P2Y Receptor-mediated Inhibition of Tumor Necrosis Factor α-stimulated Stress-activated Protein Kinase Activity in EAhY926 Endothelial Cells*

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Received for publication, January 5, 2000

In the EAhY926 endothelial cell line, UTP, ATP, and forskolin, but not UDP and epidermal growth factor, inhibited tumor necrosis factor α (TNFα)- and sorbitol stimulation of the stress-activated protein kinases, JNK, and p38 mitogen-activated protein (MAP) kinase, and MAPKAP kinase-2, the downstream target of p38 MAP kinase. In NCT254 keratinocytes, UTP and a proteinase-activated receptor-2 agonist caused similar inhibition, but in 13121N1 cells, transfected with the human P2Y2 or P2Y4 receptor, UTP stimulated JNK and p38 MAP kinase activities. This suggests that the effects mediated by P2Y receptors are cell-specific. The inhibitory effects of UTP were not due to induction of MAP kinase phosphatase-1, but were manifest upstream in the pathway of the MEK-4. The inhibitory effect of UTP was insensitive to the MEK-1 inhibitor PD 98059, changes in intracellular Ca2+ levels, or pertussis toxin. Acute phorbol 12-myristate 13-acetate pretreatment also inhibited TNFα-stimulated SAP kinase activity, while chronic pretreatment reversed the effects of UTP. Furthermore, the protein kinase C inhibitors Ro318220 and Go6983 reversed the inhibitory action of UTP, but GF109203X was ineffective. These results indicate a novel mechanism of cross-talk regulation between P2Y receptors and TNFα-stimulated SAP kinase pathways in endothelial cells, mediated by Ca2+-independent isoforms of protein kinase C.

ATP and UTP are released from many types of cells, and their acute postsynaptic effects include smooth muscle contraction, prostacyclin production, and release of nitric oxide (1–3). These effects are mediated through two receptor subfamilies that are structurally distinct: ligand-gated cation channel P2X receptors and the heptahelial, G-protein-coupled P2Y receptors (4). Five mammalian P2Y receptors have been cloned to date (P2Y1, 2, 4, 6, 11), and each has a distinct pharmacological profile for the endogenous agonists ATP, UTP, ADP, and UDP (5, 6).

In endothelial and smooth muscle cells, P2Y receptors mediate activation of many protein kinases, including protein kinase C (PKC)1 isoforms, homologues of the mitogen-activated protein kinases (p42/44 MAP kinase and c-Jun N-terminal kinase (JNK)), phosphatidylinositol 3-kinase, focal adhesion kinase pp125Src, and related focal adhesion tyrosine kinase (7–13). Activation of these kinases may underlie the sustained effects of ATP and UTP in smooth muscle and endothelial cells, such as increased cell proliferation (3, 14–16). However, another potential pro-mitogenic effect of UTP and ATP may be to prevent the pro-apoptotic actions of cytokines in cells where P2Y and cytokine receptors are co-expressed. A number of studies have shown that growth factors, in particular insulin-like growth factor-1, can negatively regulate the stress-activated protein (SAP) kinases JNK and p38 MAP kinase through activation of p42/44 MAP kinase (17–19). However, to date no study has identified a similar role for G-protein-coupled receptors, which in many cells are linked to increases in JNK and p38 MAP kinase activity (20–22).

In this study we examined the effect of P2Y receptor stimulation upon tumor necrosis factor α (TNFα)-stimulated SAP kinase activity in endothelial cells. Rather than activating SAP kinases, as shown in other cell types (9), UTP and ATP caused a marked inhibition of TNFα-stimulated JNK and p38 MAP kinase activity. This effect, which was cell type-specific, was not mediated by p42/44 MAP kinase, nor intracellular Ca2+, but required activation of atypical Ca2+-independent isoforms of PKC. This is the first study to identify such an action of a P2Y receptor.

EXPERIMENTAL PROCEDURES

Materials—The plasmid containing the cDNA encoding the GST-tagged truncated N terminus of c-Jun (GST-c-JunN-ΔN) was donated by J. R. Woodgett (Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Canada). The GST-tagged MAPKAP kinase-2 vector was a gift from C. J. Marshall (Chester Beatty Laboratories, Institute of Cancer Research, London). The MAPKAP kinase-2 antibody and the KKLN-RTLSVA peptide substrate were gifts from P. Cohen (Medical Research Council Protein Phosphorylation Unit, University of Dundee, Scotland). We thank these people for their gifts.

The JNK-1, p38 MAP kinase, and MEK-4 antibodies (Santa Cruz, Santa Cruz, CA), phospho-MEK4 and -MEK3/6 antibodies (New England Biolabs, Beverly, MA), PD 98059, Ro318220, Go6983, GF109203X, TNFα (Calbiochem-Novabiochem, Nottinghamshire, UK), reporter antibodies and the ECL detection system (Amersham, Buckinghamshire, UK) and γ[32P]ATP (3000 Ci/mmol) (NEN Life Science Products, Boston, MA) were purchased from Amersham. Protein bands were visualized by autoradiography.

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1 The abbreviations used are: PKC, protein kinase C; EGF, epidermal growth factor; IκB, inhibitor γ B; IKK, Inhibitory kappa B kinase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MAPKAP kinase-2, mitogen-activated protein kinase-activated protein kinase-2; MEK, MAP kinase kinase; NFκB, nuclear factor κ B; PAR-2, proteinase-activated receptor-2; PMA, phorbol 12-myristate 13-acetate; SAP kinase, stress-activated protein kinase; TNFα, tumor necrosis factor α; GST, glutathione S-transferase; PBS, phosphate-buffered saline; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N”-tetraacetic acid-acetoxyethyl ester.

* This work was supported by the British Heart Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 275, No. 18, Issue of May 5, pp. 13243–13249, 2000
Printed in U.S.A.
MAPKAP Kinase-2 Activity—MAPKAP kinase-2 activity in immunoprecipitates was determined by measuring incorporation of [γ-32P]ATP into the substrate peptide KKLNRILSVA as outlined previously (26).

Indirect Immunofluorescence—Cells grown on coverslips were stimulated as appropriate, and the reaction terminated by rapid aspiration of each sample subjected to electrophoresis on 11% SDS-polyacrylamide gel. Phosphorylation of GST-c-Jun was then determined by autoradiography.

RESULTS

Initially, we examined the effects of UTP (30 μM) and forskolin (10 μM) upon TNF-α-stimulated signaling events in EAhy926 cells. Preincubation with either agent for 60 min inhibited TNF-α (20 ng/ml)-induced activation of both JNK and p38 MAP kinase by about 80% (Fig. 1). When sorbitol (0.5 M) was used as the stimulus, forskolin and UTP were both less effective, only producing about 50% inhibition. ATP (30 μM) also inhibited TNF-α-induced SAP kinase activity, but UTP (30 μM) and EGF (100 nM) were ineffective. UTP had no effect upon the increase in IkKα activity or loss in IkBα expression evoked by TNF-α (not shown), indicating that the site of inhibition of SAP kinase activity by UTP is downstream of the NFκB signaling cascade.

UTP also inhibited enzyme activity downstream of p38 MAP kinase. Both sorbitol and TNF-α increased MAPKAP kinase-2 activity in EAhy926 cells by 3–5-fold (Table I). Preincubation with UTP or ATP (30 μM) reduced the increase by over 80% (Table I). Forskolin (10 μM) only inhibited the TNF-α-induced increase in MAPKAP kinase-2 activity by about 30% and had little effect on the sorbitol-induced activity. This is much less than the inhibition of p38 MAP kinase by forskolin and may be due to an indirect stimulation of MAPKAP kinase-2 by cAMP-raising agents.2

The similar effects of UTP and ATP, but not UDP, on TNF-α-stimulated SAP kinase activity suggested that P2Y2 and/or P2Y4 receptors (5, 6) could be present in EAhy926 cells. To characterize the interaction of these receptors with SAP kinases, we used 1321N1 human astrocytoma cells expressing recombinant human P2Y2 or P2Y4 receptors. However, in these cells, UTP (1–30 μM) stimulated both JNK and p38 MAP kinase activity by 3–5-fold and did not inhibit TNF-α-mediated increases in SAP kinase activity over the micromolar range (Fig. 2, panels A and B). In other respects these cells were like EAhy926 cells in that UTP (30 μM) evoked both [3H]inositol phosphate accumulation (10–20-fold) and MAP kinase activation (not shown). In another cell line, NCTC2544 keratinocytes, UTP (30 μM) inhibited the TNF-α-stimulated increase in JNK activity, and this was mimicked by the PAR-2 peptide SLIGKV.
TABLE I

| Agonist | MAPKAPK-2 activity | Pretreatment | Inhibition of agonist stimulation |
|---------|--------------------|--------------|----------------------------------|
| TNF-α   | 5.1 ± 0.8          | UTP          | 94.5 ± 1.0                       |
|         |                    | ATP          | 83.5 ± 9.3                       |
|         |                    | Forskolin    | 29.2 ± 12.8                      |
| Sorbitol| 3.217 ± 1.5        | UTP          | 86.8 ± 13.1                      |
|         |                    | ATP          | 90.3 ± 4.8                       |
|         |                    | Forskolin    | 4.1 ± 2.0                        |

FIG. 2. Effect of UTP upon TNFα-stimulated SAP activity in 1321N1 cells expressing P2Y receptors and in NCTC2544 keratinocytes. 1321N1 cells expressing the human P2Y2 (panel A), the human P2Y6 (panel B), or NCTC2544 keratinocytes expressing a human PAR-2 receptor (panel C) were incubated with 30 μM UTP (U) or ATP (A) (panel A) or increasing concentrations of UTP (panel B) or SLIGKV (SL) (200 μM) (panel C) for 30 min prior to addition of 20 ng/ml TNFα for 30 min. Samples were then assayed for JNK activity (panels A and C) or p38 MAP kinase (panel B). Each autoradiograph is representative of at least three individual experiments.

Fig. 3. Induction of MKP-1 by UTP and forskolin in EAh926 cells. EAh926 cells were incubated with vehicle control (panel A), 30 μM UTP (U) (panel B), or 10 μM forskolin (F) (panel C) for 60 min and then assessed for MKP-1 expression by indirect immunofluorescence. Each panel is representative of at least three individual experiments.
The effect of UTP on TNFα-stimulated increases in phospho-JNK and phospho-MEK-4 content. Quiescent EAhy926 cells were incubated with 30 μM UTP (U) for 30 min prior to addition of 20 ng/ml TNFα (T) for 30 min. Cell lysates were immunoblotted for phospho-JNK content (panel A) or immunoprecipitated for MEK-4 and then Western blotted for phospho-MEK-4 (panel B). Each blot is representative of at least two others.

Discussion

In this study we have identified a novel mechanism of crosstalk regulation between the P2Y receptor and TNFα receptor signaling pathways in endothelial cells. UTP exerted a strong inhibitory effect upon TNFα- and sorbitol-stimulated JNK and p38 MAP kinase activity, an effect that was also seen downstream of p38 MAP kinase, at the level of MAPKAP kinase-2. It has been shown recently that UTP and other G-protein-coupled receptor agonists, such as thrombin and angiotensin II, can stimulate both JNK and p38 MAP kinase in a number of cell types (20–22). To our knowledge this is the first study to identify an inhibitory action of a G-protein-coupled receptor, the P2Y receptor, upon TNFα-stimulated SAP kinase activation.

In these experiments, ATP had similar effects to UTP, but UDP was inactive. This rules out the P2Y4 subtype as the site of action, as UDP is the most potent agonist at this receptor (5, 6, 24). UTP is not an agonist at the P2Y1 and P2Y11 subtypes (5, 6), suggesting that the responses seen here are mediated via the P2Y2 and/or P2Y4 subtypes, consistent with our previous studies in EAhy926 cells (8, 10). Note that although ATP is not an agonist per se at the human P2Y2 receptor, in a static culture system as used here, it can donate a phosphate group to endogenous UDP to produce UTP, which will activate the P2Y4 receptor (see Refs. 30 and 31). At present, it is difficult to differentiate pharmacologically between the P2Y2 and P2Y4 receptors due to the lack of selective ligands. Also, we cannot yet rule out the possibility that more than one P2Y receptor subtype is expressed in EAhy926 cells and that UTP and ATP act at separate sites.

UTP also inhibited the TNFα-induced responses in NCTC2544 keratinocytes. However, in 1321N1 human astrocytoma cells stably expressing the recombinant human P2Y2 or P2Y4 receptors, we found that UTP alone significantly increased JNK activity and failed to reverse TNFα-mediated SAP kinase activation. This suggests that the effects mediated by P2Y2 receptors are dependent upon the cell type under study and that the P2Y receptor in EAhy926 cells and NCTC2544 keratinocytes couples to additional components. In support of this idea, the inhibitory effect of UTP is seen at lower concentrations (IC50 = 1 μM) than we and others have previously observed for P2Y receptor-mediated excitatory responses, including activation of p42/44 MAP kinase (8, 13) and JNK (9) and generation of [3H]inositol phosphates (not shown).
We sought to identify the site(s) and mechanism(s) responsible for the inhibitory action of UTP. One possibility was a role for an inducible MAP kinase phosphatase, in particular MKP-1, which we have shown previously to be induced by UTP and forskolin in EAhy926 cells (10). However, the data did not support such a role. Agonist-stimulated MKP-1 expression was located exclusively in the nucleus, while p38 MAP kinase was restricted to the cytosolic compartment and JNK was distributed evenly across the cell. TNFα and UTP did not evoke translocation of either SAP kinase to the nucleus. This was not due to a general lack of cellular responsiveness, as nuclear translocation of p42/44 MAP kinase was observed following

![Fig. 6.](image6.png) **Fig. 6.** Time course of UTP-mediated inhibition of TNFα-stimulated JNK activity. Cells were incubated with 30 μM UTP for the times indicated prior (+) or subsequent (−) to stimulation with 20 ng/ml TNFα for a further 30 min. Samples were then assayed for JNK activity. Each autoradiograph shown is representative of four individual experiments.

![Fig. 7.](image7.png) **Fig. 7.** Effect of PD 098059 upon UTP-mediated inhibition of TNFα-stimulated JNK and p38 MAP kinase activity. EAhy926 cells were incubated with or without 50 μM PD 098059 (PD) for 60 min before addition of 30 μM UTP (U) for a further 2 min (panel A) or 30 min (panels B and C). In panels B and C, cells were stimulated with 20 ng/ml TNFα (T) for a further 30 min. Samples were then assayed for p42/44 MAP kinase content (panel A), JNK (panel B), or p38 MAP kinase (panel C) activity. Each autoradiograph is representative of four individual experiments.

![Fig. 8.](image8.png) **Fig. 8.** The effect of Ca²⁺ influx on TNFα-stimulated JNK activity in EAhy926 cells. In panel A cells were washed three times in Ca²⁺-free buffer in the presence of 10 μM BAPTA-AM (B) for 60 min before preincubation with UTP (U) for 30 min prior to stimulation with TNFα. In panel B, cells were incubated with 1 μM A23187 for the times (in minutes) indicated. Samples were then assessed for JNK activity. Each autoradiograph is representative of four individual experiments.

![Fig. 9.](image9.png) **Fig. 9.** The effect of PMA and PKC down-regulation upon UTP-mediated inhibition of TNFα-stimulated JNK activity in EAhy926 cells. In panel A cells were incubated with PMA for the times indicated before addition of TNFα 20 ng/ml (T) for a further 30 min. In panel B cells were incubated with PMA for 18 h prior to incubation with 30 μM UTP (U) for 30 min followed by stimulation with 20 ng/ml TNFα (T) for a further 30 min. Samples were then assayed for JNK activity. Each autoradiograph is representative of four individual experiments.

![Fig. 10.](image10.png) **Fig. 10.** The effect of PKC inhibitors upon UTP-mediated inhibition of TNFα-stimulated JNK activity in EAhy926 cells. Cells were incubated with 500 nM GF109203X (GF) (panel A), 1 μM Ro318220 (panel B), or 1 μM Go6983 (panel C) for 30 min before addition of 100 nM PMA (P) or 30 μM UTP (U) for 30 min, followed by stimulation with 20 ng/ml TNFα (T) for a further 30 min. Samples were then assayed for JNK activity. Each autoradiograph is representative of four individual experiments.
stimulation with PMA and suggests that the normal inducible nuclear phosphates MKP-1 and MKP-2, which regulate JNK and p38 MAP kinase within the nucleus (10, 32), are not involved in the inhibitory actions of UTP.

It may be that other cytosolic MAP kinase phosphatases which display substrate specificity for JNK and p38 MAP kinase, such as M3/6 and MKP-5 (33, 34), are involved in the negative regulation of SAP kinases. However, we found that the site of action of UTP was upstream of JNK and p38 MAP kinase, at the level of MEK or above. Inhibition of TNFα-stimulated MEK-4 activation by UTP implies that dephosphorylation at serine and threonine of MEKs or MEKks, possibly by PP2A (33), or uncoupling of the pathway further upstream is a far more likely mechanism of action.

This study showed that several intracellular signaling pathways activated by UTP via P2Y receptors are not involved in its inhibitory effects. p42/44 MAP kinase is activated strongly by UTP in EAhy926 cells (8, 10) and has been implicated in the inhibition of JNK and p38 MAP kinase activity in other cell types. However, the MEK-1 inhibitor PD 098059, at concentrations that abolished MAP kinase activation (27–29), failed to reverse the effects of UTP upon JNK and p38 MAP kinase activation. Furthermore, EGF, a robust activator of the MAP kinase cascade (35, 36), did not mimic the inhibitory effects of UTP. The lack of effect of EGF also argued against a role for growth factor-mediated tyrosine kinases and/or phosphatases in the inhibition of TNFα-stimulated SAP kinase activation.

Similarly, we found no evidence for the involvement of a Ca2+-dependent mechanism in the inhibitory effects of UTP. Removal of extracellular Ca2+ and buffering of intracellular Ca2+ did not suppress the UTP inhibition of TNFα-evoked increased in JNK activity. Also, elevation of intracellular Ca2+ by A23187 gave a strong and rapid stimulation of JNK activity. This suggest that the normal cellular mechanisms by which Ca2+ can increase JNK activity are present in EAhy926 cells. This contrasts with the finding that in cells transfected with the P2Y2 receptor, UTP evokes large increases in inositol trisphosphate and intracellular Ca2+ levels (37) and increases JNK activity. Furthermore, previous studies have shown JNK activation by G-protein-coupled receptor agonists to be Ca2+-dependent (38, 39), further arguing against a role for Ca2+ in inhibitory effects of UTP.

While the inhibitory effects of forskolin imply a role for cAMP-dependent signaling events in the inhibitory effects of UTP, our previous study (10) showed that UTP does not increase cAMP in EAhy926 cells. Thus, forskolin and UTP may act by different mechanisms to inhibit SAP kinases. The present studies have revealed one such potential mechanism for UTP, involving PKC. Short term incubation with PMA mimicked the actions of UTP upon TNFα-stimulated JNK activity, while chronic pretreatment partially reversed the inhibitory action of UTP. This suggests a role for DAG-sensitive, Ca2+-independent isoforms of PKC.

We showed previously that EAhy926 cells express the PKCa (Ca2+-dependent) and PKCe (Ca2+-independent) isoforms that are down-regulated by chronic PMA treatment (8). As the inhibitory effects of UTP are independent of Ca2+, this clearly suggests that PKCe is the more likely to be involved or possibly PKCδ, which is rapidly tyrosine phosphorylated and activated by UTP in PC12 cells (13). Both possibilities were supported by the effects of PKC inhibitors. Ro318220, a nonselective PKC inhibitor (40), fully reversed the effect of UTP. GF109203X, at a concentration (500 nM) that is relatively selective for Ca2+-dependent PKC isoforms (41, 42), had no effect on UTP, but did reverse the inhibitory effect of PMA. Furthermore, GF109203X, at higher concentrations (3–10 μM) that are likely to also inhibit PKCe (42), reversed the effects of UTP (not shown).

At present we cannot rule out that other PKA-insensitive PKC isoforms are also involved, in particular atypical isoforms such as PKCζ, which is reported to be sensitive to Ro318220 in some cell types (43). Supporting this possibility is our finding that Go6983, which also inhibits PKCe (44), also reversed the inhibitory effects of UTP. If PKCe is present in EAhy926 cells and also involved in the inhibitory actions of UTP, then this may explain why the effect of PMA has a slower onset and is a less effective inhibitor of the SAP kinase responses than UTP. Alternatively, it may be that other PKC-independent mechanisms are also involved and PKC only plays a conditional role in the inhibitory effects of UTP.

The involvement of PKC isoforms in the inhibition of JNK activity distinguishes our findings from recent studies in other cell types. For example PKCβ stimulates SAP kinase activity in U-937 and HL60 cells (45). However, in the EAhy926 line used in this present study, PKCβ isoforms are poorly expressed (8). Thus, the cell-specific expression of PKC isoforms may dictate which effects are manifest upon SAP kinase signaling. In conclusion, we have identified a novel mechanism of cross-talk regulation between the P2Y receptor and TNFα receptor signaling pathways in endothelial cells, involving PKC. Activation of the recombinant human PAR-2 receptor expressed in NCTC2544 keratinocytes also suppressed TNFα receptor-mediated increases in SAP kinase activity. This suggests that such cross-talk can occur for other G-protein-coupled receptors.

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*J. Biol. Chem. 2000, 275:13243-13249.*

doi: 10.1074/jbc.275.18.13243

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