Mechanism(s) of activation of secretory phospholipase A_2s in mouse keratinocytes

Bangyan Li-Stiles and Susan M. Fischer[1]

The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, P.O. Box 389, Smithville, TX 78957

Abstract **The differential activation of different members of the phospholipase A_2 (PLA_2) superfamily and their regulation are important as one or more of them regulates the production of eicosanoids and others may contribute to the formation of other lipid mediators. We previously reported the existence of two forms of secretory or $sPLA_2$ in mouse keratinocytes, namely type I and type II $sPLA_2$. We show here that mouse keratinocyte $sPLA_2$s were potently activated by protease treatment and inhibited by protease inhibitors. We also observed that G protein effectors induced substantial release of oleic acid (OA) from prelabeled mouse keratinocytes. A G_i/G_0 protein activator significantly enhanced the hydrolysis of OA and this increase was not responsive to either pertussis toxin or cholera toxin treatment. Although there was a significant negative correlation between intracellular cAMP levels and OA hydrolysis, experimentally increasing cAMP with forskolin treatment had no effect on $sPLA_2$ activity. Arachidonic acid but not its metabolites was also shown to marginally activate keratinocyte $sPLA_2$ by 1.5-fold. These results lead to the conclusion that mouse keratinocyte $sPLA_2$s can be regulated primarily by proteolytic activation and a G protein pathway.**—Li-Stiles, B., and S. M. Fischer. **Mechanism(s) of activation of secretory phospholipase A_2s in mouse keratinocytes.** ***J. Lipid Res.*** **1999.** 40: **1701–1708.**

Supplementary key words arachidonic acid • G protein • keratinocytes • phospholipase A_2 • protease • secretory phospholipase A_2

Phospholipases A_2 are a group of enzymes that catalyze the hydrolysis of *sn*-2 fatty acids from phospholipids. One of the most important fatty acids released by this action is arachidonic acid. Arachidonic acid (AA) and its metabolites, eicosanoids, have been shown to play important roles in inflammation, cell proliferation, as well as tumor promotion (1). For example, prostaglandin E_2 (PGE_2) was shown to stimulate cell proliferation (2–4) and overexpression of prostaglandin synthase H-2 (PGHS-2) has also been observed in hyperplastic skins in SENCAR mouse as well as other strains of mice (5, 6), although its role in tumor promotion is not yet defined. However, products of other lipids may oppose the effects of AA-derived eicosanoids on tumor formation. The linoleic acid product, 13-hydroxyoctadecaenoic acid (13-HODE) has been reported to oppose the effect of AA-derived eicosanoids and inhibit cell infiltration as well as tumor cell metastasis (7). Thus, modulation of the activity of phospholipase A_2 (PLA_2), especially the different forms of PLA_2, may lead to alterations in these biological responses.

We previously reported the existence of two forms of the secretory PLA_2 ($sPLA_2$) (8) in addition to the cytosolic form of PLA_2 ($cPLA_2$) which has been reported by others as well (9). The two forms of $sPLA_2$ in mouse skin have the classical characteristics of $sPLA_2$s, and were confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) to have sequences similar to the known pancreatic type I and non-pancreatic type II $sPLA_2$ (10, 11). These $sPLA_2$s, especially the type II $sPLA_2$ have been suggested to be involved in inflammatory responses and are activated by inflammatory cytokines such as interleukin-1 (IL-1) and interleukin-6 (IL-6) as well as tumor necrosis factors (TNF) (12–14). Anti-inflammatory reagents such as dexamethasone and transforming growth factor β (TGF-β) suppress its expression and activation in many cells, providing evidence for the importance of type II $sPLA_2$ in inflammation (15–17). While some of these factors affect $sPLA_2$ activity at the level of expression, the pathways for regulation of activity by other agents are not clear.

In pancreas, the type I $sPLA_2$ is synthesized as an inactive proenzyme and is cleaved by trypsin upon release into the GI tract (18). It is not clear whether type I $sPLA_2$ activity is regulated similarly in other tissues as its functions in other tissues are not yet known. More is known about the regulation of type II $sPLA_2$. Protease cleavage does not appear to be one of the mechanisms as no evidence of a

Abbreviations: 4α-TPA, 4α-12-O-tetradecanoylphorbol 13-acetate; AA, arachidonic acid; AlF^-, aluminum fluoride; cAMP, cyclic adenosine monophosphate; $cPLA_2$, cytosolic phospholipase A_2; G protein, guanine nucleotide protein; IL-1, interleukin-1; NF κB, nuclear factor κB; OA, oleic acid; PKA, protein kinase A; PKC, protein kinase C; PLA_2, phospholipase A_2; PMSF, phenylmethylsulfonyl fluoride; $sPLA_2$, secretory phospholipase A_2; TPA, 12-O-tetradecanoylphorbol 13-acetate; TNF, tumor necrosis factor.

[1] To whom correspondence should be addressed.

proenzyme has been observed with this enzyme. The enzyme apparently is maintained in its inactive form by other mechanisms, possibly a putative inhibitor (19) or existing in granules such as in neutrophils (20, 21). Beside regulation by cytokines, the involvement of the guanine nucleotide protein (G protein) pathway has been suggested by the existence of an AP-2 response element in the promoter region of the type II $sPLA_2$ gene (22). Also, studies using forskolin and cell permeable cyclic adenosine monophosphate (cAMP), suggested that expression of the type II $sPLA_2$ is dependent on intracellular cAMP levels in smooth muscle cells as well as in some epithelial cell types (12, 15). However, the type of G protein has yet to be identified. Recently, it was suggested that a particular type of G protein, G_z was inactivated by AA and related unsaturated fatty acids. Also, AA and its metabolites may differentially regulate at least one form of the $sPLA_2$s (23). Therefore, both type I and type II $sPLA_2$ may be regulated by G proteins positively and negatively depending on the form of stimuli and the form of G proteins that the stimuli activate.

In this paper, we studied the possible mechanisms by which keratinocyte $sPLA_2$ activity can be regulated. The keratinocyte $sPLA_2$s were recently characterized but it was not clear whether the type I $sPLA_2$ could be activated in these cells by protease cleavage (8). Also, while it has been suggested that G protein may be involved in the regulation of type II $sPLA_2$, the form of G protein has not yet been identified and the pathways leading to the up-regulation of activity is not yet clear. Thus, we examined the possible regulation of keratinocyte $sPLA_2$ activity by protease, G protein/cAMP pathway as well as the possible involvement of AA and its metabolites.

We report in this paper that mouse keratinocyte $sPLA_2$s activity is potently elevated by protease treatment and inhibited by protease inhibitors. A G_0 mediated pathway also appears to lead to the activation of $sPLA_2$, independent of its effects on cAMP levels. Arachidonic acid, possibly through a G protein pathway, positively regulated $sPLA_2$ activity while the metabolites of AA were without effect.

METHODS

Cells

Primary keratinocytes obtained from SSIN (SENCAR) mice (University of Texas M.D. Anderson Veterinary Division, Bastrop, TX) were used in this study. Primary cultures of keratinocytes isolated from newborn mice by trypsinization were plated at 37°C and 5% CO_2 in an enriched Waymouth's medium containing 10% fetal bovine serum, and switched to the completely defined, serum-free SPRD-111 medium after overnight attachment (24). Cells were then treated with various stimuli or inhibitors after overnight incubation in SPRD-111 medium. With each experiment, the cell numbers for all treatment groups were the same.

Reagents

All radioisotopes were purchased from New England Nuclear (NEN, Boston, MA), including: [1-^{14}C]oleic acid and [1-^{14}C] arachidonic acid used for labeling cells as well as the dual range BIOTRAK cyclic AMP [^{125}I]enzyme immunoassay (Amersham). The synthetic G protein activator mellitin, the protease inhibitor, calpeptin, and 4α-12-O-tetradecanoylphorbol-13-acetate (4α-TPA) were purchased from LC Laboratories (Woburn, MA). The wasp venom G protein effector mastoparan-7 and its negative control peptide mastoparan-17 were purchased from Biomol (Plymouth Meeting, PA). Pertussis toxin, protein kinase A inhibitor HA1004, and lipoxygenase inhibitor eicosatetraynoic acid (ETYA) were also purchased from Biomol (Plymouth Meeting, PA). Arachidonic acid, calcium ionophore A23187, cholera toxin, forskolin, phenylmethylsulfonyl fluoride (PMSF), aluminum sulfate, and sodium fluoride were purchased from Sigma Chemical Co. (St. Louis, MO). Prostaglandin H synthase-1 specific inhibitor, resveratrol, and prostaglandin E_2 (PGE_2) were purchased from Cayman Co. (Ann Arbor, MI). The prostaglandin H synthase-2 specific inhibitor NS-398 was a generous gift from Taisho Pharmaceutical Co., Saitama, Japan.

Membrane extracts

Cells were washed with PBS and once with homogenization buffer (250 mm Tris HCl, pH = 8.5) (25, 26). The homogenization buffer also contained 150 mm NaCl with or without 1 mm Na_3VO_4, 1 mm PMSF, and 10 μg/ml leupeptin. The cells were scraped into the homogenization buffer and homogenized on ice and sonicated for 20 sec using a pulse sonicator. Membrane preparations were then prepared by treatment with 0.36 N H_2SO_4 in 2 m NaCl overnight (pH=1.6) followed by dialysis against 10 mm acetic acid–sodium acetate buffer (pH = 4.4) (19). Membranes for PLA_2 assay were stored at −20°C until use. Protein concentration of the membrane preparations was determined by the Coomassie blue reaction (Bio-Rad).

Preparation of radiolabeled *E. coli* membrane suspension

Radiolabeled *E. coli* were prepared by the method of Elsbach and Weiss (27). An overnight culture of JM109 *E. coli* was diluted 1:20 in LB broth and incubated at 37°C for 3 h in the presence of 1 mCi/ml [^{14}C]oleic acid (OA). Radiolabeled *E. coli* were then washed by incubating in fresh LB broth for 30 min followed by washing with 1% bovine serum albumin (BSA) to remove unincorporated radiolabel. The washed *E. coli* were autoclaved and resuspended in the appropriate amount of 0.85% saline to achieve 5,000 cpm/μl. Radiolabeled *E. coli* membrane suspensions were stored at −20°C until use. Typically, between 50–90% of the added [^{14}C]OA was incorporated into *E. coli*.

In vitro $sPLA_2$ assay

The $sPLA_2$ activity was measured using radiolabeled [^{14}C]OA *E. coli* membrane suspension as substrate. $sPLA_2$ assay was performed with 10 μg protein from the membrane preparation. The reaction mix contained 5 mm $CaCl_2$, 125 mm Tris HCl (pH = 8.5), 2.5 mg/ml BSA, and 50,000 cpm [^{14}C]OA-labeled *E. coli* membrane suspension. The reaction was carried out in a 37°C water bath for 30 min and was stopped by the addition of 100 μl 2 N HCl and 100 μl 20 mg/ml BSA. The reaction mix was microfuged and 250 μl supernatant was counted to monitor the [^{14}C]OA release from the *E. coli* membrane. Total counts were also obtained by counting pellets and supernatant.

PLA_2 activity in intact cells

Confluent cultures of primary keratinocytes were labeled with [1-^{14}C]oleic acid (50 mCi/mmol, 0.1 mCi/ml) at a concentration of 0.3 mCi/ml medium for 16 h (25). Dishes were then washed twice with PBS and incubated in fresh medium with or without various inhibitors or activators for 15 min. Calcium ionophore A23187, 4α-TPA or vehicle were then added to stimulate the release of incorporated fatty acids from the prelabeled cells. The

doses of stimuli used are those we have previously used, 16 μm 4α-TPA, 0.1 μm calcium ionophore A23187 or as indicated. At appropriate times, aliquots of medium were counted by liquid scintillation counting. Unless specified, data for the 3 h time point are reported in this paper. Triplicates were used at each time point.

Cyclic AMP assay

Cyclic AMP levels were measured using the ^{125}I-cAMP kit provided by Amersham. Cells were scraped into 100 μl lysis buffer and assayed according to the kit protocol. The level of unbound ^{125}I were then counted on a gamma counter and compared to a standard curve. Data reported are representative of two experiments.

Statistical analyses

Results are expressed as means ± standard errors of the mean for triplicate data or duplicate data as indicated. All data were subjected to one-way analysis of variance (ANOVA) test. When significant differences were detected, Tukey's honest significant difference test was used for multiple comparisons among the groups. Whenever applicable, Student's *t*-test was used to determine the differences between two samples. All experiments were repeated three times unless otherwise indicated. $P \leq 0.05$ was considered significant.

RESULTS

Modulation of sPLA$_2$ activities by proteolytic activation

To explore the possible activation of keratinocyte sPLA$_2$ by proteases, we treated cells with trypsin, and the activity of isolated sPLA$_2$ as well as release of incorporated radiolabeled OA from cells were evaluated. Trypsin treatment significantly enhanced the enzyme activity and increased the release of incorporated OA from mouse keratinocytes. A 3-fold increase was observed with the release of incorporated OA (**Fig. 1A**) and a little over 2-fold induction was observed in enzyme activity (Fig. 1B). The data from both approaches are in agreement and suggest that protease treatment activates keratinocyte sPLA$_2$. Thus, later experiments used only OA release as a measurement for sPLA$_2$ activity.

The trypsin data were supported by the observation that protease inhibitors diminished the release of incorporated OA from keratinocyte cell membrane in a cell-free system. The release of incorporated OA from membranes treated with protease inhibitors was half that of the untreated membranes (data not shown). Oleic acid release from intact stimulated and unstimulated cells treated with PMSF, a serine protease inhibitor, and calpeptin, a cysteine protease inhibitor, confirmed this effect (**Fig. 2**). Calpeptin produced a dose-dependent inhibition of OA release in both calcium ionophore A23187 and vehicle-treated keratinocytes (Fig. 2B). Maximum inhibition (40%) was achieved with the lowest dose of PMSF used. The basal level release of OA was also slightly (21.1%) reduced with PMSF treatment. The effects of these inhibitors were not specific to the stimulus used as PMSF also inhibited the release of incorporated OA in cells stimulated with 4α-TPA (Fig. 2C). The effects of PMSF produced a more profound (72.9%) reduction of 4α-TPA-stimulated OA release than of calcium ionophore A23187 (40%).

G protein effectors elicit the release of incorporated fatty acids in mouse keratinocytes

No proteolytic activation has been previously suggested for the type II sPLA$_2$. Mechanisms such as cytokine induction and cAMP activation have been indicated to be the primary regulatory events for the type II sPLA$_2$. Many venom components were reported to have the ability to either inhibit or activate PLA$_2$s. Mellitin, a synthetic peptide similar to the bee venom mellitin protein, was used in this experiment to modulate PLA$_2$ activities in cultured mouse keratinocytes. Mellitin was shown to elicit OA hydrolysis from prelabeled keratinocytes in a dose- and time-dependent manner over a range of 1.875 μm up to 15 μm (**Fig. 3A**).

Because mellitin is a G protein activator, we decided to explore the involvement of G protein in the modulation of PLA$_2$ activity in mouse keratinocytes. Fluoride ion, when complexed with aluminum ion, nonspecifically stimulates both the stimulatory and the inhibitory G protein. We show here that AlF^- potently stimulated the hydrolysis of incorporated OA from prelabeled mouse keratinocytes (Fig. 3B). At the 3-h time point, the lowest dose of fluoride ion (10 mm) enhanced OA release by 4-fold compared to the vehicle control. With the amount of aluminum remaining constant (5 mm), varying the amount of fluoride in the treatment from 10 to 40 mm dose-dependently enhanced the release of OA from keratinocytes. This effect is also time-dependent (Fig. 3C).

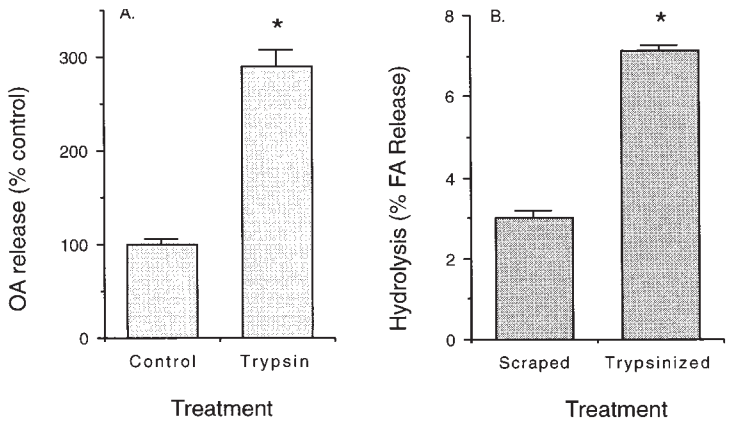


Fig. 1. Activation of keratinocyte sPLA$_2$ by trypsin. A: [^{14}C]oleic acid (OA)-labeled primary keratinocyte cultures were treated with or without 0.25% trypsin for 3 h and aliquots of 100 μl media were taken to determine the amount of [^{14}C]OA released from the cells. B: Confluent cultures of primary keratinocytes were collected by either scraping or trypsinization (0.25%). Membrane proteins were then isolated and sPLA$_2$ assays were performed. All data were expressed as mean ± SEM, n = 3. Bars with asterisks were significantly different from the control in A or from scraped in B ($P \leq 0.05$).

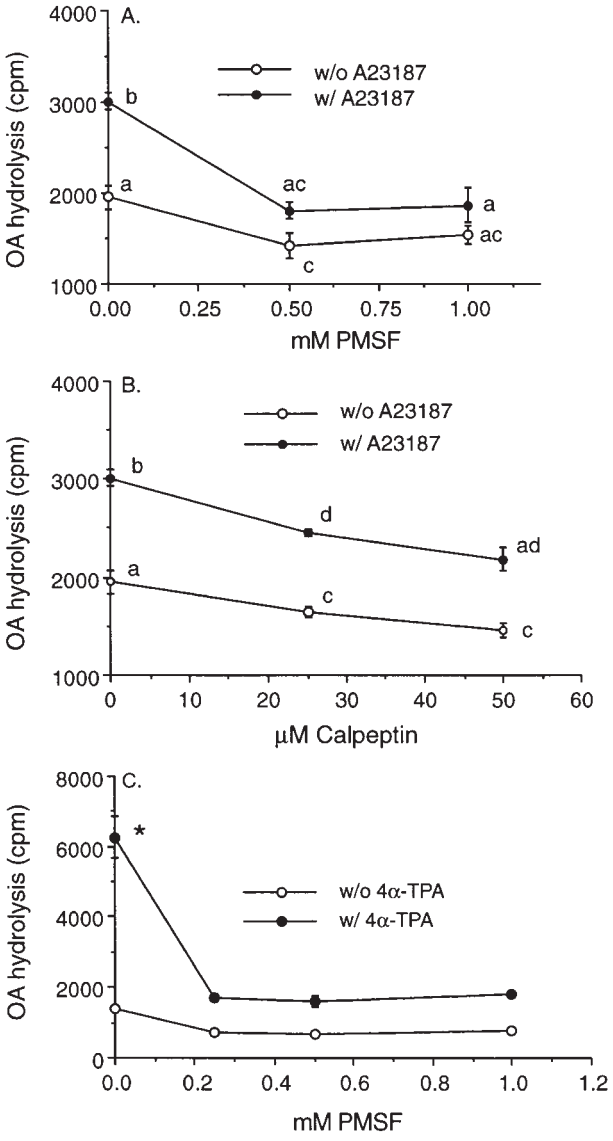


Fig. 2. Inhibition of oleic acid (OA) hydrolysis by protease inhibitors in mouse keratinocytes. Primary keratinocyte cultures were labeled with [^{14}C]OA overnight. Cells were then washed and treated with or without the indicated protease inhibitor for 15 min before the addition of stimuli. Stimuli used were either 0.1 μm calcium ionophore A23187 or 16 μm 4α-TPA. A: PMSF as protease inhibitor; calcium ionophore A23187 as stimulus; B: calpeptin as protease inhibitor; calcium ionophore A23187 as stimulus; C: PMSF as protease inhibitor; 4α-TPA as stimulus. All data were expressed as mean ± SEM, n = 3. Data points assigned different letters were significantly different from each other at $P \leq 0.05$. Data with asterisks were significantly different from the control (no treatment) at $P \leq 0.05$ in panel C.

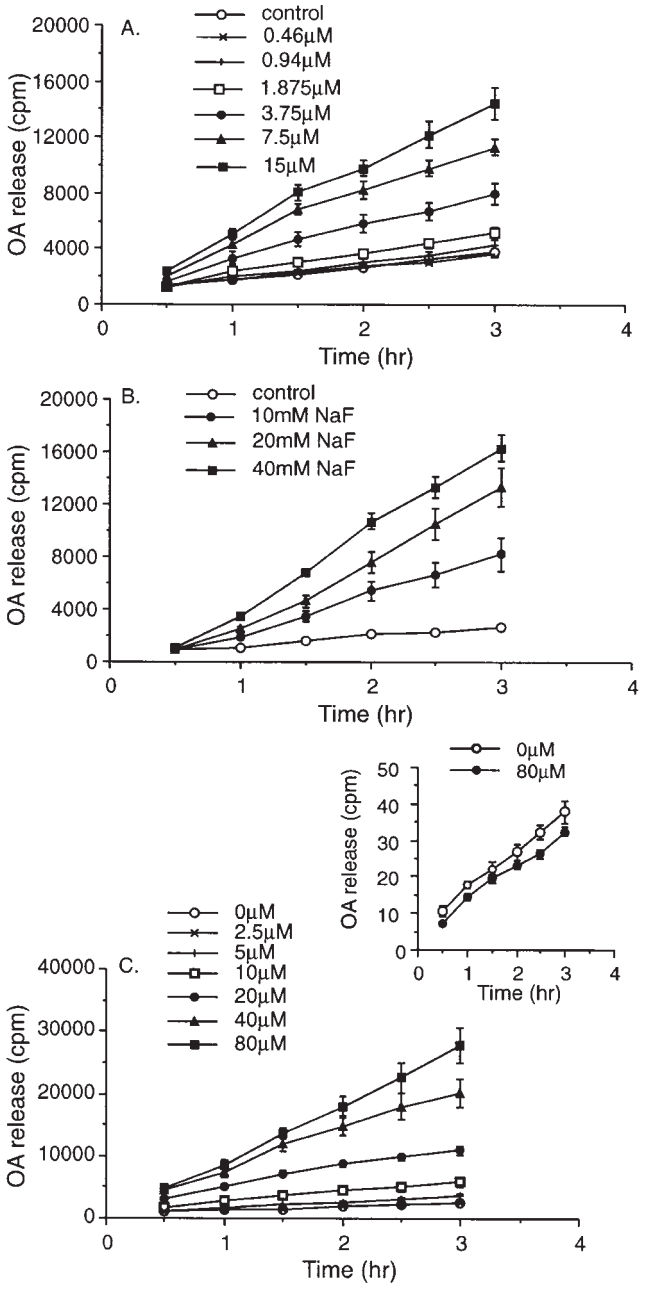


Fig. 3. Enhancement of oleic acid (OA) hydrolysis by G protein effectors in mouse keratinocytes. Mouse keratinocytes labeled with [^{14}C]OA were treated with the indicated amount of different G protein effectors: mellitin (A); fluoride ion (AlF^-, B); mastoparan-7 (Mas-7, C). Fluoride ions were generated with the simultaneous addition of 5 mm aluminum sulfate and indicated amount of sodium fluoride (NaF). At the indicated time points, aliquots of 100 μl media were taken and radioactivity was counted. Mastoparan-17 (80 μm), a negative peptide control for mastoparan-7, was also used to treat keratinocytes (panel C insert). All data were expressed as mean ± SEM, n = 3.

Using a more specific G protein effector, mastoparan-7, we were able to narrow the form of G protein to G_i/G_o (28). Mastoparan-7 was shown to potently enhance the hydrolysis of incorporated OA in a dose- and time-dependent manner (Fig. 3C). Its negative control peptide, mastoparan-17, was not able to do so at the highest dose (80 mm) used for mastoparan-7 (Fig. 3 C insert). Compared to the hydrolysis we observed previously with treatment of 4α-TPA and calcium ionophore A23187, which produce 2.5- and 1.5-fold hydrolysis, respectively, the effect with mastoparan-7 is much more substantial (2-fold at 10 μm up to more than 10-fold with 80 μm). The stimulatory effects of mastoparan-7 as well as fluoride ions and calcium ionophore A23187 were marginally inhibited by the G protein antagonist GP antagonist-2A (data not shown).

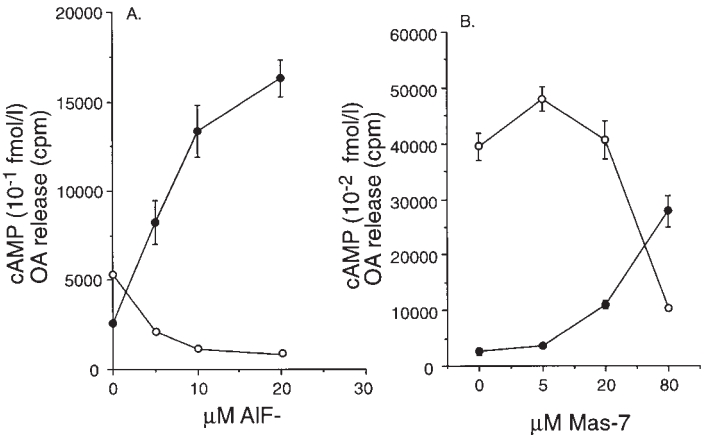


Fig. 4. Negative correlation between oleic acid (OA) release and intracellular cyclic AMP (cAMP) levels in mouse keratinocytes. [^{14}C]OA-labeled or nonlabeled primary keratinocytes were treated with fluoride ions (AlF^-, panel A) or 80 μm mastoparan-7 (Mas-7, panel B) for 3 h. Fluoride ions were generated by the simultaneous addition of 5 mm aluminum sulfate and 20 mm sodium fluoride. Aliquots of 100 μl media were taken from labeled cultures and counted for radioactivity (OA release). Nonlabeled cultures were washed and then collected in cAMP lysis buffer and assayed for intracellular cAMP levels (cAMP). Open circles are cAMP levels and solid circles are OA hydrolysis data. All data were reported as mean ± SEM with n = 3 for OA release and n = 2 for cAMP levels. The levels of cAMP and OA hydrolysis were negatively correlative with R^2 of 0.899 and 0.890 for panels A and B, respectively; $P \leq 0.05$.

To further confirm that G_i protein mediates the regulation of $sPLA_2$ in mouse keratinocytes, we applied pertussis toxin to prelabeled keratinocytes prior to treatment with various forms of stimuli. Pertussis toxin was not able to inhibit the hydrolysis of incorporated OA from these cells at any dose from 0.1 ng/ml up to 50 ng/ml with any stimuli (data not shown). The same effects were observed with cholera toxin. Up to 20 mg/ml cholera toxin failed to inhibit the stimulated release of incorporated OA from mouse keratinocytes (data not shown). To investigate whether the toxins penetrated the cell membrane and to demonstrate that the G_i/G_o and G_s pathways were not altered in these keratinocytes by cell culture, we measured the levels of cAMP in these cells in parallel with the experiments for OA release. Pertussis toxin at 0.1 ng/ml was able to elevate cAMP levels by 2-fold compared to the vehicle control. However, no further enhancement was observed at higher doses (data not shown). Cholera toxin dose-dependently increased the level of cAMP in mouse keratinocytes (data not shown).

While pertussis toxin and cholera toxin were not able to affect the hydrolysis of incorporated OA by various stimuli, the stimuli did alter the levels of cAMP in these cells. In mouse keratinocytes, both fluoride ions and mastoparan-7 dose-dependently suppressed the production of cAMP by 84.1% and 73.5%, respectively, at the highest doses used (**Fig. 4A and B**). The decline in cAMP levels was highly correlated with the enhancement of OA hydrolysis in these cells with R^2 of 0.899 and 0.890, respectively, for AlF^- and mastoparan-7. While calcium ionophore A23187 also demonstrated a similar inhibition of cAMP production (68%) (**Fig. 5A**), it did not assert the same potency on OA hydrolysis (only 1.5-fold increase in hydrolysis) (Fig. 5B). The protein kinase A inhibitor HA1004 was also used to investigate whether inhibiting protein kinase A (PKA) activity may contribute to the enhanced OA release. This inhibitor was shown to be ineffective (data not shown).

The reduction in cAMP levels after G protein activation by either AlF^- or mastoparan-7 suggested that cAMP might inhibit $sPLA_2$ activation. To test this possibility, cells were treated with 10 μm forskolin, a known activator of adenylate cyclase and mastoparan-7. We have shown that this dose of forskolin elevated cAMP levels 50-fold within 20 min (data not shown). However, forskolin treatment did not reduce OA hydrolysis by mastoparan-7 (**Fig. 6**), suggesting that the reduction of cAMP levels by this G protein activator is an event independent of $sPLA_2$ activity.

Modulation by AA and its metabolites

Many of the AA metabolites bind to cell surface receptors which are associated with one or the other form of G proteins (29, 30). Arachidonic acid itself may also function through a G protein pathway (31). We demonstrated here that treatment of prelabeled mouse keratinocytes with 125

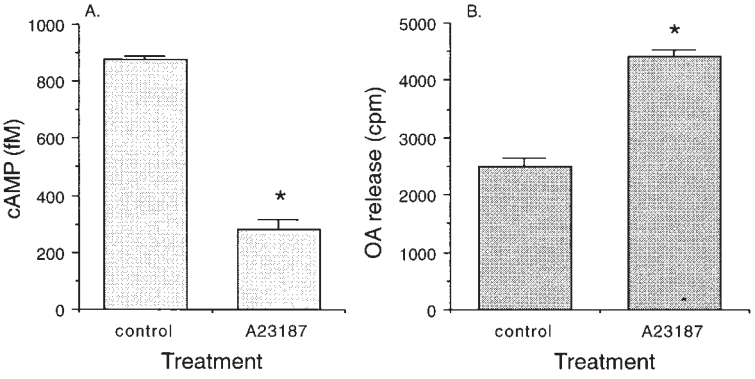


Fig. 5. Effects of calcium ionophore A23187 on intracellular cyclic AMP (cAMP) levels and the hydrolysis of incorporated oleic acid (OA) in mouse keratinocytes. Calcium ionophore A23187 (0.1 μm) was used to treat nonlabeled keratinocyte cultures or cultures labeled with [^{14}C]OA for 3 h. Nonlabeled cultures were then washed and assayed for cAMP levels (A). Aliquots of 100 μl media were taken from labeled cultures and radioactivity was counted for release of incorporated OA (B). All data were reported as mean ± SEM with n = 2 for panel A and n = 3 for panel B. Bars with asterisks were statistically different from the control group at $P \leq 0.05$.

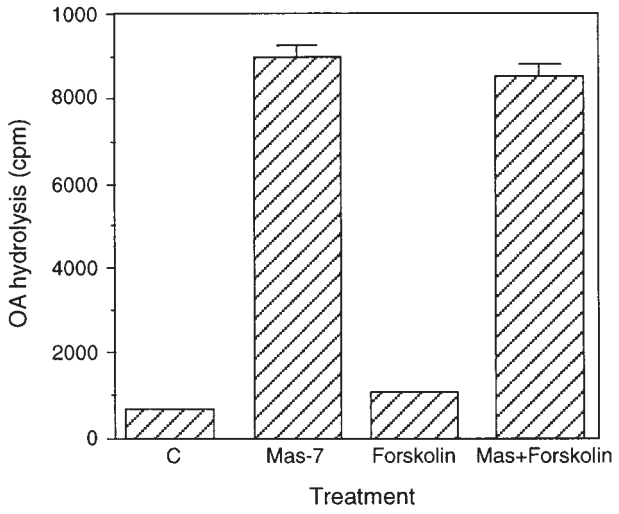


Fig. 6. Lack of an effect of cAMP on $sPLA_2$ activation. Primary keratinocytes prelabeled with [^{14}C]OA were treated with 10 μm forskolin and/or 40 μm mastoparan-7. Aliquots (50 μl) of media were collected at 3 h and radioactivity was counted for OA release. All data were calculated as mean ± SEM, n = 3. The experiment was repeated with essentially the same results.

μm AA stimulated the release of incorporated OA by 1.5-fold (**Fig. 7A**), an increase that is comparable to that observed with calcium ionophore A23187 treatment. In comparison, PGE_2 over the same dose range did not elicit the hydrolysis of OA (Fig. 7B). To investigate the possible involvement of products of the AA cascade, we used inhibitors of lipoxygenase (LOX) as well as prostaglandin H synthase (PGHS)-1 and -2. Neither class of inhibitor at low or high doses was effective in inhibiting the OA release stimulated by AA (data not shown), suggesting a role of AA rather than its metabolites in the modulation of $sPLA_2$ in mouse keratinocytes.

DISCUSSION

We previously reported the existence of two forms of $sPLA_2$ in mouse keratinocytes. These two $sPLA_2$s may be controlled by a variety of regulatory systems. In this study, we demonstrated the modulation of keratinocyte $sPLA_2$s by several mechanisms including proteolytic activation and a G protein-regulated pathway. Keratinocyte $sPLA_2$s also appeared to be regulated by the product AA but not by its metabolites. Whether these mechanisms apply to both type I and type II $sPLA_2$ is not known as it is currently not possible to distinguish between the two in keratinocytes.

The sequence for the pancreatic type I $sPLA_2$ contains both the signal peptide and the activation peptide at its amino terminal (10). These peptides confirm its ability to be secreted as a proenzyme and later activated upon release into the duodenum to function as a digestive enzyme (18). However, it is not clear what function the type I $sPLA_2$ may have in other tissues or whether it is released and activated in a similar manner. Type I $sPLA_2$ expression has been demonstrated in many tissues including the lung, kidney, stomach, spleen as well as the skin (8, 32–34). Receptors for type I $sPLA_2$ have also been identified recently in many of the same tissues (35–37). It was suggested that type I $sPLA_2$ may function through these receptors for at least some of its effects, which include regulating proliferation, contraction, and cell migration (38, 39). While eicosanoids may also elicit some of these effects, type I $sPLA_2$ might directly affect cells through a receptor-mediated mechanism. The primary effect of type I $sPLA_2$ as an acylhydrolase in tissues other than pancreas may also be a receptor-mediated event. In mouse keratinocytes, we show here that protease activates $sPLA_2$ and protease inhibitors inhibit $sPLA_2$ activity and OA hydrolysis stimulated by 4α-TPA, the non-tumor-promoting isomer of TPA, as well as calcium ionophore A23187 and the G protein activator mastoparan-7. Therefore, similar to the pancreatic type I $sPLA_2$, the keratinocyte $sPLA_2$ is activated by proteolytic cleavage. These data were supported by an earlier observation suggesting that the enhanced PLA_2 activity by calcium ionophore A23187 is associated with an increase in serine esterase activity in smooth muscle cells (40).

The most studied mechanism of type II $sPLA_2$ regulation has been the up-regulation of its expression by cytokines/cAMP and its down-regulation by dexamethasone (41). Mechanisms and pathways for its activation have not been elucidated while activation by a G protein pathway has been proposed (42, 43). Activation of PLA_2 by G protein was shown in the rod outer segment of the eye where light activation of PLA_2 is dependent on the βγ subunits of transducin (44, 45). In other tissues, primarily epithelial cell types, G protein has been shown to activate some form of PLA_2, yet it was not clear whether it was $sPLA_2$ or

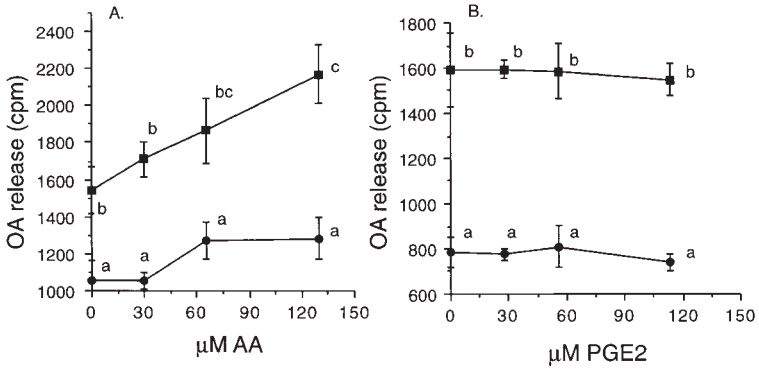


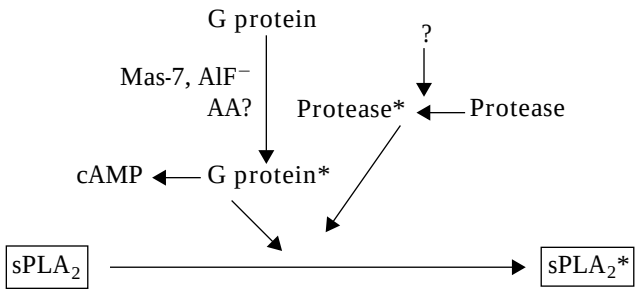
Fig. 7. Stimulatory effect of arachidonic acid (AA) on the hydrolysis of incorporated oleic acid (OA) in mouse keratinocytes. Primary keratinocytes prelabeled with [^{14}C]OA were treated with the indicated amounts of AA (panel A) or prostaglandin E_2 (panel B). Aliquots of 100 μl media were collected at 1 h (circle) and 3 h (square) after addition of the lipids, and radioactivity was counted for OA release. All data were calculated as mean ± SEM, n = 3. Data points assigned different letters were significantly different from each other, $P \leq 0.05$; representatives of two experiments.

cPLA$_2$ or one of the others in the same family (46–49). Direct association of the sPLA$_2$ with some forms of G protein has been implied (50). Reportedly, involvement of G_i, G_q, G_s, as well as the α1-adrenergic receptors has been suggested (46–49, 51). Generally, these processes of activation are inhibited by pertussis toxin, suggesting a pertussis toxin-sensitive G protein. In keratinocytes, both G_s and G_i proteins have been reported to exist in rat and human keratinocytes (52). The presence of other G proteins in keratinocytes has yet to be determined. We showed here that mouse keratinocyte sPLA$_2$ may be regulated by a pertussis toxin-insensitive G protein. As this G protein is activated by mastoparan-7, a G_i / G_o activator (28), we suggested the activation of G_o or another not yet identified G protein.

The potency of the G protein effectors on OA hydrolysis that we observed in this study suggested that this might be one of the major pathways for the activation of sPLA$_2$ in mouse keratinocytes. This pathway may also be used by AA which was shown in this study to enhance the hydrolysis of OA as well, though to a much lesser extent. Unsaturated fatty acids such as AA have been suggested to interact with some forms of G protein (31). Whether AA may bind to a G protein coupled receptor is not clear. Recently, several studies suggested that the products of one PLA$_2$, such as AA and its metabolites, may activate another PLA$_2$ (23, 53). In mast cells and osteoblastic cells, this mechanism produced a biphasic release of PGE$_2$ from these cells (53, 54). Many of the AA metabolites have cell membrane receptors that are coupled with one or more forms of G protein (29). Thus a possible role for G protein in this biphasic release of PGE$_2$ is implied. However, none of the metabolites of AA showed a stimulatory effect on keratinocyte sPLA$_2$ and inhibitors for PGHS-1 and -2 as well as lipoxygenase (LOX) were not effective at inhibiting the hydrolysis of OA elicited by AA. Therefore, the effect of AA on activating keratinocyte sPLA$_2$ is restricted to AA itself.

Studies in many tissues suggest that cAMP is a positive regulator of sPLA$_2$ due to its effect on enhancing the expression of type II sPLA$_2$ (12, 15, 55). However, the significant negative correlations between cAMP levels and OA release that we observed in mouse keratinocytes suggested a possible negative regulatory role of cAMP in this cell type. We ruled out this possibility through the use of forskolin to induce cAMP production. Thus, in keratinocytes cAMP does not appear to affect sPLA$_2$ activity.

In considering our observations on the several mechanisms that are able to modulate the activation of one or both types of sPLA$_2$, we propose the following model:



This model suggests that sPLA$_2$ can be activated (activation noted by an asterisk) by proteases and by G proteins. G protein activators also reduce the production of cAMP, but this does not affect the activation of sPLA$_2$. G protein activation also appears to cause some protease activation but this is not the principal pathway by which it activates sPLA$_2$. This model does not distinguish between the multiple forms of sPLA$_2$s and it is currently not known if they are differentially activated. Additionally, the possible involvement of phosphorylation / dephosphorylation events is not yet known. Further work is needed to more completely define a model for sPLA$_2$ activation.

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REFERENCES

1. Fischer, S. 1997. Prostaglandin and cancer. *Front. Biosci.* **2:** d482–500.
2. Bouclier, M., B. Luginbuhl, F. Delamadeleine, P. Rossio, and C. Hensby. 1989. Repeated application of arachidonic acid to the ear of mice: a model of chronic skin inflammation? *Agents Actions.* **26:** 227–228.
3. Doherty, N. S., T. H. Beaver, L. A. Rheins, and J. J. Nordlund. 1988. Multiple topical application of arachidonic acid to mouse ears induce inflammatory and proliferative changes. *J. Invest. Dermatol.* **91:** 298–302.
4. Young, J. M., B. M. Wagner, and D. A. Spires. 1983. Tachyphylaxis in 12-O-tetradecanoylphorbol acetate- and arachidonic acid-induced ear edema. *J. Invest. Dermatol.* **80:** 48–52.
5. Maldve, R., and S. M. Fischer. 1996. Multifactor regulation of prostaglandin H synthase-2 in murine keratinocytes. *Mol. Carcinog.* **17:** 207–216.
6. Muller-Decker, K., K. Scholz, F. Marks, and G. Furstenberg 1995. Differential expression of prostaglandin H synthase isozymes during multistage carcinogenesis in mouse epidermis. *Mol. Carcinog.* **12:** 31–41.
7. Honn, K. V., K. K. Nelson, C. Renaud, R. Bazaz, C. A. Diglio, and J. Timar 1992. Fatty acid modulation of tumor cell adhesion to microvessel endothelium and experimental metastasis. *Prostaglandins.* **44:** 413–429.
8. Li-Stiles, B., H-H. Lo, and S. M. Fischer. 1998. Identification and characterization of several forms of phospholipase A$_2$ in mouse epidermal keratinocytes. *J. Lipid Res.* **39:** 569–582.
9. Kast, R., G. Furstenberger, and F. Marks. 1991. Activation of a keratinocyte phospholipase A$_2$ by brady kinin and 4β-phorbol 12-myristate 13-acetate. *Eur. J. Biochem.* **202:** 941–950.
10. Seilhamer, J. J., T. L. Randall, M. Yamanaka, and L. K. Johnson. 1986. Pancreatic phospholipase A2: isolation of the human gene and cDNAs from porcine pancreas and human lung. *DNA.* **5:** 519–527.
11. Seilhamer, J. J., W. Pruzanski, P. Vadas, S. Plant, J. A. Miller, J. Kloss, and L. K. Johnson. 1989. Cloning and recombinant expression of phospholipase A2 present in rheumatoid arthritic synovial fluid. *J. Biol. Chem.* **26:** 5335–5338.
12. Nakano, T., O. Ohara, H. Teraoka, and H. Arita. 1990. Group II phospholipase A2 mRNA synthesis is stimulated by two distinct mechanisms in rat vascular smooth muscle cells. *FEBS Lett.* **261:** 171–174.
13. Vadas, P., E. Grouix, E. Stefanski, M. Wloch, W. Pruzanski, J. Schroeder, and J. Gauldie. 1997. Coordinate expression of group II phospholipase A$_2$ and the acute-phase proteins haptoglobin (HP) and α1-chymotrypsin (ACH) by HepG2 cells. *Clin. Exp. Immunol.* **198:** 175–180.
14. Schalkwijk, C., J. Pfeilschifter, F. Marki, and H. van den Bosch.

1991. Interleukin-1β, tumor necrosis factor and forskolin stimulated the synthesis and secretion of group II phospholipase A_2 in rat mesangial cells. *Biochem. Biophys. Res. Commun.* **174:** 268–275.
15. Schalkwijk, C., M. Vervoordeldonk, J. Pfeilschifter, F. Marki, and H. van den Bosch. 1991. Cytokine- and forskolin-induced synthesis of group II phospholipase A_2 and prostaglandin E_2 in rat mesangial cells is prevented by dexamethasone. *Biochem. Biophys. Res. Commun.* **180:** 46–52.
16. Jackson, B. A., R. H. Goldstein, R. Roy, M. Cozzani, L. Taylor, and P. Polgar. 1993. Effects of transforming growth factor β and interleukin-1β on expression of cyclooxygenase 1 and 2 and phospholipase A_2 mRNA in lung fibroblasts and endothelial cells in culture. *Biochem. Biophys. Res. Commun.* **197:** 1465–1474.
17. Nakano, T., O. Ohara, H. Teraoka, and H. Arita. 1990. Glucocorticoids suppress group II phospholipase A_2 production by blocking mRNA synthesis and posttranscriptional expression. *J. Biol. Chem.* **265:** 12745–12748.
18. Verheij, H. M., A. J. Slotboom, and G. H. de Haae. 1981. Structure and function of phospholipase A2. *Rev. Physiol. Biochem. Pharmacol.* **91:** 91–203.
19. Marki, F., and R. Franson. 1986. Endogenous suppression of neutral-active and calcium-dependent phospholipase A_2 in human polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* **879:** 149–156.
20. Lanni, C., and E. L. Becker. 1983. Release of phospholipase A_2 activity from rabbit peritoneal neutrophils by f-Met-Leu-Phe. *Am. J. Pathol.* **113:** 90–94.
21. Rosenthal, M. D., M. N. Gordon, E. S. Buescher, J. H. Slusser, L. K. Harris, and R. C. Franson. 1995. Human neutrophils store type II 14-kDa phospholipase A_2 in granules and secrete active enzyme in response to soluble stimuli. *Biochem. Biophys. Res. Commun.* **208:** 650–656.
22. Olivier, J. L., A. Fan, C. Salvat, M. Ziari, L. Kong, M. Mangeney, and G. Bereziat. 1994. Positive and negative hepatic regulation of the human type II phospholipase A_2 gene. *Biochemistry.* **33:** 7134–7145.
23. Prigent-Tessier, A., J. -F. Pageaux, J. -M. Fayard, M. Lagarde, C. Laugier, and H. Coheninserm. 1996. Arachidonic acid up-regulates and prostaglandin E2 down-regulates the expression of pancreatic-type phospholipase A2 and prostaglandin-endoperoxide synthase 2 in uterine stromal cells. *Eur. J. Biochem.* **241:** 872–878.
24. Morris, R. J., K. C. Tacker, J. K. Baldwin, S. M. Fischer, and T. J. Slaga. 1987. A new medium for primary cultures of adult murine epidermal cells: application to experimental carcinogenesis. *Cancer Lett.* **34:** 297–304.
25. Kast, R., G. Furstenberger, and F. Marks. 1993. Phorbol ester TPA- and bradykinin-induced arachidonic acid release from keratinocytes is catalyzed by a cytosolic phospholipase A_2 (cPLA_2). *J. Invest. Dermatol.* **101:** 567–572.
26. Kast, R., G. Furstenberger, and F. Marks. 1993. Activation of cytosolic phospholipase A_2 by transforming growth factor-α in HEL-30 keratinocytes. *J. Biol. Chem.* **268:** 16795–16802.
27. Elsbach, P., and J. Weiss. 1991. Utilization of labeled *Escherichia coli* as phospholipase substrate. *Methods Enzymol.* **1976:** 24–31.
28. Higashijima, T., J. Burnier, and E. M. Ross. 1990. Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines. *J. Biol. Chem.* **265:** 14176–14186.
29. Toh, H., A. Ichikawa, and S. Narumiya. 1995. Molecular evolution of receptors for eicosanoids. *FEBS Lett.* **36:** 17–21.
30. Goetzl, E. J., S. An, and W. L. Smith. 1995. Specificity of expression and effects of eicosanoid mediators in normal physiology and human diseases. *FASEB J.* **9:** 1051–1058.
31. Glick, J., G. Santoyo, and P. J. Casey. 1996. Arachidonate and related unsaturated fatty acids selectively inactivate and guanine nucleotide-binding regulatory protein, G_z. *J. Biol. Chem.* **271:** 2949–2954.
32. Tojo, H., T. Ono, S. Kuramitsu, H. Kagamiyama, and M. Okamoto. 1988. A phospholipase A_2 in the supernatant fraction of rat spleen—Its similarity to rat pancreatic phospholipase A_2. *J. Biol. Chem.* **263:** 5724–5731.
33. Mastsuda, Y., M. Ogawa, T. Shibata, K. Nakaguchi, J. Nishijima, C. Wakasugi, and T. Mori. 1987. Distribution of immunoreactive pancreatic phospholipase A_2 (IPPL-2) in various human tissues. *Res. Commun. Chem. Pathol. Pharmacol.* **58:** 281–284.
34. Hara, S., I. Kudo, R. Komatani, K. Takahashi, Y. Nakatani, Y. Natori, M. Ohshima, and K. Inoue. 1995. Detection and purification of two 14 kDa phospholipase A_2 isoforms in rat kidney: their role in eicosanoid synthesis. *Biochim. Biophys. Acta.* **1257:** 11–17.
35. Hanasaki, K., and H. Arita. 1992. Characterization of a high affinity binding site for pancreatic-type phospholipase A_2 in the rat. *J. Biol. Chem.* **267:** 6414–6420.
36. Lambeau, G., P. Ancian, J. Barhanin, and M. Lazdunski. 1994. Cloning and expression of a membrane receptor for secretory phospholipase A_2. *J. Biol. Chem.* **269:** 1575–1578.
37. Ishizaki, J., K. Hanasaki, K. Higashino, J. Kishino, N. Kikuchi, O. Ohara, and H. Arita. 1994. Molecular cloning of pancreatic group I phospholipase A_2 receptor. *J. Biol. Chem.* **269:** 5897–5904.
38. Arita, H., K. Hanasaki, T. Nakano, S. Oka, H. Teraoka, and K. Matsumoto. 1991. Novel proliferative effect of phospholipase A_2 in Swiss 3T3 cells via specific binding site. *J. Biol. Chem.* **266:** 19139–19141.
39. Hanada, K., E. Kinoshita, M. Itoh, M. Hirata, G. Kajiyama, and M. Sugiyama. 1995. Human pancreatic phospholipase A_2 stimulates the growth of human pancreatic cancer cell line. *FEBS Lett.* **373:** 85–87.
40. Chakraborti, S., S. K. Batabyal, J. R. Michael, and T. Sanyal. 1994. Role of membrane associated serine esterase in the activation of phospholipase A2 by calcium ionophore (A23187) in pulmonary arterial smooth muscle cells. *Mol. Cell. Biochem.* **130:** 121–127.
41. Murakami, M., Y. Nakatani, G-I. Atsumi, K. Inoue, and I. Kudo. 1997. Regulatory functions of phospholipase A_2. *Crit. Rev. Immunol.* **17:** 225–283.
42. Axelrod, J. 1990. Receptor-mediated activation of phospholipase A_2 and arachidonic acid release in signal transduction. *Biochem. Soc. Trans.* **18:** 503–507.
43. Hunt, T. W., R. C. Carroll, and E. G. Peralta 1994. Heterotrimetic G proteins containing $G_{\alpha i3}$ regulate multiple effector enzymes in the same cell-activation of phospholipases C and A_2 and inhibition of adenylyl cyclase. *J. Biol. Chem.* **269:** 29565–29570.
44. Jelsema, C. 1987. Light activation of phospholipase A_2 in rod outer segments of bovine retina and its modulation by GTP-binding proteins. *J. Biol. Chem.* **262:** 163–168.
45. Jelsema, C. L., and J. Axelrod. 1987. Stimulation of phospholipase A_2 activity in bovine rod outer segments by the βγ subunits of transducin and its inhibition by the α subunit. *Proc. Natl. Acad. Sci. USA.* **84:** 3623–3627.
46. Burch, R. M., A. Luin, D. E. Mais, D. Corda, J. Y. Vanderhoek, L. D. Kohn, and J. Axelrod. 1986. α1-adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. *J. Biol. Chem.* **261:** 11236–11241.
47. Nishio, E., H. Nakata, S. Arimura, and Y. Watanabe. 1996. α_1- Adrenergic receptor stimulation causes arachidonic acid release through pertussis toxin-sensitive GTP-binding protein and NK activation in rabbit aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* **219:** 277–282.
48. Tsunoda, Y., and C. Owyang. 1994. A newly cloned phospholipase A_2-activating protein elicits Ca^{2+} oscillations and pancreatic amylase secretion via mediation of G protein beta/phospholipase A_2/arachidonic acid cascades. *Biochem. Biophys. Res. Commun.* **203:** 1716–1724.
49. Kruger, H., S. Carr, J. C. Brennand, and J. S. McLean. 1995. Activation of phospholipase A_2 by the human endothelin receptor in Chinese hamster ovary cells involves G_i protein-mediated calcium influx. *Biochem. Biophys. Res. Commun.* **217:** 52–58.
50. Ando, M., H. Furui, K. Suzuki, F. Taki, and K. Takagi. 1992. Direct activation of phospholipase A_2 by GTP-binding protein in human peripheral polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **183:** 708–713.
51. Burch, R. M., A. Luini, and J. Axelrod. 1986. Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. *Proc. Natl. Acad. Sci. USA.* **83:** 7201–7205.
52. Takahashi, H., N. Miyokawa, M. Katagiri, and H. Iizuka. 1990. Expression of guanine nucleotide binding proteins, Gs and Gi, in mRNAs in epidermal keratinocytes. *Arch. Dermatol. Res.* **282:** 392–396.
53. Murakami, M., H. Kuwata, Y. Amakasu, S. Shimbara, Y. Nakatani, G. Atsumi, and I. Kudo. 1997. Prostaglandin E_2 amplifies cytosolic phospholipase A_2- and cycooxygenase-2-dependent delayed prostaglandin E_2 generation in mouse osteoblastic cells—enhancement by secretory phospholipase A_2. *J. Biol. Chem.* **272:** 19891–19897.
54. Reddy, S. T., M. V. Winstead, J. A. Tischfield, and H. R. Herschman. 1997. Analysis of the secretory phospholipase A_2 that mediates prostaglandin production in mast cells. *J. Biol. Chem.* **272:** 13591–13596.
55. Oka, S., and H. Arita. 1991. Inflammatory factors stimulate expression of group II phospholipase A_2 in rat cultured astrocytes. *J. Biol. Chem.* **266:** 9956–9960.