. To examine whether bradykinin stimulated the release of arachidonic acid, the \( \alpha \)-receptor-mediated activation of phospholipase A2 and not by the phospholipase C diglyceride lipase pathway, Swiss 3T3 fibroblast cells were used. Extracellular calcium was necessary for the release of arachidonic acid, but not inositol phosphates. Blocking the release of intracellular calcium reduced the production of inositol phosphates, but had little effect on the release of arachidonic acid. These findings indicated that occupation of \( \alpha \)-adrenergic receptors activated the release of arachidonic acid from neurons in primary cultures of spinal cord cells, possibly via phospholipase A2 and independent of the activation of phosphatidylinositol-specific phospholipase C. Similarly, \( \alpha \)-adrenergic receptor-mediated release of arachidonic acid via phospholipase A2 was also observed in MDCK kidney cells [15].

**Light activates phospholipase A2 in rod outer segments of the retina**

The most direct evidence showing that phospholipase A2 can be activated by G-proteins was found by examining the effect of light on the rod outer segments of bovine retina [21]. Rhodopsin is the light receptor for the rod outer segments and is coupled to cyclic GMP phosphodiesterase via the G-protein, transducin [22]. Transducin is a heterotrimeric G-protein consisting of \( \alpha \), \( \beta \)-, and \( \gamma \)-subunits. When rod outer segments are exposed to light, the \( \alpha \)-subunit dissociates from the \( \beta \gamma \)-subunit. The \( \alpha \)-subunit of transducin then stimulates the enzyme cyclic GMP phosphodiesterase, which lowers cyclic GMP levels and clamps the membrane potential, located in the disc membranes of the rod outer segments. Upon closure of the sodium channel, the cell hyperpolarizes and the visual signal is then transmitted to the brain. In dark-adapted bovine rod outer segments, light-activated phospholipase A2 was measured by the release of \( ^{14} \)C-arachidonic acid from the substrate 1-palmitoyl-2-[\( ^{14} \)C]arachidonyl-phosphatidylcholine. The addition of GTP\([S]\) to the rod outer segments in the dark also led to the activation of phospholipase A2, presumably due to the dissociation of \( \alpha \)-from the \( \beta \gamma \)-subunit of transducin [21]. Both pertussis toxin and cholera toxin inhibited the activation of phospholipase A2, by light [21], as well as the light-induced increase in cyclic GMP phosphodiesterase activity [22]. With the removal of transducin from the rod outer segments by hypotonic washings in the dark, both the light- and GTP\([S]\)-stabilized phospholipase A2 activity was markedly reduced. When purified transducin was added back to the washed rod outer segments, both the light-induced- and GTP\([S]\) [in the dark]-stabilized activity of phospholipase A2 was mostly restored [21]. These experiments demonstrated that transducin is coupled to phospholipase A2 activation in the rod outer.
segments. The addition of GTP[S] reduced phospholipase A₂ activity when added in the light. The removal of transducin by washing the rod outer segments, followed by the addition of the inhibited phospholipase A₂ activity. In experiments, the removal of transducin by washing the rod outer segments appeared to unmask a G-protein which is inhibitory to phospholipase A₂.

The effect of the α- and βγ-transducin subunits, isolated from bovine rod outer segments, on phospholipase A₂ activity was investigated [23]. The α- or βγ-subunits were added to the transducin-depleted rod outer segments in the dark. The α-subunit caused a slight increase in phospholipase A₂ activity. In contrast, the βγ-subunit markedly stimulated enzyme activity. This was a surprising result, since the α-subunit of transducin has been shown to activate cyclic GMP phosphodiesterase [22]. Addition of equipolar amounts of α- and βγ-subunits caused an inhibition of phospholipase A₂ activity in the dark-adapted rod outer segments probably due to the reassociation of the αβγ-heterotrimer of transducin [23]. This was further suggested when the addition of GTP[S], which causes the disassociation of the α- and βγ-subunits, blocked the inhibitory effect of the α-subunit. In addition, pertussis toxin treatment of the combined αγβγ-subunits added to the rod outer segments in the light, resulted in a further inhibition of phospholipase A₂. It has yet been established whether the βγ-subunit can directly stimulate phospholipase A₂ or remove an inhibitor of this enzyme. Others have shown that the βγ-subunit can stimulate calcium-dependent and voltage-sensitive potassium ion channels [24]. The βγ-subunit has also been shown to inhibit adenylyl cyclase activity and to interact with calmodulin [25], further suggesting a regulatory role for these subunits.

Interleukin 1, phospholipase A₂, and signal transduction

Interleukin 1 (IL-1) is an important messenger molecule in the immune response and appears to interact with both the nervous and endocrine systems. It is a protein growth factor (molecular mass of 17,500 Da) that is produced in many cells including macrophages, fibroblasts, and astrocytes [26]. IL-1 appears to interact with phospholipase A₂ by increasing prostaglandin (PG) synthesis and inducing the synthesis of phospholipase A₂ in many cells [27].

In view of the action of IL-1 on phospholipase A₂, we examined the effect of pretreatment of Swiss 3T3 cells with IL-1 on bradykinin receptor-mediated generation of PGE₂ [28]. Human recombinant IL-1α and IL-1β were equipotent in stimulating PGE₂ synthesis after 1 h of preincubation and the effect of IL-1 was persistent for 2 days. IL-1 augmented bradykinin-stimulated PGE₂ synthesis. In cells that had been pretreated with IL-1 for 24 h, PG synthesis after bradykinin treatment was increased about 10-fold. IL-1 pretreatment was found to induce synthesis of phospholipase A₂ and cyclo-oxygenase de novo, but not phospholipase C or PGE isomerase. This cytokine did not affect bradykinin receptor number or affinity. IL-1 also increased bradykinin-stimulated GTPase activity, indicating an induction of a putative GTP-binding protein linked to the bradykinin receptor. All of these findings suggested that IL-1 amplified the bradykinin receptor-mediated generation of PGE₂ in the same conditions. IL-1 augmented the levels of phospholipase A₂, cyclo-oxygenase and GTP-binding proteins.

Further experiments were undertaken in an AtT-20 mouse pituitary cell line to clarify the role of IL-1 in receptor-mediated transmembrane signalling. IL-1 induced the release of β-endorphin in these cells only after 18 h of pretreatment [29]. Under the same conditions, IL-1 augmented the corticoid releasing factor (CRF)- and vasoactive intestinal polypeptide (VIP)-stimulated release of β-endorphin.

Phorbol esters alone stimulated β-endorphin release and augmented secretagogue-stimulated β-endorphin release after short-term pretreatment. Desensitization of protein kinase C by prolonged treatment with phorbol esters blocked the effect of IL-1 suggesting a role for protein kinase C in its action. The potentiating effect of IL-1 on CRF-induced β-endorphin secretion was only partially abolished after desensitization of protein kinase C. In AtT 20 cells, IL-1 could induce a late response, such as the secretion of β-endorphin, by activating receptor-mediated early signals. We have found that IL-1 generates several early signals, the phosphorylation of 20 and 60 kDa proteins and an increase in mRNA for the proto-oncogenes c-fos and c-jun (M. O. Fargarasan, J. Axelrod & F. Aiello, unpublished work). c-fos and c-jun can form a heterodimer which may act on an enhancer responsive element on genes.

Cloned muscarinic receptors and signal transduction by phospholipase A₂ and C

A family of five muscarinic receptor subtypes have been cloned and are designated m₁, m₂, m₃, m₄ [30–32]. These muscarinic receptors are members of a supergene family which span the membrane seven times and are coupled to G-proteins. Each of the muscarinic receptor genes have been transfected and stably expressed in a mouse A9L fibroblast cell line [31]. The m₁- and m₄-muscarinic receptors, when stimulated with carbachol, generated inositol phosphates, arachidonic acid release and cyclic AMP generation, which were blocked by the muscarinic antagonist atropine. Carbachol-stimulated arachidonic acid release could be due to the stimulation of phospholipase A₂ or phospholipase C. To distinguish between the activation of these two phospholipases, the transfected cells were treated with phorbol ester and the release of [³H]arachidonic acid and [³H]inositol phosphates were measured. Phorbol ester pretreatment caused an inhibition of carbachol-stimulated release of inositol phosphates in these cells. These results suggest that the major source of the receptor-mediated release of arachidonic acid was due to activation of phospholipase A₂ and not phospholipase C.

As mentioned above, each of the m₁-, m₂-, and m₃-muscarinic receptors are linked to at least three second messenger systems; adenylate cyclase and cyclic AMP accumulation, phospholipase C and inositol phosphate generation, and phospholipase A₂ and arachidonic acid release. Muscarinic receptors could activate an effector enzyme either directly through a G-protein or indirectly as a consequence of activating another effector enzyme. Studies with phorbol esters previously described indicated that arachidonic acid release and inositol phosphate generation were independent second messenger pathways linked to a single receptor [33]. Arachidonic acid release is unlikely to be secondary to the elevation of the cyclic AMP levels since cyclic AMP analogues and phosphodiesterase inhibitors had no effect on basal and muscarinic receptor-stimulated arachidonic acid release in these transfected cells. Further studies were performed to determine if carbachol-stimulated cyclic AMP elevation was directly or indirectly linked to the m₁-muscarinic receptor or was mediated via release of arachidonic acid or inositol phosphate second messengers. Phorbol ester pretreatment augmented carbachol-stimulated arachidonic acid release, but blocked both carbachol-stimulated adenylate cyclase activation and inositol phosphate release with equal potency [36].
Carbachol failed to stimulate cyclic AMP in A9L cell membranes, whereas PGE₂ did. These and other observations suggested that cyclic AMP accumulation did not occur through direct m₁ muscarinic receptor coupling to adenylate cyclase via Gs, but was mediated through multiple steps involving activation of phospholipase C, the generation of inositol trisphosphate and cytosolic Ca²⁺ and stimulation of calmodulin.

Discussion

The mechanisms whereby G-proteins activate phospholipase A₂ is not known. Although a number of G-proteins have been characterized, the G-proteins that are coupled to phospholipase A₂ have not yet been identified. Cholera toxin stimulates Gₛ, but calcite by specific receptors on the cell in which phospholipase A₂, depending on the receptor or cell type investigated. Pertussis toxin inhibits Gₛ₁ [1] and transduces Gₛ₂₂, but can inhibit or stimulate phospholipase A₂ [37]. It appears that there are multiple G-proteins that are linked to phospholipase A₂ activation. There may be several phospholipase A₂ iso-enzymes that have different calcium ion requirements, different optimal intracellular locations, and may have the ability to translocate from cytosol to membrane on activation. Some G-proteins may directly couple a receptor to an effector phospholipase A₂. Other G-proteins may modulate phospholipase A₂ activity by opening calcium channels or by inositol trisphosphate-induced release of calcium from intracellular stores. Phospholipase A₂ may also be activated or inhibited by protein kinase A or protein kinase C.

Receptor generated arachidonic acid and its eicosanoid metabolites can act as second messengers. Arachidonic acid itself can mobilize cytosolic calcium, independent of inositol trisphosphate formation [38]. The γ-subspecies of protein kinase C from bovine cerebellum, is selectively activated by arachidonic acid [39]. Arachidonic acid and its lipoxigenase metabolites or on neighbouring cells [2]. They then could activate or inhibit adenylate cyclase, phospholipase C, ion channels or effect other functions on neighbouring cells.

Recent evidence has suggested an important role for arachidonic acid in nervous tissue. 5-Hydroxytryptamine-receptor-stimulated phospholipase A₂ and arachidonic acid release was demonstrated in hippocampal neurons, independent of phospholipase C [43]. N-methyl-d-aspartate (NMDA), a compound which activates excitatory amino acid receptors on nerve cells, can liberate arachidonic acid from striatal neurons in primary cultures of cerebellar granule cells, possibly by the activation of phospholipase A₂ [44]. The addition of arachidonic acid to the rat dentate gyrus causes a slow onset and persistent increase in synaptic activity, which is accompanied by an increase in the release of glutamate [45]. It is possible that arachidonic acid and/or its metabolites, produced by the NMDA-receptor-mediated activation of post-synaptic cells, could cross the synaptic cleft to act on presynaptic cells and thus might act as retrograde messenger for long-term potentiation.

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