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Inhibition of Adenylyl Cyclases by 12(*S*)-Hydroxyeicosatetraenoic Acid

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The inhibition of adenylyl cyclase (AC) by a 12-lipoxygenase metabolite of arachidonic acid, 12(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE), was investigated using three different kinds of cells: NRK-49F (normal rat kidney fibroblasts), AtT-20 (mouse pituitary tumor cell line) and HL-60 (human leukemia cells) cells. The inhibition was very obvious in NRK-49F and AtT-20 cells, but it was almost negligible in HL-60 cells. There was no difference in terms of the binding of 12-HETE to NRK-49F and HL-60 cells. Pretreatment of NRK-49F cells with pertussis toxin almost completely ADP-ribosylated G_i proteins, but it did not affect the inhibition of 12-HETE on AC in this cell. This result excludes the involvement of G_i proteins in 12-HETE-mediated inhibition of AC. It was revealed that the characteristics of ACs in these cells were quite different in response to agonists and forskolin, suggesting that these cells do have different isoforms of AC. We conclude that 12-HETE inhibits the activity of AC depending upon the isoform.

Arachidonic acid released from membrane phospholipids is converted into a family of biologically active metabolites, collectively called eicosanoids that exert various cellular reactions (1–4). Among eicosanoids, the physiological functions of hydroxyeicosatetraenoic acids (HETEs) are least well studied. The stereo specific cytosolic enzyme, lipoxygenase, inserts in platelets a single oxygen molecule into arachidonic acid at carbon 12 to yield an unstable hydroperoxide which is reduced to the corresponding monohydroxylated product. We have found that 12(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE) has two related but distinct biological effects: it inhibits collagen-induced platelet aggregation by interfering with arachidonic acid liberation from membrane phospholipids (5), but it potentiates thrombin-induced platelet aggregation by reducing the level of cAMP (6). In fact, it partially canceled the inhibitory effect of prostaglandin E_1 (PGE_1) on the platelet aggregation, and reduced the level of cAMP otherwise increased by PGE_1 in platelets (6).

cAMP, which is synthesized by adenylyl cyclases (AC) [EC 4.6.1.1], is a well-known second messenger molecule in signal transduction pathways in various cells. Intracellular cAMP stimulates the cAMP-dependent protein kinases and regulates a variety of biochemical and physiological systems in cells, including carbohydrate, lipid, protein and nucleic acid metabolisms, as well as synaptic transmission and ion channel function (7-9). To see whether the effect of 12-HETE on the level of cAMP is restricted to platelets, we have used three different kinds of unrelated cells. The results strongly indicate that the effect of 12-HETE depends on the kind of isoforms of AC present in each cell.

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Abbreviations: 12-HETE, 12(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; CRF, corticotropin-releasing factor; PGE_1, prostaglandin E_1; FSK, forskolin; IBMX, 3-isobutyl-1-methyl-xanthine; AC, adenylyl cyclase; PT, pertussis toxin.

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EXPERIMENTAL PROCEDURES

Materials

12-HETE was chemically synthesized as described previously (10), and was dissolved in ethanol. Forskolin (FSK), 3-isobutyl-1-methyl-xanthine (IBMX), isoproterenol, and corticotropin-releasing factor (CRF) were purchased from Sigma. PGE_1 and PGE_2 were from Cayman Chemical (Ann Arbor, MI). 12(*S*)-hydroxy-[5,6,8,9,11,12,14,15(n)- [^{3}H] eicosatetra-enoic acid ([^{3}H] 12-HETE) and [^{32}P]-labeled NAD (29.6 TBq/mmol) were purchased from New England Nuclear/DuPont (Boston, MA). Pertussis toxin (PT) was from Kaketsuken (Japan). Other chemicals used were of analytical grade.

Methods

Cell cultures. NRK-49F (normal rat kidney fibroblasts) and HL-60 (human leukemia cells) cells were provided from the Japanese Cancer Research Resources Bank (Tokyo, Japan). AtT-20 (mouse pituitary tumor cell line) was a generous gift from Dr. Y. Ozawa at Toranomon Hospital (Tokyo, Japan). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (for NRK-49F), RPMI 1640 medium containing 10% FCS (for HL-60), or Ham's F-10 medium supplemented with 15% FCS (for AtT-20) at 37°C, in an atmosphere of 5% CO_2 in air at 100% humidity.

NRK-49F cells were plated on 24-well culture plate (Costar 3424) and cultured several days until they reached confluence. The number of cells in each well usually reached $\sim 3.5 \times 10^5$. PT-pretreated cells were prepared by adding 100 ng/ml PT to fresh cell culture media and incubating for 4 hrs at 37°C. After washing twice with serum-free DMEM, 0.5 mM IBMX and either 12-HETE or ethanol alone were added to the cells in the serum-free DMEM, and cellls were preincubated for 30 min at 37°C in an atmosphere of 5% CO_2. The final concentration of ethanol was kept at less than 0.02% in order to avoid the deleterious effects of the alcohol. The cells were then treated with various agents for 30 min. At the end of the incubation period, the medium in each well was collected, and cells were solubilized with 0.1 N NaOH, neutralized with 1 N HCl, mixed with the medium and stored at −80°C until assay.

AtT-20 as well as HL-60 cells were grown in suspension in flasks. They were washed three times by serum-free media and were gently resuspended in serum-free media, both at a density of 3-4 $\times 10^6$ cells/ml. 12-HETE or ethanol alone was added to the cells in the presence of IBMX and preincubated at 37°C for 30 min, subsequently dispensed into Eppendorf tubes containing the appropriate test agents, and then incubated for another 30 min. The reaction was terminated by the addition of small amount of 3 N NaOH, neutralized with the same volume of 3 N HCl, and stored until assay.

cAMP determination. The concentration of cAMP in samples was quantified with a specific radioimmunoassay using a commercial kit (Yamasa Shoyu, Choshi, Japan), after removal of insoluble materials by centrifugation.

Binding assay. Binding assays were performed with NRK-49F cells in 24-well culture plate growing in monolayers, and with HL-60 cells resuspended into Eppendorf tubes. After being washed twice with serum-free media, cells were incubated with the fixed concentration of [^{3}H] 12-HETE (specific activity, 7289 GBq/mmol; final concentration, 0.078 nM) and the increasing concentrations of non-radioactive 12-HETE in the range of 0-189 μM in a final volume of 500 μl per well or tube. The incubation was performed for 30 min at 37°C, and the reaction was terminated by washing cells with ice-cold PBS. Cells were then solubilized with 300 μl of 0.1 N NaOH, and the cell-bound radioactivity was counted by a liquid scintillation counter (Beckman Scintillation System LS 6500, Fullerton, CA).

Membrane preparation. NRK-49F cells with or without PT-pretreatment were harvested and washed with a phosphate buffer (1.47 mM KH_2PO_4, 8.06 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.2) containing protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin) at the cell numbers of $\sim 10^8$. Total membranes were prepared by hypotonic lysis of cells in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) containing the protease inhibitors, followed by homogenization, centrifugation at 40,000 $\times$ *g*, and re-suspension in 25 mM Tris buffer (pH 7.5) containing the protease inhibitors to give a final protein concentration of about 1-2 mg/ml, as described by McKenzie (11). They were then stored at −80°C until used.

PT-catalyzed ADP-ribosylation of Gi. The heterodimeric PT was preactivated by incubation at 30°C for 25 min in 50 mM Tris-HCl buffer, pH 7.5, containing 100 mM DTT and 0.1 mM ATP. Membranes were incubated at 30°C for 40 min with the preactivated PT (10 μg/ml) in 40 μl of reaction mixture consisting of 100 mM Tris-HCl, pH 7.5, 40 μM GDP, 0.1 mM NADP, 1 mM thymidine, 1 mM ADP-ribose, and 20 mM nicotinamide, as well as 1 μM [^{32}P]NAD (12). The reaction was terminated by adding 10 μl of 3-fold concentrated Laemmli-buffer, followed by heating at 90°C for 3 min. Samples were subjected to SDS-polyacrylamide gel (10%) electrophoresis. The [^{32}P]ADP-ribose incorporated into G_i proteins were visualized by an imaging analyzer, BAS 2000 (FUJIX Bio-imaging analyzer).

RESULTS AND DISCUSSION

We first investigated the effect of 12-HETE on PGE_1-stimulated cAMP production in NRK-49F cells. During the 30 min stimulation with 10 μM PGE_1, total cAMP level was increased

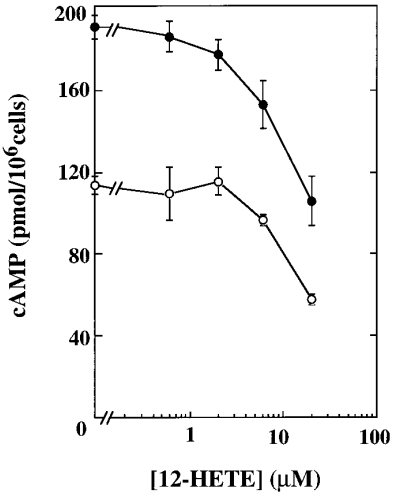

FIG. 1. 12-HETE inhibits the synthesis of cAMP induced by PGE_1 and isoproterenol in NRK-49F cells. Confluent cells were preincubated at 37°C for 30 min with 0.5 mM IBMX and 12-HETE at indicated concentrations, followed by stimulation with 10 μM PGE_1 (-●-) as well as with 10 μM isoproterenol (-○-) for 30 min. Amounts of cAMP synthesized were determined as described under *Experimental Procedures.* Data are the mean ± S.E. of four determinations from a representative of three experiments.

significantly from the basal level (from 8.8 to 191 pmol/10^6 cells). As is clearly depicted in Fig. 1, incubation with 12-HETE strikingly reduced in a dose-dependent manner the level of cAMP produced. This result was quite similar to that obtained with a β-adrenergic agonist, isoproterenol, whose receptor is also coupled to G_s-protein to activate AC. The concentration of cAMP is determined by the balance between the synthesis by AC and the degradation by phosphodiesterase. As the latter process is inhibited by IBMX in this experiment, it is solely determined by the former process. The concentration required for the inhibition was in the micromolar range, which was comparable to that observed with platelets (6). In both cases, about 50% inhibition was obtained with 20 μM 12-HETE.

In order to further investigate the specificity of the effect of 12-HETE, it was necessary to use other cells. We employed a peptide hormone and AtT-20 cells. CRF, a 41-residue hypophysiotropic peptide, binds to the specific receptor of AtT-20 cells and stimulates cAMP synthesis by AC *via* G-protein coupled receptors (13, 14). As shown in Fig. 2, 20 μM 12-HETE lowered the level of cAMP induced by 10 -1000 nM CRF. The lower the concentration of CRF, the more obvious the effect of 12-HETE was in terms of the inhibition. About 50% inhibition was obtained with 20 μM 12-HETE when 10 nM CRF was used. Since NRK-49F cells do not respond to CRF and AtT-20 cells do not respond well to PGE_1, we then decided to investigate further using HL-60 cells, which are known to respond to PGE_1 (and PGE_2) to produce cAMP. Interestingly enough, 12-HETE did not inhibit PGE_1- or PGE_2-induced cAMP production in HL-60 cells, although the extent of the stimulation of AC by PGE_1 and PGE_2 in HL-60 cells was reasonably high compared with NRK-49F cells (Table 1 and Fig. 1). Even though we have tried experiments with HL-60 cells using various agonists, such as isoproterenol, which stimulate the synthesis of cAMP, inhibitory (or stimulatory) effect of 12-HETE was not observed in any cases.

Results so far obtained have revealed that the effect of 12-HETE varies depending upon the cells used. The difference can not be clearly explained by the agonists used, because very different results were obtained with NRK-49F and HL-60 cells using PGE_1 as the agonist. How could the differing effect of 12-HETE on different cells be explained? A

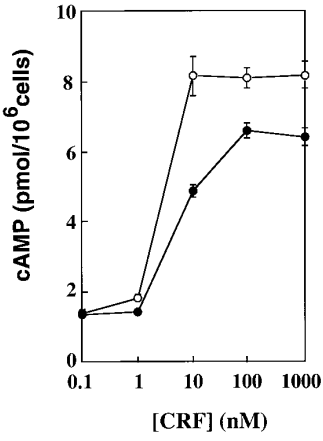

FIG. 2. Effect of 12-HETE on CRF-induced cAMP synthesis in AtT-20 cells. AtT-20 cells were preincubated with 0.5 mM IBMX at 37°C for 30 min either in the presence (-●-) or absence (-○-) of 20 μM 12-HETE, followed by stimulation with CRF at indicated concentrations for another 30 min. Values are expressed as the mean ± S.E. obtained from four experiments.

plausible explanation is that 12-HETE does not bind to HL-60 cells in contrast to other cells. We investigated this point using [^{3}H] 12-HETE. This fatty acid actually bound to HL-60 even a bit more than to NRK-49F cells. Furthermore, the binding was steadily reduced by the competition with the non-radioactive 12-HETE in a dose-dependent manner in both cells, as is clearly shown in Fig. 3. These results strongly indicate that there are specific binding sites in both cells.

Another possibility is the difference in inhibitory G proteins, G_i. Based on the hypothesis that the inhibition of 12-HETE is mediated by a specific G_i protein(s), the difference between NRK-49F and HL-60 cells might be explained by the difference in the kind of G_i protein(s) present in these cells. In order to determine the presence of G_i proteins as well as the receptors for PT in NRK-49F cells, the intact cells were treated with extracellularly added PT and further

TABLE 1
Effects of 12-HETE on PGE_1 and PGE_2-Induced cAMP Accumulation in HL-60 Cells

Treatment	cAMP (pmol/10^6 cells)
Control	4.72 ± 0.08
+ 12-HETE	6.19 ± 0.38
PGE_1	96.9 ± 1.70
+ 12-HETE	91.1 ± 5.17
PGE_2	96.2 ± 1.32
+ 12-HETE	101 ± 7.42

Note. HL-60 cells were preincubated with 0.5 mM IBMX at 37°C for 30 min in either the presence or absence of 20 μM 12-HETE, followed by stimulation with 10 μM PGE_1 or PGE_2. The amount of cAMP formed in 30 min was then determined. Values are expressed as the mean ± S.E. of four determinations from a representative of two independent experiments.

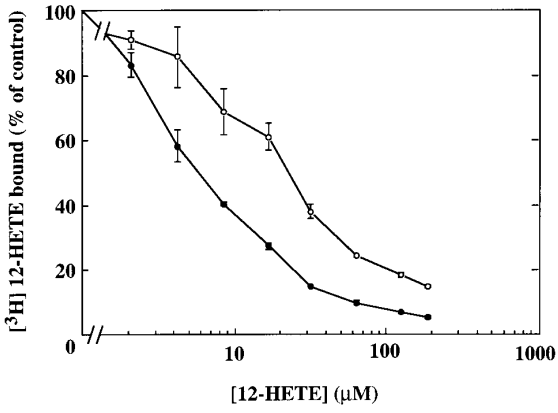

FIG. 3. Specific binding of 12-HETE to NRK-49F and HL-60 cells. NRK-49F (-●-) and HL-60 cells (-○-) were incubated with a fixed concentration (0.078 nM) of [^{3}H] 12-HETE in the presence of varied concentrations of non-radioactive 12-HETE for 30 min at 37°C. In the absence of non-radioactive 12-HETE, the radioactivity bound to NRK-49F and HL-60 cells was 7000 and 12000 dpm/cell, respectively, under the experimental condition. Values are expressed as the mean ± S.E. obtained from four experiments.

ADP-ribosylation of G_i proteins in the membrane preparation was studied. As clearly shown in Fig. 4A, the pretreatment of cells with PT completely abolished ADP-ribosylation of G_i proteins in the membrane preparation, indicating the presence of G_i proteins as well as receptors for PT in the cell. To investigate whether the G_i proteins are indeed involved in the inhibition by 12-HETE, the effect of 12-HETE on the cAMP production by PT-pretreated cells was determined. As it is unambiguously depicted in Fig. 4B, 12-HETE did inhibit cAMP production regardless of the pretreatment of cells with PT, thus excluding the possible involvement of G_i

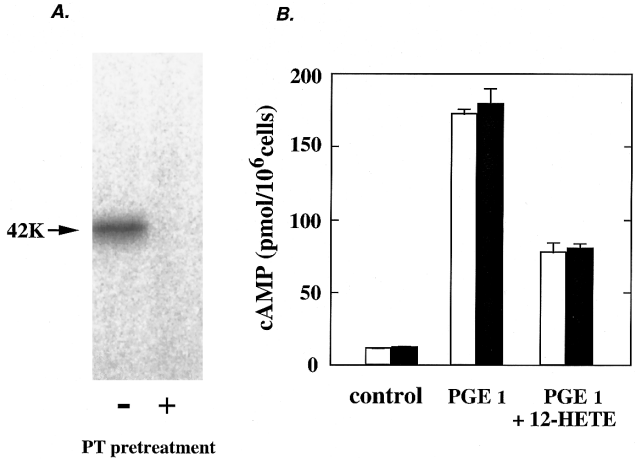

FIG. 4. The effect of pertussis toxin on the inhibition of cAMP production by 12-HETE. **A**. PT-pretreated NRK-49F cells were prepared by incubating cells for 4 hrs at 37°C with PT at 100 ng/ml in fresh culture media. Membranes were prepared from cells with or without the pretreatment, followed by incubation with [^{32}P] NAD and visualization as described under Experimental Procedures. **B**. The effect of 12-HETE on the cAMP production was determined using normal NRK-49F (open columns) and the PT-pretreated NRK-49F (closed columns) cells as described under *Experimental Procedures.* The concentrations of PGE_1 and 12-HETE were 10 μM and 20 μM, respectively. Values are expressed as the mean ± S.E. of four determinations from a representative of two experiments.

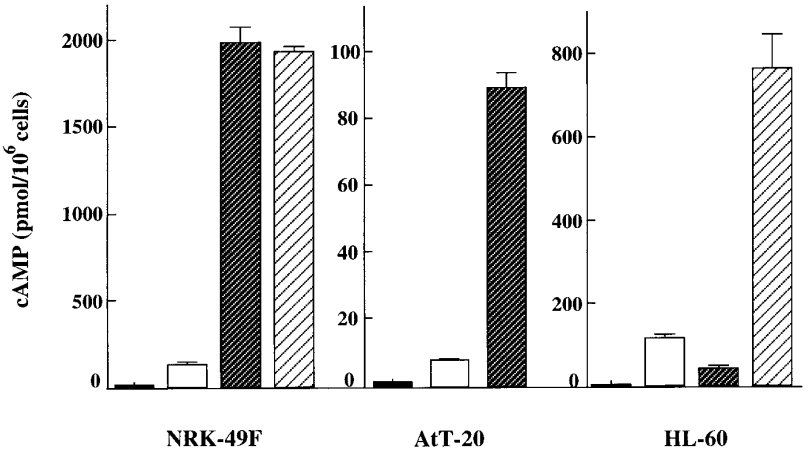

FIG. 5. Characterization of AC present in various cells. Agonist- and FSK-stimulated cAMP syntheses were compared among three different cells. Cells were pre-incubated with IBMX as described under *Experimental Procedures* and further incubated with agonists (open columns), *i.e.* 10 μM PGE_1 (NRK-49F and HL-60 cells) and 1 μM CRF (AtT-20 cells), or 100 μM FSK (dark hatched columns). The incubation of cells was also continued with IBMX alone to give the control values (closed columns). Synergistic effects between PGE_1 and FSK were investigated using NRK-49F and HL-60 cells. Cells were stimulated with FSK (100 μM) as well as PGE_1 (10 μM) simultaneously (light hatched columns). Values are expressed as the mean $\pm$ S.E. obtained from four experiments.

proteins in the inhibition by 12-HETE. The difference between NRK-49F and HL-60 cells, therefore, can not be explained by the difference in G_i proteins in these cells.

Further, it is possible that different cells may have different isoforms of AC, and that some isoforms do respond to 12-HETE while some do not. It is known that there are at least eight to nine isoforms in the family of AC; they share similarities but are substantially different in some aspects, such as type-specific stimulatory and inhibitory regulation by G-protein α and $\beta\gamma$ subunits, Ca^{2+}-calmodulin, and forskolin (15). The following data will strongly indicate that the AC present in NRK-49F and HL-60 cells are indeed very different each other. First of all, we have compared the effects of agonists and FSK on the activity of AC. The agonists used here are PGE_1 and CRF, both of which are known to activate AC through G-protein ($G_s\alpha$ subunit). In contrast, FSK is known to directly activate AC probably by causing specific conformational change in AC through specific interaction (16). As is clearly demonstrated in Fig. 5, in the case of NRK-49F cells, PGE_1 elevated the production of cAMP from the basal value of 11.5 to 136 pmol/10^6 cells at 10 μM. This effect was maximal since higher concentration of PGE_1 failed to increase cAMP levels further. On the other hand, the effect of FSK was much more profound than that of PGE_1. cAMP was elevated to almost 2000 pmol/10^6 cells. A similar result was obtained with AtT-20 cells treated with CRF or FSK, although the activity of AC in this cell was generally much less than that observed in NRK-49F cells in all cases. A strikingly different result, however, was obtained with HL-60 cells. PGE_1 (10 μM) stimulated the activity of AC efficiently; the cAMP level was increased from the basal value of 2.1 to 115 pmol/10^6 cells. In contrast, FSK only poorly activated the enzyme; the stimulatory effect of FSK (100 μM) was less than 40% of that obtained with PGE_1 (10 μM). This makes a very sharp contrast to that obtained with other two kinds of cells, in which the effects of FSK were more than 10 times stronger than the agonists. We then investigated the combined effect of PGE_1 and FSK using NRK-49F and HL-60 cells. With NRK-49F cells the basal level of cAMP was 11.5, and the levels of cAMP obtained by the stimulation with PGE_1 (10 μM) and FSK (10 and 100 μM) were 136, 1030, and 1980 pmol/10^6 cells, respectively.

Simultaneous addition of PGE_1 and FSK did not increase the level of cAMP from the sum of the levels obtained by each alone, as presented in the left panel of Fig. 5. A totally different result was obtained with HL-60 cells (the right panel of Fig. 5). The basal level of cAMP was 2.1, and those obtained with PGE_1 (10 μM) and FSK (10 μM and 100 μM) were 115, 12.8 and 43.8 pmol/10^6 cells, respectively. When 10 μM PGE_1 and 10 μM FSK were added together, cAMP was increased to 292 pmol/10^6 cells. The value is more than the additive effects of each compound alone. This synergism became even more striking when the concentration of FSK was increased to 100 μM; the value obtained with the simultaneous addition was 764 instead of 159 pmol/10^6 cells, which might be the value expected if there is no synergism at all. It is of particular interest to note that type II isoform, in sharp contrast to type I isoform, was reported to respond synergistically to $G_s\alpha$ and FSK but respond very weakly to FSK alone in a recombinant baculovirus expression system in Sf9 cells (17). Pieroni *et al.* (18), using the same system found that type II isoform expressed a very weak response to FSK. They also reported that type II isoform had much stronger basal activities in the presence of Mg^{2+} or Mn^{2+} relative to type VI isoform.

In conclusion, our results have shown that 12-HETE inhibition of AC may depend upon the type of AC present. The classification of isoforms in this regard remains to be elucidated and the detailed mechanism for the inhibition also remains to be clarified in near future.

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