Nucleotide Receptors Involved in UTP-Induced Rat Arterial Smooth Muscle Cell Migration

Xavier Pillois, Hervé Chaulet, Isabelle Belloc, Françoise Dupuch, Claude Desgranges, Alain-Pierre Gadeau

Abstract—Many factors have been shown to be involved in the development of hyperplasic lesions of vessels, but the role of extracellular nucleotides remains largely unknown. The presence of P2Y and P2X nucleotide receptors on arterial endothelial and smooth muscle cells suggests a potential role for nucleotides in the vessel pathophysiology. Although the role of P2X in physiology of vessels is well documented, that of P2Y is not completely understood. We recently demonstrated that extracellular nucleotides, and particularly UTP, induced migration of cultured arterial smooth muscle cells (ASMCs). This migration is dependent on osteopontin expression and involves the Rho and mitogen-activated protein (MAP) kinase pathways. An important question is to determine the specific role of the different P2Y receptors of rat ASMCs in the UTP-induced migration process. Therefore, we first quantified mRNA levels of P2Y2, P2Y4, and P2Y6 nucleotide receptors in cultured rat ASMCs by a competitive RT-PCR approach and demonstrated that P2Y2 is the most highly expressed among these receptors potentially involved in the UTP-mediated response. In addition to UTP, UDP also induced ASMC migration even when UTP regeneration was inhibited, suggesting the involvement of UDP receptor P2Y6. Moreover, suramin, a specific antagonist of rat P2Y2 receptor, acted as an inhibitor of UTP-induced migration. Taken together, these results suggest a prominent role for the UTP receptor, P2Y2, and for the UDP receptor, P2Y6, in UTP-induced rat ASMC migration. (Circ Res. 2002;90:678-681.)

Key Words: purinergic receptors  migration  UTP  smooth muscle cells

The important role of arterial smooth muscle cell (ASMC) migration and proliferation in arterial hyperplasia is well documented in experimental models for atherosclerosis and restenosis. Although proliferation can be easily demonstrated in arterial injury models, evidence for in vivo ASMC migration is suggested only by the presence of these cells in the intima. Many factors have been shown to be involved in the development of hyperplasic lesions of vessels. Among these, the role of extracellular nucleotides remains largely unknown. Two families of receptors have been identified for these compounds: inotropic P2X receptors and metabotropic P2Y receptors. The presence of P2Y and P2X receptors on endothelial and ASMCs suggests a potential role for nucleotides in the arterial pathophysiology. Although the role of P2X receptors in vasomotoricity of vessels is well documented, that of P2Y receptors in the vessel wall is still under investigation.

Several studies have shown that ATP and UTP binding to P2Y G protein–coupled receptors mediates ASMC activation, cell-cycle progression, and cell proliferation. Moreover, we recently demonstrated that UTP induces ASMC migration and that this migration is dependent on osteopontin expression and involves the Rho and mitogen-activated protein (MAP) kinase pathways. The overexpression of the ATP/UTP P2Y2 receptor in ASMCs of rat aortic intimal lesion and in ASMCs of human coronary atherosclerotic/restenotic lesions (C. Seye and C. Desgranges, unpublished results, 1997) underlines the potential role of nucleotides in pathological processes.

In mammals, the P2Y family currently includes six receptors P2Y1, P2Y2, P2Y4, P2Y6, P2Y11 (for review, see Reference 3) and the recently cloned platelet receptor P2Y12. Their nucleotide agonist specificity has been extensively studied mainly on calcium flux responses of transfected cells, and these studies have demonstrated a selective purine response of ADP for P2Y1 and P2Y11, of ATP for P2Y11, a selective UDP response for P2Y6, and a mixed purine and pyrimide selectivity for P2Y2 and the rat P2Y2.

A further issue concerns the specific role of the different P2Y receptors of rat ASMCs in UTP-induced migration. For this purpose, we first evaluated the expression of UTP/UDP-activated receptors by measuring the level of mRNA of P2Y2, P2Y6, and P2Y8 receptors in rat ASMCs using competitive RT-PCR. Then, we compared the ability of UTP and UDP to induce ASMC migration with the aim of discriminating the specific role of these receptors in this process. Taken together, these results suggest a prominent role of P2Y2 and P2Y6 in UTP-induced rat ASMC migration.
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added to the upper chamber of the Transwells. The lower chamber

UTP or UDP solutions (10−6 mol/L) were preincubated during 1 hour at 37°C. Migration tests were performed in the presence of 10 mmol/L pretreated nucleotide solution and 1 U/mL hexokinase. Full nucleotide transformation by NDPK plus CTP or by hexokinase was coamplified with respective cellular cDNA. PCR products from competitor (black arrowheads) were separated from specific restriction enzymes. Equipotent ethidium bromide fluorescence of competitor and cellular cDNA PCR products indicates identical template concentration in the starting solution. Each experiment was done in triplicate.

Materials and Methods

Cell Culture

Rat ASMCs were prepared from thoracic aortas of Wistar rats as previously described.10 Cells were cultured in DMEM containing 5% FCS, 150 mmol/L HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Cergy Pontoise, France). ASMCs from passages 6 to 12 were used.

Migration Assay

Cell migration was performed using the Transwell system (Costar), which allows cells to migrate through 8-μm-pore sized polycarbonate membrane as previously described. Briefly, 50 000 cells were added to the upper chamber of the Transwells. The lower chamber was filled with 100 μL serum-free DMEM containing or not containing nucleotides (ATP, ADP, UTP, UDP, UMP, α,β-methylene-ATP) or uridine in appropriate concentrations. After a 6-hour incubation, cells present beneath the membrane were stained with hemalum and counted in 10 high-power microscopic fields. Analysis was performed on 3 wells for each condition and each experiment was repeated at least 3 times.

In experiments using nucleoside-5′-diphosphate kinase (NDPK), UTP or UDP solutions (10 μmol/L) were preincubated 15 minutes at room temperature with 2.5 U/mL NDPK and 100 μmol/L CTP before addition in the lower chamber of Transwells and migration assay. In experiments using hexokinase, UTP or UDP solutions (1 mmol/L) were preincubated during 1 hour at 37°C with 10 U/mL hexokinase. Migration tests were performed in the presence of 10 mmol/L pretreated nucleotide solution and 1 U/mL hexokinase. Full nucleotide transformation by NDPK plus CTP or by hexokinase was checked by HPLC. In experiments using suramin, ASMCs were preincubated 10 minutes with the inhibitor before loading in the upper chamber and performing migration test as described above. Enzymes, nucleotides, nucleosides, and suramin were from Sigma (St Quentin Fallavier, France).

Competitive RT-PCR

Purification of mRNA, cDNA synthesis, and PCR were performed as previously described. For competitive PCR, the specific competitor of P2Y receptor cDNA at various concentrations and the cellular cDNA were added to the PCR mix in a final volume of 25 μL and coamplified. Competitive templates were either mutated DNA obtained by site-directed mutagenesis or orthologues of the target cDNA that differ from the respective rat P2Y receptor sequence by the presence or absence of a specific restriction site (Table). Thirty to forty amplification cycles were used with an annealing temperature of 66°C for P2Y1, P2Y6, and P2Y4 and of 62°C for P2Y2 receptors. Cellular cDNA and competitor PCR products were separated on a 1.5% agarose gel electrophoresis after hydrolysis by specific restriction enzymes. Equipotent ethidium bromide fluorescence of competitor and cellular cDNA PCR products indicates identical template concentration in the starting solution. Each experiment was done in triplicate.

Statistics

ANOVA and unpaired Student’s t test were performed for statistical analysis. A probability value less than 0.05 was considered statistically significant. Data are expressed as mean±SD.

Results

Quantification of P2Y mRNA Expression

The mRNA amount of P2Y1, P2Y2, P2Y4, and P2Y6 receptors in cultured ASMCs was evaluated by competitive RT-PCR. Figure 1 shows gel electrophoresis of PCR products obtained with different competitor concentrations and demonstrated that P2Y receptor mRNA was expressed at very different levels in the cells. The estimated amount of P2Y2 receptor mRNA was 10-fold that of P2Y4 receptor, itself 10-fold higher than that of P2Y1 receptor. P2Y1 receptor expression was 100-fold lower than that of P2Y4 receptor.

Figure 1. P2Y1, P2Y2, P2Y4, and P2Y6 receptor expression in cultured rat ASMCs. Purinergic receptor cDNA quantification was performed by competitive PCR. Competitor of each P2Y receptor, added from 10−12 to 10−5 μg/μL to the PCR mix test, was coamplified with respective cellular cDNA. PCR products from competitor (black arrowheads) were separated from specific cDNA (white arrowheads) by 1.5% agarose gel electrophoresis. The white rectangle on each image delineates the reversal point of amplification of specific cDNA versus competitor. L indicates 100-bp ladder; C, control lane without competitor added in the test tube.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5′ → 3′)</th>
<th>Competitor Characteristics</th>
<th>PCR Product Lengths, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y1</td>
<td>F: TCACTGCTACGGACTCCAGC</td>
<td>Rat P2Y1, cDNA, site-directed mutation for the BglII site</td>
<td>431 or (143 + 288)*</td>
</tr>
<tr>
<td></td>
<td>R: AGCTGATCGGTCGTCTGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2Y2</td>
<td>F: TGTCGTCCTATATCTTCAGTCGCC</td>
<td>Human P2Y2, cDNA, sensitive to BglII digestion</td>
<td>428 or (159 + 269)‡</td>
</tr>
<tr>
<td></td>
<td>R: CAAGGGCGGAGAGACCACTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P2Y6</td>
<td>F: CCTTCCTCTTCTAGCCAC</td>
<td>Mouse P2Y6, cDNA, sensitive to Smal digestion</td>
<td>391 or (127 + 264)†</td>
</tr>
<tr>
<td></td>
<td>R: TACGCTGTCCTGCTTGTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*After BglII digestion; †after Smal digestion; ‡after BglI digestion; §after PpuMI digestion.
Effect of UTP and UDP on ASMC Migration

At least three receptors, P2Y2, P2Y4, and P2Y6, are known to respond to pyrimidine nucleotides and could mediate the ASMC migration response induced by UTP or its immediate catabolite UDP. To determine the receptor(s) involved in this process, we compared the effect of the uridylic family UTP, UDP, UMP, and uridine and that of ATP and ADP on ASMC migration. Figure 2 demonstrates that neither UMP nor uridine has migratory potential. In contrast, UDP induced ASMC migration but to a lesser degree than UTP. The UDP response was ~50% that of UTP. The specific potential of UTP and UDP by themselves needs to be evaluated because UTP and UDP can be converted to each other. Indeed UTP can be catabolized by ectonucleotidases into UDP, and UDP can be converted into UTP by extracellular ectonucleotide diphosphokinase.11

To confirm the involvement of UTP-specific receptors, the migration test was performed in conditions limiting the involvement of UDP. To this end, the migration test was done in the presence of NDKP and CTP in the cultured medium so that UDP generated by UTP catabolism was immediately transformed into UTP. The efficiency of this process was assessed by determining the nucleotide concentration in the cultured medium by HPLC (not shown). The UTP solution added to the bottom of the migration chamber was also treated with NDKP to eliminate UDP contamination. In these conditions, UTP-mediated ASMC migration could be entirely attributed to this nucleotide. Moreover, addition of NDKP to the medium of UDP-stimulated ASMCs led to an increased chemotactic activity, demonstrating that UTP is a more potent chemoattractant than UDP for ASMCs (Figure 3A).

Conversely, to evaluate the potential involvement of P2Y6 receptor in UDP-induced ASMC migration, we added hexokinase to the culture medium to avoid UTP contamination or formation as previously described.11 Figure 3B shows that the chemotactic activity of UDP was maintained in the absence of UTP, destroyed by hexokinase treatment, suggesting that the P2Y6 receptor was also involved in ASMC migration.

ATP, but not ADP, also demonstrated a chemotactic activity (Figure 4), suggesting that ATP-induced ASMC migration was not due to P2Y1 activation but rather to the activation of other receptor(s), ie, the P2Y2 or P2Y4 receptors.
hypothesis, α,β-methylene-ATP was added to ATP to protect it from ectonucleotidases. Figure 4 shows that α,β-methylene-ATP strongly increased the ATP-induced migration while inducing only a small nonsignificant response by itself. Because agonists could not discriminate P2Y₆ and P2Y₄, rat receptors, we used suramin, a P2Y₂ but not P2Y₄ receptor antagonist in the rat on UTP-induced ASM migration. Figure 4B shows that suramin inhibited the UTP-induced migration dose dependently. This result is consistent with a major role of P2Y₂ receptor in this process.

Discussion
In previous experiments, we demonstrated that extracellular UTP is able to induce migration of cultured rat ASMCs in a dose-dependent manner. In the present work, we demonstrate that UTP can also induce ASM migration. Because UTP and UDP can activate different P2Y receptors, the aim was to define the role of P2Y₂, P2Y₆, and P2Y₄ receptors in UTP-induced ASM migration. First, we determined that the P2Y₂ receptor is 10- and 100-fold more highly expressed than P2Y₆ and P2Y₄ receptors, respectively. The expression of the P2Y₂ receptor was lower than that of the P2Y₄ receptor.

The very low level of P2Y₄ receptor mRNA was confirmed by the absence of ADP-induced migration of cultured ASMCs, demonstrating that P2Y₄ is not involved in this process.

Both UTP and UDP induced ASM migration. The reversible conversion of UTP to UDP made it difficult to determine the P2Y receptor(s) involved in the UTP-induced migratory response. We found that a commercial solution of UDP and hexokinase-treated UDP solution (UDP-free) induced ASM migration equally, thereby demonstrating that UDP is chemotactic by itself for ASMCs and that P2Y₄ receptor is thus able to mediate migration. Conversely, the migration experiment done in conditions limiting UTP degradation by NDPK treatment demonstrated the involvement of P2Y₂ and/or P2Y₄ receptor(s). The lower migration induced by UDP than by UTP could be the result of reduced receptor efficiency in this process, but it also could be due to the reduced P2Y₄ receptor expression as suggested by the smaller amount of P2Y₂ than P2Y₄ receptor mRNA found in cultured ASMCs.

ATP and UTP have been found to have an equal effect on the calcium fluxes resulting from stimulation of rat P2Y₂ and P2Y₄ receptors in transfected cells. In the present study, ATP demonstrated a migratory effect lower than UTP. However, this difference decreased and became nonsignificant when ATP was protected from ectonucleotidase degradation by α,β-methylene-ATP. This result suggests that the lower effect of ATP alone is certainly related to the ATP conversion in its metabolites. Although agonist-induced experiments could not allow the discrimination between P2Y₅ and P2Y₆-mediated response, the inhibitory effect of the rat P2Y₁ receptor antagonist, suramin, on UTP-induced migration and the fact that P2Y₂ mRNA content is 100-fold higher than that of P2Y₄ strongly suggest a prominent involvement of the P2Y₂ receptor in UTP-induced rat ASM migration.

Taken together, these results suggest that UTP-induced migration is mainly due to the P2Y₂ receptor. Moreover, this work also demonstrated for the first time a functional role of P2Y₂ in ASMCs and that this receptor could be involved in UTP-mediated ASM migration after UTP conversion in UDP.

The findings that P2Y₂ receptor is overexpressed in experimentally induced arterial intimal thickening and that UTP induces significant ASM migration at concentrations as low as 10 nmol/L close to in vivo concentration suggest that UTP could play a significant role in pathophysiological conditions by inducing ASM migration.

Acknowledgments
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References
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