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12(S) Hydroxyeicosatetraenoic acid induces cAMP production via increasing intracellular calcium concentration

Go Hasegawa[a], Satoru Kumagai[a], Masato Yano[a], Yong-Gang Wang[b], Yuichi Kobayashi[b], Yuji Saito[a,*]

[a] *Department of Biological Sciences, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan*

[b] *Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan*

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Abstract **We have found that a 12-lipoxygenase metabolite of arachidonic acid, 12(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE), induces cAMP production in human normal fibroblast TIG-1 cells. This phenomenon was not observed in other cells tested including human embryonic kidney HEK293 cells. We have speculated that this specific response might be influenced by the kinds of isoform of adenylyl cyclase (AC) present in cells. We found that TIG-1 cells specifically expressed type VIII AC. As type VIII AC is known to be activated by an increase of calcium concentration, we determined the change of intracellular Ca^{2+} concentration after the addition of 12-HETE. It was elevated not only in TIG-1 cells, but also HEK293 cells, which did not respond to 12-HETE to produce cAMP. The addition of a calcium ionophore elevated the concentration of intracellular cAMP in TIG-1 cells, but it was without effect in HEK293 cells. To show that the expression of this particular isoform of AC is responsible for the positive response to 12-HETE, we transfected this AC isoform into HEK293 cells. The type VIII AC-transfected cells, in contrast to the mock-transfected ones, became very responsive to 12-HETE to produce cAMP. Taken all together the data would strongly suggest that 12-HETE specifically activates type VIII AC via increasing intracellular Ca^{2+} concentration.**

Key words: Hydroxyeicosatetraenoic acid; Adenylyl cyclase; Calcium; cAMP

1. Introduction

Arachidonic acid released from membrane phospholipids by phospholipases is metabolized into various eicosanoids [1] in the cells. There are two metabolic pathways for arachidonic acid: the lipoxygenase and cyclooxygenase pathways. Stereoactive lipoxygenases insert a hydroxyperoxyl group, which is subsequently reduced to a stable hydroxyl group to produce hydroxyeicosatetraenoic acids (HETEs). HETEs are known to be involved in cellular signaling and various biological events [2]. 12(*S*)-Hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid (12-HETE) is also a biologically active eicosanoid formed by 12-lipoxygenase. It is synthesized in platelets, leukocytes, macrophages, endothelial cells and smooth muscle cells [2,3]. 12-HETE is the major arachidonic acid metabolite in platelets, and plays an important role in the regulation of platelet aggregation [4,5]. It has also been reported to regulate corneal epithelial cell proliferation [6], enhance interleukin production in human bone marrow cells [7], promote angiogenesis in microvascular endothelial cells [8], act as a neutrophil chemoattractant [9] and induce the expression of heat shock proteins in human leukocytes [10].

cAMP is a well-known second messenger molecule in signal transduction pathways in various cells, and it is synthesized by adenylyl cyclases (ACs) (EC 4.6.1.1). AC is a membrane-spanning enzyme that catalyzes the conversion of intracellular ATP to cAMP. To date, it is known that AC has nine isoforms in mammalian cells. The mechanism of the regulation of these ACs has been reviewed [11,12]. All mammalian ACs are activated by GTP-bound stimulatory G protein α subunit ($G\alpha_s$). Agonist-induced receptor activation affects $G\alpha_s$, and consequently induces AC-mediated cAMP production. The plant-derived diterpene, forskolin, also directly activates all AC isoforms except type IX. On the other hand, inhibitory G protein α subunit inhibits all AC isoforms except types II and IV. Protein kinase C has a positive effect on type I–V and VII ACs, although protein kinase C as well as A have negative effects on type VI AC. Effects of Ca^{2+} are very diverse depending on the AC isoform. The activities of type I and VIII ACs are enhanced by Ca^{2+}, although those of types V and VI are inhibited. From these results it would be concluded that ACs are regulated by complicated regulatory signal pathways.

We have already reported that 12-HETE enhances cAMP production in fibroblast cells [13]. In the present study, we have clarified the mechanism of this 12-HETE-induced cAMP production. We report that 12-HETE enhances cAMP production by specifically activating type VIII AC via increasing intracellular Ca^{2+} concentration.

*Corresponding author. Fax: (81)-45-924 5808.
E-mail address: ysaito@bio.titech.ac.jp (Y. Saito).

Abbreviations: 12-HETE, 12(*S*)-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; AC, adenylyl cyclase; PMA, phorbol 12-myristate 13-acetate; IBMX, 3-isobutyl-1-methylxanthine; W-13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; PGE_1, prostaglandin E_1; RT-PCR, reverse transcriptase-polymerase chain reaction

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2. Materials and methods

2.1. Materials

12-HETE was chemically synthesized as described previously [14], and dissolved in ethanol. Phorbol 12-myristate 13-acetate (PMA) was purchased from Nacalai Tesque (Kyoto, Japan). Anti-cAMP antiserum was purchased from Gramsch Laboratories (Schwabhausen, Germany). Fura-2 AM was purchased from Dojindo Laboratories (Kumamoto, Japan). Oligonucleotide primers were purchased from Invitrogen (Carlsbad, CA, USA). 3-Isobutyl-1-methylxanthine (IBMX), *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W-13), prostaglandin E_1 (PGE_1), 2′-*O*-monosuccinyladenosine 3′,5′-cyclic monophosphate, the calcium ionophore A23187 and all other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell cultures

G361 (human melanoma cells), HT1080 (human fibrosarcoma cells), HEK293 (human embryonic kidney cells) and TIG-1 (human normal fibroblasts) were provided by the Human Science Research Resources Bank (Osaka, Japan). HT1080, TIG-1 and HEK293 cells were cultured with Dulbecco's modified Eagle's medium supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), 10% fetal bovine serum and non-essential amino acids. G361 cells were cultured with RPMI 1640 medium supplemented with penicillin, streptomycin and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Preparation of samples for cAMP determination

Cultured cells were seeded on 24-well culture plates and cultured for a few days until they reached confluence. Each well was extensively washed with a serum-free medium, incubated with 0.5 mM IBMX at 37°C for 30 min, and further incubated with 12-HETE dissolved in ethanol or ethanol alone as the control at 37°C for another 30 min. The final concentration of ethanol was kept at less than 0.5%. When PMA and W-13 were used, these reagents were added simultaneously with IBMX. After incubation, 20 μl of 3 M NaOH was added to stop the reaction and to dissolve the cells, and HCl was then added to neutralize. The synthesized cAMP was succinylated as follows prior to the determination. Aliquots were reacted with succinic anhydride dissolved in dry dioxane in the presence of triethylamine at room temperature for 10 min, and the mixtures were then diluted in 0.3 M imidazole buffer (pH 6.4). These samples were stored at −80°C until use.

2.4. cAMP determination

cAMP–protein conjugates were prepared using 2′-*O*-monosuccinyladenosine 3′,5′-cyclic monophosphate by the method described [15]. In the initial stage of the investigation we used yeast β-galactosidase as the protein, which facilitated the measurement of coating of the conjugate. Later we used ovalbumin as the protein. Fifty μl of the 10 μg/ml cAMP–protein conjugate was added onto 96-well Nunc-Immuno Plates (Nalge Nunc, Rochester, NY, USA) and left at room temperature for 1 h to coat the surface. The unbound protein was removed, and the surface of wells was blocked with 2% ovalbumin at room temperature for 1 h. At first we established the standard curve for the determination. cAMP at varying concentrations was succinylated as described above and then it was incubated with a fixed amount of anti-cAMP antiserum in separate tubes at room temperature for 1 h with rotation. Fifty μl of the mixtures was then added to plates coated with the cAMP–protein conjugate. Only the antibody, which did not bind to cAMP, should be able to bind to the conjugate. The higher the concentration of cAMP the lower the amount of the antibody bound to the coated cAMP–protein conjugate. Each well was washed three times with 10 mM Tris–HCl buffer (pH 7.4) in 150 mM NaCl (TBS), and incubated with alkaline phosphatase-labeled anti-rabbit IgG at room temperature for 1 h. After washing the wells three times with TBS, the activity of the bound alkaline phosphatase was determined using 250 μM 4-methylumbelliferone phosphate as a substrate. The release of 4-methylumbelliferone was monitored fluorometrically by fluorescence plate reader (CytoFluor Multi-well Plate Reader Series 4000, Applied Biosystems, Foster City, CA, USA). Having established the standard curve as described above, we determined the concentration of cAMP present in various samples by the procedure described above for the development of the standard curve.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

mRNAs were extracted from about 1×10^7 cells with QuickPrep Micro mRNA purification kit (Amersham Biosciences, Piscataway, NJ, USA). They were then subjected to first strand cDNA synthesis with Ready-To-Go T-Primed First-Strand kit (Amersham Biosciences). Up to the synthesis of cDNAs, all experiments were carried out under RNase-free condition. First-strand cDNAs were then used as templates in PCR. The oligonucleotide primers synthesized were 5′-TGCCTTATTTGGCCTTGTCTACC-3′ and 5′-GACACCCGGAAAAATATGGCTAG-3′ for type I AC; 5′-GCGTGATAGAAGAAAAGCACTGG-3′ and 5′-TACAAAACTGGTCCTAGGCTGTG-3′ for type II AC; 5′-TTGAAACTGAAGGAAGTCCCGAC-3′ and 5′-TTCAAAATGGGAGCCATGTCCTG-3′ for type III AC; 5′-TGGAGTATGGGATGCTGAGAGA-3′ and 5′-CAATCCAAATGCTGGTGACAACC-3′ for type IV AC; 5′-TGAGCTTCTACCTGTCGTGTTTC-3′ and 5′-TGTAGGTGAAGTACTCTGGGAAG-3′ for type V AC; 5′-GTAACTGTGTGTGCATGTTGGTC-3′ and 5′-TCAAACCCCAGACAGTTCAAGTG-3′ for type VI AC; 5′-GGGCTGAAATTAAGAGTACAGC-3′ and 5′-TCATGTGAAACCAGCCCTTCATG-3′ for type VII AC; 5′-GAAGACAAAGTACTCTGGGTAGG-3′ and 5′-GTTCAAGTCAAACTTGGTCTGTGC-3′ for type VIII AC; 5′-GTGTGGATCACAGTTATTCAGGG-3′ and 5′-TGTGTGCGCATGTGTGCTTACAT-3′ for type IX AC. PCR was carried out with the following experimental conditions: 95°C for 4 min (1 cycle); 95°C for 1 min, 70°C for 1 min, 72°C for 1 min (2 cycles); 95°C for 1 min, 68°C for 1 min, 72°C for 1 min (2 cycles); 95°C for 1 min, 66°C for 1 min, 72°C for 1 min (2 cycles); 95°C for 1 min, 64°C for 1 min, 72°C for 1 min (2 cycles); 95°C for 1 min, 62°C for 1 min, 72°C for 1 min (2 cycles); 95°C for 1 min, 60°C for 1 min, 72°C for 1 min (19 cycles); 72°C for 9 min (1 cycle). PCR products were electrophoresed on 4% polyacrylamide or 2% agarose gel, and stained with ethidium bromide.

2.6. Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was monitored using a fluorescent probe, fura-2 AM, as described previously [16]. Briefly, cultured cells were incubated with 3 μM fura-2 AM at 37°C for 45 min. Subsequently, cells were washed extensively with TBS and suspended in TBS containing 1.8 mM $CaCl_2$. 12-HETE (20 μM) was then added to the cell suspension (3×10^6 cells/ml). The fluorescence emission was measured at 505 nm in response to excitation at 340 nm ($_{340}F^{505}$) and 380 nm ($_{380}F^{505}$) by Caf-100 (JASCO, Tokyo, Japan), and time-dependent changes in $_{340}F^{505}/_{380}F^{505}$ were monitored. Intracellular Ca^{2+} concentration was calculated using the equation of Grynkiewicz et al. [16], where $K_d = 224$ nM.

2.7. Membrane preparation

Membrane fractions were prepared as described previously [17]. In brief, TIG-1 cells (10^8 cells) were harvested and washed with phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A and 10 μg/ml leupeptin. Total membrane fractions were prepared by freeze-thawing of cells, followed by hypotonic lysis in 10 mM Tris–HCl buffer containing 0.1 mM EDTA (pH 7.5), homogenization, centrifugation at $40\,000\times g$ and resuspension in 25 mM Tris–HCl buffer (pH 7.5). They were then stored at −80°C until use.

2.8. Transfection of type VIII AC into HEK293 cells

Transient transfection into HEK293 cells was performed using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One μg of plasmid encoding type VIII AC was transfected into 1×10^5 cells, and following experiments were performed 2 days after the transfection.

3. Results and discussion

The effect of 12-HETE on the production of cAMP was investigated using various cells. The result is shown in Fig. 1. Among these cells tested, only TIG-1 cells responded positively to 12-HETE. As we pretreated cells with the phosphodiesterase inhibitor IBMX in this experiment to prevent the breakdown of cAMP formed, the effect of 12-HETE observed

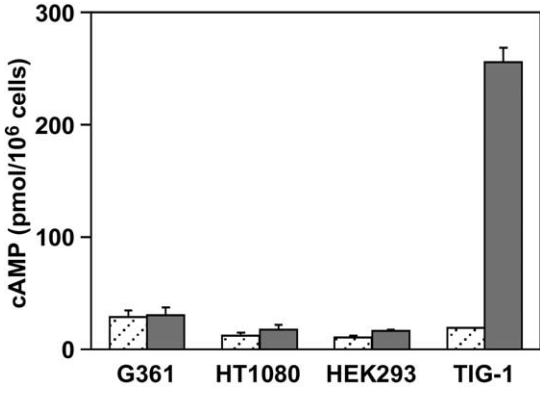

Fig. 1. The effect of 12-HETE on the production of cAMP in various cells. G361 (human melanoma cells), HT1080 (human fibrosarcoma cells), HEK293 (human embryonic kidney cells) and TIG-1 (human normal fibroblasts) were pretreated with 0.5 mM IBMX at 37°C for 30 min. They were then incubated with 25 μM 12-HETE (closed columns) or ethanol alone (hatched columns) at 37°C for another 30 min. cAMP produced was determined as described in Section 2. The data shown are the mean ± S.E.M. of duplicate determinations from a representative of three experiments.

here might be solely regarded as the effect on the activity of AC. We previously presented the specific effect of 12-HETE on TIG-1 cells among various cells other than those cells used in the present study [13]. We also showed there that a calcium chelator and a calmodulin inhibitor inhibited the effect of 12-HETE on the production of cAMP. These results would imply that the effect of 12-HETE might have something to do with the change of intracellular concentration of Ca^{2+}. Previously we showed that 12-HETE inhibited the agonist-induced cAMP production in platelets [5] as well as cultured cells such as rat kidney fibroblast NRK-49F and mouse pituitary tumor AtT-20 cells [17]. We also showed that 12-HETE was without effect on the agonist-induced cAMP production in HL-60 human leukemia cells [17]. The question then arose why the effect of 12-HETE differed so much depending on the cells we used to investigate. Generally speaking, an agonist should activate a specific receptor and it in turn activates the downstream effector. However, if there are multiple kinds of downstream effectors present in different cells, it would be

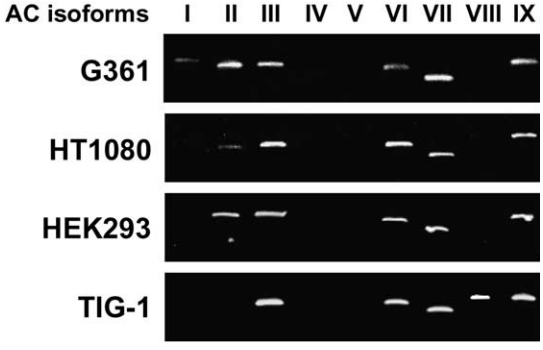

Fig. 2. Expression of different AC isoforms in various cells. RT-PCR was performed using specific primers for various AC isoforms as described in Section 2. PCR products were electrophoresed on 4% polyacrylamide gels and stained with ethidium bromide. The data shown are from one experiment that is representative of at least three separate experiments.

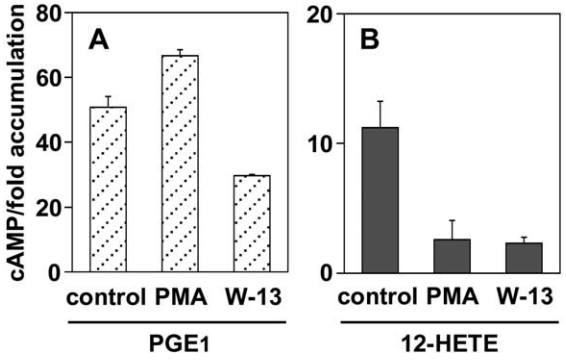

Fig. 3. Activation of the specific AC isoform by 12-HETE. TIG-1 cells were pretreated with 0.5 mM IBMX plus either 10 nM PMA or 10 μM W-13, or with IBMX alone at 37°C for 30 min. The cells were then incubated either with 10 μM PGE_1 (A) or with 25 μM 12-HETE (B) at 37°C for 30 min, cAMP produced was then measured. The data shown are the mean ± S.E.M. of duplicate determinations from a representative of three experiments.

possible that the final outcome might be quite different depending on the cells we investigate.

It is known that there are various isoforms of AC expressed in different cells and tissues [11,18]. Although all of them have a common basic structure with 12 membrane-spanning domains, there are subtle structural differences among them. Because of this the activity of these isoforms is differently

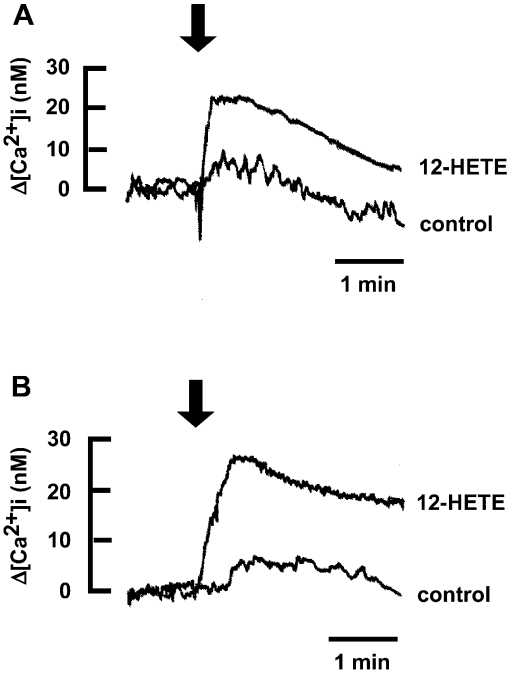

Fig. 4. 12-HETE induces the rise of intracellular Ca^{2+} concentration. TIG-1 (A) or HEK293 (B) cells were treated with 3 μM fura-2 AM at 37°C for 45 min in a calcium-free medium. 12-HETE-induced changes in intracellular Ca^{2+} concentration were then monitored in the medium containing 1.8 mM $CaCl_2$ by measuring the fluorescence emission at 505 nm in response to excitation at 340 nm and 380 nm. 12-HETE (20 μM) was added at times indicated by the arrows. The concentrations of Ca^{2+} were calculated according to the procedure described in Section 2. The data shown are from one experiment that is representative of at least three separate experiments.

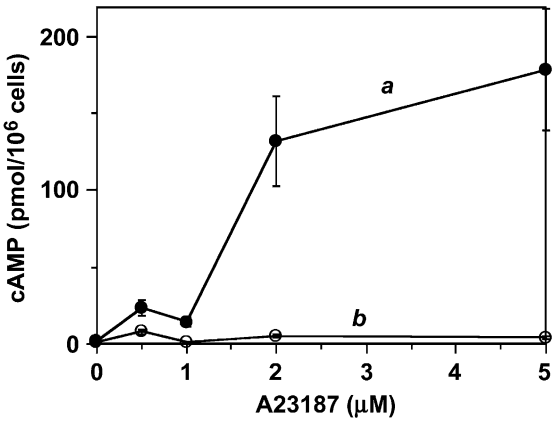

Fig. 5. Effects of a calcium ionophore on cAMP production in TIG-1 and HEK293 cells. TIG-1 (curve a) or HEK293 (curve b) cells were pretreated with 0.5 mM IBMX, and were then incubated with the calcium ionophore A23187 at varying concentrations in the presence of 1.5 mM $CaCl_2$ at 37°C for 30 min. After the incubation, the concentration of cAMP produced was measured as described in Section 2. The data shown are the mean ± S.E.M. of duplicate determinations from a representative of three experiments.

regulated [11,12]. We, therefore, investigated the kinds of isoforms of AC present in these cells. Each isoform contains short and variable amino- and carboxy-termini, and relatively homologous long cytoplasmic catalytic domains denoted C_1 and C_2 [12]. Therefore specific primers for various AC isoforms for RT-PCR were synthesized, which corresponded to the C-terminal region and transmembrane spans. The results of RT-PCR presented in Fig. 2 show that types II, III, VI, VII and IX AC are expressed in all cells tested. On the other hand, type VIII AC is expressed specifically in TIG-1 cells. Additionally, another human fibroblast cell line, MRC-5, also reacted positively with 12-HETE, and expressed type VIII AC (data not shown). These data suggest that type VIII AC might be expressed specifically in fibroblast cells. It is of interest and also of importance to note that this particular isoform is known to be activated by Ca^{2+} [19,20]. It is possible that this isoform is specifically activated in TIG-1 cells after the addition of 12-HETE. Furthermore, it would become a possibility that 12-HETE would elevate the concentration of intracellular Ca^{2+} prior to giving an effect on AC. The final outcome from the addition of 12-HETE on cAMP production would differ solely depending on the isoforms of AC present in various cells.

To confirm that the specific kind of AC isoform present in TIG-1 cells was activated by 12-HETE, we studied the effect of an activator of protein kinase C, PMA, and the calmodulin inhibitor W-13, on PGE_1- and 12-HETE-induced activation of AC in TIG-1 cells. It is known that PGE_1 binds to the specific receptor and activates $G\alpha_s$, which in turn activates all isoforms of AC [11]. As shown in Fig. 3, PGE_1-induced activation of AC was further enhanced by PMA. On the other hand, 12-HETE-induced activity was very strongly inhibited by PMA. W-13 inhibited the PGE_1-induced AC activity. It is obvious, however, that it inhibited the 12-HETE-induced activity much more strongly. These obvious differences could be expected if we assume that 12-HETE activates the very specific isoform present in TIG-1 cells that is type VIII AC.

To further substantiate the speculation described above, we actually determined the change of intracellular Ca^{2+} concentration after the addition of 12-HETE to the cell suspension using the method described in Section 2. In this experiment we employed not only TIG-1 cells but also HEK293 cells, which did not respond positively to 12-HETE in terms of cAMP production probably due to the lack of the type VIII AC isoform. As shown in Fig. 4, it is quite obvious that 12-HETE induced the increase of intracellular Ca^{2+} concentration in both TIG-1 and HEK293 cells under this experimental condition. The maximum increases of intracellular Ca^{2+} concentration induced by 12-HETE in TIG-1 and HEK293 cells were very comparable at 22.7 nM and 25.5 nM, respectively. These data indicate that 12-HETE increased the intracellular Ca^{2+} concentration to the same extent in both TIG-1 and HEK293 cells. It then became even more plausible that the difference of response to 12-HETE in terms of cAMP production in various cells could be accounted for by the presence of the specific isoform of AC present in cells.

We then used the calcium ionophore A23187 to artificially elevate the concentration of intracellular Ca^{2+} using two different cell lines, TIG-1 and HEK293 cells. In this experiment we kept the concentration of extracellular Ca^{2+} concentration at 1.5 mM, and varied the concentration of the ionophore. We then determined the change of intracellular concentration of cAMP in these cells. The results are shown in Fig. 5. As expected it was increased in TIG-1 cells in a dose-dependent manner, probably due to the presence of type VIII AC. On the other hand, it was not increased at all in HEK293 cells.

If the effect of 12-HETE on the production of cAMP is really mediated by the increase of intracellular Ca^{2+} concentration, we would need whole intact cells to see this effect. This effect should not be observed with membrane preparations, as there is no way to increase the Ca^{2+} concentration in the membrane suspension. This notion was found to be the case. First of all we confirmed the integrity of the membrane prepared from TIG-1 cells by showing the positive effect of PGE_1 on cAMP production in the presence of GTP and ATP (Fig. 6A). It is known that PGE_1 binds to activate the specific receptor on plasma membrane and stimulates the binding of GTP to the α subunit of G_s protein, which subsequently activates AC [11,21]. On the other hand, 12-HETE did not induce

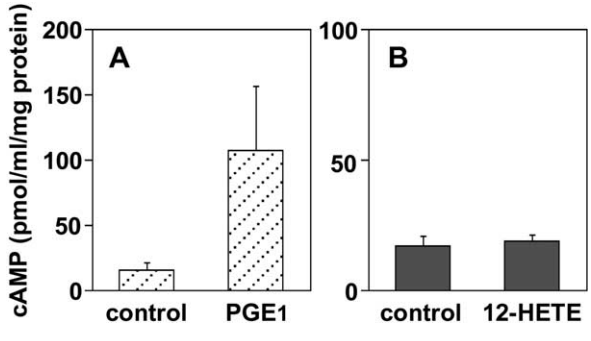

Fig. 6. Lack of stimulation of cAMP production by 12-HETE with membrane. Plasma membrane fractions were prepared from TIG-1 cells as described in Section 2. The membrane preparation was added to a reaction mixture containing 70 mM Tris–HCl buffer (pH 7.4), 0.5 mM IBMX, 1 mM $MgCl_2$, 50 μM ATP, 40 μM GTP, 12 mM phosphocreatine, 2.5 U creatine phosphokinase and PGE_1 (10 μM, A) or 12-HETE (25 μM, B). They were incubated at 30°C for 20 min, and the produced cAMP was then determined as described in Section 2. The data shown are the mean ± S.E.M. of duplicate determinations from a representative of three experiments.

cAMP production with membrane preparation (Fig. 6B), as was expected.

Our notion that the effect of 12-HETE on cAMP production is mediated by the increase of intracellular Ca^{2+} and that its final manifestation depends solely on the kind of isoforms of AC present could be substantiated by a transfection experiment. We have already shown that HEK293 cells do not respond positively to 12-HETE in terms of cAMP production. We have, however, also shown that 12-HETE induces the increase of intracellular Ca^{2+} in this cell line. It is quite reasonable to expect that HEK293 cells would become positively responsive to 12-HETE and produce cAMP after transfection of type VIII AC. Transient transfection of type VIII AC to HEK293 cells was performed, and the effect of 12-HETE on cAMP production was subsequently assessed. First of all we checked the expression of type VIII AC in transfected HEK293 cells. As is unambiguously shown in the inset of Fig. 7, the results of PT-PCR demonstrate the expression of the new isoform of AC in this cell. The effect of the transfection is very clearly depicted in this figure. Contrary to the mock-transfected cells, the transfected HEK293 cells became positively responsive to 12-HETE. They responded to 12-HETE in a dose-dependent manner, as if they were TIG-1 cells.

In this study, we have reported that 12-HETE stimulates cAMP production in human fibroblast TIG-1 cells via increasing intracellular Ca^{2+} concentration and the following activation of type VIII AC. On the other hand, we reported previously that 12-HETE inhibited PGE_1-induced cAMP production in platelets [5], rat kidney fibroblast NRK-49F and mouse pituitary tumor AtT-20 cells [17]. Based on the results of the present investigation we speculate that these cells might express isoforms of AC inhibited by Ca^{2+} such as types V and VI [22]. The results of RT-PCR experiments, which are demonstrated in Fig. 2, show that type VI AC is expressed fairly commonly among cells tested including TIG-1 cells. The presence of this isoform, however, does not interfere with the effect of 12-HETE on type VIII AC. This is because the basal activity of any isoform of AC is very low, and it is almost impossible to show the inhibitory effect there. The classification of AC isoforms and the mechanism of inhibition of AC by 12-HETE in those cells remain to be elucidated. The mechanism of increase of intracellular Ca^{2+} concentration by 12-HETE also remains to be elucidated in the future.

12-HETE increased the intracellular Ca^{2+} concentration in TIG-1 and HEK293 cells in the presence of extracellular Ca^{2+}, as shown in Fig. 4. These data coincide well with the report of a 12-HETE-induced increase of intracellular Ca^{2+} concentration in human neutrophils [23]. In conclusion, our results have shown that 12-HETE increases intracellular Ca^{2+} concentration and specifically activates type VIII AC.

Not only type VIII but also type I AC is known to be activated by Ca^{2+}. The distribution of the latter is quite specific in the neural system including brain, retina, and adrenal medulla [24]. Thrombotic tendency in the brain is a big problem for aged people, and 12-HETE could be released from platelets in the microthrombi. It would be possible then that 12-HETE increases the intracellular Ca^{2+} concentration in the brain cells, which in turn activates type I AC. The produced cAMP in the brain cells might stimulate cAMP-dependent protein kinase to phosphorylate tau protein and might accelerate the development of Alzheimer's disease [25].

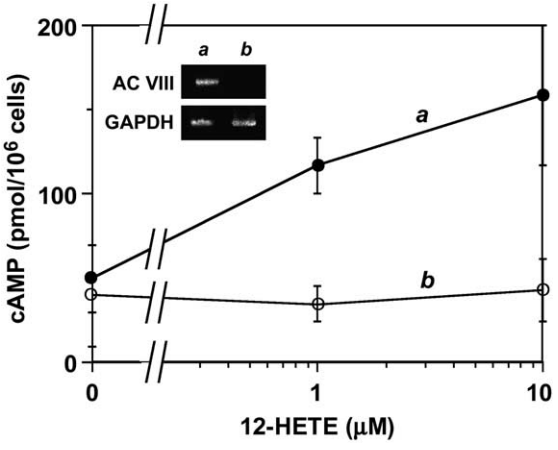

Fig. 7. cAMP production in HEK293 cells transiently expressing type VIII AC. HEK293 cells were transfected with type VIII AC expression construct (curve a) or with vector alone (curve b). After 2 days of transfection they were incubated with 0.5 mM IBMX at 37°C for 30 min. They were then incubated with 12-HETE at increasing concentrations at 37°C for another 30 min, and the production of cAMP was measured. The data shown are the mean ± S.E.M. of duplicate determinations from a representative of three experiments. The transfection of type VIII AC was confirmed by RT-PCR amplification using specific primers for type VIII AC (inset). PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide.

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