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PHOSPHOLIPASE A_2 ACTIVITY IN CARRAGEENIN-INDUCED INFLAMMATORY TISSUE OF RATS

KAZUO OHUCHI*, MASAKO WATANABE, NORIKO NUMAJIRI and SUSUMU TSURUFUJI
Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Sendai 980, Japan

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Abstract—Phospholipase A_2 activity was detected in 7-day-old carrageenin-induced inflammatory tissue of rats using a synthetic substrate, 1-acyl-2-[^{3}H]arachidonyl-phosphatidylcholine. The inflammatory tissue was homogenized in saline containing 1 M KCl, and the 105,000 *g* supernatant fraction was placed on a Sephadex G-100 column. The partially purified phospholipase A_2 had a pH optimum at 6–7 and was Ca^{2+} dependent. *p*-Bromophenacyl bromide was strongly inhibitory to the partially purified phospholipase A_2 ($IC_{50} = 1.44 \times 10^{-5}$ M). A moderate inhibition was observed with indomethacin. Cycloheximide and dexamethasone, which inhibit prostaglandin production in inflammatory tissue, exerted no direct inhibitory action on the phospholipase A_2. There were no direct inhibitory effects of quinacrine, bradykinin, or actinomycin D. The cell-free supernatant fraction of the inflammatory exudate of 7-day-old carrageenin-induced granulation tissue was found to have no phospholipase A_2 activity.

During a series of investigations of prostaglandin production in the carrageenin air-pouch inflammatory tissue of rats [1–3], we found that prostaglandin production by the minced 7-day-old air-pouch inflammatory tissues of rats was inhibited by a cycloheximide, a protein synthesis inhibitor [4]. A glucocorticoid, dexamethasone, was also inhibitory [4]. Inhibition of prostaglandin production by cycloheximide has been reported also in methylcholanthrene-transformed fibroblasts [5], Madin–Darby canine kidney cells stimulated with tumor-promoting phorbol diesters [6], and renomedullary interstitial cells [7]. Phospholipase A_2 (phosphatide 2-acylhydrolase, EC 3.1.1.4) is widely accepted as having a regulatory role in the release of polyunsaturated fatty acids from phospholipids for prostaglandin production [8]. The present investigation was undertaken to characterize the partially purified phospholipase A_2 of carrageenin-induced inflammatory tissue and to determine whether cycloheximide and dexamethasone, which inhibit prostaglandin production of the inflammatory tissue [4], exert any direct inhibitory action on the partially purified phospholipase A_2 of inflammatory tissue. The effects of other drugs which have been reported to modulate arachidonic acid metabolism were also examined.

MATERIALS AND METHODS

Carrageenin-induced inflammatory tissue. Male rats of the Sprague–Dawley strain (Charles River Japan, Kanagawa, Japan), specific pathogen free, 5-weeks-old and weighing 150–180 g, were injected subcutaneously with 8 ml of air on the dorsum to make an air-pouch. After 24 hr, 4 ml of a 2% (w/v) solution of carrageenin (Seakem No. 202, Marine Colloid Inc., Springfield, NJ, U.S.A.) in 0.9% NaCl was injected [9]. The carrageenin solution was sterilized by autoclaving at 120° for 15 min and injected after cooling to 40–45°. Immediately before the injection, penicillin and streptomycin were added to the carrageenin solution (0.1 mg each per ml of the solution). Seven days after the carrageenin injection, the rats were killed by cutting the carotid artery, and the granulation tissue was carefully dissected free from the surrounding fat, muscle and nongranulomatous subcutaneous tissue. The dissected granulation tissue was washed with ice-cold 0.9% NaCl solution and stored at −40° until use. The inflammatory exudate was centrifuged at 1000 *g* for 15 min at 4°, and the supernatant fraction was kept at −40° until use.

Preparation of the substrate for phospholipase A_2 assay. Preparation of the substrate was carried out according to the methods described by Smith and Silver [10] with a slight modification. Two solutions were prepared. Solution I: 50.5 mg adenosine tri-phosphate (Wako Pure Chemical Industries, Tokyo, Japan), 0.8 mg coenzyme A (Kohjin, Tokyo, Japan), and 10.2 mg $MgCl_2 \cdot 6H_2O$ were dissolved in 5 ml of 0.1 M phosphate buffer (pH 7.5). Solution II: 20 μg lysophosphatidylcholine (Sigma Chemical Co., St. Louis, MO, U.S.A.), 2 μg arachidonic acid (Sigma), 10 μCi of [^{3}H]arachidonic acid ([5,6,8,9,11,12,14,15^{3}H(N)]arachidonic acid, 61 Ci/mmole, New England Nuclear Corp., Boston, MA, U.S.A.), and 2 μg stearic acid (Sigma) were dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.5) by sonication at 4°. Fresh microsomal fraction was obtained from rat liver (5 g) and was homogenized in 5 ml of 0.1 M phosphate buffer (pH 7.5). One milliliter each of solutions I and II and 0.3 ml of the fresh microsomal solution were mixed and incubated for 3 hr at 37°. After 30 and 120 min of incubation, an additional 1 ml of solution I and 0.3 ml of the micro-

* Address correspondence to: Kazuo Ohuchi, Ph.D., Department of Biochemistry, Faculty of Pharmaceutical Sciences, Tohoku University, Aoba, Aramaki, Sendai 980, Japan.

somal solution were added and mixed well, and the incubation was continued. After 3 hr of incubation, 10 ml of $CHCl_3$–MeOH (2:1, v/v) was added and mixed well. After centrifugation, the lower phase was removed and evaporated to dryness under reduced pressure and dissolved in 0.5 ml of $CHCl_3$. Silicic acid column chromatography was used to separate unreacted [^{3}H]arachidonic acid from [^{3}H]phosphatidylcholine. Silicic acid (Sigma) (3.2 g) and Hyflo (Wako) (0.8 g) were mixed, suspended in 10% MeOH in $CHCl_3$ (v/v), and slurried into a glass column (20 mm in diameter). The extracted residue, dissolved in 0.5 ml of $CHCl_3$, was applied on the column and eluted with 30 ml of 10% MeOH in $CHCl_3$ (v/v). Unreacted [^{3}H]arachidonic acid was eluted in this first fraction. Elution was continued with 30 ml of 40% MeOH in $CHCl_3$ and then 30 ml of 80% MeOH in $CHCl_3$ (v/v). [^{3}H]Phosphatidylcholine was eluted with 80% MeOH in $CHCl_3$ (v/v). Radioactivity in each 5-ml fraction of 80% MeOH in $CHCl_3$ (v/v) eluate was monitored, and fractions 2 and 3 were combined. An aliquot of this solution was used as a substrate. The substrate solution was stocked at −40° in a sealed tube under N_2 gas until use. To determine the positional specificity of [^{3}H]arachidonic acid, a portion of the combined solution was evaporated to dryness and dissolved in glycylglycine buffer (pH 8.0) containing 10 mM $CaCl_2$ and incubated with or without phospholipase A_2 from *Crotalus adamanteus* venom (Sigma) at 37° for 3 hr. After extraction with $CHCl_3$–MeOH (2:1, v/v), the $CHCl_3$ layer was evaporated to dryness. The lipid residue and each 20 μg of phosphatidylcholine and arachidonic acid as carriers were dissolved in 20 μl of $CHCl_3$ and separated by thin-layer chromatography using silica gel G thin-layer plates (E. Merck, West Germany) in the solvent system $CHCl_3$–MeOH–AcOH (90:5:5, by vol.). More than 99% of the radioactivity in the synthesized substrate moved with free arachidonic acid after treatment with the venom phospholipase A_2. Less than 1% of the radioactivity remained at the origin in the position of phosphatidylcholine. When the substrate was incubated without phospholipase A_2 from *C. adamanteus*, no detectable amount of radioactivity was found at any position except phosphatidylcholine after the separation by thin-layer chromatography. The specific radioactivity of the labeled phosphatidylcholine was calculated as 560 mCi/mmole.

Partial purification of phospholipase A_2 from carrageenin-induced inflammatory tissue. After thawing, the inflammatory tissue was cut into small pieces and homogenized in ice-cold 0.9% NaCl solution (1:2, w/v) containing 1 M KCl using a Vir-Tis 45 homogenizer (4 min at maximum speed). The homogenate was centrifuged at 105,000 *g* for 90 min at 4°, and 5 ml of the supernatant fraction was applied to a Sephadex G-100 column (20 × 250 mm). The column was eluted with 0.9% NaCl solution containing 1 M KCl (flow rate, 16 ml/hr; fraction volume, 4 ml). Fractions 22–27 were combined and used as a partially purified enzyme source. Protein content was determined by the method of Lowry *et al.* [11] with a slight modification.

Phospholipase A_2 assay. Phospholipase A_2 assay was carried out according to the method described by Smith and Silver [10]. The standard assay mixture contained 0.1 M Tris (pH 6.5), 10 mM $CaCl_2$, and 1.1×10^{-2} μCi of the labeled phosphatidylcholine, and the enzyme source in a final volume of 3 ml. After incubation at 37° for 2 hr, 10 ml of $CHCl_3$–MeOH (2:1, v/v) were added, mixed well, and centrifuged to separate the $CHCl_3$ layer. The $CHCl_3$ layer was evaporated to dryness, and the residue was dissolved in an aliquot of $CHCl_3$–MeOH (1:1, v/v) containing unlabeled standard solutions of phosphatidylcholine (25 μg) and arachidonic acid (25 μg) as carriers. The residue was then subjected to thin-layer chromatography on silica gel G (E. Merck) with a solvent system of $CHCl_3$–MeOH–AcOH (90:5:5, by vol.). The spots were visualized by exposure to iodine vapour. After evaporating the I_2, the zones corresponding to arachidonic acid, phosphatidylcholine and the remnants were scraped out and transferred to the counting vials containing 5 ml of scintillation mixture. The radioactivity was measured in an Aloka LSC-671R liquid scintillation spectrometer (Aloka, Tokyo, Japan) with appropriate quench correction. Blanks without enzyme source were included in all experiments. The solvent system that was employed separates [^{3}H]arachidonic acid from [^{3}H]phosphatidylcholine with no radioactive interference by each other.

The drugs examined were cycloheximide, *p*-bromophenacyl bromide (Wako), dexamethasone, quinacrine, actinomycin D (Sigma), indomethacin (Nihon Merck Banyu, Tokyo, Japan) and bradykinin (Peptide Institute Protein Research Foundation, Mino-o, Japan). With the exception of bradykinin, the drugs were dissolved in ethanol and were added to the standard assay mixture described above. The same amount of ethanol was added to the control as the vehicle. Bradykinin was dissolved in 0.1 M Tris buffer (pH 6.5) and added to the standard assay mixture.

RESULTS

Characterization of phospholipase A_2 activity in 7-day-old granuloma. As shown in Fig. 1, when the homogenate of 7-day-old carrageenin-induced inflammatory tissue was incubated with the labeled phosphatidylcholine, the release of [^{3}H]arachidonic acid was observed as a function of time. This result indicates the presence of phospholipase A_2 activity in the carrageenin-induced inflammatory tissues, since [^{3}H]arachidonic acid was specifically labeled at the 2-position of the labeled substrate. The distribution of phospholipase A_2 activity after homogenization of the carrageenin-induced inflammatory tissue is shown in Table 1. The inflammatory tissue was homogenized in 0.9% NaCl solution containing 1 M KCl instead of 0.9% NaCl solution for the purpose of solubilization of membrane bound protein. By the addition of 1 M KCl, about a 1.7-fold increase in protein content was recovered in the 105,000 *g* supernatant fraction (not shown), in which 76.9% of total phospholipase A_2 activity was distributed. However, in the 105,000 *g* supernatant fraction, only a slight increase in the specific activity of phospholipase A_2 was observed compared with that hom-

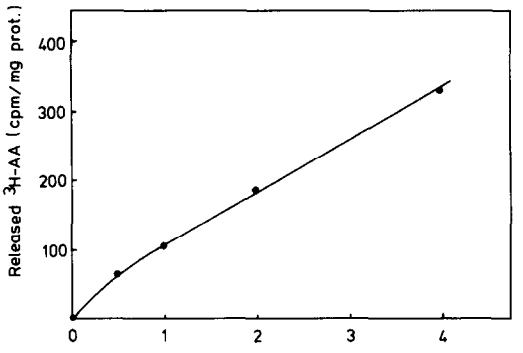

Fig. 1. Release of [^{3}H]arachidonic acid from labeled substrate as a function of time. Seven-day-old carrageenin-induced inflammatory tissue was homogenized, in ice-cold 0.9% NaCl solution (1:2, w/v), by a Vir-Tis 45 homogenizer at maximum speed for 4 min. One milliliter of the homogenate (13.3 mg protein) was incubated with 1.1×10^{-2} μCi of the labeled substrate at 37° for the indicated times. The release of [^{3}H]arachidonic acid was detected as described in Materials and Methods. The results are corrected for non-enzymatic release, which was less than 1.5% of total label.

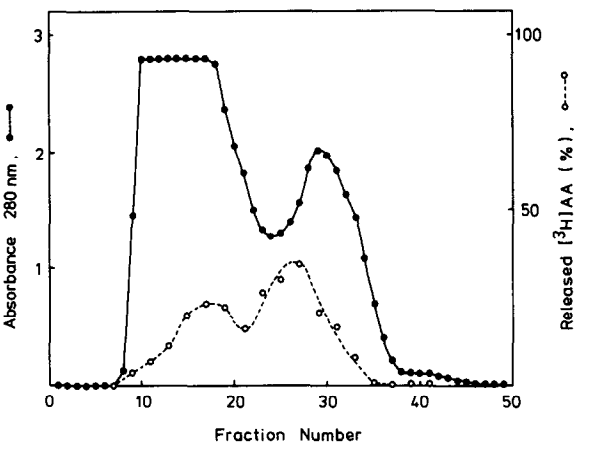

Fig. 2. Elution pattern of total protein and phospholipase A_2 activity from a Sephadex G-100 chromatography column with 0.9% NaCl solution containing 1 M KCl as eluent. Seven-day-old carrageenin-induced inflammatory tissue was homogenized in ice-cold 0.9% NaCl solution containing 1 M KCl (1:2, w/v). Five milliliters of the 105,000 *g* supernatant fraction of the homogenate was applied on a Sephadex G-100 column (20 × 250 mm). The column was eluted at 4° with 0.9% NaCl solution containing 1 M KCl (flow rate, 16 ml/hr; fraction volume, 4 ml). Three milliliters of each fraction was used for assaying phospholipase A_2 activity. The assay mixture contained 1.1×10^{-2} μCi of the labeled substrate and 10 mM $CaCl_2$ and was incubated for 3 hr at 37°. The reproducibility of the results was examined by repeated experiments.

ogenized in 0.9% NaCl solution (not shown). To increase the specific activity of phospholipase A_2, the 105,000 *g* supernatant fraction was subjected to Sephadex G-100 column chromatography. As shown in Fig. 2, two major peaks of protein were observed. The higher phospholipase A_2 activity was found in fractions just before the second peak of protein. These fractions (22–27) were pooled for further experiments. The specific activities of phospholipase A_2 in the pooled fractions were increased to about fifty times compared with that of the homogenate in 0.9% NaCl containing 1 M KCl. Effects of pH on the hydrolysis of the substrate by the partially purified phospholipase A_2 are shown in Fig. 3. The optimum pH of phospholipase A_2 of the carrageenin-induced inflammatory tissue ranged between 6.0 and 7.0; therefore, subsequent incubations to assay phospholipase A_2 activity were carried out at pH 6.5. Figure 4 depicts the inhibition of the partially purified phospholipase A_2 in the presence of various amounts of EDTA. The requirement for Ca^{2+} by these enzyme fractions was tested.

It was found that the phospholipase A_2 activity was increased by the addition of $CaCl_2$ up to 15 mM with a broad plateau (data not shown). Therefore, 10 mM $CaCl_2$ in the assay mixture was found to be optimal for the assay conditions of the phospholipase A_2 activity. In the standard assay mixture, 0.1 M Tris–HCl buffer (pH 6.5), 10 mM $CaCl_2$ in a final volume of 3 ml containing the partially purified enzyme source and 1.1×10^{-2} μCi of the synthetic substrate, effects of protein concentration and incubation time were examined. The hydrolysis of the substrate was proportional to protein content up to 550 μg protein and for 4 hr of incubation time (not shown).

To examine the activity of phospholipase A_2 in the cell-free supernatant fraction of the inflammatory

Table 1. Distribution of phospholipase A_2 activity after homogenization of carrageenin-induced inflammatory tissue*

	Total protein (mg)	Phospholipase A_2 activity	
		Released [^{3}H]arachidonic acid (cpm/hr × 10^{-3})	%
Homogenate	475	38.1	100
105,000 *g*			
Supernatant	369	28.3	74.3
Precipitate	116	8.5	22.3

* About 10 g of 7-day-old carrageenin-induced inflammatory tissue was homogenized, in ice-cold 0.9% NaCl solution containing 1 M KCl, by a Vir-Tis 45 homogenizer at maximum speed for 4 min. The homogenate was centrifuged at 105,000 *g* for 1.5 hr at 4°. Precipitate was resuspended in the same solution using a Teflon-pestle homogenizer. Aliquots of the homogenate, the 105,000 *g* supernatant fraction and the resuspended precipitate were incubated for 3 hr at 37° with 1.1×10^{-2} μCi of the labeled substrate in a volume of 2 ml.

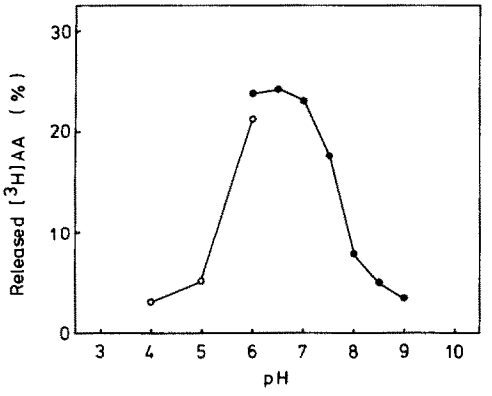

Fig. 3. Effect of pH on the release of [^{3}H]arachidonic acid from the labeled substrate. To 2 ml of the pooled samples after the fractionation by Sephadex G-100 column chromatography was added 1 ml of 0.3 M sodium acetate buffer (pH 4.0 to 6.0) or 0.3 M Tris–HCl buffer (pH 6.0 to 9.0) and the mixture was incubated with 1.1×10^{-2} μCi of the labeled substrate at 37° for 2 hr. Key: (○——○) final 0.1 M sodium acetate buffer containing 10 mM $CaCl_2$; and (●——●) final 0.1 M Tris–HCl buffer containing 10 mM $CaCl_2$. Each point is the mean of two assays. The results have been corrected for non-enzymatic release which was less than 1.3% of total label.

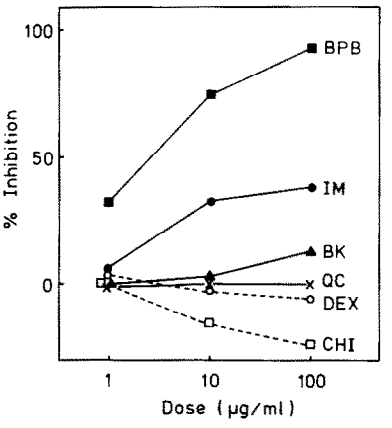

Fig. 5. Effects of drugs on the partially purified phospholipase A_2 of carrageenin-induced inflammatory tissue. Two milliliters of the pooled fraction after Sephadex G-100 column chromatography was incubated for 2 hr at 37° in a volume of 3 ml containing 1.1×10^{-2} μCi of the labeled substrate, 10 mM $CaCl_2$, 0.1 M Tris–HCl buffer (pH 6.5) and indicated doses of drugs. Each drug was dissolved in ethanol and added to the mixture. The control contained the same amount of the vehicle. In the case of bradykinin, it was dissolved in Tris–HCl buffer (pH 6.5) and added to the incubation mixture. Total protein in 2 ml of the pooled fraction varied from 320 to 410 μg from experiment to experiment. The results are the means of duplicate or triplicate assays and the values agreed within 10% of the means. Abbreviations: BPB; *p*-bromophenacyl bromide; IM, indomethacin; DEX, dexamethasone; CHI, cycloheximide; QC, quinacrine; and BK, bradykinin. The lack of effect of actinomycin D is not depicted in the figure.

exudate, up to 2 ml of the fraction was incubated at 37° with 1.1×10^{-2} μCi of the synthetic substrate in a final volume of 3 ml containing 0.1 M Tris (pH 6.5) and 10 mM $CaCl_2$. After 2 hr of incubation, there was no significant release of [^{3}H]arachidonic acid from the substrate.

Effects of drugs on phospholipase A_2 activity. The effects of several drugs on the partially purified phospholipase A_2 are summarized in Fig. 5. Among the drugs examined, *p*-bromophenacyl bromide showed the highest inhibitory activity. Its IC_{50} value was calculated to be 1.44×10^{-5} M. Indomethacin at a dose of 1 μg/ml showed no inhibitory activity on the phospholipase A_2. At the same dose of indomethacin, prostaglandin production by the carrageenin-induced inflammatory tissue was inhibited more than 95% [4]. However, moderate inhibition by indomethacin at doses of 10 and 100 μg per ml was observed. Cycloheximide, which inhibits prostaglandin production by carrageenin-induced inflammatory tissue at doses of 0.1 to 10 μg/ml [4], showed no direct inhibitory activity on the partially purified phospholipase A_2 at any of the doses examined. On the other hand, a tendency for stimulation of hydrolysis of the substrate was observed at a dose of 100 μg/ml. Bradykinin, quinacrine and the steroidal anti-inflammatory drug, dexamethasone, also had no direct inhibitory effect on the partially purified phospholipase A_2. There was no inhibitory effect of actinomycin D up to a dose of 100 μg/ml (not shown). Further, no direct inhibitory effect by cycloheximide or dexamethasone was observed when the labeled substrate was incubated for 2 hr at 37° with the homogenate of the tissue instead of the partially purified phospholipase A_2 (not shown).

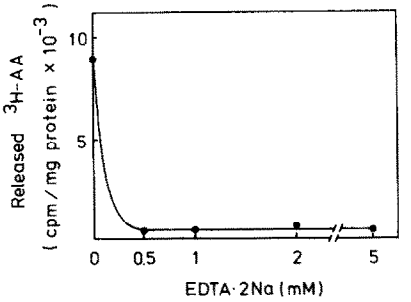

Fig. 4. Effect of EDTA on the release of [^{3}H]arachidonic acid from the labeled substrate. Two milliliters of the pooled fractions after Sephadex G-100 column chromatography was added to 1 ml of 0.3 M Tris–HCl buffer (pH 6.5) containing 1.1×10^{-2} μCi of the labeled substrate and indicated doses of EDTA–2Na. The incubation was carried out at 37° for 2 hr.

DISCUSSION

Except for one report dealing with peritoneal exudate in rabbits [12], there have been no observations made on the characteristics of phospholipase A_2 of inflammatory tissue. The present investigation was undertaken to clarify the characteristics of the phospholipase A_2 of the proliferative inflammatory tissue that is induced by carrageenin. Seven days after the injection of a 2% carrageenin solution into rat dorsum, the oval capsule of granulomatous inflammatory tissue retaining the inflammatory exudate was well developed, and the wet weight of the inflammatory tissue and the volume of the

exudate reached about 5 g and 20 ml respectively. The main components of the 7-day-old tissue are fibrocytes, macrophages and collagen fiber. We have reported that the 7-day-old carrageenin-induced inflammatory tissue synthesizes and releases large amounts of prostaglandins when the tissue is minced and incubated in Eagle's minimal essential medium containing 10% calf serum [4]. The minced inflammatory tissue synthesizes mainly 6-keto-prostaglandin $F_{1\alpha}$ and prostaglandin E_2 and small amounts of prostaglandin $F_{2\alpha}$ and thromboxane B_2. Bragt and Bonta [13] and Higgs and Salmon [14] also reported prostaglandin production in carrageenin-induced inflammatory tissue with contrasting results.

From the positional specificity experiment of [^{3}H]arachidonic acid in the substrate, it was demonstrated that most of the radioactivity released from the substrate was associated with the 2-position of phosphatidylcholine and not with lysophosphatidylcholine. Therefore, we concluded that the enzyme activity in our preparations was due to phospholipase A_2 rather than phospholipase A_1. Since unsaturated fatty acids can be released not only by phospholipase A_2 alone, but also by the combined action of either phospholipase A_1 and lysophospholipase or phospholipase C and diacylglycerol lipase, the partially purified phospholipase A_2 may not be the acylhydrolase responsible for the biosynthesis of the prostaglandins in the carrageenin air-pouch inflammation.

Recently, Wightman *et al.* [15] reported two phospholipase A_2 activities in homogenates of mouse peritoneal macrophages. The first is active at pH 4.5, not dependent on Ca^{2+}, and presumably of lysosomal origin; the second is Ca^{2+} dependent and is optimally active at pH 8.5. In the present investigation, the partially purified phospholipase A_2 from carrageenin-induced inflammatory tissue showed only one pH optimum, between 6 and 7. It may be natural since, in the present investigation, the homogenate of the inflammatory tissue was centrifuged at 105,000 *g* for 90 min to remove lysosomal particles. Indirect evidence for a role of lysosomal phospholipase A_2 in prostaglandin production in carrageenin-induced inflammation was offered by Anderson *et al.* [16]. As to an optimum pH of phospholipase A_2, not a lysosomal acylhydrolase, there are many reports with different tissues using different assay conditions. The highest optimum pH, pH 9.5, was reported with rabbit platelets [17]. With sheep red cell membrane [18] and porcine pancreas phospholiphase A [19], the optimum pH is 8.0, while with rat myocardium [20], skin [21] and mammary tumor phospholipase A_2 [22], and human seminal plasma phospholipase A_2 [23], the optimum pH was reported to be 7.5. In rabbit peritoneal inflammatory fluid [12], a broad pH range (pH 6–8) was reported. An optimum pH around 7.0 was also reported for rat spleen phospholipase A [24] and BCG-induced rabbit alveolar macrophages [25]. In the present investigation, the optimum pH of the partially purified phospholipase A_2 from carrageenin-induced inflammatory tissue was shown to be 6.5. This is consistent with the value obtained in rat lung 105,000 *g* supernatant fraction [26]. The partially purified phospholipase A_2 from the inflammatory tissue was sensitive to Ca^{2+} concentration and was inhibited by EDTA. The Ca^{2+} requirement for optimal activity of the purified phospholipase A_2 from the inflammatory tissue was similar to those from other sources such as rat skin [21], glycogen-induced rabbit peritoneal exudate [12], porcine pancreas [19], human seminal plasma [23], sheep red cell membranes [18], and others [17, 24].

p-Bromophenacyl bromide, which inhibits porcine pancreatic phospholipase A_2 [27] by alkylating the imidazole side-chain of the histidine residue at position 53 of the molecule, strongly inhibited the partially purified phospholipase A_2 of the inflammatory tissue. The IC_{50} was calculated to be 1.44×10^{-5} M. *p*-Bromophenacyl bromide inhibition of purified phospholipase A_2 from *Vipera russelli* was also reported by Vallee *et al.* [28], and the IC_{50} was shown to be 1.25×10^{-5} M, which is close to our value. Quinacrine, which was reported to be an inhibitor of phospholipase A_2 by Vargaftig and Dao Hai [29], failed to inhibit the partially purified phospholipase A_2 from the inflammatory tissue. This is consistent with the finding by Vallee *et al.* [28] that quinacrine is unable to inactivate venom phospholipase A_2. Bradykinin was reported to stimulate prostaglandin production by activating phospholipase A_2 [30], but no direct stimulatory effect on the partially purified phospholipase A_2 was observed in the present investigation. Actinomycin D, an inhibitor of DNA-dependent RNA synthesis, also inhibits prostaglandin production [5, 7] but had no direct inhibitory effect on the partially purified phospholipase A_2. In an earlier paper [4], we have shown that the non-steroidal anti-inflammatory drug, indomethacin, and the steroidal anti-inflammatory drug, dexamethasone, inhibit the production of prostglandins and thromboxane B_2 by the carrageenin-induced inflammatory tissue. Cycloheximide, a protein synthesis inhibitor, also inhibited prostaglandin E_2 production in a dose-dependent manner [4]. Indomethacin inhibition of phospholipase A_2 activity of rabbit polymorphonuclear leukocytes was reported by Kaplan *et al.* [31]. They showed that 50 μM indomethacin failed to inhibit the phospholipase A_2 from the venoms of the Russell viper, *C. adamanteus*, and the bee, and from pig pancreas. However, 70% of the phospholipase A_2 activity of leukocytes was inhibited at this dose. Franson *et al.* [32] also reported that indomethacin inhibits highly purified phospholipase A_2 isolated from human platelets, rabbit alveolar macrophages, and peritoneal polymorphonuclear leukocytes and that the IC_{50} is 75 μM in the presence of 5 mM Ca^{2+}. In the present investigation, indomethacin did inhibit phospholipase A_2 activity at concentrations higher than 28 μM (10 μg/ml). As reported previously [4], however, significant inhibition of prostaglandin production by indomethacin in the minced inflammatory tissue was apparent at concentrations much lower than that required to block phospholipase A_2 activity. Therefore, at low doses of indomethacin, inhibition of prostaglandin synthesis in the minced inflammatory tissue is a consequence of the drug acting on cyclooxygenase, not on phospholipase A_2. However, at low doses, indomethacin did inhibit release of [^{3}H]arachidonic acid from prelabeled canine kidney cells stimulated by tumor-promoting phorbol diesters, but the effect

was only observed 12 hr after the addition of indomethacin [33].

As to the mechanism of the inhibitory effects of the steroidal anti-inflammatory drugs on prostaglandin production, it has been suggested [34, 35] that the anti-inflammatory steroids induce biosynthesis of a protein which inhibits phospholipase A_2. This protein has been named "macrocortin" by Blackwell *et al.* [36] or "lipomodulin" by Hirata *et al.* [37]. According to their findings, there should be no direct inhibitory effects of the steroidal anti-inflammatory drugs on the isolated phospholipase A_2. Consistent with their findings, the present investigation has revealed that the steroidal anti-inflammatory drug, dexamethasone, at doses up to 100 μg/ml failed to directly inhibit the partially purified phospholipase A_2 of the inflammatory tissue. There is no direct inhibitory effect on rabbit platelet phospholipase A_2 by the steroidal anti-inflammatory drugs, prednisolone and hydrocortisone, at a dose of 200 μg/ml [17]. However, Ziboh and Lord [21] demonstrated a direct inhibitory effect on a phospholipase A_2 by hydrocortisone and triamcinolone acetonide at concentrations of 50 μM, 18 and 22 μg per ml respectively.

The release of [^{3}H]arachidonic acid from the prelabeled peritoneal macrophages of rats was inhibited by cycloheximide (unpublished observations). As shown in Fig. 5, cycloheximide had no direct inhibitory effect on the phospholipase A_2 activity of the inflammatory tissue. Since no direct inhibition by cycloheximide of cyclooxygenase activity has been reported [5], our results suggest that cycloheximide inhibits prostaglandin production by some indirect action, not by direct inhibition of phospholipase A_2 or cyclooxygenase.

No phospholipase A_2 activity was detected in a cell-free supernatant fraction of the inflammatory exudate of carrageenin-induced granulation tissue. In contrast to our results, Franson *et al.* [12] reported the presence of a phospholipase A_2 in the peritoneal inflammatory exudate produced in rabbits injected with 1% glycogen. In the earlier stages of inflammation, prostaglandin E_2 content in the exudate was reported to be higher than that in 7- or 8-day-old inflammatory exudate [1, 2, 14]. Consequently, phospholipase A_2 may be present in the exudate during the early stage of inflammation. There is also the possibility that in the exudate of carrageenin-induced inflammatory tissue, some as yet unknown factors which inhibit phospholipase A_2 may exist. In this regard, further investigations are under way in our laboratory.

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