Capacity for Purinergic Control of Renin Promoter via P2Y$_{11}$ Receptor and cAMP Pathways

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**Abstract**—Renin secretion can be stimulated by ATP via purinergic P2Y receptors. ATP is a cotransmitter with norepinephrine and is released from the cytosol during cell damage. Such release could account for the de novo renin expression seen in the proximal tubule in renal disease and in myocardial infarct borders. Whereas most P2Y purinoceptor subtypes utilize phosphoinositide signal-transduction pathways, the effector mechanisms of the subtype P2Y$_{11}$ also involve increases in cAMP, a well-known renin secretagogue and stimulus to renin production. The present study tested the effect of ATP on human renin gene (REN) promoter activity and the role of P2Y$_{11}$. By means of reverse transcriptase–polymerase chain reaction, we found that renin-expressing Calu-6 cells express P2Y$_{11}$ mRNA. Expression was also detected in the brain, kidney, testis, muscle, liver, and spleen. We made a novel cell line (Calu-6/P2Y$_{11}$) in which P2Y$_{11}$ cDNA, under the control of a strong promoter, was stably integrated into genomic DNA. These cells produced P2Y$_{11}$ mRNA during culture. Treatment of Calu-6/P2Y$_{11}$ cells with 1 mmol/L ATP caused a 3-fold increase in renin mRNA and protein over 36 hours. Transient transfection of Calu-6/P2Y$_{11}$ cells with constructs containing 896 bp of human REN 5′-flanking DNA linked to the luciferase reporter gene led to a 5.8±0.6-fold increase (mean±SEM) in reporter activity in response to ATP ($P=0.0015$). In contrast, UTP produced only a 1.4±0.1-fold increase ($P=0.016$). For ADP, it was 1.7±0.1-fold ($P=0.011$). The response profile was ATP>ADP>AMP=adenosine=0, consistent with a P2Y$_{11}$ effect. Mutation of the cAMP response element (CRE) located at −222 in the REN promoter DNA abolished the effect of ATP. Furthermore, ATP induced a rapid, time-dependent increase in the phosphorylation of CRE binding protein (CREB) and activating transcription factor-1. These data implicate a cAMP pathway in mediation of the P2Y$_{11}$ effect. In conclusion, we have made a novel cell line that overexpresses the P2Y$_{11}$ purinoceptor. Stimulation of these cells by ATP activates a cAMP signal-transduction pathway that phosphorylates CREB and stimulates renin promoter activity via the CRE at −222. The data raise the possibility of a contribution of ATP/P2Y$_{11}$ effects to sympathetic stimulation of renin, as well as to responses in renin seen after tissue damage, such as in kidney disease and myocardial infarction. (Hypertension. 2000;36:1093-1098.)

**Key Words:** renin gene | human | purinergic receptor P2Y$_{11}$ | ATP | Calu-6 cells | cAMP | cAMP response element | CREB | ATF-1

ATP is present with norepinephrine in renal sympathetic nerves and sympathetic terminals that innervate smooth muscle and is released in response to increased vascular flow rate. The renin-secreting juxtaglomerular cells in the kidney are modified vascular smooth muscle cells that are innervated by sympathetic neurons, stimulation of which leads to renin secretion. In addition, high concentrations (3 to 5 mmol/L) of ATP are present in the cytosol of cells, from where it is released during cell damage, such as occurs in renal disease and blood vessel rupture, leading to extracellular concentrations of 1 mmol/L. It is also released in response to hypoxia and stressful stimuli. The kidney produces 10% of total ATP in the body and contains very high levels of mitochondrial transcripts. ATP stimulates renin secretion from rat renal cortical slices, the relative response to ATP analogues being in accord with a P2Y, rather than a P1, purinergic effect. Moreover, increased release of nitric oxide may be involved in eliciting this response in renin.

Relative agonist potencies define P2 purinoceptors and their subtypes. The signal-transduction pathways used by P2Y purinoceptors P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y$_6$ involve G-protein activation that stimulates phospholipase C, inositol triphosphate, and Ca$^{2+}$ release. These P2Y receptors are expressed in a variety of tissues and can be differentiated pharmacologically on the basis of their selectivity for adenosine (ATP, ADP) and uridine (UTP, UDP) nucleotides. A more recently cloned purinoceptor, P2Y$_{11}$, differs from other members of the P2Y family in that it is coupled not only to the phosphoinositide pathway but also to the adenyl cyclase pathway. In the placenta, spleen, small intestines of tissue culture experiments it was shown that the P2Y$_{11}$ response was due to the release of cAMP and via adenylate cyclase. The P2Y$_{11}$ receptor is also found in the brain, kidney, testis, muscle, liver, and spleen. We have made a novel cell line (Calu-6/P2Y$_{11}$) in which P2Y$_{11}$ cDNA, under the control of a strong promoter, was stably integrated into genomic DNA. These cells produced P2Y$_{11}$ mRNA during culture. Treatment of Calu-6/P2Y$_{11}$ cells with 1 mmol/L ATP caused a 3-fold increase in renin mRNA and protein over 36 hours. Transient transfection of Calu-6/P2Y$_{11}$ cells with constructs containing 896 bp of human REN 5′-flanking DNA linked to the luciferase reporter gene led to a 5.8±0.6-fold increase (mean±SEM) in reporter activity in response to ATP ($P=0.0015$). In contrast, UTP produced only a 1.4±0.1-fold increase ($P=0.016$). For ADP, it was 1.7±0.1-fold ($P=0.011$). The response profile was ATP>ADP>AMP=adenosine=0, consistent with a P2Y$_{11}$ effect. Mutation of the cAMP response element (CRE) located at −222 in the REN promoter DNA abolished the effect of ATP. Furthermore, ATP induced a rapid, time-dependent increase in the phosphorylation of CRE binding protein (CREB) and activating transcription factor-1. These data implicate a cAMP pathway in mediation of the P2Y$_{11}$ effect. In conclusion, we have made a novel cell line that overexpresses the P2Y$_{11}$ purinoceptor. Stimulation of these cells by ATP activates a cAMP signal-transduction pathway that phosphorylates CREB and stimulates renin promoter activity via the CRE at −222. The data raise the possibility of a contribution of ATP/P2Y$_{11}$ effects to sympathetic stimulation of renin, as well as to responses in renin seen after tissue damage, such as in kidney disease and myocardial infarction.
tine, myocardial tissue, dendritic cells, and macrophages. Overexpression of P2Y11 has been achieved in CHO-K1 cells and yielded a relative potency for cAMP production that was as follows: adenosine 5′-O-(3-thiotriphosphate) = 2′,3′-O-(4-benzoylbenzoyl)-ATP (BzATP) > ATP > 2-thethylthio-ATP (MeSATP) > ADP > UTP.

cAMP is the second messenger in pathways that trigger renin secretion and synthesis. Effects on the human renin gene (REN) promoter involve a cAMP response element (CRE) located 222 bp upstream of the transcription start site. Studies of a renin-expressing cell line, Calu-6, derived from a pulmonary adenoma have shown that cAMP activates protein kinase A, which phosphorylates CRE binding protein (CREB), and that CREB then acts on the CRE to stimulate REN promoter activity.

As well as having a possible role in stimulating renin output from traditional sources such as juxtaglomerular cells under physiological or pathophysiological circumstances, we hypothesized that the de novo renin expression seen in proximal tubule cells in kidney disease could involve an effect of ATP on P2Y11 purinoceptors. We therefore developed a new line of Calu-6 cells that overexpressed P2Y11 and performed experiments to test whether ATP stimulated the promoter, as well as elucidating whether the REN CRE and phosphorylation of CREB were involved.

### Methods

#### Cell Culture

Calu-6 cells were grown in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, 1 mmol/L sodium pyruvate, and 0.1 mmol/L nonessential amino acids, as described previously.

#### Constructs

Full-length P2Y11 receptor cDNA (GenBank AF030335) was cloned by polymerase chain reaction (PCR) of cDNA reverse-transcribed (RT) from the promyelocytic leukemia cell line NB4. The PCR mixture consisted of 0.5 μmol/L of each primer (forward, 5′-ATG GAT CGA GGT GCC AAC TGC T-3′; reverse, 5′-TCA TIG GCT CAG CTC AGG GGA C TG-3′; synthesized by GeneWorks). 2.4 μg of NB4 cell cDNA, 5 U of BIO-X-ACT DNA polymerase (Fisher Biotec), 50 mmol/L MgCl2, 20 mmol/L dNTP, and Opti-buffer (Fisher Biotec). PCR was performed for 30 cycles of 94°C for 30 seconds, 60°C for 90 seconds, and 72°C for 120 seconds. This gave a 1.1-kb PCR product, which was then cloned into the pGEM-T Easy vector (Promega) and termed pGEM-P2Y11.

This was sequenced to confirm its identity and to ensure that the PCR had not introduced any mutations. A construct termed pLEN was created by insertion of the neo cassette (SpeI-BsaI blunt-ended fragment from pEGFP-1; Clontech) into the SpeI-MluI site of pIREs-EGFP (Clontech). A construct termed pLEN-P2Y11-S was then made by insertion of the P2Y11 fragment from pGEM-P2Y11 into the multiple-cloning site of pLEN. This gave a construct in which P2Y11 cDNA was under the control of the strong, ubiquitously utilized human cytomegalovirus (CMV) promoter. The ~900-1uc and ~900mut constructs were produced in collaboration with Dr Curt Sigmund. The construct CMV-SEAP (secreted alkaline phosphatase gene under the control of the CMV promoter) was made from BglII/EcoRI-cut pSEAP-Basic and pIREs-EGFP.

#### Creation of Calu-6/P2Y11 Cell Line

Calu-6 cells in the log phase of growth were transduced with 20 μg of the pLEN-P2Y11-S construct by using 20 μL of Fugene-6 (Boehringer Mannheim). After 48 hours, the transfectants were selected for stable integrants by culturing the cells for 4 weeks in medium containing 500 μg/mL genetin (G418 sulfate, Gibco BRL). Thereafter, cells were maintained in 200 μg/mL of this antibiotic. The stably transfected cells were named Calu-6/P2Y11.

### RT-PCR of P2Y11 mRNA

RNA was extracted as described previously from Calu-6, Calu-6/P2Y11, and human embryonic kidney (HEK) 293 cells. cDNA was made with Superscript RT (Gibco BRL) by using 5 μg of DNase I-treated RNA (Ambion), according to the manufacturer’s instructions. PCR was performed by using cDNA prepared in both the presence and absence of RT to control for genomic DNA contamination. In addition, cDNA made from poly(A) RNA taken from human fetal liver, kidney, tests, liver, muscle, and spleen (Multiple First-strand cDNA) was purchased from OriGene Technologies and used for PCR. The PCR mixture contained 45 μL of PCR Supermix (Gibco BRL) and 0.5 μmol/L of PCR primers (forward, 5′-AGT CTA CAT CCT CAG C-3′; reverse, 5′-GGT ATT TTT CCG CTC C-3′; designed by using GenBank sequence number X00063). After an initial 2-minute hot start at 95°C for 2 minutes, PCR was performed for 30 cycles by the following protocol: 94°C for 45 seconds, 42°C for 45 seconds, and 72°C for 120 seconds. The resulting 273-bp PCR product was then gel-purified for 45 seconds, 57°C for 45 seconds, and 72°C for 60 seconds, followed by a final 10 minutes at 72°C. This yielded a 273-bp PCR product. cDNA purified from Calu-6/P2Y11 cells was used as a positive control. To control for the quality of the cDNA prepared from cell lines, PCR was performed with primers designed against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. In the case of human tissue samples, cDNA quality control involved PCR of β-actin cDNA with primers supplied by Origin. Laser densitometry was used to determine the relative intensity of each ethidium bromide-stained band on Polyaroid photographs of polyacrylamide gel electrophoresis (PAGE) gels.

### Northern Blotting

Total RNA was extracted from Calu-6 and Calu-6/P2Y11 cells as described above. Northern blot analysis with 40 μg of total RNA was performed as described previously. The REN probe was generated from human kidney cDNA by PCR with TaqGold (Perkin-Elmer) and 50 ng of the primers (forward, 5′-AAC TCA CCC TTC GCT ATT C-3′; reverse, 5′-GGG TGT ATT CTT TGG CTC C-3′; designed by using GenBank sequence number X00063). After an initial 2-minute hot start at 95°C for 2 minutes, PCR was performed for 30 cycles by the following protocol: 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 45 seconds, followed by a final 5 minutes at 72°C. The resulting 600-bp product was then gel-purified (BRESASpin gel extraction kit, GeneWorks), sequenced to confirm that it was correct, and labeled with [32P]dCTP (GeneWorks) by random-hexamer labeling (Megaprime DNA labeling system, Amersham). All blots were stripped and rehybridized with a probe for GAPDH mRNA, as described previously.

### Western Blotting

Western blot analysis was performed on whole-cell lysates of Calu-6 and Calu-6/P2Y11 cells collected by resuspending 4 × 10^6 cells in 2× SDS-PAGE loading buffer (8 M urea, 14% SDS, 4% Triton X-100, pH 8.2), and 10% vol/vol glycerol; 4% vol/vol SDS; 0.01% bromophenol blue; and 5 mmol/L DTT). The samples were boiled before being loaded onto a 10% SDS-PAGE gel. Protein on the gel was then transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was blocked overnight and then incubated for 2 hours with mouse monoclonal antibody to human renin (Hoffman–La Roche) to a rabbit antibody to the phosphorylated form of CREB (New England Biolabs) or to the CREB of phosphorylation-independent status (New England Biolabs). For detection of renin, the membrane was then washed and incubated with a rabbit anti-mouse antibody conjugated to alkaline phosphatase (Sigma) for 2 hours. In the case of phosphoCREB and CREB, the relevant blot was washed and incubated with a goat anti-rabbit IgG conjugated to alkaline phosphatase for 2 hours. The blots were developed by exposure to Sigma....
Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution for 2 to 5 minutes.

Transfection and Luciferase Assays
Calu-6 or Calu-6/P2Y11 cells (2×10^5 per well) were plated into a 12-well plate and transfected with 5 μg of −900luc or −900mut vector, plus 0.1 μg of CMV-SEAP as an internal positive control. The cells were transfected with 7 μL of Fugene-6 as described above. At 24 hours after transfection, the medium was replaced and 1 mmol/L ATP was added. After 24-hour incubation, the cells were harvested by scraping and a luciferase assay was performed by using a commercially available kit (luciferase assay system, Promega). All luciferase assays were performed in duplicate and the values averaged to obtain n=1. The data shown represent the mean±SEM of 3 independent experiments. SEAP activity was measured in the medium of transfected cells by using a kit (Great EscAPE SEAP, Clontech). The data were normalized with respect to the luciferase activity of cells transfected with −900luc. Standard curves for luciferase and SEAP assays were constructed to ensure that all samples were analyzed within the linear range of the assay.

Results

P2Y_{11} mRNA in Calu-6/P2Y11 Cells and Other Tissues
A strong RT-PCR product of the expected size was obtained for Calu-6/P2Y11 cells (Figure 1A), indicating overexpression of P2Y_{11} mRNA. A band of similar size but of weaker intensity was also seen for Calu-6 cells (Figure 1A). An RT-PCR product indicative of P2Y_{11} mRNA was also seen in HEK 293 cells (Figure 1A), as well as in (in order of intensity) human fetal brain, kidney, testis, muscle, liver, and very weakly, in spleen (Figure 1B).

ATP Upregulates Renin Expression in Calu-6/P2Y11 Cells
In response to 1 mmol/L ATP, Calu-6/P2Y11 cells showed a time-dependent upregulation of renin mRNA, as seen on Northern blots (Figure 2A). The elevation in renin transcript was 2.0-fold after 24 hours and reached 2.4-fold by 36 hours. Western blot analysis with renin antibody showed the presence of a band of ≈45 kDa, a size similar to that of mature renin protein (Figure 2B). In response to ATP, renin increased 3.0-fold within 16 hours and remained elevated for the duration of the experiment (36 hours). In contrast, Calu-6 cells did not show an increase in renin mRNA or protein in response to ATP (Figure 2B).

Renin Upregulation Occurs via Activation of the REN Promoter
To determine whether binding of ATP to P2Y_{11} served to activate signal transduction pathways that stimulated the REN promoter, we performed transient transfection analyses in-
volving Calu-6/P2Y11 cells and a construct containing 896 bp of the human REN promoter fused to the luciferase gene as the reporter. We tested constructs that contained either the native (–900luc) or a nonfunctional (–900mut) CRE. Calu-6/P2Y11 cells transfected with –900luc showed a 5.7-fold enhancement of luciferase production over unstimulated cells when exposed to 1 mmol/L ATP for 24 hours (Figure 3A). In contrast, the P2Y2 agonist UTP had little effect (Figure 3A). Metabolites of ATP, viz, ADP, AMP, and adenosine (each at 1 mmol/L) also had a much lower or no effect in stimulation of reporter gene activity (Figure 3B). For ATP-treated Calu-6/P2Y11 cells transfected with –900mut, no enhancement in luciferase activity was seen; rather, a decrease was noted (Figure 3C). This finding is consistent with involvement of the CRE in stimulation of the REN promoter under basal conditions present during culture of these cells. ATP elicited an increase in reporter expression for –900mut. This attained a level similar to that seen for the unstimulated native construct –900luc. Such an increase may reflect the contribution to the overall cAMP response of a Pit-1 site at –77 to –67, as reported for transient transfection studies of Calu-6 cells, as well as chorion cells in primary culture and non–renin-expressing pituitary GC cells. In contrast to Calu-6/P2Y11 cells, ATP had no effect on luciferase reporter activity in Calu-6 cells transfected with –900luc (Figure 3D).

Figure 3. Transient transfection results showing response of the –900luc-REN promoter-luciferase construct to ATP, as well as metabolites and analogues in Calu-6/P2Y11 or Calu-6 cells (n=3). A, Reporter activity of Calu-6/P2Y11 cells after 24-hour exposure to 1 mmol/L ATP or UTP. B, Reporter activity after 24-hour exposure to 1 mmol/L ATP, ADP, AMP, or adenosine (Ado). C, Effect of mutation of the CRE in REN promoter DNA (–900mut) in untreated cells and after 24-hour treatment with 1 mmol/L ATP. D, Effect of ATP on reporter activity in –900luc-transfected Calu-6 cells. Luciferase activities were corrected for transfection efficiency by means of cotransfected SEAP gene under control of the CMV promoter and then normalized with respect to the activity obtained for untreated –900luc-transfected cells in each experiment. All experiments were performed 3 times in duplicate, and values shown are mean±SEM. Probability values shown are for t tests vs control. In C, we also compared data for –900mut with –900mut + ATP. This gave P=0.0054.

Renin Upregulation Occurs via Phosphorylation of CREB
To further assess the downstream effects of ATP in Calu-6/P2Y11 cells, we performed Western blotting to determine whether there was a response in phosphorylation of CREB. This revealed a time-dependent phosphorylation of both CREB and activating transcription factor-1 (ATF-1) to 10- and 5-fold, respectively, of basal levels by 15 minutes,
returning to baseline 2 hours after ATP was added (Figure 4). The same antibody binds CREB and ATF-1; ie, it does not discriminate between these closely related transcription factors, but each could be distinguished on PAGE by size difference: 38 versus 43 kDa for ATF-1 and CREB, respectively.

**Discussion**

We report the production of a new cell line involving transfection of Calu-6 cells, a popular line used to study renin expression, with a stably integrated P2Y11 cDNA under control of the CMV promoter. This line, Calu-6/P2Y11, was found to produce much higher concentrations of P2Y11 mRNA than the parent line of Calu-6 cells. Moreover, expression in Calu-6 cells appeared to be of a similar order of magnitude as in several other tissues, some of which (testis, muscle, and liver) have not previously been reported to express P2Y11 mRNA. We also found evidence of P2Y11 mRNA expression in HEK 293 cells, and others have observed P2Y11 in MDCK-D1 cells, a well-differentiated kidney epithelial cell line derived from distal tubule/collecting duct.

Consistent with their expression of P2Y11 purinoceptors, Calu-6/P2Y11 cells showed increases in renin and renin mRNA in response to ATP, and in transient transfection experiments, the relative response to different ATP metabolites was in accord with their order of potency in binding to this purinoceptor subtype. We found, however, that ATP did not affect renin mRNA concentration in Calu-6 cells, despite the presence of P2Y11 mRNA, albeit at a level lower than in Calu-6/P2Y11 cells. This finding suggests that P2Y11 mRNA is not translated or that there is some other impediment to P2Y11 production, a problem with insertion into the plasma membrane of the cell, or in P2Y11 function in Calu-6 cells. Although the presence of P2Y receptors in Calu-6 cells has not been investigated previously, the closely related Calu-3 cell line has been reported to express the ADP-selective P2Y1 receptor.

Stimulation of the P2Y1 purinoceptor has been shown to result in elevations in cAMP in MDCK-D1 cells. However, this occurred by indirect stimulation of adenylyl cyclase; since it was inhibited by indomethacin, the pathway utilized involved the generation of arachidonic acid, whose conversion to prostaglandins leads to prostaglandin receptor-mediated activation of adenylyl cyclase. Moreover, this mechanism has only been reported to exist in MDCK-D1 cells, which do not express renin. Furthermore, the activation of P2Y2 cannot explain the indomethacin-insensitive 2-methylthio-ATP-mediated elevation of cAMP in these cells, leading the authors to suggest that 2-methylthio-ATP could be functioning via the P2Y11 receptor, which is expressed in MDCK-D1 cells.

The present study supports the likelihood that the Calu-6/P2Y11 cell line may prove to have general utility in studies of various aspects of P2Y11 receptor function. In our experiments, the increase in renin mRNA and protein was confined to Calu-6/P2Y11 cells that we made to overexpress P2Y11 mRNA, being absent in the parent Calu-6 line. Thus, the effects of ATP on renin expression that we observed would have to involve the P2Y11 purinoceptor.

Previously, forskolin, which stimulates adenylyl cyclase in a receptor-independent manner, has been found to activate the human renin promoter in Calu-6 cells by a mechanism involving the CRE at –222 and phosphorylation of CREB. A number of receptors, such as beta-adrenoceptors and adenosine A1 receptors, utilize cAMP pathways in triggering a response in renin secretion or expression. The present work shows that P2Y11 purinoceptor stimulation by ATP leads to phosphorylation of both CREB and ATF-1, as well as an increase in renin promoter activity that is primarily dependent on the renin CRE. CREB and ATF-1 are present in cells as homodimers or heterodimers. The rapid effect in bringing about CREB/ATF-1 phosphorylation suggests that P2Y11 is capable of being responsive to acute stimuli, such as are incurred by vascular injury or trauma. Furthermore, the detection of P2Y11 in human myocardium and kidney (Figure 1) shows that P2Y11 has the potential to play a role in the pathogenesis of cardiomyopathy and renal disease, conditions that are associated with an elevated release of ATP by damaged cells. A role for the renin-angiotensin system has been invoked in such pathological conditions, and renin expression, which is normally absent in cardiac and renal tubular structures, appears de novo under conditions of strong stimulation to renin expression, as well as in myocardial infarct borders and proximal tubule cells in diabetic nephropathy. The latter pathological conditions are associated with cell damage and release of high concentrations of ATP. The elevated extracellular ATP in renal disease may increase renin expression from juxtaglomerular cells and also by activating local renin systems in the proximal tubule, which accounts for 60% of cells in the kidney, as well as in extrarenal tissues, and which could contribute to elevations in local and systemic angiotensin.

There is suggestive evidence for the presence of P2Y11 on juxtaglomerular cells that could mediate the stimulation of renin secretion by ATP. P2Y11 has been implicated in granulocytic differentiation of HL-60 and NB4 cells. Thus, the possibility of a role for P2Y11 in the development and maturation of afferent arterioles of the kidney, where renin is located, and in the metaplasia of renin-expressing cells merits consideration.

In conclusion, ATP can stimulate the renin promoter via a P2Y11 purinergic mechanism. We suggest that this involves the cAMP signal-transduction pathway. Such transcriptional
effects, together with possible posttranscriptional effects of cAMP that increase renin mRNA stability, could be of physiological and pathophysiological significance in eliciting renin production. Further work will, however, be needed to establish that such a mechanism operates in native renin-producing cells.

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