# Evidence for Basolateral $P2Y_6$ Receptors along the Rat Proximal Tubule: Functional and Molecular Characterization

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*Abstract.* In this study, the distribution of $P2Y_6$ receptor mRNA in rat nephron segments was investigated and a functional approach was used to analyze basolateral protein expression. Reverse transcription-PCR studies revealed more intense expression of $P2Y_6$ receptor mRNA in the proximal tubule and the thick ascending limb of Henle's loop, less intense expression in the thin descending limb and the cortical and outer medullary collecting ducts, and no detectable expression in either the thin ascending limb or the inner medullary collecting duct. Dose-dependent calcium responses to basolateral administration of UDP (a selective agonist for the $P2Y_6$ receptor) were observed in the proximal tubule but not in any of the other segments studied. In the proximal tubule, intracellular calcium concentration changes induced by UDP were associated with increased production of inositol phosphates, as were those induced by ATP and norepinephrine. However, UDP-induced intracellular calcium concentration changes were different, exhibiting no plateau after the initial peak; moreover, a single stimulation with a high concentration of UDP induced full desensitization of the UDP-sensitive calcium pathway but did not alter the responsiveness of the proximal tubule to ADP (a specific $P2Y_1$ receptor agonist), ATP, or norepinephrine. In summary, this report demonstrates that $P2Y_6$ receptor mRNA is expressed in most segments of the rat nephron but that basolateral expression of the protein is restricted to the proximal tubule, where the receptor is coexpressed with the $P2Y_1$ receptor. The differences in the distributions of $P2Y_6$ receptor mRNA and UDP responses may indicate the presence of luminal receptors in other nephron segments.

A recent report demonstrated that several cell types, including epithelial cells, release significant amounts of UTP (1). This suggests that uridine compounds, such as UTP and UDP, are potentially important extracellular signaling molecules. The uridine-activated receptors, *i.e.*, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors, are subtypes within the larger P2Y receptor family, and these receptors are widely distributed in many tissues. UTP and ATP are equipotent at $P2Y_2$ and $P2Y_4$ receptors (2–4), whereas $P2Y_6$ receptors are preferentially activated by UDP, with ATP and ADP being only weakly active (5–7). Functionally, all of these receptors are coupled to phospholipase C (PLC), and activation leads to transient increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) (8,9). As demonstrated by molecular and functional studies of cultured (10–17) and native (18–22) epithelial cells, several different P2Y subtypes are located in the luminal membrane, the basolateral membrane, or both membranes; these subtypes can be coexpressed in the same cells, all participating in the regulation of fluid and/or electrolyte transport.

In the kidney, reverse transcription-PCR (RT-PCR) studies have demonstrated the presence of $P2Y_2$ and $P2Y_4$ receptor mRNA in most segments of the nephron (23). Functional studies have also demonstrated that UTP and ATP generate calcium signals along the whole renal tubule (23–26); however, these agonists are equipotent at the rat $P2Y_2$ and $P2Y_4$ receptors, and whether the signals involve one or the other (or both) of these receptor subtypes cannot be determined. It is also not possible to know whether responses are mediated via apical or basolateral membrane receptors, because $P2Y_2$ receptor-specific immunoreactive sites were recently observed in both membranes of inner medullary collecting duct (IMCD) cells (27). In addition, ATP- and UTP-sensitive receptors were detected in both the luminal and basolateral membranes of the mouse cortical collecting duct (CCD) (28).

Relatively little is known regarding the function of $P2Y_6$ receptors, although recent reports suggested that UDP-sensitive receptors can activate ion channels in a variety of polarized epithelia, such as sweat gland (12,15,29), gallbladder (20), pancreatic duct (22), and nasal (13) cells. Northern hybridization studies detected $P2Y_6$ receptor mRNA expression in several different tissues (*e.g.*, spleen, placenta, thymus, intestine, and blood leukocytes), but expression was observed at a very low level in the kidney (5,6). However, it is possible that this receptor has only limited distribution within the nephron and

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that Northern analysis of whole-kidney extracts could not detect this. Therefore, the aim of this study was to screen for $P2Y_6$ receptors in segments of the rat nephron. The RT-PCR technique was used to study the distribution of mRNA; because specific antibodies directed against the rat $P2Y_6$ receptor are not yet available, we analyzed the functional expression of the protein by measuring inositol phosphate (IP) production and associated changes in $[Ca^{2+}]_i$ in response to UDP. Our results demonstrate that $P2Y_6$ receptor mRNA is expressed in most segments of the rat nephron but basolateral expression of the protein, as assessed functionally, is restricted to the proximal tubule, where the receptor is coexpressed with the $P2Y_1$ receptor.

## Materials and Methods

### *Isolation of Rat Nephron Segments*

The left kidneys of male Sprague-Dawley rats anesthetized with sodium pentobarbital (Nembutal; 50 mg/kg body wt, administered intraperitoneally) were perfused, via the renal artery, with 5 ml of Hepes-buffered saline solution (HBSS; 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4$, 0.33 mM $Na_2HPO_4$, 0.44 mM $NaH_2PO_4$, 1.0 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 5 mM NaOH, 5 mM glucose [pH 7.4]), followed by the same volume of a 0.16% collagenase solution (Serva; Boehringer Mannheim, Mannheim, Germany). Thin corticomedullary slices were then incubated in 0.12% collagenase solution for 20 min at 30°C before segments were isolated in microdissection medium (HBSS to which 0.1% bovine serum albumin had been added) at 4°C. For proximal tubules, only non-collagenase-treated kidneys were used (isolated from rats of 90 to 100 g body wt), because collagenase treatment adversely affects responses to calcium-mobilizing agents. Pyruvate (1 mM), glycine (4 mM), and glutamate (2 mM) were included in the proximal tubule microdissection medium. All tubule samples were then stored in a droplet of microdissection medium (2 $\mu$l) at 0 to 4°C until use.

The following segments were studied: nonlocalized proximal convoluted tubules (PCT) taken from the kidney cortex or, when specified, S1 segments identified on the basis of functional criteria (see the Results section), proximal straight tubules taken from medullary rays along the S3 portion of the proximal tubules, type II thin descending limbs (DTL) of juxtamedullary nephrons, thin ascending limbs (ATL), cortical thick ascending limbs, medullary thick ascending limbs (MTAL), CCD, outer medullary collecting ducts (OMCD), and IMCD. DTL, MTAL, and OMCD were all isolated from the inner stripe of the outer medulla.

### *Extraction of mRNA*

RNA was extracted from microdissected segments by using a micromethod (30) adapted from the guanidinium thiocyanate-phenol/chloroform method (31). Briefly, pools of individual segments were photographed for length determinations before being transferred, with 5 to 10 $\mu$l of microdissection medium, into 400 $\mu$l of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 mM $\beta$-mercaptoethanol, 20 $\mu$g of yeast tRNA). After phenol/chloroform extraction and isopropyl alcohol precipitation, the final pellet was vacuum-dried and resuspended in RNA dilution buffer [10 mM Tris-HCl [pH 7.6], 1 mM ethylenediaminetetraacetate, 2 mM dithiothreitol, 40 U/ml ribonuclease inhibitor (RNasin; Promega, Madison, WI)]. It was previously determined that the yield of this extraction procedure was >90% (30).

### *RT-PCR*

Specific primers were selected from the sequence of the rat $P2Y_6$ receptor cDNA and from the sequence of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA by using Oligo Primer analysis software (Medprobe, Oslo, Norway). The sequences of the $P2Y_6$ receptor primers were 5'-TGCTTGGGTGGTATGTGGAGTC-3' (positions 868 to 889) for the forward primer and 5'-TGGAAAGGCAGGAAGCTGATAAC-3' (positions 1206 to 1184) for the reverse primer. The sequences of the GAPDH primers were 5'-GCCATCAATGACCCCTTCAT-3' (positions 154 to 173) for the forward primer and 5'-GAGGGGGCAGAGATGATGAC-3' (positions 434 to 415) for the reverse primer.

For each segment, mRNA extracted from 1-mm tubule length (approximately 60 pg of mRNA) was reverse-transcribed for 50 min at 42°C with 0.5 $\mu$g of oligo$(dT)_{12–18}$ primer, using a first-strand cDNA synthesis kit for RT-PCR (Superscript II RNase $H^-$ reverse transcriptase; Invitrogen Ltd., Paisley, UK). After denaturation at 95°C for 3 min, the resulting product was used as a template with the PCR Core System I (Promega). For the $P2Y_6$ receptor, 40 PCR cycles were performed under the following conditions: 95°C for 30 s, 62°C for 1 min (annealing), and 72°C for 1 min (extension) for five cycles; 95°C for 30 s, 56°C for 1 min, and 72°C for 1 min for 34 cycles, with a last cycle with a 10-min extension stage. For GAPDH, only 30 cycles were performed, as follows: 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min for 29 cycles, plus a last cycle with a 10-min extension stage.

The resulting PCR products were resolved on a 2% (wt/vol) agarose gel containing 10 $\mu$g/ml ethidium bromide and were observed under ultraviolet illumination. For the $P2Y_6$ receptor, the nature of the PCR product (expected size, 339 bp) was confirmed by sequencing (Oswel DNA Sequencing Laboratories, Southampton, UK). In all experiments, the presence of possible contaminants was investigated using control RT-PCR assays of samples in which either mRNA had been excluded (blank) or reverse transcriptase had been omitted from the RT mixture.

### *Measurement of $[Ca^{2+}]_i$*

$[Ca^{2+}]_i$ was measured using previously described methods (32). Each tubular segment was loaded with fura2/acetoxymethyl ester (10 $\mu$M in microdissection medium; Molecular Probes, Eugene, OR) for 1 h at room temperature and was subsequently transferred to a superfusion chamber, where the ends of the segment were aspirated into two glass holding pipettes (to prevent possible perfusion of the luminal membrane). The segment was then superfused with HBSS, at a rate of 10 ml/min, for a 5-min equilibration period. Fura2 fluorescence was measured in a tubular portion of 20 to 30 cells, using a standard photometric configuration (model MSP 21; Zeiss, Jena, Germany), during superfusion with either HBSS alone or HBSS to which agonists had been added. All solutions were stored in individual reservoirs at room temperature until use; the temperature of each solution was increased to 37°C just before introduction into the perfusion chamber.

After subtraction of tubular autofluorescence from the fura2 fluorescence intensities at 340 and 380 nm, $[Ca^{2+}]_i$ was calculated using the equation presented by Grynkiewicz *et al.* (33), with a calcium dissociation constant for fura2 of 224 nM. The calibration parameters were determined from internal calibration assays using solutions containing 10 $\mu$M ionomycin and both 0 mM $Ca^{2+}$ and 1 mM ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid (for the minimal ratio) or 3 mM $Ca^{2+}$ (for the maximal ratio).

### *Measurement of Phosphoinositide Metabolism*

The method used was previously described in detail (34,35). Briefly, well preserved pieces of proximal tubules (*i.e.*, a mixture of S1, S2, and S3 cortical fragments) were sorted from tubule suspensions prepared, by sieving, from non-collagenase-treated rat kidney cortex (35). OMCD were microdissected from collagenase-treated rat kidneys, as described above. All tubules were labeled for 2 h in HBSS (2 mM $Ca^{2+}$) containing 1 mCi/ml *myo*-[$^3$H]inositol (Amersham, Buckinghamshire, UK), in a humid atmosphere at 37°C.

After labeling, segments were extensively rinsed at room temperature under stereomicroscopic observation, for removal of extracellular *myo*-[$^3$H]inositol, and then batches of 10 segments (approximately 5-mm tubule length) were transferred, in 2 $\mu$l of HBSS, to glass test tubes containing 48 $\mu$l of HBSS (1 mM $Ca^{2+}$). Incubations with agonists were initiated by the addition of 50 $\mu$l of HBSS (1 mM $Ca^{2+}$) containing the agent (at double concentration) and LiCl (final concentration, 10 mM).

Reactions were terminated after 15 min by the addition of 940 $\mu$l of chloroform/methanol (1:2, vol/vol) and 200 $\mu$l of 5 mM Tris-ethylenediaminetetraacetate (pH 7.0). Water and chloroform (300 $\mu$l of each) were added to the reaction tube, which was then centrifuged for 5 min at 3000 $\times$ *g* at 4°C for separation of the phases. For each sample, the upper hydrophilic phase was removed for chromatography, whereas 500 $\mu$l of water and 500 $\mu$l of methanol were added to the remaining hydrophobic phase. After vortex-mixing and repeat centrifugation, the lower phase, containing phosphoinositides (PI), was recovered and evaporated to dryness in counting vials before measurement of radioactivity.

Radioactivities associated with free inositol, glycerophosphoinositol (GPI), and IP were separated by chromatography, as described previously (35). Samples were applied to columns containing 0.25 g of Dowex AG1-X8 resin, and free inositol, GPI, and IP were successively and respectively eluted with the following: (*1*) 3 mM Hepes-buffered water (pH 7.0), (*2*) 30 mM ammonium formate, and (*3*) 1 M ammonium formate and 0.1 M formic acid. The radioactivity contained in each eluate and in the PI extract was counted by $\beta$-emission spectrometry, after the addition of 15 ml of Aquasol-2 scintillation cocktail (Packard Bioscience Ltd., Pangbourne, UK) to each vial. IP production was expressed as a percentage of the total radioactivity present in the sample (inositol plus GPI plus IP plus PI), because it was previously demonstrated that there is a good correlation between IP formation expressed in this way and that expressed per unit of tubule length (35,36).

### *Statistical Analyses*

All data are presented as mean $\pm$ SEM. Statistical comparisons were made using *t* tests for paired and unpaired samples and ANOVA, as appropriate. Statistical significance was taken as $P < 0.05$.

### *Chemicals*

UDP, ADP, and ATP (disodium salts), as well as arterenol bitartrate (norepinephrine), carbamyl choline chloride (carbachol), angiotensin II, and 8-arginine vasopressin, were purchased from Sigma (Poole, Dorset, UK). Because commercially available UDP contains as much as 10% UTP, the UDP stock solution was treated for 1 h with hexokinase (10 U/ml), in the presence of 25 mM glucose.

## Results

### *Distribution of $P2Y_6$ Receptor mRNA along the Rat Nephron*

For each segment, $P2Y_6$ receptor mRNA expression was investigated in three or four extracts. Results obtained from one extract are illustrated in Figure 1. Strong signals were repeatedly observed in both the proximal tubule (PCT and proximal straight tubule) and the thick ascending limb (cortical thick ascending limb and MTAL), and less intense bands were observed in the DTL, CCD, and OMCD. No signal could be observed in the ATL or IMCD (Figure 1). The absence of detectable $P2Y_6$ signal in the ATL was not attributable to its thin diameter (and thus a smaller amount of mRNA in each PCR tube), because comparable GAPDH signals were obtained for the DTL, ATL, and OMCD (Figure 1B).

### *Effects of UDP on $[Ca^{2+}]_i$ in Isolated Nephron Segments*

RT-PCR data indicated that $P2Y_6$ receptor mRNA is present in segments of the renal tubule, but they provide no information regarding expression of the protein. In the absence of an antibody against the $P2Y_6$ receptor, we measured UDP-stimulated changes in $[Ca^{2+}]_i$ in most segments containing mRNA, *i.e.*, the proximal tubule, the DTL, the MTAL, and the OMCD. As shown in Figure 2A, significant increases in $[Ca^{2+}]_i$ occurred in the proximal tubule. In contrast, the other segments were poorly responsive or unresponsive, although they were sensitive to other PLC-stimulating agonists used as controls (ATP or UTP for DTL and OMCD and angiotensin II for MTAL; results not shown). Compared with the results we obtained previously with ATP and ADP (23), the response to UDP in the proximal tubule was characterized by more intra- and interanimal variation (mean $[Ca^{2+}]_i$ increase, 365 $\pm$ 40 nM; $n = 33$). Nine of 42 tubules were observed to be insensitive to UDP, although they responded normally to either ATP (441 $\pm$ 74 nM, $n = 4$) or ADP (460 $\pm$ 81 nM, $n = 5$). The effect of UDP dose-dependently increased between 10 and 30 $\mu$M (Figure 2B). At all concentrations tested, the response was monophasic, with a single sharp peak before $[Ca^{2+}]_i$ returned to resting values (Figure 2C). In contrast, the responses to ATP and ADP were biphasic, consisting of a peak and then a lower plateau phase that was sustained throughout exposure to the agonist. Similarly, the responses to the other agonists used in this set of experiments (carbachol and norepinephrine) were biphasic (Figures 2C, 3, and 4).

Because the proximal tubule segments used in these initial experiments were microdissected at random from the kidney cortex and were not anatomically localized, it was possible that the scatter of UDP-induced $[Ca^{2+}]_i$ changes was physiologic and reflected different sensitivities of S1, S2, and S3 segments of the proximal tubule to this nucleotide. Therefore, subsequent experiments were performed with localized segments of proximal tubules. As stated in the Materials and Methods section, S3 segments were identified by anatomic criteria that could not be applied to S1 segments, because it is very difficult to microdissect proximal tubules attached to glomeruli in non-

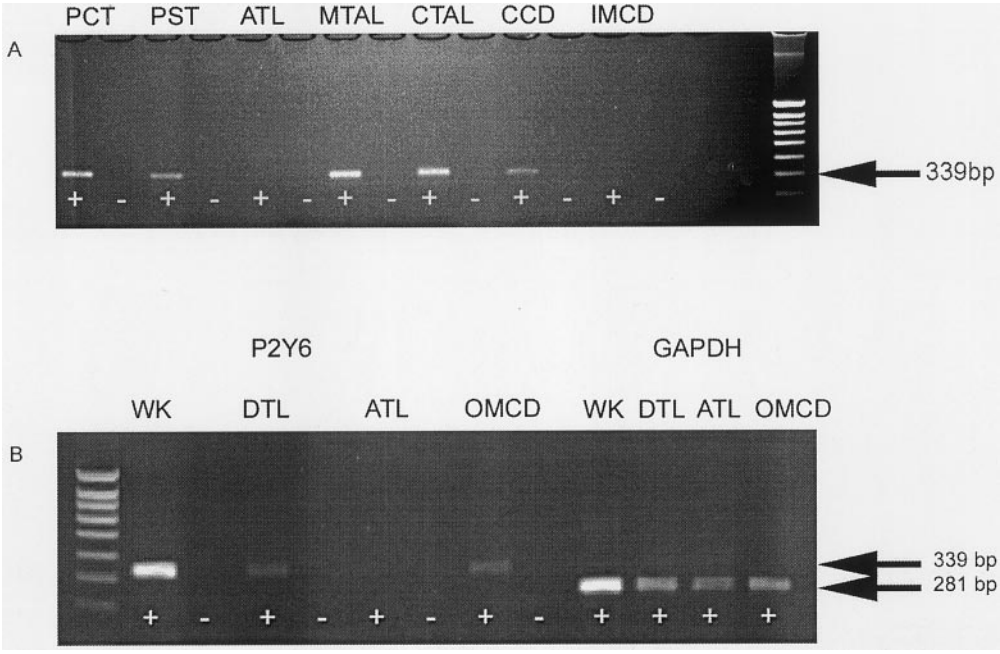


*Figure 1.* P2Y$_6$ receptor mRNA analysis. (A) Sample gel from one reverse transcription (RT)-PCR experiment, showing the distribution of P2Y$_6$ receptor mRNA in isolated segments of the rat nephron. PCT, proximal convoluted tubule; PST, proximal straight tubule; ATL, thin ascending limb; MTAL, medullary thick ascending limb; CTAL, cortical thick ascending limb; CCD, cortical collecting duct; IMCD, inner medullary collecting duct. + and −, samples assayed in the presence and absence of reverse transcriptase, respectively. (B) Comparison of P2Y$_6$ receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in whole-kidney (WK), thin descending limb of Henle's loop (DTL), thin ascending limb of Henle's loop (ATL), and outer medullary collecting duct (OMCD) extracts. For whole-kidney samples, 1 $\mu$g of total RNA (approximately 20 ng of mRNA) was used for RT-PCR; for DTL, ATL, and OMCD samples, we used 3 $\mu$l of extract (*i.e.*, the mRNA extracted from 1-mm tubule length) in each tube. Note that the GAPDH signal was greater in whole-kidney samples than in tubule samples, whereas signals in ATL, DTL, and OMCD samples were comparable. This finding indicates that GAPDH signals did not reach saturation in the tubule samples and that comparable amounts of mRNA were used for RT-PCR for DTL, ATL, and OMCD samples.

collagenase-treated rat kidneys. As an alternative, we used a functional criterion based on results from previous studies, which demonstrated that changes in $[Ca^{2+}]_i$ in response to carbachol decrease from S1 to S3, whereas responses to norepinephrine remain constant (35). Therefore, responses to UDP, carbachol, and norepinephrine were always measured in the same segments, using carbachol as an index of localization and norepinephrine as an index of tubule viability. Figure 3 presents sample traces obtained for the S1, S2, and S3 segments. The mean UDP-induced responses were not statistically different between the S1 segment ($\Delta[Ca^{2+}]_i = 340 \pm 73$ nM, $n = 8$) and the S3 segment ($\Delta[Ca^{2+}]_i = 425 \pm 30$ nM, $n = 10$), a finding that suggests that the proximal tubule is responsive to UDP along its entire length.

Finally, we observed that two consecutive responses to UDP (30 $\mu$M) did not occur in the same proximal tubule, even when stimulations were separated by 15 to 20 min of superfusion with control medium (data not shown). We took advantage of this result to examine whether this desensitization was specific for UDP or also applied to other nucleotides that had been previously demonstrated to stimulate PLC in this tubule segment (23). As illustrated by the trace in Figure 4, previous stimulation by UDP ($\Delta[Ca^{2+}]_i = 374 \pm 116$ nM, $n = 9$) prevented the effect of a second basolateral application of the nucleotide. However, these tubules remained sensitive to ADP ($\Delta[Ca^{2+}]_i = 440 \pm 118$ nM, $n = 6$), ATP ($\Delta[Ca^{2+}]_i = 333 \pm 89$ nM, $n = 3$), and norepinephrine ($\Delta[Ca^{2+}]_i = 404 \pm 63$ nM, $n = 5$), all of which were tested within the period of desensitization to UDP.

### *Effects of UDP on PI Metabolism in the PCT*

To confirm that the effects of UDP on $[Ca^{2+}]_i$ were mediated via G-protein-coupled receptor activation of the PLC pathway, we assessed the effects of this agent on PI signaling in isolated segments of the proximal tubule. Production of IP was also investigated in the OMCD, a segment that expresses P2Y$_6$ receptor mRNA (Figure 1) but weakly responds to basolateral UDP superfusion (Figure 2). As previously reported for the PCT (35), approximately 80% of the total radioactivity was incorporated as PI. In contrast, only 5% of the total radioactivity appeared in the PI fraction in the OMCD, as reported for the thick ascending limb of Henle's loop (36).

For both segments, the percentages of radioactivity present in the IP pool under different experimental conditions are presented in Table 1. In the PCT, UDP (30 $\mu$M) typically caused a fivefold increase in IP production, compared with basal values ($P < 0.01$). This increase was slightly, but not significantly, larger than that evoked by either ATP (used at the same concentration) or norepinephrine. In the OMCD, the P2Y$_6$ agonist also promoted a significant increase in the accu-

mulation of IP (three- to fourfold, $P < 0.05$), as did ATP (sixfold, $P < 0.01$) and arginine vasopressin (sevenfold, $P < 0.01$).

## Discussion

In this study, we used the RT-PCR technique to demonstrate the expression of $P2Y_6$ receptor mRNA in whole kidney and we mapped the distribution of this receptor in specific renal tubular segments. $P2Y_6$ receptor mRNA was expressed mainly in the proximal tubule and thick ascending limb of Henle's loop, but receptor transcripts were also identified in the DTL, the CCD, and the OMCD. Under our experimental conditions, there was no evidence for $P2Y_6$ receptor mRNA in either the ATL or the IMCD. The absence of a positive $P2Y_6$ receptor mRNA signal in the ATL is likely to be a real finding, because it was confirmed with different mRNA extracts, all of which demonstrated positive signals for GAPDH (see the example in Figure 1); moreover, the same extracts were previously demonstrated to be positive for $P2Y_2$ and $P2Y_4$ receptor mRNA (23).

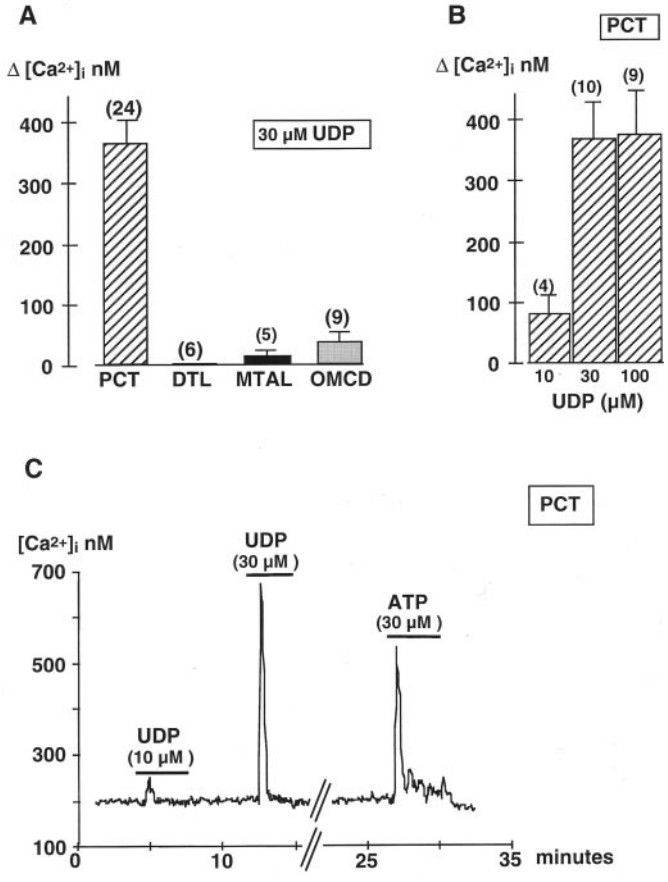


*Figure 2.* Pattern of UDP-induced intracellular calcium concentration ($[Ca^{2+}]_i$) changes along the nephron. (A) $[Ca^{2+}]_i$ increases measured in nonlocalized PCT, DTL, MTAL, and OMCD. Bars correspond to mean ± SEM $[Ca^{2+}]_i$ increases (peak − basal levels) calculated for several tubules (numbers in parentheses) from at least four different rats. (B) Dose-dependent effects of UDP on $[Ca^{2+}]_i$ in PCT (mean ± SEM, *n* in parentheses). (C) Representative trace comparing the effects of UDP and ATP on $[Ca^{2+}]_i$ in PCT.

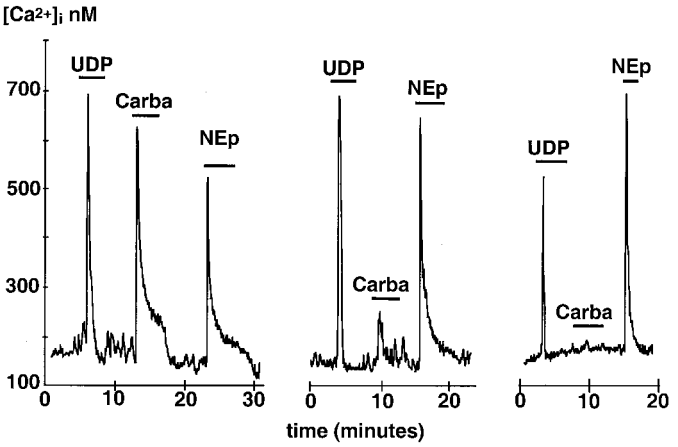


*Figure 3.* Representative traces, illustrating the effects of 30 μM UDP on $[Ca^{2+}]_i$ in different portions of the proximal tubule (S1, S2, and S3 segments). S3 segments were isolated from the cortex on the basis of anatomic criteria (see the Materials and Methods section); as explained in the Results section, S1 and S2 segments were identified on the basis of their different responsiveness to carbachol (Carba). Norepinephrine (NEp) was used as an index of tubule functional integrity.

The $P2Y_6$ receptor is functionally coupled to PLC, and receptor activation leads to transient increases in $[Ca^{2+}]_i$ in several cell types (5–7). We used this coupling to examine the functional expression of renal $P2Y_6$ receptors, by measuring changes in $[Ca^{2+}]_i$ during the application of UDP to the basolateral membrane. In all segments of the proximal tubule (S1,

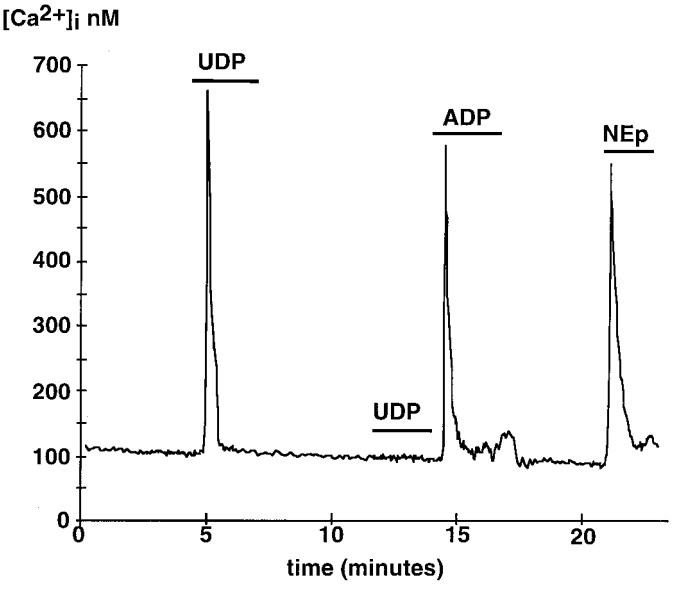


*Figure 4.* Representative trace, illustrating homologous desensitization of the calcium response to UDP after a single stimulation with the nucleotide (30 μM). Note that the responses to 30 μM ADP (an agonist specific for $P2Y_1$ receptors) and to 10 μM norepinephrine (NEp) were not blunted.

*Table 1.* Effects of phospholipase C-coupled agonists on IP accumulation in proximal tubules and OMCD[a]

| Condition | IP Production (% of Total Radioactivity) | |
|---|---|---|
| | Proximal Tubules | OMCD |
| Basal | 1.76 ± 0.22 | 0.22 ± 0.04 |
| UDP (30 $\mu$M) | 9.82 ± 2.43[b] | 0.79 ± 0.40[c] |
| ATP (30 $\mu$M) | 7.06 ± 1.53[c] | 1.44 ± 0.40[b] |
| Norepinephrine (10 $\mu$M) | 6.78 ± 1.28[c] | Not tested |
| AVP (100 nM) | Not tested | 1.61 ± 0.36[b] |

[a] Values are means ± SEM of 20 determinations (four rats) from samples incubated in the absence (basal level) or presence of UDP, ATP, or a positive control agent [norepinephrine and arginine vasopressin (AVP) for proximal tubules and outer medullary collecting ducts (OMCD), respectively] before determination of radioactivity in [$^3$H]inositol-containing pools. For each experimental condition, inositol phosphate (IP) production was expressed as the percentage of total radioactivity incorporated in the sample.

[b] $P < 0.01$ *versus* basal levels (one-way ANOVA).

[c] $P < 0.05$.

S2, and S3), expression in the basolateral membrane was confirmed by the finding that peritubular UDP caused a dose-dependent increase in $[Ca^{2+}]_i$ (Figures 2 and 3). This probably represents (at least in part) release of calcium from intracellular stores, because IP production was also stimulated by UDP (Table 1). Unlike the proximal tubule, more distal tubular segments that expressed $P2Y_6$ receptor mRNA (Figure 1) did not respond to basolateral UDP superfusion with clear-cut increases in $[Ca^{2+}]_i$ (Figure 2); these findings were noted for the DTL, the whole thick ascending limb, the CCD, the IMCD, and the OMCD. These data may indicate that the receptor is only weakly coupled to PLC in these segments or that it is expressed exclusively in the luminal membrane. In support of the latter possibility, several studies have established the presence of luminal $P2Y_6$ receptors (12,13,15,22). However, our study did not allow us to address this question, because UDP had no access to the luminal membrane in our calcium experiments (see the Materials and Methods section). In contrast, the tubules used for the measurement of IP generation did have free open ends and were incubated for up to 15 min in the presence of UDP. Therefore, it is possible that the fourfold stimulation of IP production by UDP in the OMCD reflects activation of luminal receptors (Table 1), but contrary to this interpretation is the recent finding that UDP did not alter $[Ca^{2+}]_i$ when applied to the lumen of microperfused mouse CCD (28). However, there are species differences in the distribution of other P2Y receptors along the nephron, as well as their cellular localization. For example, levels of functional expression of $P2Y_2$ receptors in the basolateral membrane of the thick ascending limb are high in mice (25) but low in rats, despite the presence of mRNA (23), and evidence for luminal expression of $P2Y_2$ receptors has been observed in mouse CCD but not in rabbits (28). Such species differences could also apply to the $P2Y_6$ receptor, and the development of specific antibodies to the $P2Y_6$ receptor would help address this issue.

Data in the literature have established that $P2Y_1$ receptors (23,26) and, to a lesser extent, $P2Y_2$ and/or $P2Y_4$ receptors (23) are expressed in the basolateral membrane of rat proximal tubules. This raises the question of whether UDP responses actually reveal the presence of an additional P2 receptor (Y or X) or whether they result from interactions with other P2 receptor subtypes. Four pieces of evidence support UDP-induced $[Ca^{2+}]_i$ changes as reliable indicators of the presence of the $P2Y_6$ receptor. The first is that UDP exposure was associated with increased IP production, a finding that cannot be accounted for by UDP interactions with ionotropic P2X receptors. The second is that tubular segments that were previously demonstrated to express PLC-coupled $P2Y_2/P2Y_4$ receptors (DTL and OMCD) and $P2Y_1$ receptors (OMCD) in their basolateral membranes (23) were insensitive to UDP (Figure 1). The third is that the transient monophasic increases in $[Ca^{2+}]_i$ elicited by UDP in proximal tubules are unlike the biphasic responses to other P2Y receptor agonists, such as ATP and ADP (Figures 2 to 4). This suggests that renal $P2Y_6$ receptors may be coupled to different effector pathways, compared with $P2Y_1$ and $P2Y_2$ receptors. Indeed, a recent study reported that $P2Y_6$ receptors expressed in *Xenopus* oocytes are coupled to only one class of G-protein, whereas $P2Y_2$ receptors are coupled to two (37). There is also a large body of evidence indicating that different P2Y receptor subtypes expressed in the same cells activate different signal transduction pathways [see the report by Communi *et al.* (9)]. The fourth piece of evidence is our previous demonstration that no desensitization occurred when several successive stimulations with maximal concentrations of ATP and/or ADP were administered to proximal tubules (23). In contrast, in our present experiments we observed that a single UDP stimulation prevented further responses to this nucleotide but did not affect the ability of the proximal tubule to respond to ATP or ADP (Figure 4). Taken together, the data suggest that the response to UDP involves a receptor that is distinct from the ADP- and ATP-sensitive $P2Y_1$ subtype.

We have not analyzed the mechanisms underlying the desensitization of the calcium response to UDP, *i.e.*, whether it occurs at the receptor level or further along the PLC/$[Ca^{2+}]_i$ pathway. Whatever the mechanism, this desensitization process, which seems to be specific for the $P2Y_6$ receptor, may explain why the effects of UDP on $[Ca^{2+}]_i$ were more variable in magnitude than were those of ATP and ADP (see the Results section). Little is presently known regarding the source and identity of the natural ligands for the $P2Y_6$ receptor (1,9). It could be that abnormal nucleotide release, because of the conditions of the study animals and/or the tubule preparations, accounts for the variability in UDP-induced calcium effects; such variation in UDP effects has already been described for pancreatic duct cells (22). Preferential desensitization of $P2Y_6$ receptor-induced responses, compared with $P2Y_2$ receptor-induced calcium responses, has also been reported for nasal epithelium (13).

The maximal ATP-induced effects on IP production and

$[Ca^{2+}]_i$ were in the range of those induced by UDP or ADP alone, which may mean that the $P2Y_6$ receptor is only weakly sensitive to ATP in rat proximal tubules, as it is when expressed in *Xenopus* oocytes (5) and other cell types (6,15,20,38). If this is true, then the effects of ATP must reflect mainly interactions with the basolateral $P2Y_1$ receptor. Another possibility is that the $P2Y_6$ and $P2Y_1$ receptor subtypes are present in the same cells and hydrolyze the same pool of membrane PI and that a single stimulation of either one at high concentrations is sufficient to induce maximal effects on IP production and intracellular calcium release. These alternatives are not mutually exclusive, because we already have evidence for such a coupling between $P2Y_1$ and $P2Y_2$ receptors in the same cell type in the rat OMCD (23). If $P2Y_6$, $P2Y_1$, $P2Y_2$, and/or $P2Y_4$ receptors are coexpressed in the basolateral membrane of the same proximal tubule cells, then the important question is whether these nucleotides can induce similar or distinct physiologic responses (9). Perhaps there is some necessary functional overlap or redundancy. Published data from studies on the secretory epithelium of upper respiratory airways and exocrine glands indicate that different P2Y receptors coexpressed in the same cells all stimulated PLC and activated chloride currents (9). Results obtained in $P2Y_2$ receptor-deficient mice indicate that the apical $P2Y_6$ receptor can substitute for the inactivated $P2Y_2$ receptor to regulate chloride secretion in the gallbladder (20). However, in addition to this common regulatory pathway, other data indicate that different (10,37) or the same (38) P2Y receptors can couple to different G-proteins in the same cell to activate different isoforms of PLC (39) and thus regulate different effectors (37,38). This may result in synergistic or opposing effects on cell functions. G-protein-dependent inhibition of calcium and potassium currents by $P2Y_6$ receptors is thought to exert synergistic effects on neuronal excitability in rat sympathetic neurons. Conversely, ATP and ADP are known to induce opposite effects on platelet aggregation. From these observations, it is clear that the interactions among different P2Y receptors are specific for each cell type, depending on the nature of cellular components and on whether the receptor is targeted to the luminal or basolateral membrane.

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