Kallikrein stimulates arachidonic acid release and production of prostaglandins from TEA3A1 endocrine thymic epithelial cells

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Using TEA3A1 rat endocrine thymic epithelial cells, we demonstrated that kallikrein (EC 3.4.21.35) not only stimulated the release of arachidonic acid (AA) and its metabolites from TEA3A1 cells but also enhanced the intracellular synthesis of prostaglandin E₂ (PGE₂) and thromboxane B₂ (TXB₂) by approx. 2-fold. The stimulatory effect of kallikrein was dose- and time-dependent and could be blocked by aprotinin, a kallikrein inhibitor. It was found that the phospholipase A inhibitors ONO RS082 [2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid], and mepacrine {6-chloro-9-[(4-dimethylamino)-1-methyl]amino-2-methoxyacridine; quinacrine} also inhibited the kallikrein-stimulated release of AA and its metabolites. It is suggested that the kallikrein-induced stimulatory effect might be mediated through a phospholipase A₂ pathway. The effect of bradykinin was studied and no significant stimulation was observed, even at a high dose (10 µg/ml). This suggested that the formation of kinin does not have a role in the kallikrein-induced stimulation of AA release from TEA3A1 cells. Furthermore, the effect of kallikrein was also totally abolished by adding pepstatin A, a known inhibitor of renin, pepsin and cathepsin D which does not inhibit kallikrein itself. This indicates that kallikrein did not act on the phospholipase-like enzyme directly. There is at least one more enzyme, a pepstatin A-inhibitable proteinase, that acts as a mediator for kallikrein-induced regulation of AA release.

INTRODUCTION

Arachidonic acid (AA) metabolites play an important role in the regulation of the immune response by modulating lymphocyte proliferation and function. Prostaglandin E (PGE) inhibits T-cell growth and interleukin 2 production [1–3]. Products of the lipoxygenase pathway are involved in the interleukin-1 mediated response in T cells and in lectin-stimulated T-cell proliferation [4]. Moreover, it is believed that PGs have an important function in T-cell development in the thymus. It has been shown that T-cell development in the thymus was severely impaired by the administration of anti-PGE₂ *in vivo* [5]. In normal thymus, levels of PGs are 4–10-fold higher than in plasma [6,7] indicating that PGs are generated *in situ*. In addition, it has been shown that the levels of PGE and PGF in the thymuses of lupus-prone NZB mice are abnormally low, which may relate to the disruption of early T-cell differentiation in these animals [6–8]. In spite of these findings, the regulatory mechanism of PG production in the thymus is still unclear.

Recently, a rat thymic endocrine epithelial cell line, TEA3A1, has been established and cloned [9]. Using this cell line we have found that the cells are constitutively producing PGs and thromboxane (TX). Moreover, the production of these AA metabolites was regulated by exogenous hormones. In the present paper, we demonstrate that kallikrein (EC 3.4.21.35) stimulates the release of AA and its metabolites from TEA3A1 cells, and enhances the accumulation of PGE₂ and TXB₂ in the

cells. Kallikrein-mediated stimulation of PG production has been reported in kidney [10,11] and in bovine vascular endothelial cells [12]. The kallikrein effect on PG production is generally considered to be due to the formation of kinin from kininogen. However, in vascular endothelial cells, the stimulatory effect of kallikrein was shown to be independent of the formation of kinin [12]. Our study is in agreement with this observation.

Based on our findings, a possible pathway of the kallikrein effect on the regulation of PG and TXB₂ production in TEA3A1 cells is proposed.

MATERIALS AND METHODS

Materials

Kallikrein was purchased from Calbiochem (La Jolla, CA, U.S.A.). Pepstatin A, trypsin (EC 3.4.21.4), bradykinin, bovine insulin, transferrin, dexamethasone and mepacrine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [5,6,8,9,11,12,14,15-³H(n)]-arachidonic acid and ³H-labelled PGE₂, PGF_{2α}, 6-oxo-PGF_{1α} and TXB₂ were obtained from New England Nuclear (Boston, MA, U.S.A.). ONO RS082 was a gift from ONO Pharmaceutical Co. Ltd. (Osaka, Japan). Unlabelled PGs and TXB₂ were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). Ca²⁺-free WJJC404 medium was a gift from Kyokuto Pharmaceutical Co. Ltd. (Tokyo, Japan). Iron-supplemented calf serum was obtained from Hyclone Laboratory

Abbreviations used: AA, arachidonic acid; PG, prostaglandin; TX, thromboxane; ONO RS082, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid; mepacrine, 6-chloro-9-[(4-dimethylamino)-1-methyl]amino-2-methoxyacridine; DMEM, Dulbecco's modified Eagle's medium; DW-ITD, DMEM: WJJC404A (1:1) with added insulin (I) (10 µg/ml), transferrin (T) (10 µg/ml) and dexamethasone (D) (10 µM); sCS, supplemented calf serum; RIA, radioimmunoassay; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; BAEE, benzoylarginine ethyl ester; KIU, kallikrein inhibitory units.

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Cell culture

The rat endocrine thymic epithelial cell line (TEA3A1) was developed in our laboratory [9]. The stock cells were maintained in WJ404A (7.5% DMEM in Ca^{2+} -free WJ404) medium supplemented with 10 μg of insulin (I)/ml, 10 μg of transferrin (T)/ml, 10 μM -dexamethasone (D) and 2% supplemented calf serum (sCS). Experimental cells were seeded in 35 mm-diam. dishes and cultured in DMEM:WJ404A (1:1) medium with added insulin, transferrin and dexamethasone (DW-ITD) and 2% sCS to confluency.

[^3H]Arachidonic acid release assay

Confluent TEA3A1 cells were prelabelled with 0.5 μCi of [^3H]AA/ml/dish in fresh DW-ITD medium containing 2% sCS overnight at 37 °C. More than 95% of the [^3H]AA was incorporated into the TEA3A1 cells. After washing with 3 $\times$ 1.0 ml of DMEM, the [^3H]AA-labelled cells were incubated with or without different factor(s) in fresh medium for varying lengths of time at 37 °C. At the end of the incubations, the media were collected and the radioactivity released into the media was counted on a scintillation counter.

Radioimmunoassay of arachidonic acid metabolites

Confluent cells were incubated in DMEM with or without kallikrein for 3 h at 37 °C. After the incubation, cells were washed with fresh medium and collected into 1.0 ml of water and disrupted by sonication (Tekmar 40 W output). The cell sonicates were centrifuged in a microcentrifuge (12000 g for 3 min) and supernatants were collected. AA metabolites in the supernatants were quantified by radioimmunoassay (RIA), essentially according to Campbell & Ojeda, [13]. Briefly, specific antibodies, ^3H -labelled tracer and unlabelled standards were dissolved in assay buffer (phosphate-buffered saline, pH 7.4, 0.1% gelatin). Antibody (100 μl), plus [^3H]prostaglandins or [^3H]thromboxane (15000 c.p.m., 50 pg in 100 μl) and unlabelled prostaglandins or thromboxane standards or samples (100 μl) were mixed well and incubated at 4 °C for 18 h. At the end of incubation, 0.3 ml of dextran-coated charcoal suspension in water (at 4 °C) was added to the tube. The mixture was centrifuged at 1650 g for 15 min at 4 °C, and then 0.5 ml of the supernatant was taken into a scintillation vial and mixed with 5.0 ml of scintillation cocktail. The radioactivity was counted on a scintillation counter. A standard curve (0–10 ng) was included in each assay. The cross-reactivity of the antibodies (anti-PGE $_2$, anti-PGF $_{2\alpha}$ and anti-TXB $_2$) with other PGs (PGE $_2$, PGF $_{2\alpha}$, PGA $_2$, PGD $_2$, 6-oxo-PGF $_{1\alpha}$ and TXB $_2$) were also determined. Cross-reactivity was less than 0.5% except for PGA $_2$, which was recognized 100% by anti-PGE $_2$. In addition, a serial dilution of sample was parallel to the standard curve and the recovery of PGs added to the sample was > 90%. This strongly indicated that RIAs used in our studies were specific and the samples contained no interfering materials that might affect the quantification of antigens.

RESULTS

Kallikrein stimulates arachidonic acid release

The [^3H]AA-prelabelled TEA3A1 cells were treated with various concentrations of kallikrein or trypsin (0–2.0 BAEE units/ml) in DMEM at 37 °C for 3 h. As shown in Fig. 1, no stimulation was observed as a result of trypsin treatment, even at a concentration as high as 2.0 BAEE units/ml. On the contrary, kallikrein treatment resulted in a concentration-dependent stimulation of the release of [^3H]AA and its metabolites from [^3H]AA-prelabelled TEA3A1 cells. A 2-fold increase in the release was observed in the presence of 0.05 BAEE units of kallikrein/ml. The optimum concentration of kallikrein was 1.0 BAEE unit/ml, which resulted in a 3-fold stimulation of release.

Aprotinin, a kallikrein inhibitor, was added to the medium along with 1.0 BAEE unit of kallikrein/ml. As Fig. 2 shows, aprotinin abolished the kallikrein-induced release of [^3H]AA and its metabolites in a concentration-dependent manner. As the aprotinin concentration was increased to 100 KIU/ml, the kallikrein-induced stimulation was totally inhibited. This strongly indicated that the production of AA and its metabolites in the TEA3A1 cells could be activated by the proteolytic action of kallikrein.

Time-course studies showed that a significant stimulation of [^3H]AA release by kallikrein was observed after a 10 min incubation time, and a linear stimulation was observed up to 3 h incubation (Fig. 3). In addition, when aprotinin (100 KIU/well) was added to the culture after 30 min of kallikrein treatment, no further stimulation by kallikrein was observed. These results indicated that the stimulation by kallikrein was time-

Fig. 1. Effect of kallikrein or trypsin treatment on [^3H]AA release

TEA3A1 cells were seeded in 35 mm culture wells and cultured to confluence in DW-ITD medium containing 2% sCS. The confluent cells were pre-labelled with 0.5 μCi of [^3H]AA in 1.0 ml of fresh culture medium containing 2% sCS overnight at 37 °C. After washing, cells were treated with various concentrations of kallikrein (●) or trypsin (○) (0–2.0 BAEE units/ml) at 37 °C for 3 h in DMEM. At the end of the treatment, the medium was collected and the radioactivity in the medium was counted. Each point on the figure represents the mean of three individual wells of culture $\pm$ S.E.M.

Fig. 2. Effect of aprotinin on kallikrein-induced [3 H]AA release

[3 H]AA-prelabelled TEA3A1 cells were incubated with or without kallikrein (1.0 BAEE unit/ml) in the presence of various concentrations of aprotinin (0–100 KIU/ml) for 3 h at 37 °C in DMEM. The radioactivity released into the medium was counted at the end of the incubation. The results were expressed as the percentage inhibition of kallikrein-induced [3 H]AA release. Each point represents the mean of three individual culture wells of cells $\pm$ S.E.M.

Fig. 3. Time course of kallikrein treatment

[3 H]AA-prelabelled TEA3A1 cells were incubated with (a, b) or without (c) kallikrein (1.0 BAEE unit/ml) in DMEM at 37 °C. After different lengths of incubation, the medium was collected for the measurement of the release of [3 H]AA and its metabolites. For b, 100 KIU of aprotinin was added into the culture medium after 30 min of kallikrein treatment. Each point in the Figure represents the mean of three individual wells of cell culture $\pm$ S.E.M.

dependent and required the continuous action of kallikrein.

Phospholipase A₂ inhibitors (ONO RS082 and mepacrine) block the stimulation of [3 H]AA release by kallikrein

The [3 H]AA-prelabelled TEA3A1 cells were treated

Fig. 4. Inhibition of kallikrein-induced [3 H]AA release by ONO RS082 and mepacrine

[3 H]AA-prelabelled TEA3A1 cells were treated with 1.0 BAEE unit of kallikrein in 1.0 ml of DMEM for 3 h at 37 °C in the presence of various concentrations of ONO RS082 (0–20 μ M) ($\bullet$) and mepacrine (0–20 μ M) ($\circ$) added 30 min prior to the addition of kallikrein. After the incubation, the medium was collected for the measurement of the release of [3 H]AA and its metabolites. The results are expressed as the percentage inhibition of kallikrein-induced [3 H]AA release. Each point represents the mean of three individual wells of cell culture $\pm$ S.E.M.

with kallikrein (1.0 BAEE unit/ml) in the presence of various concentrations of ONO RS082 or mepacrine at 37 °C for 3 h. As shown in Fig. 4, the kallikrein-induced release of [3 H]AA and its metabolites was decreased in a concentration-dependent manner by the phospholipase A₂ inhibitors ONO RS082 and mepacrine [14,15]. As the concentration of ONO RS082 was increased to 10 μ M, the stimulatory effect of kallikrein on the release of [3 H]AA and its metabolites was totally inhibited. On the other hand, a maximum of 80 % inhibition was observed with mepacrine, even at concentrations above 2 μ M. At the same concentration, ONO RS082 could not block TPA-induced [3 H]AA release (results not shown). This suggests that the kallikrein effect may be mediated through the phospholipase A₂ pathway, stimulating AA release and the synthesis of PGs and TXs in TEA3A1 cells. Moreover, when TEA3A1 cells prelabelled with [14 C]stearic acid were treated with kallikrein, no measurable release of radioactivity was observed, indicating that the phospholipase A₁ pathway is not involved (results not shown).

Bradykinins have no effect on AA release from TEA3A1 cells

[3 H]AA-prelabelled TEA3A1 cells were treated with various amounts of bradykinin and Ile-Ser-bradykinin for 3 h at 37 °C. Even at a high concentration (10 μ g/ml), no significant stimulation of the release of [3 H]AA and its metabolites from TEA3A1 cells was observed. Bradykinin could not mimic the stimulatory effect of kallikrein. This fact suggests that the action of kallikrein

in the release of AA and its metabolites from TEA3A1 cells is independent of the formation of kinin.

Pepstatin A can abolish kallikrein-induced release of AA and its metabolites

Pepstatin A, an inhibitor of renin, cathepsin D and pepsin, was added to the [^3H]AA-prelabelled TEA3A1 cells treated with kallikrein (1.0 BAEE unit/ml) for 3 h at 37 °C. It was found that pepstatin A inhibited the kallikrein-mediated release of [^3H]AA and its metabolites, in a concentration-dependent manner. A complete inhibition was observed when 5 μg of pepstatin A/ml was added to the culture medium (Fig. 5). Only kallikrein-mediated activation was inhibited by pepstatin A; the basal level of AA release was not affected. This result strongly indicated that kallikrein does not act directly and that there is at least one other pepstatin A-sensitive proteinase which mediates the effect of kallikrein.

Kallikrein stimulates the intracellular production of prostaglandins and thromboxane in TEA3A1 cells

The effect of kallikrein on the synthesis of prostaglandins and thromboxanes within TEA3A1 cells was also studied. In this experiment, confluent TEA3A1 cells were incubated in the absence or presence of 1.0 BAEE units of kallikrein/ml for 3 h. At the end of the treatment, both the media and the cells were collected. The amount of prostaglandins E_2 , $\text{F}_{2\alpha}$, 6-oxo- $\text{F}_{1\alpha}$ and thromboxane B_2 in the media and the cells were determined by RIA as described. It was found that kallikrein treatment increases the amounts of PGE_2 and TXB_2 by 2-fold both in the media and in the cells. However, no significant effect was

Fig. 5. Inhibition of kallikrein-induced [^3H]AA release by pepstatin A

[^3H]AA-prelabelled TEA3A1 cells were treated with kallikrein (1.0 BAEE unit/ml) in DMEM for 3 h at 37 °C in the presence of various concentrations of pepstatin A. The radioactivity released into the medium was counted at the end of the incubation. The results are expressed as the percentage inhibition of kallikrein-induced [^3H]AA release from the cells. Each point represents the mean of three individual wells of cell culture $\pm$ S.E.M.

Table 1. Effect of kallikrein on intracellular prostaglandin production in TEA3A1 cells

Confluent TEA3A1 cells cultured in DW-ITD medium containing 2% sCS were incubated with or without kallikrein (1.0 BAEE unit/ml) in DMEM for 1 h at 37 °C. The amounts of intracellular PGs were assayed by RIA as described in the Materials and methods section. Means $\pm$ S.E.M. of six individual wells of culture are given. n.d., not detectable.

Treatment	PGE_2	$\text{PGF}_{2\alpha}$	TXB_2	6-Oxo- $\text{PGF}_{1\alpha}$
Control	2.0 ± 0.3	1.1 ± 0.05	0.16 ± 0.01	n.d.
Kallikrein	4.3 ± 0.4	1.7 ± 0.06	0.28 ± 0.01	n.d.

Table 2. Effect of enzyme inhibitors on kallikrein-mediated generation of prostaglandin E_2

Confluent TEA3A1 cells cultured in DW-ITD medium containing 2% sCS were incubated with or without kallikrein (1.0 BAEE units/ml) in DMEM for 3 h in the presence or absence of several different inhibitors. The amounts of PGE_2 both in the medium and in the cells were measured by RIA as described in the Materials and methods section. The means $\pm$ S.E.M. of three individual wells of culture are given.

Treatment	PGE_2 concn.	
	In cells (ng/well)	In media (ng/ml)
Control	2.60 ± 0.18	0.24 ± 0.02
Kallikrein	4.20 ± 0.30	0.50 ± 0.05
Kallikrein + pepstatin A	2.50 ± 0.18	0.36 ± 0.03
Kallikrein + aprotinin	3.10 ± 0.18	0.28 ± 0.02
Kallikrein + ONO RS082	2.50 ± 0.18	0.30 ± 0.02

observed on the levels of $\text{PGF}_{2\alpha}$ and 6-oxo- $\text{PGF}_{1\alpha}$ (see Table 1). Furthermore, kallikrein-induced intracellular PG and TX synthesis could be inhibited by aprotinin, pepstatin A and ONO RS082 (Table 2).

DISCUSSION

It has been reported that in several tissues phospholipase A_2 is the key enzyme in the regulation of the synthesis of prostaglandins and other arachidonic acid metabolites. Activation of prostaglandin synthesis by various external stimuli is associated with the activation of phospholipase A_2 [16–19]. In the present study, it was found that the phospholipase A_2 inhibitors ONO RS082 and mepacrine could abolish the stimulatory effect of kallikrein on the release of [^3H]AA and its metabolites from [^3H]AA-prelabelled TEA3A1 cells at concentrations of 10 μM and 2 μM , respectively. This result suggests that phospholipase A_2 may play an important role in the kallikrein-induced release of AA from the cellular phospholipid pool. Furthermore, the findings that kallikrein had no effect on the release of radioactivity from [^{14}C]stearic acid-labelled cells and that ONO RS082 could not block TPA-induced [^3H]AA release, confirm that kallikrein is indeed activating phospholipase A_2 .

It is generally understood that the function of kallikrein in the regulation of the synthesis of PGs is mediated through the formation of kinin from kininogen [10,11]. However, it has been reported by Morita *et al.* [12] that the stimulatory effect of kallikrein on the release of prostacyclin from vascular endothelial cells was independent of the formation of kinin. Our study is consistent with this observation. Bradykinin, even at a high concentration (10 $\mu\text{g/ml}$), could not stimulate the release of [^3H]AA from [^3H]AA-prelabelled TEA3A1 cells. Moreover, since all of our assays were done in serum-free medium, it is unlikely that the kallikrein-mediated effect could be due to endogenously generated kinins (unless the cells are producing kininogen or there is a significant carry-over of kininogen from the 2% sCS in which TEA3A1 cells are grown to confluence, even after 3 washes). Our observation suggests the existence of another mechanism for the kallikrein-induced activation of AA metabolism. Our study also suggests that kallikrein not only stimulates the release of [^3H]AA and its metabolites but also enhances the intracellular levels of PGE_2 and TXB_2 . Our data suggest that the intracellularly-generated AA metabolites may not be released from the cells, and may have as yet unknown functions. However, the possibility that the intracellular generation of eicosanoids occurs due to the mechanical perturbation of the membranes during the extraction process is not excluded. Whatever the mechanism may be, the effect of kallikrein on the stimulation of eicosanoid release is evident. AA metabolism, at least in TEA3A1 cells, should therefore be studied further in both the intra- and the extracellular compartments in order to obtain a complete picture of cellular regulation.

Another very interesting finding was that pepstatin A, an inhibitor of renin, pepsin and cathepsin D [20], could also completely inhibit the stimulatory effect of kallikrein on the release of [^3H]AA and its metabolites from TEA3A1 cells. This finding strongly indicates that kallikrein-mediated activation of arachidonic acid metabolism requires the presence of an as yet unidentified proteinase which can be inhibited by pepstatin A.

Since kallikrein is known to convert prorenin into renin, and renin can convert angiotensinogen to produce angiotensin I, which can in turn be converted into angiotensin II by angiotensin-converting enzyme (provided that both the substrate and enzyme are present in TEA3A1 cells) it is possible that angiotensin II may participate in the activation of eicosanoid production [21].

Based on our results, a possible pathway for kallikrein-induced activation of AA metabolism is proposed: (1) kallikrein activates an unidentified proteinase, which can be inhibited by pepstatin A; (2) the unidentified proteinase directly or indirectly results in the activation of AA metabolism, possibly mediated through the phospholipase A_2 pathway.

We do not know how general this proposed 'proteinase cascade' mechanism is for phospholipase A_2 activation by kallikrein among various cell types. Although the

involvement of serine proteases in the activation of phospholipase A_2 [12] and phospholipase C [22] has been reported, involvement of a 'proteinase cascade' in the activation of phospholipase A is quite intriguing.

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