Purine and pyrimidine nucleotide-sensitive phospholipase A$_2$ in ampulla from frog semicircular canal

MARIE TEIXEIRA,$^1$ CHRISTIAN BERNARD,$^2$ EVELYNE FERRARY,$^1$ AND DANIEL BUTLEN$^1$

$^1$Institut National de la Santé et de la Recherche Médicale, Unité 426, Faculté de Médecine Xavier Bichat, 75870 Paris Cedex 18; and $^2$Laboratoire de Neurophysiologie Sensorielle, Université de Rouen, 76821 Mont-Saint-Aignan Cedex, France

Received 26 May 2000; accepted in final form 20 September 2000

EXTENSIVE FINDINGS PERFORMED on cultured cell lines have clearly established that extracellular triphosphonucleotides stimulate phospholipase A$_2$ (PLA$_2$) activity, leading to increases in arachidonic acid (AA) release and subsequent synthesis and secretion of prostaglandins through activation of cyclooxygenases (9, 15, 22, 25, 33).

For Tetrapod inner ear organs, evidence has been provided for regulating roles of extracellular ATP and UTP on neurotransmission and endolymph homeostasis (1, 16, 29). ATP was found in perilymphatic and endolymphatic fluids of the cochlea (21), and it was reported that prostaglandins are released by Rana esculenta semicircular canal (8).

Biochemically, two types of P2 purinoceptors functionally coupled to phospholipase C (PLC) activation have been characterized in ampulla from R. ridibunda semicircular canal (3, 4, 28). On the basis of their rank orders of potencies for recognition of ATP structural analogs, these receptors resembled to mammalian P2Y$_1$ and P2(Y) receptors (11) and were called P2Y$_1$-like and P2(Y)-like receptors (4, 28). In the gerbil vestibule and cochlea, it was assumed that the UTP-induced inhibition of the vectorial transport of K$^+$ toward the endolymphatic compartment is mediated by PLC stimulation followed by protein kinase C (PKC) activation through diacylglycerol-dependent and inositol triphosphate- and Ca$_{2+}$-independent mechanisms (16–18).

The aim of the present work was to screen, using a series of structural UTP analogs more selective for the P2Y receptors linked to G protein signal pathways or P2X ligand-gated ion channels (10, 11), the pharmacological properties of the P2 receptors triggering PLA$_2$ stimulation in isolated ampullas microdissected from R. ridibunda semicircular canals. Our results indicate that the nucleotide-induced PLA$_2$ activation requires occupancy of P2Y$_1$-like and/or P2(Y)-like receptors (11), and PLC and PKC stimulation. Basal PLA$_2$ activity is inhibited by agonists acting through cAMP-dependent pathways.

MATERIALS AND METHODS

Products Used

Arachidonic acid [5,6,8,9,11,12,14,15-$^3$H(N)] ([$^3$H]AA; 8.03 TBq/mmol) was provided by New England Nuclear (Le Blanc Mesnil, France).

Other chemicals were purchased from the following sources: adenosine, AMP, cAMP, 8-bromoadenosine 3’,5’-cyclic monophosphate (8-BrcAMP), ADP, adenosine 5’-O-(2-thiodiphosphate) (ADP$_{T_{2P}}$), ATP, adenosine 5’-O-(3-thiotriphosphate) (ATP$_{T_{3P}}$), α,β-methyleneadenosine 5’-triphosphate (α,β-MeATP), 2’- and 3’-O-(4-benzoylbenzoyl)-adenosine 5’-triphosphate (Bz-ATP), CTP, diadenosine tetraphosphate (Ap$_4$A),

Address for reprint requests and other correspondence: D. Butlen, INSERM U. 426, Faculté de Médecine Xavier Bichat, 16, rue Henri Huchard, BP 416, 75870 Paris Cedex 18, France (E-mail: u426@bichat.inserm.fr).

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

http://www.ajpregu.org 0363-6119/01 $5.00 Copyright © 2001 the American Physiological Society
uridine, UMP, UDP, UTP, arginine vasotocin (AVT), bradykinin, epinephrine, isoproterenol, phenylephrine, phorbol 12-myristate 13-acetate (PMA), prostaglandin E2 (PGE2), N-[2-[(p-bromocinnamylamino)-ethyl]-5-isoquinolinesulfonamide (H-89), DIDS, reactive blue 2 (basilen blue E-3G), bacitracin, and forskolin from Sigma (St. Louis, MO); 2-methyl-(H-89), DIDS, reactive blue 2 (basilen blue E-3G), bacitracin, (b) dolylmaleimide I (GF-109203X), methyl arachidonyl fluoro-

Research Biochemicals International (Natick, MA); bisin-

head was split in two halves and the brain was destroyed

tigne Breeding Center (France). Frogs were kept in tanks

R. ridibunda

sexes of the species

Calbiochem (Meudon, France); suramin from Miles (Naper-

3-methoxyestra)-1,3,5(10)-

4°C did not induce supplementary release of [3H]AA and

stimulated PLA2 activities (%total radioactivity incorpo-

Control experiments. It was checked that 1) both basal and

0.1 mM UTP-stimulated enzyme activities increased linearly

with incubation time up to 30 min at 26°C; 2) at the end of the

PLA2 assay, a second washing of ampullas with medium D at

4°C did not induce supplementary release of [3H]AA and

[3H]-labeled-derived metabolites. Basal and 50 μM UTP-

stimulated PLA2 activities (%total radioactivity incorpo-

rated; means ± SE of 6 replicates) of control samples were

4.2 ± 0.4 and 7.9 ± 0.7 and those of washed samples were

4.5 ± 0.6 and 8.1 ± 0.8, respectively (differences between

corresponding basal or UTP-stimulated activities are not

significant; Student's t-test); and 3) the presence of 2.5% dimethyl sulfoxide (DMSO) in the incubate enhanced basal

PLA2 activity. The latter were (%total radioactivity incorpo-

rated; means ± SE of mean data from number of experi-

ments) 5.7 ± 0.7, N = 6, and 3.9 ± 0.2, N = 36 for DMSO-

treated and control samples, respectively (P < 0.025,

Student’s t-test).

Statistical Analysis

Results were given as means ± SE of n (replicates performed

in the same experiment) or as means ± SE of mean data from N (different experiments). When appropriate, differences were analyzed using Student’s t-test; the latter were considered significant at P < 0.05.

For each analog tested, the 95% confidence interval variation range of pK_a value (pK_a = −log K_a, in which K_a was expressed as molar concentration) for nucleotide-induced PLA2 activation was calculated by computerized analysis of the corresponding dose-response curves (GraphPad Prism Software; sigmoidal dose-response fit with variable slope). It was assumed at the 95% probability level that two analogs stimulate PLA2 with differing apparent affinities, only when the lower limit of pK_a variation range of the best nucleotide is higher than the upper limit of pK_a variation range of the worst analog.

Animals

Experiments were performed using adult frogs of both sexes of the species R. ridibunda purchased from the Conti-

tigne Breeding Center (France). Frogs were kept in tanks

containing tap water at 8°C. They were decapitated. The head was split in two halves and the brain was destroyed

(approval of the Ministère de l’Agriculture et de la Pêche, No.

5521).

Microdissection of Ampullas From Frog

Semicircular Canals

Microdissection of the three semicircular canals from each

inner ear was performed at 4°C under stereomicroscopic observation in a modified amphibian Ringer solution, me-

dium A (in mM, 20 HEPES-NaOH, pH 7.5, 82 NaCl, 3 KCl,

1.8 CaCl₂, 1 MgCl₂, 0.33 NaH₂PO₄, 0.44 Na₂HPO₄, 5 glucose,

and 10 sodium acetate, and 0.1% BSA). The ampullas were

separated from the adjacent regions of the semicircular canals and opened by sagittal incision of their dorsal aspects.

In some experiment, the dorsal ampullary region formed of undifferentiated epithelial cells was separated from the ven-

tral region containing secretory dark cells, transitional cells, sensory hair cells, and undifferentiated cells (23) by cutting the higher frontal level of ampulla. Secretory dark cell and sensory hair cell areas with their adjacent connective tissue were microdissected from other structures in the ventral ampullary region. Epithelial structures were kept at 4°C until use.

PLA2 Studies

[3H]AA release. Assays were performed using a microtech-
nique adapted to frog ampullas derived from the method described for cultured cell lines (9, 24). Ampullas were la-

beled in 40 μl of medium B (medium A containing 18.5 kBq

[3H]AA for 3 h at 27.5°C in a humid atmosphere and then

extensively washed in a large volume of medium A at room temperature to eliminate extracellular [3H]AA.

One ampulla was used for each individual determination. The incubation was started by transferring ampulla onto a

10-μl droplet of medium C (medium A containing 25 mM

HEPES-NaOH, pH 7.5, 0.5% BSA, 0.1% bacitracin, various amounts of UTP or structural analogs, agents of interest or vehicles) put on the hollow of a siliconized bacteriological slide, and the sample was tightly covered with a petroleum jelly-coated slide to obtain a water-tight seal. The reaction was carried out for 30 min at 26°C and stopped by adding 200 μl of chilled medium D (medium C plus 5 mM EGTA-NaOH, pH 7.5, and 5 mM EDTA-NaOH, pH 7.5). The ampulla was removed, treated with 10 μl NaOH 1 N, and counted for 10 min for determination of tissular-associated radioactivity.

The medium was recovered and counted twice for 20 min for measurement of released [3H]AA and derived [3H]-labeled metabolites.

Generally for each experiment, six to eight concentrations of a given UTP analog were tested in six replicates. Therefore, each dose-response curve was drawn from data obtained with 36–48 ampullas microdissected from six to eight frogs.

Calculations. Results were expressed as ratios between released radioactivity and total radioactivity incorporated (the latter was 3,437 ± 242 counts·min⁻¹·ampulla⁻¹; means ± SE of mean data from 42 experiments with 30–64 individual determinations).

The kinetics of PLA2 activation induced by potent agonists are adequately described by the following relation

\[ V = V_{\text{max}} \times L^n H/(L^n H + K_a^n H) \]

in which V and V_{\text{max}} are enzyme activations (above basal velocity) measured in the presence of submaximal and satu-

rating agonist concentrations (L), K_a and nH are the appa-

rent activation constant (nucleotide concentration leading to half-maximal enzyme activation) and Hill coefficient of the agonist, respectively. K_a and nH were computed from x-intercept and slope of corresponding Hill plot of dose-response curve. Intrinsic activities of agonists (i.e., magnitude of maximal PLA2 activation) were expressed as ratios between enzyme activations induced by saturating amounts of a given nucleotide (Δ_{\text{max}}) and of UTP (Δ_{\text{max UTP}}) measured in the same experiment.

Results were given as means ± SE of n (replicates performed in the same experiment) or as means ± SE of mean data from N (different experiments). When appropriate, differences were analyzed using Student’s t-test; the latter were considered significant at P < 0.05.
RESULTS

Figure 1 shows that incubation of \([\text{3H}]\text{AA}\)-labeled ampullas with UTP increased the basal release of \([\text{3H}]\text{AA}\) and derived \(^3\text{H}\)-labeled metabolites. The UTP-induced \(\text{PLA}_2\) activation was dose-dependent, saturable with nucleotide concentration, and characterized by the following parameters (means ± SE of 6 experiments): 1) a threshold response for about 0.1 \(\mu\text{M}\) UTP; 2) an apparent activation constant \(K_a = 1.3 ± 0.4 \mu\text{M}\), \(N = 6; 3) a Hill coefficient \(nH = 0.86 ± 0.15, N = 6; 4)\) a maximal response observed for about 50 \(\mu\text{M}\) UTP (maximal stimulating factor = 1.98 ± 0.09, \(N = 23\)); and 5) self-inhibitory effects for nucleotide concentrations higher than 50 \(\mu\text{M}\).

The sensitivity of \(\text{PLA}_2\) of ampulla from frog semicircular canal to structural UTP analogs is illustrated by typical experiments depicted in Fig. 2, and all results are summarized in Table 1. It is worth noting that the nucleotides tested were able to stimulate \([\text{3H}]\text{AA}\) release with differing potencies as regards 1) their apparent activation constants, the \(K_a\) values of the P2(Y) agonists UTP and UDP were smaller than those of the long-acting analogs ADPβS and ATPγS; those of the natural nucleotides ATP, ADP, and CTP; and those of the potent markers of the P2Y1 receptors (2-MeS-ATP), P2Y<sub>Ap4A</sub> receptors [Ap4A (11)], and P2X<sub>1</sub> and P2X<sub>9</sub> receptors (α,β-Me-ATP), respectively. They were also far lower than that of the selective P2<sub>X</sub>7 agonist (Bz-ATP) and those of AMP and UMP; 2) their Hill coefficients (ADPβS, ATPγS, Ap4A, and α,β-Me-ATP stimulated enzyme activity with negative cooperativity phenomena, UTP, UDP, ATP, ADP, CTP, and Bz-ATP according to Michaelian kinetics and 2-MeS-ATP with slight positive cooperativity phenomena); and 3) their intrinsic activities or magnitude of the maximal responses observed (ATP was about 1.6 times more potent than UTP; 2-MeS-ATP, Ap4A, CTP, Bz-ATP, ATP, and UMP exhibited partial agonistic potencies, whereas 10 mM of uridine or adenosine were inactive).

Data from a competitive experiment between the potent P2(Y) agonist UTP and the selective P2Y<sub>1</sub> agonist 2-MeS-ATP (10, 11) clearly indicate that the responses to saturating amounts of both nucleotides were not additive. The increases in \([\text{3H}]\text{AA}\) release (Δ, %total radioactivity incorporated; means ± SE of 8 experiments): UDP, \(y = 1.06x + 6.16, r = 0.98\); CTP, \(y = 0.94x + 3.63, r = 0.90\); ATP, \(y = 0.946x + 9.50, r = 0.99\); 2-MeS-ATP, \(y = 1.51x + 6.95, r = 0.98\); ADP, \(y = 1.06x + 4.50, r = 0.98\). Kinetic parameters of nucleotides for stimulation of \([\text{3H}]\text{AA}\) release are summarized in Table 1.

---

Fig. 1. Dose-dependent UTP-induced stimulation of \([\text{3H}]\text{arachidonic acid (}\text{3H}\text{AA}\) release in ampulla from frog semicircular canal. Values are means ± SE of 6 replicates performed in the same experiment. \([\text{3H}]\text{AA}\)-labeled ampullas were incubated for 30 min at 26°C in presence of the indicated amounts of UTP. Releases of \([\text{3H}]\text{AA}\) and derived \(^3\text{H}\)-labeled metabolites were expressed as %total radioactivity incorporated, corrected for corresponding basal activities and further expressed as %activations induced by 50 \(\mu\text{M}\) UTP (UTP<sub>max</sub>) observed in the same experiments. Fitting data in Hill coordinates generates linear plots whose equations of linear regression are as follows: UDP, \(y = 1.06x + 6.16, r = 0.98\); CTP, \(y = 0.94x + 3.63, r = 0.90\); ATP, \(y = 0.946x + 9.50, r = 0.99\); 2-MeS-ATP, \(y = 1.51x + 6.95, r = 0.98\); ADP, \(y = 1.06x + 4.50, r = 0.98\). Kinetic parameters of nucleotides for stimulation of \([\text{3H}]\text{AA}\) release are summarized in Table 1.

---

Fig. 2. Dose-dependent activations of \([\text{3H}]\text{AA}\) release by structural UTP analogs in ampulla from frog semicircular canal. Values are means ± SE of 6 replicates performed in the same experiments. \([\text{3H}]\text{AA}\)-labeled ampullas were incubated for 30 min at 26°C in presence of increasing concentrations of either pyrimidine nucleotides (UDP, CTP and UMP; A) or purine nucleotides [ATP, 2-methylthio-ATP (2-MeS-ATP), ADP and AMP; B]. Releases of \([\text{3H}]\text{AA}\) and derived \(^3\text{H}\)-labeled metabolites were expressed as %total radioactivity incorporated, corrected for corresponding basal activities and further expressed as activations induced by 50 \(\mu\text{M}\) UTP (UTP<sub>max</sub>) observed in the same experiments. Fitting data in Hill coordinates generates linear plots whose equations of linear regression are as follows: UDP, \(y = 1.06x + 6.16, r = 0.98\); CTP, \(y = 0.94x + 3.63, r = 0.90\); ATP, \(y = 0.946x + 9.50, r = 0.99\); 2-MeS-ATP, \(y = 1.51x + 6.95, r = 0.98\); ADP, \(y = 1.06x + 4.50, r = 0.98\).
Table 1. Kinetic parameters of structural UTP analogs for PLÁ₂ activation in ampulla from frog semicircular canal

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>pKᵃ</th>
<th>95% Confidence Interval</th>
<th>Hill Coefficient</th>
<th>Δ₉₅/Δmax/UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP</td>
<td>5.9 ± 0.1</td>
<td>5.7–6.2</td>
<td>0.9 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>UDP</td>
<td>5.6 ± 0.1</td>
<td>5.4–5.9</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>ADP₅S</td>
<td>5.1 ± 0.1</td>
<td>4.9–5.2</td>
<td>0.5</td>
<td>0.9</td>
</tr>
<tr>
<td>ATP₅S</td>
<td>5.0 ± 0.1</td>
<td>4.7–5.3</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>ATP</td>
<td>4.6 ± 0.1</td>
<td>4.3–4.9</td>
<td>0.8 ± 0.2</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>2-MeS-ATP</td>
<td>4.5 ± 0.1</td>
<td>4.3–4.8</td>
<td>1.6 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>ADP</td>
<td>4.2 ± 0.1</td>
<td>4.0–4.5</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Ap₅A</td>
<td>4.1 ± 0.1</td>
<td>4.0–4.3</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>α,β-Me-ATP</td>
<td>4.0 ± 0.1</td>
<td>3.8–4.1</td>
<td>0.7</td>
<td>0.9</td>
</tr>
<tr>
<td>CTP</td>
<td>3.8 ± 0.1</td>
<td>3.4–4.2</td>
<td>0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Bz-ATP</td>
<td>2.9 ± 0.1</td>
<td>2.8–3.0</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>AMP</td>
<td>&lt;3</td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>UMP</td>
<td>&lt;3</td>
<td></td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>Uridine</td>
<td>&lt;2</td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>Adenosine</td>
<td>&lt;2</td>
<td></td>
<td></td>
<td>0.0</td>
</tr>
</tbody>
</table>

Values of kinetic parameters for nucleotide-induced phospholipase A₂ (PLÁ₂) activation are mean data from 2 experiments or means ± SE of mean results from 6 (†) or 3 (‡) experiments. ADP₅S; adenosine 5’-O-(2-thiodiphosphate); ATP₅S; adenosine 5’-O-(3-thiotriphosphate); 2-MeS-ATP; 2-methylthio-ATP; Ap₅A, diadenosine tetraphosphate; α,β-Me-ATP, α,β-methylene-ATP; Bz-ATP, 2’- and 3’-O-(4-benzoylbenzoyl)-ATP. Apparent activation constants are given as pKᵃ = −log Kᵃ, in which Kᵃ values are expressed as molar concentration. For each analog tested, the 95% confidence interval of the variation range of pKᵃ value was calculated by computerized analysis of the corresponding dose-response curves (see MATERIALS AND METHODS). The lowest and highest r² values of the fits for calculations of 95% confidence intervals are 0.90 for CTP and 0.99 for Bz-ATP. Computation gives the following rank order of stereospecificity: UTP > ADP₅S = ATP₅S ≥ ATP > 2-MeS-ATP = ADP > Ap₅A ≥ α,β-Me-ATP = CTP > Bz-ATP ≥ AMP = UMP >> uridine and adenosine. Δ₉₅/Δmax/UTP ratios between maximal enzyme activations induced by saturating amounts of a given analog (Δ₉₅/Δmax) and of UTP (Δmax/UTP) measured in the same experiments.

replicates) above basal activity (2.2 ± 0.2) induced by 50 μM UTP, 0.3 mM 2-MeS-ATP, and 50 μM UTP + 0.3 mM 2-MeS-ATP were, respectively, 4.2 ± 0.6, 2.8 ± 0.6, and 4.7 ± 0.4; the latter value is significantly lower than the sum of both activations (computed Δ, 7.0 ± 0.8; P < 0.01; Student’s t-test). These data demonstrate that UTP and 2-MeS-ATP stimulate the same pool of PLÁ₂.

As expected, the unrelated nucleotide chemicals revealing antagonistic properties (3, 4, 10, 11, 28) were able to inhibit UTP-induced PLÁ₂ activation (Table 2).

In the presence of antagonist concentrations leading to total inhibition of specific UTP binding (28) (5 mM DIDS, 2 mM reactive blue 2, 10 mM PPADS, and 10 mM suramin), the basal enzyme activity was decreased by about 50% by DIDS and not significantly impaired by reactive blue 2, PPADS, or suramin, whereas the activation of [³H]AA release elicited by 1 μM UTP vanished in the presence of the four antagonists tested.

Results depicted in Fig. 3 show the effects of inhibitors of either PLC [U-73122 (12)], PKC [GF-109203X (31)], or cytosolic PLÁ₂ [MAFP (19)] on PLÁ₂ activity. In the assay medium of 50 μM U-73122, 10 μM GF-109203X, or 50 μM MAFP did not alter basal [³H]AA release but decreased by about 50% the

Table 2. Inhibitions induced by antagonists of UTP-stimulated PLÁ₂ activity in ampulla from frog semicircular canal

<table>
<thead>
<tr>
<th>[³H]Arachidonic Acid Release</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>Control</td>
<td>U73122</td>
</tr>
<tr>
<td>UTP</td>
<td>4.0 ± 0.4</td>
<td>6.2 ± 0.6</td>
</tr>
<tr>
<td>Reactive blue 2</td>
<td>5.5 ± 0.8</td>
<td>5.5 ± 0.5 NS</td>
</tr>
<tr>
<td>DIDS</td>
<td>1.9 ± 0.4</td>
<td>2.0 ± 0.2 NS</td>
</tr>
<tr>
<td>PPADS</td>
<td>5.7 ± 0.8</td>
<td>5.7 ± 0.4 NS</td>
</tr>
<tr>
<td>Suramin</td>
<td>4.1 ± 0.6</td>
<td>4.7 ± 0.7 NS</td>
</tr>
</tbody>
</table>

Fig. 3. Inhibitory effects of U-73122, GF-109203X, and methyl arachidonyl fluorophosphate (MAFP) on UTP- and 2-MeS-ATP-induced activations of [³H]AA release in ampulla from frog semicircular canal. Values are means ± SE of 6 replicates performed in the same experiment. [³H]AA-labeled ampullas were incubated for 30 min at 26°C without (basal) or with 1 μM UTP, in absence (control) or presence of either 2 mM reactive blue 2, 5 mM DIDS, 10 mM pyridoxal-phosphate-6-azophenyl-2’,4’-disulfonic acid (PPADS) or 10 mM suramin.

Releases of [³H]AA and derived 3H-labeled metabolites were expressed as %total radioactivity incorporated. Differences between basal activities of control and antagonist-treated samples: reactive blue 2, PPADS, and suramin, not significant; DIDS, ↑P < 0.005 (Student’s t-test). Differences between UTP-stimulated and corresponding basal activities of control or antagonist-treated samples, *P < 0.025, NS, not significant (Student’s t-test). This experiment was performed twice and similar results were obtained.
It was checked that the basal release of $[^3]$H]AA was stimulated about 1.3, 1.4, and 1.8 times by 0.1 mM phenylephrine, 0.1 uM PMA, and 0.1 mM epinephrine, respectively, but was not modified by 10 uM bradykinin. Interestingly, it was found that 10 uM AVT, 10 uM isoproterenol, 0.1 uM PGE$_2$, or 10 mM cAMP reduced basal enzyme activity by 21, 31, 49, and 42%, respectively ($P < 0.05$, Student's $t$-test) and for H-89-treated samples, activities measured in absence or presence of 8-BrcAMP or forskolin are significantly lower than that determined in their absence ($^{*}P < 0.05$, $^{**}P < 0.025$; Student's $t$-test) for control samples, activities measured in presence of 8-BrcAMP or forskolin are significantly lower than that determined in their absence ($^{*}P < 0.05$, $^{**}P < 0.025$; Student's $t$-test) and for H-89-treated samples, activities measured in absence or presence of 8-BrcAMP or forskolin do not differ significantly (NS, not significant; Student's $t$-test). This experiment was performed twice and similar results were obtained.

Finally, the distribution of nucleotide-sensitive PLA$_2$ activities in the different regions of ampulla from frog semicircular canal is presented in Table 3. Results show clearly that UTP or 2-MeS-ATP stimulated $[^3]$H]AA release in the dorsal region, the ventral region, and in the dark cell areas, whereas neither agonist enhanced basal activity in the hair cell area.

**DISCUSSION**

The experiments described provide evidence for the expression of a nucleotide-sensitive PLA$_2$ activity in ampulla of *R. ridibunda* semicircular canal. Data also show that basal release of $[^3]$H]AA and derived $[^3]$H-labeled metabolites was stimulated by the selective P2(Y) receptor agonist (UTP) and the potent P2Y$_1$ receptor marker (2-MeS-ATP) (10, 11) in the dorsal region, the ventral region, and in the dark cell areas but not in the hair cell area of the ampullary epithelium.

Despite the minute amounts of tissue used [about 2.5 $\mu$g total protein/whole ampulla (28)], the validity of the microassay employed was verified by the following experiments: 1) basal and UTP-stimulated releases of $[^3]$H]AA and derived $[^3]$H-labeled metabolites increased linearly with incubation time up to 30 min; 2) agonist-induced PLA$_2$ activations were dose dependent and saturable; 3) purinoceptor antagonists inhibited UTP-stimulated enzyme activity; and 4) the selective cytosolic PLA$_2$ inhibitor MAFP decreased the activations induced by maximal concentrations of UTP and 2-MeS-ATP.

It seems unlikely that the differences found between $K_c$ values of agonists for PLA$_2$ stimulation result from ecto-ATPase and ecto-nucleotidase activities (5, 6, 30) because it was reported earlier that a triphosphonucleotide-regenerating system did not impair basal, sub-maximal, and maximal ATP- and UTP-stimulated PLC activities in frog ampulla (3, 28).

It must be pointed out that data from a competition experiment between UTP and 2-MeS-ATP show that the PLA$_2$ responses to saturating amounts of both agonists were not additive. This observation indicates that occupancies of P2(Y)-like receptors by UTP and of P2Y$_1$-like receptors by 2-MeS-ATP lead to stimulation of the same pool of PLA$_2$ in frog ampulla.

Results of pharmacological investigations demonstrate that nucleotide-induced PLA$_2$ activation is mediated by P2Y$_1$-like and/or P2(Y)-like receptor occupancy. Indeed 1) the potent marker of P1 receptors (adenosine) was inactive and the selective ligands for P2X$_1$ and P2X$_2$ receptors (a,b-ATP-Me) and for P2X$_7$ receptors (Bz-ATP) stimulated PLA$_2$ with only low

<table>
<thead>
<tr>
<th>Target Structure</th>
<th>[3H]AA release Basal</th>
<th>UTP</th>
<th>2-MeS-ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal region</td>
<td>7.6 ± 0.7</td>
<td>12.8 ± 1.9*</td>
<td>13.0 ± 0.9†</td>
</tr>
<tr>
<td>Ventral region</td>
<td>6.1 ± 1.1</td>
<td>10.7 ± 0.6†</td>
<td>9.2 ± 0.8*</td>
</tr>
<tr>
<td>Dark cell area</td>
<td>5.6 ± 0.8</td>
<td>11.4 ± 2.0*</td>
<td>10.2 ± 0.9†</td>
</tr>
<tr>
<td>Hair cell area</td>
<td>14.2 ± 3.9</td>
<td>14.2 ± 0.5NS</td>
<td>16.1 ± 3.0NS</td>
</tr>
</tbody>
</table>

Values are means ± SE of 5 replicates performed in the same experiment. The indicated structures microdissected from ampullas of frog semicircular canals were labeled with $[^3]$H]AA and incubated for 30 min at 26°C in absence (basal) or presence of either 50 $\mu$M UTP or 0.3 $\mu$M 2-MeS-ATP. Releases of $[^3]$H]AA and derived $[^3]$H-labeled metabolites were expressed as %total radioactivity incorporated. The latter were 669 ± 64, 2,681 ± 208, 1,327 ± 162, and 1,136 ± 132 counts·min$^{-1}$·structure$^{-1}$ (means ± SE of 15 determinations) for dorsal region, ventral region, dark cell area, and hair cell area, respectively. Differences between UTP- or 2-MeS-ATP-stimulated and corresponding basal activities, *$P < 0.05$; †$P < 0.01$, NS, not significant (Student's $t$-test).
The affinities and the antagonists reactive blue 2, DIDS, PPADS, and suramin that inhibit competitively UTP binding and UTP-induced PLC activation in frog ampulla (28) abolished UTP-stimulated PLA2 activity.

On the one hand, both the occurrence of a close linear relationship between the $pK_a$ values of 11 potent structural UTP analogs for PLA2 activation and their corresponding values for PLC stimulation (Fig. 5) and the observed decreases of UTP- and 2-MeS-ATP-induced PLA2 activations in the presence of the selective PLC inhibitor U-73122, suggest strongly that PLC activation is involved in the purine- and pyrimidine-dependent mechanisms triggering PLA2 stimulation. In the other systems so far studied, it was reported that PLC activates PLA2 via PKC (9, 15, 22, 25, 33). In frog ampulla, a similar pathway may be suggested on the basis of the following observations: 1) the PKC activator PMA increased basal PLA2 activity and 2) the PKC inhibitor GF-109203X reduced UTP- and 2-MeS-ATP-induced PLA2 activations.

On the other hand, it should be stressed that cAMP and forskolin, as well as AVT, isoproterenol, and PGE2 that enhance intracellular cAMP levels in frog ampulla (7) were able to decrease basal PLA2 activity. These agonist-induced inhibitory effects are likely mediated by intracellular cAMP-dependent pathways rather than by occupancy of P2Y-like receptors by the extracellular cAMP released from intracellular stores because 1) cAMP did not interact with [35S]ADPβS- and [3H]UTP-labeled binding sites and did not impair basal PLA2 activity in frog ampulla (3, 4, 28) and 2) the PKA inhibitor H-89 restored to control level the 8-BrcAMP- and forskolin-induced inhibitions of basal PLA2 activity. This latter observation suggests that PKA activation is implicated in the cAMP-dependent mechanisms triggering PLA2 inhibition.

On these grounds, it might be assumed that the nucleotide-dependent PLA2 stimulation and cAMP-dependent enzyme inhibition involve the following biochemical steps: 1) occupancy by extracellular purines and pyrimidines of the P2Y1-like and/or P2-Y-like receptors coupled to PLC activation triggering direct activation of PKC through diacylglycerol-dependent mechanisms as observed in the gerbil vestibule and cochlea (17, 18); 2) phosphorylation of cytosolic PLA2 by PKC leading to increases in AA release and subsequent synthesis and secretion of prostaglandins through cyclooxygenase activation as reported in Ma-
Purine- and pyrimidine-sensitive PLA2 activities were cellular localization(s) and physiological function(s). Activity and likely in the synthesis and release of prostanoids. Moreover, in streptomycin treatment of ampullae, suggesting that hair cells produce low amounts of prostaglandins. Indeed, prostaglandin synthesis (8). More importantly, prostaglandin synthesis is increased by ATP and is consistent with the known effects of extracellular nucleotides on prostaglandin release, intracellular cAMP levels, and adenylate cyclase sensitivity to AVT, isoproterenol, and PGE2.

**Perspectives**

The expression in frog ampulla of P2Y1-like and P2(Y)-like receptors involves through PLC- and PKC-dependent mechanisms in the regulation of PLA2 activity and likely in the synthesis and release of prostaglandins. These observations suggest that the P2Y receptors borne by ampullary hair cells trigger prostaglandin-independent biological effects and strengthen data suggesting that hair cells produce low amounts of prostaglandins. Indeed, streptomycin treatment of ampullae that destroys sensory cells, did not alter significantly prostaglandin synthesis (8). Moreover, in sensory structures of the inner ear, ATP increased the spontaneous electrical activity of afferent fibers from R. pipiens semicircular canal and Xenopus laevis lateral line (1, 20), it induced lysis of outer hair cells, and altered gross cochlea, auditory nerve potential, and distortion product otoacoustic emissions in guinea pig cochlea (2, 13, 14, 29). Therefore, the molecular subtype(s) and physiological significance(s) of the P2Y receptors expressed in Amphibia sensory cells call for additional electrophysiological, biochemical, and molecular cloning experiments.

Finally, the presence of a purine- and pyrimidine-sensitive PLA2 activity in the dorsal region and in the dark cell areas of the ventral region of frog ampulla might reflect an ubiquitous localization of P2Y1-like and P2(Y)-like receptors mediating AA and prostaglandin releases as reported for many other systems (9, 15, 22, 25, 30). This nucleotide-dependent PLA2 activity also might be involved in the functions of secretory dark cells. Indeed, it was proposed that prostaglandins could be implicated in the regulation of endolymphatic fluid composition (26), and it was reported that both ATP and UTP decrease the vectorial transport of K+ toward the endolymphatic space in the gerbil vestibule and cochlea, whereas cAMP increases K+ secretion via activation of I_{Ks}/KvLQT1 channels in strial marginal cells (16, 27). Thus further electrophysiological experiments will be necessary to investigate the effects of purine and pyrimidine nucleotides, cAMP, and prostaglandins on the electrogenic K+ secretion by ampulla from frog semicircular canal.

We are indebted to Prof. Gérard Friedlander for critical advice and stimulating discussions.

This work was supported by grants from Institut National de la Santé et de la Recherche Médicale and Université Paris 7.

**REFERENCES**


