Volume 244, number 1, 51-56 FEB 06773 February 1989

A phospholipase A_2 hydrolyzing arachidonoyl-phospholipids in mouse peritoneal macrophages

Jonny Wijkander and Roger Sundler

Department of Physiological Chemistry, University of Lund, PO Box 94, S-221 00 Lund, Sweden

Received 28 November 1988

A calcium-dependent phospholipase A_2 with half-maximal activity at approx. 0.7 μM free Ca^{2+} has been identified in the cytosolic fraction from macrophages. The enzyme eluted as a 70 kDa protein upon gel chromatography and showed increased activity after 10 min pretreatment of the cells with 10 nM phorbol myristate acetate. No significant activity could be detected in the membrane fraction. The enzyme hydrolyzed arachidonic acid-containing phosphatidylcholine and -ethanolamine as well as phosphatidylinositol. The release of arachidonic acid in the in vitro assay was inhibited in a dose-dependent manner by nordihydroguaiaretic acid and quercetin that are also potent inhibitors of the mobilization of arachidonic acid in intact macrophages.

Phospholipase A_2; Arachidonic acid; Phorbol myristate acetate; Quercetin; Nordihydroguaiaretic acid; (Mouse macrophage)

1. INTRODUCTION

Macrophages from the mouse peritoneal cavity respond to activators of protein kinase C, such as phorbol myristate acetate (PMA), by releasing prostaglandin E_2 [1,2]. The arachidonic acid used is mobilized from phosphatidylinositol by enhancement of a phospholipase A pathway that may also be responsible for the mobilization from phosphatidylcholine and -ethanolamine [3,4]. However, the signal-transducing pathway from kinase C to the lipolytic step, as well as the phospholipase enzyme(s) involved, have remained unidentified. We have therefore investigated kinase C-dependent protein phosphorylation in the macrophages and found a close correlation with the release of arachidonic acid [5] and have now undertaken to identify, isolate and characterize the phospholipase(s) involved. This paper describes the identification and initial characterization of a soluble, calcium-dependent PLA_2 activity with an apparent molecular mass of approx. 70 kDa. The present results strongly suggest that the enzyme is responsible for the kinase C-related mobilization of arachidonic acid observed in intact macrophages.

Correspondence address: R. Sundler, Department of Physiological Chemistry, University of Lund, PO Box 94, S-221 00 Lund, Sweden

Abbreviations: PMA, 4-β-phorbol 12-myristate 13-acetate; PLA_2, phospholipase A_2

2. EXPERIMENTAL

Mouse peritoneal macrophages were isolated and cultured (2×10^6 cells/35 mm dish) as described [3]. Cells were scraped off the dishes and homogenized in a buffer consisting of 80 mM KCl, 5 mM dithioerythritol, 1 mM EDTA and 10 mM Hepes, pH 7.4 (buffer A). The homogenate from 12 dishes was centrifuged at $700 \times g$ for 5 min and the resulting supernatant was further centrifuged at $10^5 \times g$ for 60 min to obtain a cytosolic fraction and a membrane pellet. The cytosolic fraction was then run on a column (1 × 48 cm) of either Sephadex G-200 superfine or Sephacryl S-300 HR (LKB-Pharmacia), equilibrated in buffer A containing 10% (v/v) glycerol.

Assays for PLA_2 contained 100 pmol phospholipid (5–15 × 10^3 dpm), solubilized by sonication for 8 min, enzyme (30–60 μl), $CaCl_2$ (625 nmol total, 290 μM free Ca^{2+}) and fatty acid-depleted bovine serum albumin (100 μg) in a total volume of 525 μl buffer A (without dithioerythritol). Labeled phospholipids (Amersham) were; 1-stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine (117 Ci/mmol), 1-acyl-2-[^{14}C]arachidonoylphosphatidylethanolamine (58 mCi/mmol), 1-stearoyl-2-[^{14}C]arachi-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

donoylphosphatidylinositol (58 mCi/mmol) and 1-palmitoyl-2-[^{14}C]oleoylphosphatidylcholine (52 mCi/mmol). Nonlabeled phospholipids were from Sigma. When the concentration of calcium was varied, the concentration of free Ca^{2+} (in the range 0.1–20 μM) was determined in an SLM 8000 C fluorimeter (SLM Instruments) in parallel incubation mixtures also containing 0.1 μM fura-2 (Molecular Probes, Eugene, OR), by the use of dual-wavelength excitation (at 340 and 380 nm), the fluorescence ratio at 492 nm and a K_d for Ca^{2+} of 135 nM [6].

After incubation for 30 min at 37°C the reaction was stopped by the addition of 0.1 ml of 250 mM HCl and carrier lipids followed by extraction according to Bligh and Dyer. Lipid extracts were separated by TLC in a solvent system of diethyl ether/light petroleum (b.p. 40–60°C)/acetic acid (50:50:2, by vol.) and radioactivity in areas corresponding to free fatty acids and phospholipids was determined. In later experiments the TLC analysis was replaced by a simplified method that gave identical results. The lipid phase was then applied to a column containing 100 mg silicic acid equilibrated in chloroform. Fatty acids were eluted with 1 ml chloroform and phospholipids with 2.5 ml methanol. In order to assess the formation of radiolabeled lysophosphatidylcholine, samples were analyzed by TLC, using as solvent chloroform/methanol/4 M NH_4OH (65:35:5, by vol.). Nordihydroguaiaretic acid, quercetin, taxifolin and *p*-bromophenacylbromide (Sigma) were dissolved in ethanol/H_2O (1:1) and added to assay mixtures in a volume of 2–5 μl. This amount of ethanol had no effect on PLA_2 activity. Albumin was omitted in experiments with inhibitors.

3. RESULTS

The cytosolic fraction from cultured mouse macrophages contained a phospholipase activity that released arachidonic acid from phosphatidylcholine. Upon gel chromatography the phospholipase activity eluted as a single peak with a K_{av} corresponding to approx. 70 kDa (fig.1). This activity was increased to 163 ± 41% (mean ± SD, $n = 7$) of that in controls (25.0 ± 9.0 pmol hydrolysis/10^6 cells per 30 min (mean ± SD, $n = 7$)) when the cells had been pretreated with 10 nM PMA for 10 min (fig.1). In two of the above experiments, 10 mM NaF was included in the buffer and in those experiments the increase in activity in PMA-treated cells was well above the average (217 and 218% of control, respectively). Because of the aggregated state of the substrate and the fact that it was used at subsaturating concentration, we have not undertaken any analysis of how individual kinetic parameters are affected. The enzyme was identified as a PLA_2 since: (i) labeled arachidonic acid was released from 1-stearoyl-2-[^{3}H]arachidonoylphos-

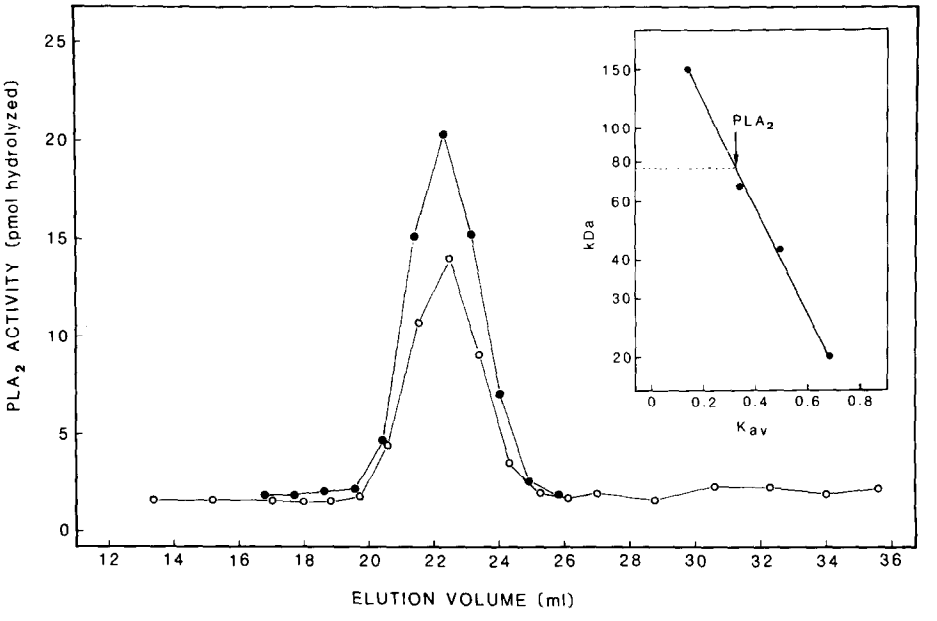

Fig.1. Gel chromatography of the cytosolic fraction from mouse macrophages. Cytosolic fractions were run on Sephadex G-200 and 60 μl of each fraction was assayed for PLA_2 activity using 1-stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine as substrate. Cells pretreated with 10 nM PMA for 10 min (●) were compared to control cells (○). There was no difference in protein concentration, as determined by A_{280}. The void volume of the column (13.5 ml) was determined using Blue dextran 2000. For further details see text. (Inset) K_{av} of PLA_2 compared to standard proteins [immunoglobulin G (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and soybean trypsin inhibitor (20 kDa)].

phatidylcholine without detectable formation of labeled lysophosphatidylcholine and (ii) the use of phosphatidyl[^{3}H]choline from macrophages as substrate yielded labeled lysophosphatidylcholine as product (not shown). Furthermore, the presence of deoxycholate (50 μg/ml) and albumin (4 mg/ml) in the assay mixture at concentrations shown previously to inhibit severely lysophospholipase [7] caused no inhibition of the present enzyme activity. When the unfractionated cytosol was assayed for PLA_2 activity the arachidonic acid released was, to a large extent, further metabolized to more polar metabolites. Attempts to identify any PLA_2 activity in the membrane fraction, with or without sonication or treatment with detergent, have not been successful.

The PLA_2 identified here was totally dependent on calcium (fig.2), showed significant activity at 350 nM free Ca^{2+} and was fully activated at micromolar concentrations, since no further increase in activity occurred at 1 mM (fig.2) or 5 mM Ca^{2+} (not shown). We have not been able to detect any difference in calcium dependence between enzyme from control and PMA-treated cells. The pH dependence of the PLA_2 activity was also determined and a broad optimum was found between pH 7 and at least 10, while the activity dropped drastically when pH was reduced below 6 (fig.3). The drop at acidic pH could, at least in part, be due to irreversible loss of activity, since preincubation for 15 min at pH 5, in contrast to pH 7.4, resulted in considerable loss of activity. The stability of the enzyme depended on the presence of glycerol also at neutral pH. When the cytosolic fraction was stored at 4°C in the presence of 10% glycerol (pH 7.4) the enzyme retained considerable activity for at least 1 month.

The enzyme was found to hydrolyze arachidonic acid-containing phosphatidylcholine and phosphatidylethanolamine equally well (table 1). The hydrolysis of arachidonic acid-containing phosphatidylinositol dependend on how this substrate was presented. Pure phosphatidylinositol vesicles were not hydrolyzed at all, but when 75 mol% phosphatidylcholine or -ethanolamine was included, the hydrolysis of phosphatidylinositol was only

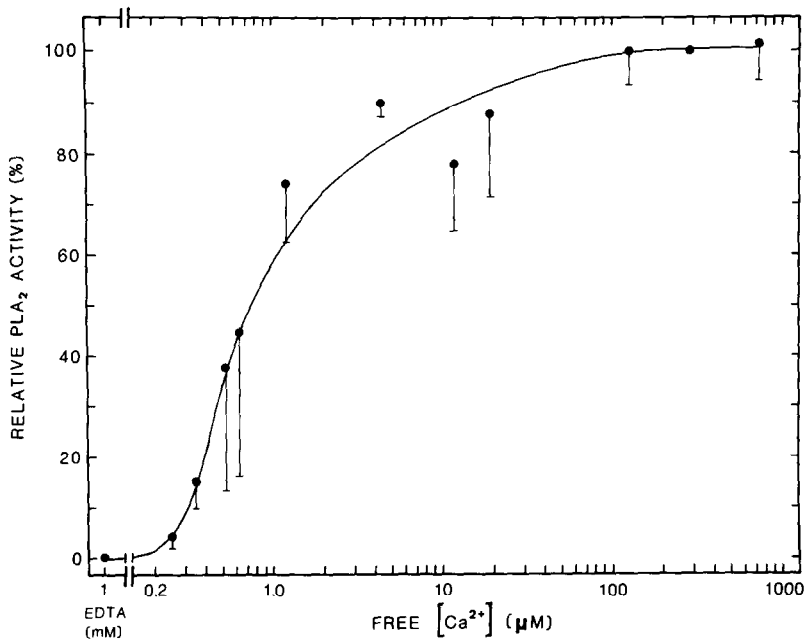

Fig.2. Calcium dependence of macrophage PLA_2. PLA_2 activity against 1-stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine was measured with enzyme taken through gel chromatography (fig.1) in the presence of various concentrations of free Ca^{2+}. The concentration of free Ca^{2+} in the EDTA-buffered incubation mixtures was determined using the fluorescent probe fura-2 (see section 2). Results obtained with different preparations of PLA_2 are expressed as percent of the activity at 290 μM Ca^{2+} (10–15% hydrolysis) and represent means ± SD ($n \geq 2$).

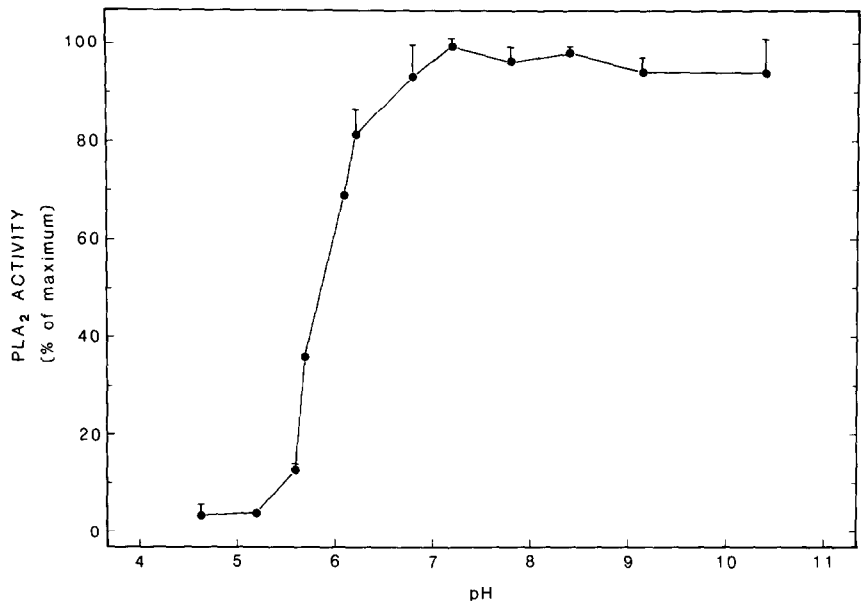

Fig.3. pH dependence of the PLA_2 activity. Enzyme from gel chromatography was assayed for PLA_2 activity vs 1-stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine at the pH values shown. Results are expressed as percent of maximal activity and represent means ± SD ($n \geq 3$), except at pH 5.2, 5.7 and 6.1 which represent single observations.

somewhat lower than that of phosphatidylcholine (table 1). It should be mentioned that in a mixture of 25 mol% phosphatidylcholine and 75 mol% phosphatidylinositol the hydrolysis not only of phosphatidylinositol but also of phosphatidylcholine was severely inhibited (not shown). We feel confident that the hydrolysis of labeled arachidonoylphosphatidylinositol is due to the PLA_2 rather than to phosphoinositide phospholipase C, since arachidonic acid was released rather than diacylglycerol and the activity against phosphatidylinositol and -choline coeluted exactly during gel chromatography. Furthermore, the activity vs both phospholipids was increased to the same extent by pretreatment of cells with PMA. It is also clear from table 1 that the PLA_2 enzyme showed a high preference for arachidonic acid-compared to oleic acid-containing phosphatidylcholine. Omission of albumin from the standard assay mixture reduced the hydrolysis of arachidonoylphosphatidylcholine by 20–40%, most likely because of inhibition by reaction products.

Table 1

Substrate specificity of macrophage PLA_2

Substrate	Hydrolysis			
	20:4-PC	20:4-PE	20:4-PI	18:1-PC
20:4-PC/20:4-PE[a]	25.0	31.7		
20:4-PC/20:4-PI[b]	27.7		19.1	
20:4-PC/18:1-PC[c]	25.0			7.0

[a]1-Stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine/1-acyl-2-[^{14}C]arachidonoylphosphatidylethanolamine (50:50, mol %).
[b]1-Stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine/1-steaoryl-2- [^{14}C]arachidonoylphosphatidylinositol (75:25, mol%).
[c]1-Stearoyl-2-[^{3}H]arachidonoylphosphatidylcholine/1-palmitoyl-2- [^{14}C]oleoylphosphatidylcholine (50:50, mol %).
(see m.s.)

The enzyme was inhibited in a dose-dependent manner by nordihydroguaiaretic acid (fig.4A), an agent known to inhibit the release of arachidonic acid metabolites from macrophages [8] and by the flavonoid quercetin, but not by the structurally related flavonoid taxifolin (fig.4B). The latter result argues against a non-specific inhibitory effect of quercetin, such as interaction with the phospholipid substrate. *p*-Bromophenacylbromide, a well known but far from specific inhibitor of PLA_2, also inhibited the enzyme in a dose-dependent manner with half-maximal effect at 15 μM (not shown).

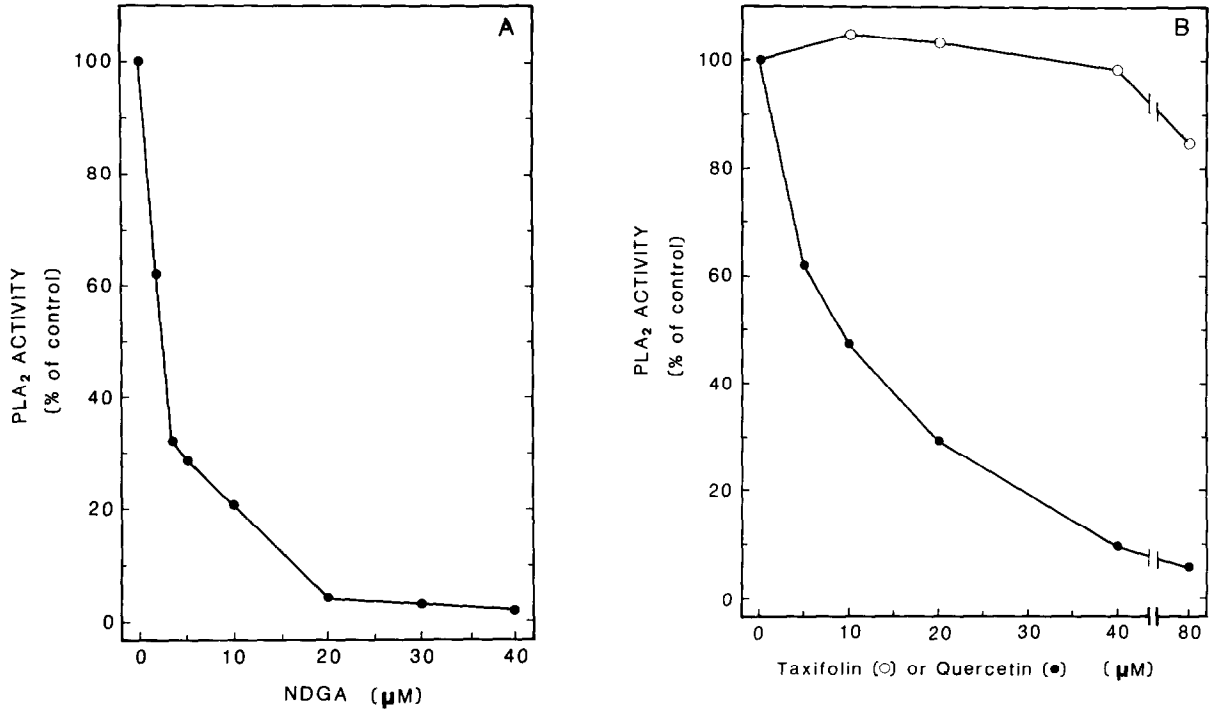

Fig.4. Effect of inbibitors on macrophage PLA_2. Enzyme purified by gel chromatography was assayed against 1-stearoyl-2-[^{3}H]-arachidonoylphosphatidylcholine in the presence of various concentrations of inhibitor. Results (from ≥2 separate experiments) are expressed as percent of control (without inhibitor). (a) Nordihydroguaiaretic acid (NDGA) and (b) quercetin and taxifolin.

4. DISCUSSION

It has been demonstrated earlier that treatment of macrophages with PMA or other agents that cause activation of protein kinase C [5] induces release of arachidonic acid from phosphatidylcholine, -ethanolamine and -inositol [3,4]. Here, we have identified a cytosolic PLA_2 that releases arachidonic acid from all three of these phospholipids and whose activity is increased after treatment of the cells with PMA. The enzyme has an apparent molecular mass of 70 kDa and is the only detectable enzyme activity in macrophage homogenate that releases arachidonic acid from phosphatidylcholine under our assay conditions. It is therefore likely that investigations on homogenate of the same cell type [9] or homogenate and soluble fraction of bone marrow-derived mouse macrophages [7] deal with the same 70 kDa PLA_2. This suggestion is supported in particular by the similarities in substrate specificity, with activity against both phosphatidylcholine and -ethanolamine [7,9] and the preference for arachidonoyl over oleoylphosphatidylcholine [7]. Preferential hydrolysis of arachidonoylphosphatidylcholine has also been clearly indicated from substrate competition experiments for the PLA_2 activity in the cytosol of human neutrophils [10]. Furthermore, an increase in PLA_2 activity in response to PMA treatment, as shown here, has been observed in bone marrow-derived macrophages by assay on homogenates [11] although treatment for at least 30 min was reported to be necessary.

The calcium dependence of the 70 kDa PLA_2, with half-maximal activation at approx. 0.7 μM free Ca^{2+}, indicates potential regulation by calcium-mobilizing ligands and is fully consistent with the previous finding [5] that calcium ionophores also cause liberation of arachidonic acid in intact macrophages after down-regulation of protein kinase C.

The inhibition of the enzyme by nordihydroguaiaretic acid and quercetin showed dose dependences that were remarkably similar to those for

the release of arachidonic acid in intact cells (IC_{50} values 2 and 10 μM, respectively in the latter system; Emilsson, A. and Sundler, R. unpublished), while taxifolin was ineffective in both systems. Nordihydroguaiaretic acid and quercetin have also been shown to inhibit the PLA_2 activity in acid extracts and sonicates of rabbit neutrophils, although with somewhat higher IC_{50} values [12].

In conclusion, the results indicate that the 70 kDa PLA_2 is likely to be responsible for stimulus-induced mobilization of arachidonic acid in mouse macrophages. Since the same, or a closely related, enzyme is also present in various solid organs, it is now being purified and further characterised from such sources.

Acknowledgement: This work was supported by grants from the Swedish Medical Research Council (nos 5410, 7830 and 8553), the Albert Påhlsson Foundation, the Alfred Österlund Foundation and the Medical Faculty, University of Lund. We thank Marianne Petersson for excellent technical assistance.

REFERENCES

[1] Brune, K., Glatt, M., Kälin, H. and Peskar, B.A. (1978) Nature 274, 261–263.
[2] Bonney, R.J., Wightman, P.D., Dahlgren, M.E., Davis, P., Kuehl, F.A. jr and Humes, J.L. (1980) Biochim. Biophys. Acta 633, 410–421.
[3] Emilsson, A. and Sundler, R. (1986) Biochim. Biophys. Acta 876, 533–542.
[4] Emilsson, A., Wijkander, J. and Sundler, R. (1986) Biochem. J. 239, 685–690.
[5] Wijkander, J. and Sundler, R. (1988) Biochim. Biophys. Acta, in press.
[6] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[7] Flesch, I., Schmidt, B. and Ferber, E. (1985) Z. Naturforsch. 40c, 356–363.
[8] Humes, J.L., Sadowski, S., Galavage, M., Goldenberg, M., Subers, F.A., Kuehl, Jr. and Bonney, R.J. (1983) Biochem. Pharmacol. 32, 2319–2322.
[9] Wightman, P.D., Humes, J.L., Davies, P. and Bonney, R.J. (1981) Biochem. J. 195, 427–433.
[10] Alonso, F., Henson, P.M. and Leslie, C.C. (1986) Biochim. Biophys. Acta 878, 273–280.
[11] Flesch, I. and Ferber, E. (1986) Biochim. Biophys. Acta 889, 6–14.
[12] Lanni, C. and Becker, E.L. (1985) Int. Arch. Allergy Appl. Immunol. 76, 214–217.