Signaling pathways downstream of P2 receptors in human neutrophils

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Received: 12 October 2005 / Accepted: 10 March 2006 / Published online: 20 June 2006
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Abstract Extracellular nucleotides stimulate human neutrophils by activating the purinergic P2Y2 receptor. However, it is not completely understood which types of G proteins are activated downstream of this P2 receptor subtype. We investigated the G-protein coupling to P2Y2 receptors and several subsequent signaling events. Treatment of neutrophils with pertussis toxin (PTX), a Gi protein inhibitor, caused only ~75% loss of nucleotide-induced Ca2+ mobilization indicating that nucleotides cause Ca2+ mobilization both through Gi-dependent and Gi-independent pathways. However, the PLC inhibitor U73122 almost completely inhibited Ca2+ mobilization in both nucleotide- and fMLP-stimulated neutrophils, strongly supporting the view that both the PTX-sensitive and the PTX-insensitive mechanism of Ca2+ increase require activation of PLC. We investigated the dependence of ERK phosphorylation on the Gi pathway. Treatment of neutrophils with PTX caused almost complete inhibition of ERK phosphorylation in nucleotide or fMLP activated neutrophils. U73122 caused inhibition of nucleotide- or fMLP-stimulated ERK phosphorylation, suggesting that although pertussis toxin-insensitive pathways cause measurable Ca2+ mobilization, they are not sufficient for causing ERK phosphorylation. Since PLC activation leads to intracellular Ca2+ increase and PKC activation, we investigated if these intracellular events are necessary for ERK phosphorylation. Exposure of cells to the Ca2+ chelator BAPTA had no effect on nucleotide- or fMLP-induced ERK phosphorylation. However, the PKC inhibitor GF109203X was able to almost completely inhibit nucleotide- or fMLP-induced ERK phosphorylation. We conclude that the P2Y2 receptor can cause Ca2+ mobilization through a PTX-insensitive but PLC-dependent pathway and ERK phosphorylation is highly dependent on activation of the Gi proteins.

Key words ATP · calcium · fMLP · G protein · mitogen-activated protein kinases · P2 receptor · reactive oxygen species · UTP

Abbreviations
ERK extracellular signal-regulated kinase
fMLP formyl methionyl-leucyl-phenylalanine
GPCR G protein-coupled receptor
HBSS Hank’s balanced salt solution
MAPK mitogen-activated protein kinase
PTX pertussis toxin
ROS reactive oxygen species

Introduction

Extracellular nucleotides can induce a wide variety of responses in many cell types, including muscle contraction and relaxation, vasodilation, neurotransmission, platelet aggregation, ion transport regulation, and cell growth. In the vasculature, platelets and endothelial cells release nucleotides when exposed to stimuli such as ischemia, hypoxia, and chemical or mechanical stress [23]. The receptors mediating these effects are termed P2 receptors, which are activated by a variety
of nucleotides including ATP and UTP, and P1 receptors, which are activated by adenosine. There are two types of P2 purinoceptors: P2Y type G protein-coupled receptors (GPCRs), and P2X type ligand-gated ion channels. Vascular cells have been shown to express both the P2Y and P2X receptors [8].

The P2Y receptors possess seven transmembrane hydrophobic domains with short extracellular amino and intracellular carboxyl terminals that have been linked to activation of the phosphoinositide pathway [1, 14, 26]. The P2Y receptors are subdivided into Gq-coupled subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11) and Gi-coupled subtypes (P2Y12, P2Y13, P2Y14). The P2Y11 receptor additionally couples to Gs and activates adenylyl cyclase.

Early work indicated that biological responses triggered in neutrophils by ATP and UTP are mediated by a P2Y2-like receptor, previously described as the P2U receptor [11]. Recently, we demonstrated that, in human neutrophils, the P2Y2 receptor mediates nucleotide-induced Ca2+ mobilization, primary granule release and extracellular signal-regulated kinase ERK 1/2 activation [19].

Conflicting reports exist regarding the coupling of the P2Y2 receptor to heterotrimeric G proteins. Initial evidence based on the use of pertussis toxin strongly suggested that P2Y2 receptors are coupled to Gi proteins [17, 25, 28]. However, in certain cell types, P2Y2 receptor can couple to other G proteins as well. For example, HEL cells have been shown to express P2Y2 receptors [4]; when Gα16 is suppressed in HEL cells using antisense RNA, Ca2+ mobilization by UTP or ATP was completely abrogated. This finding led to the conclusion that stimulation of P2Y2 purinoceptors leads to the mobilization of intracellular Ca2+ by a mechanism that strictly depends on Gα16, and that P2Y2 purinoceptors can communicate with two distinct signaling pathways diverging at the G protein level [4, 10].

We therefore investigated the G protein coupling of the P2Y2 receptor in human neutrophils. Because ERK 1/2 activation is essential for many neutrophil processes such as degranulation, ROS production, and migration [9, 20, 24, 33], we investigated the role of the Gi protein on ERK activation.

**Materials and methods**

**Reagents**

ATP, UTP, fMLP, U73122, GF109203X, 1,2-Bis(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl) ester (BAPTA), phorbol 12-myristate 13-acetate (PMA), dimethyl sulfoxide (DMSO) and bovine serum albumin (fraction V) were obtained from Sigma (St. Louis, Mo.). Dextran T500, and Ficol-Paque, were from Amersham Biosciences (Piscataway, N.J.). Fura-2 AM was from Molecular Probes (Eugene, Ore.). Nucleotides were solubilized in water. Stock solutions of fMLP, U73122, GF109203X, PMA, and fura-2 AM were prepared in DMSO. Polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) and anti-p44/42 MAP kinase were from Cell Signaling Technology (Beverly, Mass.). CDP-star was from Tropix (Bedford, Mass.).

**Neutrophil isolation**

Venous blood was collected from healthy subjects in polypropylene tubes containing ACD anticoagulant (1.5% citric acid, 2.5% sodium citrate, 2% dextrose). Blood was mixed with an equal volume of 3% dextran T500 in saline. Erythrocytes were allowed to sediment for 20 min, and then leukocyte rich plasma was subjected to centrifugation on Ficol-Paque at 400 x g for 45 min. The pellet was collected and the contaminating erythrocytes were removed by hypotonic lysis. Isolated neutrophils were resuspended in Hank’s balanced salt solution (HBSS) containing bovine serum albumin (BSA) 0.2%. Neutrophils were counted using a Reichert-Jung hemacytometer (Hauser Scientific, Horsham, Pa.). Cell viability was checked by the Trypan blue exclusion method and was routinely found greater than 96%.

**Measurement of intracellular Ca2+ concentration**

Isolated neutrophils were incubated for 45 min at room temperature with 1 μM Fura-2 AM (Molecular Probes, Eugene, Ore.). The cells were washed 3 times and then resuspended at a concentration of 3×106/ml in HBSS containing 0.5 mM EGTA and BSA 0.2%. Aliquots of 0.5 ml of cells were placed in a quartz cuvette maintained at 37°C. Intracellular calcium concentrations were assayed during agonist stimulation using excitation wavelengths of 340 nm and 380 nm and the emission was monitored at 510 nm using an AB2 spectrofluorometer (Spectronic Instruments, Rochester, N.Y.). Calcium concentrations were calculated according to Tsien’s ratiometric method [16].

**Western blotting**

Neutrophils (6×10⁶ cells/ml) were preincubated at 37°C with 2 mM CaCl₂ and 1 mM MgCl₂ for 10 min.
Aliquots of 0.5 ml of cells were then stimulated with agonist for 3 min at 37°C. The reaction was terminated by addition of 0.5 ml of cold lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM sodium vanadate, 0.1 mM calyculin A). Lysis was allowed to occur for 20 min on ice. Lysates were centrifuged for 5 min at 14,000 g. Proteins from the supernatants were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane and incubated for 1 h in Tris buffered saline (TBS, 20 mM Tris-HCL, 150 mM NaCl, 0.05% Tween 20, pH 7.5) containing 1% BSA. Primary antibodies were diluted in TBS containing 1% BSA and membranes were incubated overnight at 4°C in the presence of the indicated primary antibody. Membranes were washed 3 times for 5 min at room temperature. The appropriate secondary antibody conjugated with alkaline phosphatase was diluted (1:5,000) in TBS containing 1% BSA. The membranes were incubated with the secondary antibody for 1 h at room temperature. Chemiluminescent reagents were used to visualize the reactive proteins on a Fuji LAS1000 digital camera.

Measurement of neutrophil respiratory burst activity

The release of ROS was measured in aliquots of 500 µl cell suspension (3×10⁶ cells /ml) which were stimulated with agonists at 37°C in a glass cuvette under stirring conditions. The oxidative burst was measured using isoluminol-enhanced chemiluminescence in a lumi-aggregometer model 560-CA (Chironog, Havertown, Pa.). Isoluminol (10 µM) and HRP (4 U/ml) were added. The cells were allowed to equilibrate for 2 min before stimulation with reagents and the luminescence signal was recorded on a chart recorder. A standard solution of hydrogen peroxide was used to determine the sensitivity of the peroxidase-isoluminol system and for normalization of data.

Results

Characterization of the G protein coupled to P2Y receptor in human neutrophils

To identify the role of Gi/o family of G proteins in nucleotide-mediated intracellular Ca²⁺ increase, neutrophils were either treated with buffer or pertussis toxin, an inhibitor of Gi/o proteins. The cells were then resuspended in a Ca²⁺-free solution, to rule out any contribution of Ca²⁺-influx through Ca²⁺ channels to increases in intracellular calcium. As can be seen by the fluorescence intensities in Figure 1, the response to stimulation of neutrophils with 10 nM fMLP, 10 µM ATP or 10 µM UTP leads to increases in intracellular Ca²⁺ concentrations. All three agonists produce similar levels of Ca²⁺ increase. Treatment with 2 µg/ml pertussis toxin for 2 h (Figure 1, gray tracing) abolished the Ca²⁺ increase caused by fMLP. On the other hand, pertussis toxin-treated cells stimulated with ATP or UTP caused a partial inhibition of the Ca²⁺ responses, both inhibited by about 70%, compared to non-treated cells. These data indicate that the Ca²⁺ increase caused by nucleotides is partially resistant to PTX treatment, suggesting that nucleotides may not be signaling solely through the PTX-sensitive Gi-proteins. ROS production was measured to check the viability of the cells following pertussis toxin treatment (Figure 2). Even after treatment with pertussis toxin, neutrophils stimulated with 10 nM PMA are still able to produce ROS.
whereas PTX treatment abolished the ROS production caused by fMLP.

Role of PLC in intracellular Ca\(^{2+}\) increase in human neutrophils

PLC converts PIP\(_2\) to DAG and IP\(_3\), and IP\(_3\) leads to Ca\(^{2+}\) mobilization from intracellular stores. There are also other candidate second messengers (i.e., NAADP-nicotinic acid adenine dinucleotide phosphate, cyclic ADP-ribose) that can also lead to Ca\(^{2+}\) mobilization [15]. We investigated the role of PLC in fMLP- and nucleotide-induced Ca\(^{2+}\) increase in human neutrophils. Incubation with the PLC inhibitor U73122 abolished fMLP- or nucleotide-induced intracellular Ca\(^{2+}\) increase (Figure 3). These results indicate that both the PTX-sensitive and PTX-insensitive mechanism of Ca\(^{2+}\) increase require activation of PLC.

Effect of Pertussis toxin on fMLP or nucleotide-induced ERK phosphorylation

We have previously shown that extracellular nucleotides cause p44/42 (ERK) MAPK phosphorylation and that ERK phosphorylation participates in nucleotide induced-elastase release from neutrophils [19]. Hence, we investigated the dependence of the Gi pathway on ERK phosphorylation. PTX blocks ERK phosphorylation caused by nucleotides or fMLP (Figure 4). These data indicate that pertussis toxin-

Figure 2 Effect of PTX on fMLP- or PMA-induced ROS production in isolated human neutrophils. Isolated human neutrophils were pretreated with 2 \(\mu\)g/ml pertussis toxin or vehicle for 2 h and stimulated with the indicated agonists. Cells were incubated for 1 min with isoluminol and horseradish peroxidase and ROS production was recorded during neutrophil stimulation with 10 nM fMLP or 10 nM PMA, as described under Materials and methods. Tracings representative for at least three independent experiments are shown

Figure 3 Effect of U73122 on fMLP- or nucleotide-induced intracellular Ca\(^{2+}\) increase in isolated human neutrophils. Isolated human neutrophils were loaded with Fura-2 and the intracellular Ca\(^{2+}\) was measured as described under Materials and methods. Intracellular Ca\(^{2+}\) tracings were obtained after pretreatment of neutrophils with 4 \(\mu\)M U73122 (gray) or vehicle (black) for 2 min at 37\(^\circ\)C, followed by stimulation with 10 nM fMLP, 10 \(\mu\)M ATP or 10 \(\mu\)M UTP. Representative tracings for at least three independent experiments are shown
insensitive pathways are not sufficient for causing ERK phosphorylation.

Role of the PLC-Ca2+ pathway on ERK phosphorylation

An important step in intracellular Ca2+ increases is activation of PLC. To investigate whether PLC activation was essential for phosphorylation of ERK by neutrophils, the cells were either preincubated with 4 μM U73122 or vehicle before being activated with 10 nM fMLP, 10 μM ATP or 10 μM UTP. Cells were lysed for 20 min on ice in the presence of protease and phosphatase inhibitors. The samples were run on SDS-PAGE and the proteins transferred on PVDF membranes. The membranes were probed with an anti-phospho-ERK antibody to detect activated ERK.

![Figure 5 Effect of the PLC inhibitor U73122 on ERK phosphorylation. Isolated neutrophils were pretreated with 4 μM U73122 or vehicle for 2 min and then stimulated for 3 min with 10 nM fMLP, 10 μM ATP or 10 μM UTP. Cells were lysed for 20 min on ice in the presence of protease and phosphatase inhibitors. The samples were run on SDS-PAGE and the proteins transferred on PVDF membranes. The membranes were probed with an anti-phospho-ERK antibody to detect activated ERK]

We investigated whether Ca2+ release from internal stores is required for ERK activation. Neutrophils were preincubated with 20 μM of BAPTA/AM, an intracellular calcium chelator, or vehicle for 15 min at 37°C prior to activation. ERK activation induced by 10 μM nucleotides or 10 nM fMLP was not inhibited by treatment with BAPTA (Figure 6). Additionally, the ability of nucleotides to induce an increase in the intracellular free calcium concentration was inhibited by treatment with BAPTA (not shown), indicating that the BAPTA treatment was effective in chelating intracellular Ca2+.

![Figure 6 Effect of the BAPTA on ERK phosphorylation. Isolated neutrophils were pretreated with 20 μM BAPTA or vehicle for 15 min at 37°C and then stimulated for 3 min with 10 nM fMLP, 10 μM ATP or 10 μM UTP. Cells were lysed for 20 min on ice in the presence of protease and phosphatase inhibitors. The samples were run on SDS-PAGE and the proteins transferred on PVDF membranes. The membranes were probed with an anti-phospho-ERK antibody to detect activated ERK]

Effect of the PKC inhibitor GF109203X on ERK phosphorylation

Next, we tested for the involvement of PKC on ERK activation of nucleotide-stimulated neutrophils. To investigate this hypothesis, neutrophils were pretreated with 10 μM GF109203X, a pan-PKC inhibitor, or vehicle for 2 min at 37°C. ERK phosphorylation of neutrophils stimulated with 10 μM ATP or 10 μM UTP for 3 min was inhibited by GF109203X (Figure 7). GF109203X (10 μM) abolished ROS production induced by fMLP (data not shown) confirming that the inhibitor effectively blocked PKC.

![Figure 7 Effect of the PKC inhibitor GF109203X on ERK phosphorylation. Isolated neutrophils were pretreated with 10 μM GF109203X for 2 min and then stimulated for 3 min with 10 nM fMLP, 10 μM ATP or 10 μM UTP. Cells were lysed for 20 min on ice in the presence of protease and phosphatase inhibitors. The samples were run on SDS-PAGE and the proteins transferred on PVDF membranes. The membranes were probed with an anti-phospho-ERK Ab to detect activated ERK]

Discussion

The P2Y2 receptor has been shown to be sensitive to pertussis toxin [13], which inactivates Gi and Go.
classes of G proteins. However, several reports support the view that P2Y2 receptors can also couple to G proteins belonging to the Gq family [4, 10]. The coupling between P2Y2 receptors and heterotrimeric G proteins is insufficiently described in human neutrophils, although nucleotides have been shown to play important roles in modulating fundamental neutrophil responses, such as superoxide production [27], chemotaxis [12, 31], adherence to endothelial cells [22], and degranulation [19].

It is conceivable that P2Y2 couples both to Gq and Gi proteins in a manner similar to how P2Y11 couple to multiple G proteins [7]. Because the Gi protein can be inactivated by pertussis toxin this provides a valuable tool for isolating the effect of the two G proteins. We have previously demonstrated that human neutrophils express the P2Y2 receptor, which is responsible for both nucleotide-induced intracellular Ca2+ mobilization and primary granule release. The sensitivity to PTX can aid in clarification of the G protein family of proteins plays a role in increase of Ca2+. The inability of pertussis toxin to completely inhibit the Ca2+ increase is an indication that there are additional pathways being activated. The fact that the fMLP-induced calcium mobilization, which is known to couple to Gi proteins, is completely inhibited by the same treatment with pertussis toxin indicates that the PTX treatment completely blocked the Gi proteins in neutrophils. This is in agreement with the work in HL-60 cells that showed fMLP receptors are completely inhibited by treatment with pertussis toxin [30].

The Gq family proteins are known to activate PLCs and mobilize Ca2+ from inositol 1,4,5-trisphosphate (IP3)-sensitive intracellular Ca2+ stores, and are thus a candidate for causing the observed PTX-insensitive Ca2+ mobilization. In particular, the α-subunit of G16 (a member of the Gq family of G proteins) has been shown to be expressed in HL-60 cells, though this protein is decreased when differentiated toward neutrophil line by treatment with DMSO [2]. Human erythroleukemia cells expressing the P2Y2 receptor have been shown to engage the G16 protein to mobilize intracellular Ca2+ [4]. Another possibility is that the P2Y2 receptor interacts with Gq11, which has been shown to be present on many hematopoietic cell lines [34] and, in human embryonic kidney cells, endogenously expressing P2Y2 nucleotide receptors [32].

It has been well established that the activation of PLC leads to generation of two second messengers, IP3 and DAG. IP3 induces a Ca2+ release from the intracellular calcium stores [29]. Intracellular Ca2+ increases can also be the result of influx through Ca2+ channels. For example, arachidonic acid can induce Ca2+ mobilization from IP3-insensitive stores [18]. In the present study, when U73122 was used to inhibit PLC activity and thus inhibit Ca2+ released from IP3-sensitive stores, the increase in intracellular Ca2+ was abolished (Figure 3). The fact that U73122 abolished any intracellular Ca2+ changes indicates that the nucleotide-induced Ca2+ mobilization in neutrophils is due to activation of PLC (both the PTX-sensitive and PTX-insensitive pathways require PLC activation to increase intracellular Ca2+), and that the PTX-insensitive pathway is not due to Ca2+ influx.

Previous studies have shown that nucleotide or fMLP stimulation of neutrophils activates ERK 1/2 [5, 19]. In addition, stimulation of the P2Y2 receptor has been shown in endothelial cells to activate ERK 1/2 [6]. In this study, we show that both nucleotide- and fMLP-induced ERK 1/2 activation in human neutrophils can be blocked by pertussis toxin treatment. This suggests that the signaling events activated by Gi proteins are required for ERK 1/2 activation. The signaling of the PTX-insensitive pathway, though sufficient to cause activation of PLC and intracellular Ca2+ mobilization, does not cause ERK 1/2 phosphorylation.

Stimulation of neutrophils by nucleotides leads to activation of PLC by PTX-sensitive and PTX-insensitive pathway. Inhibition of PLC activity by U73122 did inhibit ERK 1/2 activation; Figure 5). We have found that concentrations of U73122 as high as 4 μM did not change PMA-induced ROS production in neutrophils, suggesting that neutrophil viability was not affected. PLC inhibition has been reported in a previous study to inhibit ERK activation by fMLP [5]. The observation that inhibition of PLC prevents intracellular Ca2+ mobilization suggests that ERK 1/2 phosphorylation depends on Ca2+ mobilization or PKC activation. Treatment of neutrophils with BAPTA, an intracellular Ca2+ chelator, was insufficient to inhibit ERK 1/2 phosphorylation (Figure 6). This suggests that ERK activation is mediated by an event downstream of PLC that is not dependent on intracellular Ca2+ increase.

Protein kinase C (PKC) comprise a family of phospholipid-dependent serine-threonine kinases that play a crucial role in signal transduction by numerous agonists [21]. Pretreatment of neutrophils with GF109203X (10 μM) attenuated the nucleotide-induced ERK 1/2 phosphorylation (Figure 7). In preliminary experiments, concentrations of GF109203X as high as 10 μM did not alter the intracellular calcium...
increase caused by fMLP or by nucleotides, suggesting that this reagent does not alter the viability of granulocytes. The inhibition of ERK 1/2 phosphorylation by GF109203X suggests that PKC mediates nucleotide-induced ERK 1/2 phosphorylation, which is consistent with reports that PKC stimulates ERK phosphorylation in rat neutrophils depends on PKC activation [5]. Furthermore, it has been shown that fMLP-stimulated ERK phosphorylation in rat neutrophils depends on PKC activation [5]. Also GF109203X inhibits fMLP-induced phosphorylation of ERK 1/2 in human neutrophils [3]. The concept of multiple G protein pathways coupling to a single receptor is not unique. The P2Y11 receptor is known to couple to both Gq and Gs proteins [7, 10]. There are hundreds of G protein-coupled receptors that transduce signals through G proteins and the ability of a single receptor to activate multiple G proteins provides an additional level of control of signaling pathways. This also leads to a difficulty in unraveling the signaling events downstream of receptor activation.

In summary, this study brings evidence suggesting that P2Y2 receptor activation in human neutrophils activates multiple G proteins, both a PTX-sensitive G protein and a PTX-insensitive G protein (outlined in Figure 8). The two G proteins lead to activation of PLC and an increase in intracellular Ca²⁺, as well as ERK activation. The activation of ERK is Ca²⁺-independent but requires the activation of PKC. These results may help to illuminate the signaling events downstream of the P2Y2 receptor and the development of therapeutic strategies to limit activation of nucleotide-induced activation of neutrophils as a means to reduce inflammation.

Acknowledgement This work was supported by a Research Grant HL63933 from the National Institutes of Health.

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