Mg^{2+} Activates 5-Lipoxygenase in Vitro: Dependency on Concentrations of Phosphatidylcholine and Arachidonic Acid†

Konda Veera Reddy, Tove Hammarberg, and Olof Rådmark*

Department of Medical Biochemistry and Biophysics, Division of Chemistry II, Karolinska Institute, S-17177, Stockholm, Sweden

Received August 17, 1999; Revised Manuscript Received November 29, 1999

ABSTRACT: Mg^{2+} gave dose-dependent activation of 5-lipoxygenase (5LO) in vitro. As for Ca^{2+}, the activation depended on the presence of phosphatidylcholine (PC) vesicles, and the activation response was different at various combinations of arachidonate and PC. Stimulation of 5LO activity was observed with Mg^{2+} concentrations of 0.1−1 mM, similar to the concentration range of free Mg^{2+} in mammalian cells. However, to observe a clear increase in 5LO hydrophobicity, a higher concentration of Mg^{2+} (4 mM) was required, and at this concentration also 5LO activation was optimal. Combinations of Mg^{2+} with ATP (containing free Mg^{2+} and $MgATP^{2-}$ complex) gave better activation of 5LO than either agent alone. This effect of Mg^{2+} (and ATP) could be of interest in relation to basal 5LO activity in cells not subjected to a particular stimulus.

5-Lipoxygenase (5LO)[1] is the key enzyme in leukotriene biosynthesis, responsible for the oxidation of arachidonic acid to 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE), and the subsequent conversion to 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid [leukotriene A_4 (LTA_4)]. LTA_4 is further converted to the chemotactic dihydroxy acid LTB_4, and to the peptidoleukotrienes (LTC_4, LTD_4, and LTE_4) which are potent mediators in inflammation and allergy reactions (*1*).

It is firmly established that the divalent cation calcium stimulates 5LO enzyme activity. First, calcium ionophore A23187 was shown to stimulate 5LO activity in human PMNL (*2*). Subsequently, the relevance of elevated intracellular calcium levels (evoked by ionophores and other stimuli) for activation of 5LO has been studied in more detail (*3*−*6*). Also, in cell-free systems and after purification, calcium stimulates 5LO activity (*7*, *14*) although it should be observed that 5LO has some activity in vitro also without addition of calcium (*8*−*10*). For purified 5LO, the concentration of calcium giving half-maximal activation was 1−2 μM while full activity was reached at 4−10 μM (*11*, *12*). We recently demonstrated that 5LO actually binds calcium in a reversible manner (K_d ca. 6 μM) with an apparent maximum calcium binding of two Ca^{2+} per 5LO (*13*). Calcium increases the hydrophobicity of 5LO in vitro (*12*, *13*), and 5LO activity is low unless a membrane fraction or phosphatidylcholine (PC) is included in in vitro assays (*9*, *14*, *15*). Among the phospholipids that have been tested (PC, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, diacylglycerol), only PC promoted 5LO activity, although 5LO could associate also with phosphatidylethanolamine and phosphatidylserine (*5*, *12*, *16*). Also, studies on 5LO activity in the presence of PC or the detergent Tween 20 indicated that 5LO performs its catalysis at a lipid/water interface (*15*, *17*). Furthermore, after homogenization of leukocytes in the presence of calcium, 5LO was recovered in the 100000*g* pellet (cellular membranes), but not when the homogenization was done with EDTA (*18*−*20*). When intact cells were stimulated to produce leukotrienes (by ionophore, *N*-formylmethionylleucinylphenylalanine, or by IgE−antigen complexes), membrane association of 5LO occurred as well (*6*, *19*, *21*, *22*). Thus, it is generally agreed that calcium activates 5LO by promoting membrane association.

ATP has been found to stimulate 5LO in the presence of calcium (*23*). In the presence of calcium, K_a values for ATP were 30−100 μM (*8*, *24*). For crude guinea pig neutrophil 5LO, there was an increase in the V_{max} and a reduction in the lag phase to less than 5 s (*8*). However, 5LO can be activated by ATP without simultaneous addition of Ca^{2+} (Ying-Yi Zhang, personal communication) (*10*), and when ATP was combined with 20 nM calcium (well below the concentration of calcium required to activate 5LO), there was also a stimulatory effect (*25*).

Mg^{2+} is the second most abundant intracellular cation. Of the total intracellular magnesium (10−30 mM), a large part forms chelates with various anionic ligands including ATP, and Mg^{2+} activates phosphate groups and reactions that involve ATP (for reviews, see refs *26*−*28*). The concentration range for free Mg^{2+} in mammalian cells is 0.1−1 mM at rest, and transient and limited changes in Mg^{2+} concentration may occur upon cell activation. Mg^{2+} is a critical cofactor for more than 300 enzymatic reactions, involving energy metabolism and protein and nucleic acid synthesis.

† This study was supported by grants from the Swedish Medical Research Council (03X-217), from the European Union (BMH4-CT96-0229), and from the Verum Foundation.

* Corresponding author. Telephone: +46 8 7287624. Telefax: +46 8 7360439. E-mail: Olof.Radmark@mbb.ki.se.

[1] Abbreviations: 5LO, 5-lipoxygenase (EC 1.13.11.34); 5-H(P)ETE, 5-hydro(pero)xyeicosatetraenoic acid; 13-HPODE, 13-hydroperoxyoctadecadienoic acid; PC, L-α-phosphatidylcholine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi9919246 CCC: $19.00
Published on Web 01/29/2000

Also, Mg^{2+} can compete with Ca^{2+} for binding sites on membranes and proteins, and modulates Ca^{2+} influx in pathophysiologic states.

In previous studies, it was first shown that Ca^{2+}, Mg^{2+}, and Mn^{2+} activated both 5LO and 12LO in RBL-1 cell 100000*g* supernatant (*29*). Subsequently, it was described that in addition to Ca^{2+}, also Ba^{2+}, Sr^{2+}, and Mn^{2+} (concentration ranges 25−200 μM) could activate purified recombinant 5LO. However, there was no effect of Mg^{2+} at concentrations up to 400 μM (*11*, *12*). In a fourth study, an inhibitory effect of Mg^{2+} (concentrations above 10 mM) on 5LO activity was described (*30*). In the present report, we clarify the effect of Mg^{2+} on 5LO in vitro. It is compared to activating effects of calcium and ATP, and the influence of concentrations of phosphatidylcholine and arachidonate on 5LO activation by Mg^{2+}, Ca^{2+}, and ATP is described.

MATERIALS AND METHODS

The following chemicals were from Sigma: ATP−agarose (A2767), isopropyl-β-D-thiogalactopyranoside (IPTG), ampicillin, L-α-phosphatidylcholine type XVI E from egg yolk (P3556), ADP, ATP, PMSF, dithiothreitol, soybean trypsin inhibitor, glutathione peroxidase (0.67 unit/μg), superoxide dismutase (4.2 units/μg). Arachidonic acid was from Nu-Check Prep., Elysian, MN. Magnesium salts were from Merck.

Expression of Recombinant Human 5LO. *E. coli* MV1190, transformed with expression vector pT3-5LO, was grown in modified M9CA medium containing 5 mM $FeSO_4$ (*31*) at 27 °C. IPTG (0.2 mM) was added after reaching OD 0.2 (620 nm), and the culture was continued overnight (OD 3−4). Cells were harvested at 5000*g* for 12 min, resuspended in homogenization buffer (50 mM TEA buffer, pH 8, 2 mM EDTA, 2 mM dithiothreitol, 1 mM PMSF, and 60 μg/mL soybean trypsin inhibitor) containing 0.5 mg/mL lysozyme, and kept on ice for about 30 min. After sonication, the sample was centrifuged at 10000*g* for 30 min. Fresh homogenization buffer was added to the pellet, which was resonicated and centrifuged again. The supernatants were pooled.

Purification of 5LO. To the *E. coli* supernatant pool was added 60% ammonium sulfate (1 h on ice), and after centrifugation, the pelleted protein was dispersed in buffer A (50 mM TEA/HCl, pH 7.35, 1 mM EDTA, 10 mM 2-mercaptoethanol). The sample was ultracentrifuged at 100000*g* for 1 h before purification on an ATP−agarose affinity column (8 mL) attached to a FPLC chromatography system, as described (*31*). The bound protein was eluted with 12 mM ATP in buffer B (50 mM TEA/HCl, pH 7.35, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 M NaCl), giving 90−95% pure 5LO protein. ATP was removed by gel filtration on a G-75 column (1.5 × 12 cm) eluted with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 14 mM 2-mercaptoethanol. Alternatively, for small sample volumes, a PD-10 column (Pharmacia) was used. The desalted sample was subsequently loaded onto a MonoQ HR 5/5 column. Buffer A was 50 mM Tris-HCl, pH 7.5, 14 mM 2-mercaptoethanol. Buffer B was 50 mM Tris-HCl, pH 7.5, 1 M NaCl, 14 mM 2-mercaptoethanol. The 5LO protein eluted at around 220 mM NaCl; it was apparently pure as judged by SDS−PAGE. For stabilization of 5LO (*32*), glutathione peroxidase (0.1 unit/mL) and superoxide dismutase (4.2 units/mL) were added immediately. The enzyme was stable (up to 10% loss of activity) at 0 °C for 2−3 days. In the later phase of our studies, the total time for purification from homogenization to the Mono Q column was shortened to 10 h, which gave an increase in 5LO specific activity, up to 80 μmol mg^{-1} (10 min)$^{-1}$.

Protein Estimation. Protein concentration was estimated according to Bradford (*33*) using reagents from BioRad, with bovine serum albumin as standard protein.

Preparation of Substrate Mix. Stock solutions of arachidonic acid and phosphatidylcholine were kept under argon, with ethanol as solvent. After evaporation of solvent, arachidonate and PC were redissolved separately in 75 mM Tris-HCl, pH 7.5, at 1250 μM and 1250 μg/mL, respectively. The required volumes were mixed and sonicated at 0 °C for 3 × 20 s, using an MSE 150 W sonicator at 40% power level. The obtained solution could be stored at 4 °C for a few days.

Assay of 5LO Enzyme Activity. Incubations (100 μL total volume) were performed in siliconized 0.65 mL microcentrifuge tubes at room temperature. The incubation buffer contained 75 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 μM 13-HPODE, 10 mM 2-mercaptoethanol, and additional factors (arachidonate, phosphatidylcholine, Ca^{2+}, Mg^{2+}, ATP) as described in the figure legends. 5LO (typically 100 ng) was added, and the mixture was preincubated for 1 min. Concentrations of free divalent cations and of various complexes ($MgEDTA^{2-}$, $MgCl^{1+}$, $MgATP^{2-}$, Mg_2ATP, $CaEDTA^{2-}$, $CaATP^{2-}$, Ca_2ATP) were determined according to Robertson and Potter (*34*) and using the program BAD (*35*). The results of these calculations are given in the figures.

The reaction was initiated by adding 40 μL of substrate mix containing arachidonate and phosphatidylcholine in 75 mM Tris-HCl, pH 7.5. After 10 min at room temperature, the incubation was terminated by addition of 300 μL of ice-cold stop solution [80% acetonitrile, 20% H_2O, 0.2% acetic acid (v/v)] containing 1 nmol of the internal standard 17(*S*)-hydroxy-13,19(*Z*)-15(*E*)-docosatrienoic acid. After mixing and centrifugation (10 min), 100 μL of the sample supernatant was subjected to HPLC analysis. For HPLC, a C_{18} column (Waters Nova Radial Pak) was eluted with acetonitrile/water/acetic acid (80/20/0.2, v/v), and the eluate was monitored at 234 nm. The enzyme activity was calculated from the sum of 5(S)-HPETE and 5(S)-HETE.

Spectrophotometric Assay of 5-Lipoxygenase. Incubation was performed in a quartz cuvette at room temperature, and formation of lipoxygenase products containing a conjugated diene was monitored at 235 nm. The incubation buffer was the same as for the HPLC assay. All components (including substrate mix) were added to both sample and reference cuvettes (volume 1 mL). The reaction was started by addition of 5LO (1.2 μg) only to the sample cuvette.

Determination of Hydrophobicity of 5LO. Bordier (*36*) described the nonionic detergent Triton X-114 which remains in solution at low temperature, but forms micelles and undergoes phase separation at 30 °C. The micelles can be separated by centrifugation along with bound hydrophobic proteins.

A solution of MonoQ-purified 5LO protein (150−200 μg/mL 5LO in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 μM 2-mercaptoehanol) was prepared. Ovalbumin was added to the same concentration, and also 1% (w/v) Triton X-114.

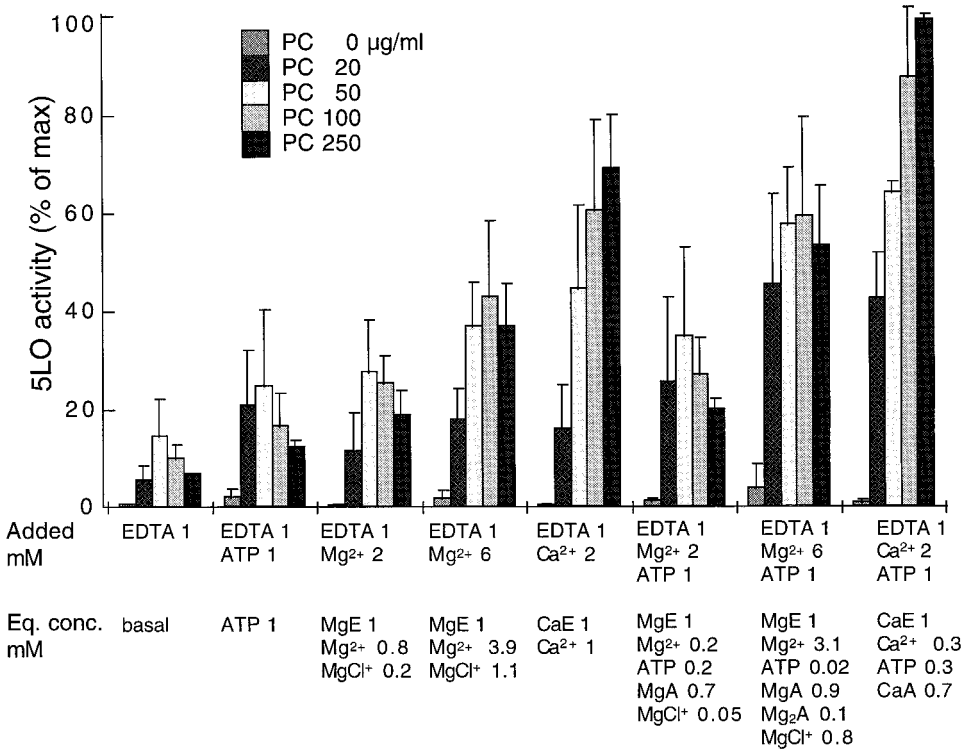


FIGURE 1: 5LO activities with different stimuli, at different concentrations of PC. The concentration of arachidonate was 100 μM. Phosphatidylcholine, but not arachidonate, was sonicated. Enzyme activity was determined by HPLC assay after 10 min incubations, as described under Materials and Methods. Data are from three incubation series; for each series, activities were determined in triplicate. Results are given as mean ± SD. The added concentrations of EDTA, ATP, Ca^{2+}, and Mg^{2+} are given, as well as the calculated equilibrium concentrations (compare references *34* and *35*) of divalent cations and various complexes. Abbreviations: MgE, $MgEDTA^{2-}$; MgA, $MgATP^{2-}$; CaE, $CaEDTA^{2-}$; MgA, $MgATP^{2-}$; Mg_2A, Mg_2ATP.

The clear sample aliquot (200 μL) was added onto 300 μL of sucrose cushion buffer containing 6% (w/v) sucrose, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.06% Triton X-114, on the bottom of a 0.65 mL siliconized Eppendorf tube. Tubes were incubated at 30 °C for 5 min for phase separation, and centrifuged immediately at 1200 rpm for 3 min in a preheated swingout bucket rotor. The tube was carefully removed from the bucket, and the upper phase was transferred to a separate tube. Then, the sucrose phase was decanted carefully without disturbing the barely visible micelle droplet at the bottom of the tube. The droplet (less than 15 μL) containing hydrophobic 5LO was diluted, to get a sample of 50 μL containing 1× SDS−PAGE sample loading buffer. The upper phase was cooled to 0 °C, and Triton X-114 was added again (to 1%). After incubation at 30 °C for 5 min, the tube was centrifuged to eliminate remaining hydrophobic protein from the sample. The supernatant containing hydrophilic protein was diluted with an equal volume of 2× SDS−PAGE sample loading buffer. The presence of 5LO and ovalbumin in the upper aqueous (hydrophilic) and micelle (hydrophobic) phases was analyzed by SDS−PAGE using a Pharmacia Phast system.

RESULTS

Mg^{2+} Activates 5LO: Comparison to Effects of Ca^{2+} and ATP. Effects of different 5LO activating agents are compared in Figure 1. For these enzyme activity determinations, purified recombinant human 5LO was incubated at room temperature for 10 min. The concentration of arachidonic acid was 100 μM, and phosphatidylcholine (PC, 0−250 μg/mL) was added as vesicles (obtained by sonication). Products [5-H(P)ETE] were analyzed by HPLC. The data in Figure 1 were compiled from two series (A and B) of incubations with one enzyme batch, and a third incubation series (C) with another enzyme batch. Since there is always some variation in 5LO activities between differerent incubation series, the results are given as percentages of the maximum activity, in each incubation series. The maximum activities (always with Ca^{2+} and ATP) were as follows: for A at [PC] 250 μg/mL, 82 μmol mg^{-1} $(10\ min)^{-1}$; for B at PC 250 μg/mL, 57 μmol mg^{-1} $(10\ min)^{-1}$; for C at PC 100 μg/mL, 67 μmol mg^{-1} $(10\ min)^{-1}$.

As shown, Mg^{2+} (0.8 or 3.9 mM) stimulates 5LO considerably, giving about half the activity obtained with a saturating dose of calcium (1 mM). According to the BAD program (*35*), a significant part of the added Mg^{2+} is present as the complex ion $MgCl^+$, which probably does not contribute to the activating effect. As with Ca^{2+}, the activating effect of Mg^{2+} depended on the presence of PC; in the absence of PC, there was no or very little 5LO activity. To acertain that the activation by Mg^{2+} was not due to Ca^{2+} contamination, three different Mg^{2+} salts [$MgCl_2$ or $Mg(OAc)_2$] were used for the three incubation series. For one of these, the low Ca^{2+} content was confirmed by atomic absorption spectroscopy (compare below, Mg^{2+} dose−response).

The data in Figure 1 also confirm some previous observations. First, 5LO has a basal activity without addition of divalent cations or ATP (*8*−*10*). Also this basal activity depends on the presence of PC. Second, 5LO can be activated by ATP without simultaneous addition of Ca^{2+} (*10*) (Ying-Yi Zhang, personal communication). Under our experimental

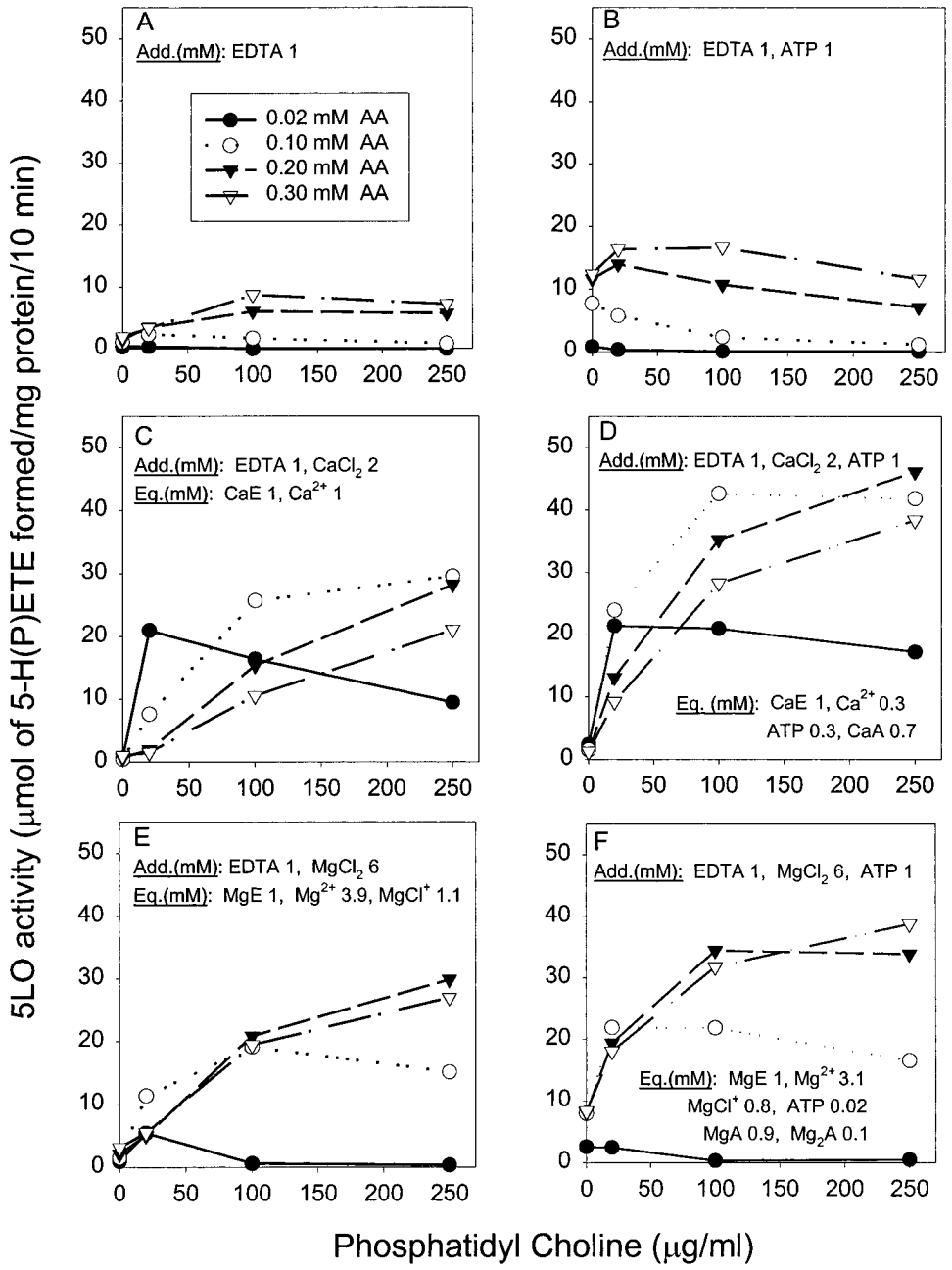


FIGURE 2: 5LO activities at 16 different combinations of arachidonate (20, 100, 200, and 300 μM) and PC (0, 20, 100, and 250 μg/mL). Activity was determined with different stimuli, as indicated. Phosphatidylcholine and arachidonate were sonicated together. Enzyme activity was determined by HPLC assay after 10 min incubations, as described under Materials and Methods. Data are from single incubations. The added concentrations of EDTA, ATP, Ca^{2+}, and Mg^{2+} are given, as well as the calculated equilibrium concentrations of divalent cations and various complexes. Abbreviations: MgE, $MgEDTA^{2-}$; MgA, $MgATP^{2-}$; CaE, $CaEDTA^{2-}$; MgA, $MgATP^{2-}$; Mg_2A, Mg_2ATP.

conditions, also the ATP-induced 5LO activity was considerably augmented by PC. At a low concentration of PC (20 μg/mL), ATP alone (1 mM) was actually the most efficient stimulus, slightly better than the divalent cations. When ATP was added together with Mg^{2+}, there was an increased activation of 5LO, as found previously for ATP together with calcium. In these incubations, most of the ATP was present in complex with Mg^{2+} or Ca^{2+}. Particularly at the high Mg^{2+} concentration, the concentration of free ATP was quite low (20 μM), indicating that the $MgATP^{2-}$ complex (approximately 1 mM) can replace ATP regarding activation of 5LO.

PC vesicles in vitro mimic the in vivo membrane interfaces, at which 5LO catalysis most probably occurs (*15*, *17*). As shown in Figure 1, the optimum PC concentration for the in vitro incubations was not the same with all stimuli (5LO activated at 100 μM arachidonic acid). At basal conditions, or when stimulated with ATP or Mg^{2+}, PC at 50−100 μg/mL was best. However, for 5LO stimulated with calcium, high PC (250 μg/mL) gave the highest 5LO activity. When ATP was added together with Mg^{2+} or Ca^{2+}, optimum PC concentrations were the same as with the respective divalent cation alone. The reason for the variation in the PC optimum is unclear. It has been claimed that the ratio of arachidonate to PC is important for 5LO activity (*15*). This was further studied in the next experiment.

Effect of Arachidonate and Phosphatidylcholine on 5LO Activity with Various Stimuli. In Figure 2, 5LO activities at

16 different combinations of fatty acid substrate and PC are shown. Additions of EDTA, ATP, Ca^{2+}, Mg^{2+}, as well as equilibrium concentrations of free cations and various complexes are given. For these experiments, arachidonate and phosphatidylcholine were sonicated together to give mixed vesicles. The data in Figure 2 are from one experiment; however, the same pattern of activities was observed in several other experiments with other enzyme batches (data not shown). Please observe that the given concentrations of arachidonate and PC would be present if arachidonate and PC were truly dissolved in the incubation mixture. Instead, vesicles are formed, and the indicated concentrations reflect the composition of these vesicles.

At basal conditions (no divalent cation or ATP added), activity was most prominent when arachidonate was 200−300 μM, combined with high PC content (100 or 250 μg/mL) (see Figure 2A). There was no substrate inhibition at high concentrations of arachidonate. These results are in accordance with a recent paper by Skorey (*10*), in the sense that Ca^{2+} is not required for 5LO activity at high concentrations of PC. However, in contrast to ref *10*, in our hands Ca^{2+} (or Mg^{2+}) led to considerable activation of 5LO also at high concentrations of arachidonate and PC.

With ATP (1 mM) as stimulus, activity was most prominent at 300 μM arachidonate combined with 20 or 100 μg/mL PC (Figure 2B). As under basal conditions, there was no substrate inhibition. One difference between the results of this experiment (Figure 2) and the data in Figure 1 is that here ATP gave considerable 5LO activity also in the absence of PC (at arachidonate concentrations of 100, 200, and 300 μM). A presumable reason for this discrepancy is that in this experiment the arachidonate was included in the sonication, leading to vesicles which favor 5LO catalysis. For the Figure 1 experiments, only the PC (not the arachidonic acid) was sonicated.

When 5LO was stimulated with Ca^{2+} at a saturating concentration (1 mM), activity was most prominent at 100 μM arachidonate combined with 100 or 250 μg/mL PC (Figure 2C). Also, particularly for calcium-stimulated 5LO, there was considerable activity with only 20 μM arachidonate in combination with low PC (20 μg/mL). This may indicate that calcium is the most efficient of the tested stimuli, regarding promotion of 5LO association to vesicles. Also, it has previously been shown that calcium reduces the K_m of 5LO toward arachidonic acid (*8*). Another finding for calcium-stimulated 5LO was that at higher concentrations of arachidonate, substrate inhibition was apparent. When Ca^{2+} was combined with ATP (Figure 2D), the activities were generally increased. However, at zero PC, activities were still low, lower than with ATP alone. Note, however, that in Figure 2D most of the ATP is in complex with Ca^{2+}. Apparently, ATP in the presence of Ca^{2+} did not have the same activating effect at zero PC as ATP alone. When Ca^{2+} was combined with ATP, substrate inhibition was present, as with Ca^{2+} alone.

With 6 mM Mg^{2+} total concentration added (leading to 3.9 mM free Mg^{2+}) as stimulus, activity was most prominent at 200 μM arachidonate, combined with 250 μg/mL PC (Figure 2E). Substrate inhibition was not as apparent as with Ca^{2+} as stimulus. At low concentration of arachidonate (20 μM) combined with low concentration of PC (20 μg/mL), the 5LO activity was about 25% of that obtained with Ca^{2+}.

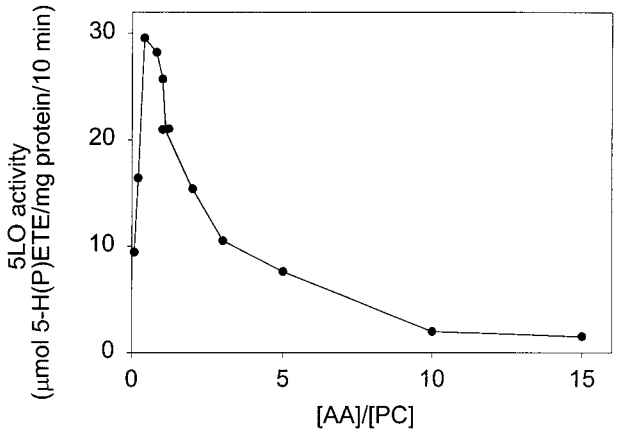
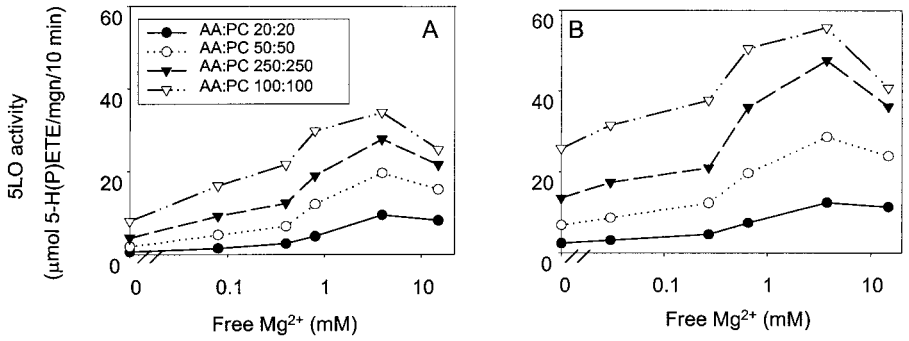


FIGURE 3: 5LO activity as function of arachidonate/PC molar ratio for 5LO stimulated with 1 mM Ca^{2+} (data from Figure 2C).

When Mg^{2+} was combined with ATP (giving mostly free Mg^{2+} and $MgATP^{2-}$ complex), principally the same pattern as with Mg^{2+} alone was obtained, but activities were generally increased (Figure 2F).

When the above activity data were plotted in relation to the molar ratio [arachidonate]/[PC], a correlation could be found only for calcium-stimulated 5LO; the highest activities were obtained at ratios of 0.4−1.2 (Figure 3). For this calculation, the molecular mass of PC was estimated as 1000 Da. However, under basal conditions or with ATP as stimulus, no correlation was found. Instead, activity increased with increasing concentrations of arachidonate. With Mg^{2+} as stimulus, for each concentration of PC the optimum activity was found at a particular concentration of arachidonate, but there was no general correlation between activity and the ratio of arachidonate to PC (as found for Ca^{2+}). Furthermore, depending on the choice of combination of arachidonate and PC, any of the three tested stimulating agents (Ca^{2+}, Mg^{2+}, ATP) can be found to be the best. Thus, ATP was the most efficient 5LO stimulus at high concentrations of arachidonate in combination with low PC (0 or 20 μg/mL). Mg^{2+} was most efficient at high concentrations of arachidonate in combination with high PC. Finally, Ca^{2+} was most efficient at 100 μM arachidonate in combination with high PC, and particularly at 20 μM arachidonate combined with 20 μg/mL PC.

Dose−Response of Mg^{2+} Activation. Dose−response curves for Mg^{2+} at different combinations of arachidonate and PC are shown in Figure 4. For these experiments, the incubation buffer was slightly modified (75 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10 μM 13-HPODE, 14 mM 2-mercaptoethanol). Additions of EDTA, ATP, and Mg^{2+} as well as equilibrium concentrations of free Mg^{2+} and various complexes are given in tabular form. On the abscissas, the concentrations of free Mg^{2+} are indicated. When ATP (0.2 mM) was present in the incubations (Figure 4B), the pattern of activities resembled the results without ATP (Figure 4A), except that activities were generally higher. Activities were most prominent at added Mg^{2+} concentrations of 5.1 mM, leading to free Mg^{2+} concentrations close to 4 mM. However, at the higher concentrations of arachidonate and PC, free Mg^{2+} concentrations of 0.8 mM (Figure 4A) and 0.65 mM (Figure 4B) were sufficient for considerable activation. At the highest concentration of Mg^{2+}, activities decreased. In the presence of ATP (Figure 4B), formation



Added (mM):												
Mg^{2+}	-	0.20	0.60	1.10	5.10	20.10	-	0.20	0.60	1.10	5.10	20.10
EDTA	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
ATP	-	-	-	-	-	-	0.20	0.20	0.20	0.20	0.20	0.20
Eq.conc.(mM):												
Mg^{2+}	0	0.08	0.40	0.80	3.9	15.0	0	0.03	0.27	0.65	3.76	14.8
$MgCl^{+}$	0	0.02	0.10	0.20	1.1	5.0	0	0.01	0.07	0.16	1.0	4.9
ATP	0	0	0	0	0	0	0.20	0.13	0.04	0.01	0.002	0.001
$MgATP^{2-}$	0	0	0	0	0	0	0	0.07	0.16	0.18	0.17	0.12
Mg_2ATP	0	0	0	0	0	0	0	0	0	0.01	0.03	0.07
$MgEDTA^{2-}$	0	0.10	0.10	0.10	0.10	0.10	0	0.10	0.10	0.10	0.10	0.10

FIGURE 4: Dose-dependent activation of 5LO by $MgCl_2$, at different concentrations of arachidonate (20, 50, 100, and 200 μM) and PC (20, 50, 100, and 200 μg/mL). Phosphatidylcholine and arachidonate were sonicated together. Enzyme activity was determined by HPLC assay after 10 min incubations, as described under Materials and Methods. Data are from single incubations. Without other activating agents (A), addition also of 200 μM ATP (B). The added concentrations of Mg^{2+}, EDTA, and ATP are given, as well as the calculated equilibrium concentrations of free Mg^{2+} and various complexes. On the abscissas, the concentrations of free Mg^{2+} are indicated.

of the $MgATP^{2-}$ complex became more prominent at the higher Mg^{2+} concentrations (addition of 1.1 mM or more); in parallel, the concentration of free ATP became quite low (10 μM or less). These low concentrations of free ATP could be compared to published K_a values for ATP (determined in the presence of calcium) which were 30−100 μM (*8*, *24*). Nevertheless, 5LO activities were higher than without addition of ATP. Again, this indicates that the $MgATP^{2-}$ complex has a similar effect as free ATP, regarding activation of 5LO.

When Ca^{2+} (0.2 mM), or Ca^{2+} and ATP were present in the incubation series (data not shown), Mg^{2+} had no appreciable effect, except for the slight inhibition at the highest Mg^{2+} concentration. In these incubations, a variety of complexes were formed, but the concentration of free Ca^{2+} was 60−100 μM. This is sufficient to activate 5LO, and addition also of Mg^{2+} did not augment the activity further.

According to the manufacturers' specifications, the $MgCl_2$ used for the experiments in Figure 4 contained 0.003% Ca^{2+}, leading to [Ca^{2+}] of 0.15 μM at an addition of 5 mM Mg^{2+}. Also, the concentrations of other divalent cations which can activate 5LO (Sr^{2+}, Ba^{2+}, and Mn^{2+}) were below 0.0005%, leading to at most 25 nM at 5 mM Mg^{2+}. This $MgCl_2$ solution was also checked by atomic absorption spectroscopy, confirming a Ca^{2+} content below the specified value. Since 1 μM Ca^{2+} is required for significant 5LO activation (*11*, *12*), we conclude that the activation obtained with Mg^{2+} was not due to Ca^{2+} contamination.

Effect of Mg^{2+} on 5LO Initial Velocity. The data described above were obtained by HPLC assay of 5-H(P)ETE formation in 10 min incubations. Continuous monitoring of 5-H(P)-ETE formation (absorbance at 234 nm) during incubations was also performed. Different concentrations of arachidonate (25, 50, 75, 100, 150, and 200 μM) were tested in combination with 100 μg/mL PC. Only the PC (not arachidonate) was sonicated. In Figure 5A, six examples of time courses with Ca^{2+} or Mg^{2+} as stimuli, at arachidonate concentrations of 50 and 100 μM, are shown. As described previously, time curves can contain an initial lag-phase, and self-inactivation occurs (*8*, *10*, *11*). The plateaus of the time courses thus reflect enzyme inactivation and not depletion of substrate. For example, for the curve corresponding to 1 mM Ca^{2+} and 50 μM arachidonate, 50 nmol of substrate was added, and 12 nmol of product 5-H(P)ETE (extinction coefficient 25 000) was formed.

A plot of initial velocities versus substrate concentration (Figure 5B) shows that addition of Ca^{2+} (1 mM free Ca^{2+}) and Mg^{2+} (3.9 mM free Mg^{2+}) can give similar v_{init}, but that for the calcium-stimulated enzyme this occurs at a lower concentration of arachidonate. Apparently, Mg^{2+} cannot lower the K_m of 5LO for arachidonate, as previously found for Ca^{2+} (*8*). Also, total product formation was most prominent at 50 μM arachidonate for 5LO stimulated with Ca^{2+}, while 100 μM arachidonate was best for Mg^{2+}-stimulated 5LO. Lag-phases were more evident and catalysis tended to persist for longer times for Mg^{2+}-stimulated 5LO (Figure 5A,B). Notably, Mg^{2+} at the lower concentration (0.8 mM free Mg^{2+}) did not give significant 5LO activity, when the concentration of arachidonate was low (25 or 50 μM). Substrate inhibition was apparent for calcium-stimulated 5LO, seen also with Mg^{2+} (3.9 mM), but absent with Mg^{2+} (0.8 mM).

At 20 μg/mL PC, a similar pattern of initial velocities versus concentration of arachidonate was found (data not shown). Maximum initial velocities were not as high as with 100 μg/mL PC, but they were obtained at lower concentrations of arachidonic acid.

Dose−Response Effect of ATP on Mg^{2+} Activation. Mg^{2+} is known to form chelates with anionic ligands including ATP. Since both Mg^{2+} and ATP could activate 5LO, we examined the response to Mg^{2+} together with different concentrations of ATP. For these experiments, the incubation

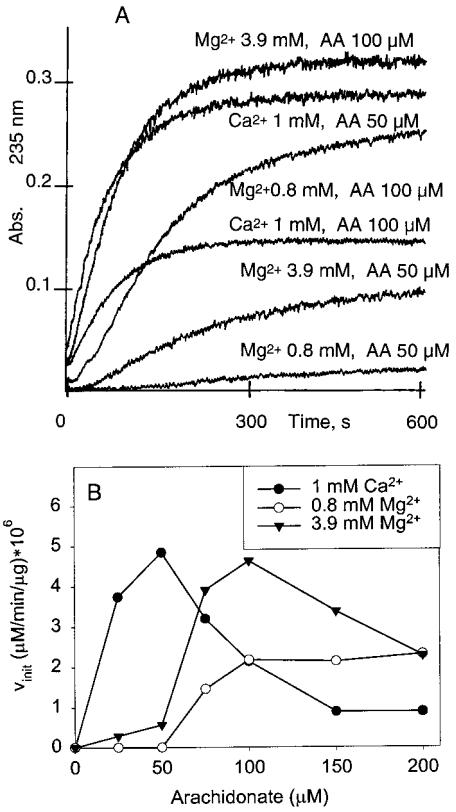
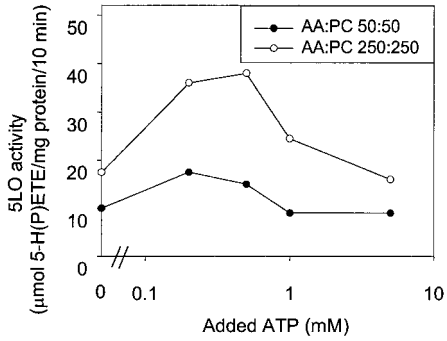


FIGURE 5: (A) Time courses of 5LO catalysis were monitored at 235 nm, as described under Materials and Methods. Ca^{2+} or Mg^{2+} were used as stimuli, and results obtained at arachidonate concentrations of 50 and 100 μM are shown. The indicated cation concentrations are free Ca^{2+} or Mg^{2+}. To obtain 3.9 mM free Mg^{2+}, the actual additions were: Mg^{2+} 6 mM, EDTA 1 mM. To obtain 0.8 mM free Mg^{2+}, the actual additions were: Mg^{2+} 2 mM, EDTA 1 mM. To obtain 1 mM free Ca^{2+}, the actual additions were: Ca^{2+} 2 mM, EDTA 1 mM. The concentrations of PC and 5LO were 100 and 1.2 μg/mL, respectively. (B) Effect of arachidonate concentration on initial velocity of 5LO catalysis. Ca^{2+} (1 mM) or Mg^{2+} (0.8 or 3.9 mM) were present as stimuli; the concentrations of PC and 5LO were 100 and 1.2 μg/mL, respectively.



Added (mM):					
ATP	-	0.20	0.50	1.00	5.00
EDTA	0.10	0.10	0.10	0.10	0.10
Mg^{2+}	1.10	1.10	1.10	1.10	1.10
Eq. conc. (mM):					
ATP	0	0.01	0.06	0.23	3.25
$MgATP^{2-}$	0	0.18	0.43	0.74	0.97
Mg_2ATP	0	0.01	0.01	0.01	0
$NaATP^{3-}$	0	0	0	0.01	0.78
$MgEDTA^{2-}$	0.10	0.10	0.10	0.10	0.10
Mg^{2+}:	0.80	0.65	0.44	0.19	0.02
$MgCl^{+}$	0.20	0.16	0.11	0.05	0.005

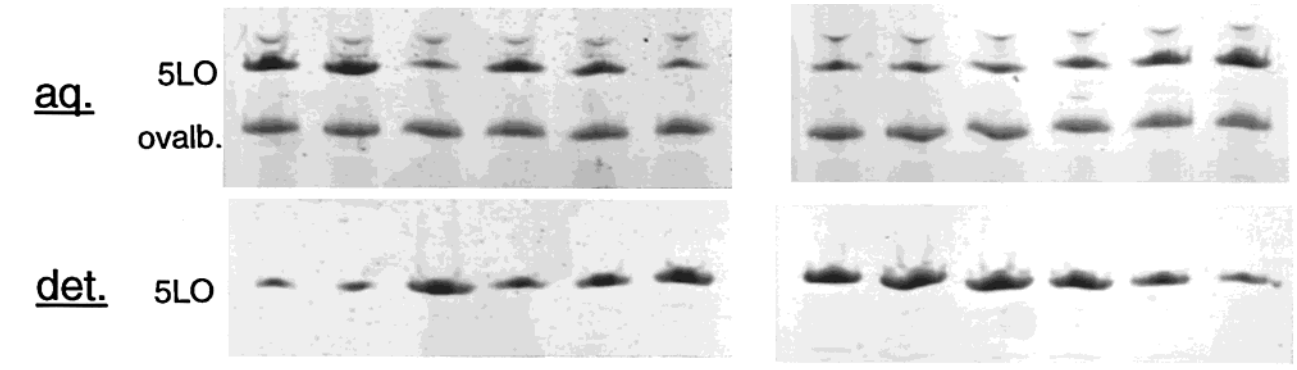
FIGURE 6: Effect of different additions of ATP on 5LO activity, in the presence of 1.1 mM added Mg^{2+}. Concentrations of arachidonate and PC (both sonicated) were 250 μM and 250 μg/mL (open circles) or 50 μM and 50 μg/mL (black circles). Enzyme activity was determined by HPLC assay after 10 min incubations, as described under Materials and Methods. Given data are averages of two enzyme activity determinations. The added concentrations of ATP, EDTA, and Mg^{2+} are given, as well as the calculated equilibrium concentrations of free Mg^{2+} and various complexes. On the abscissa, the concentrations of total added ATP are indicated.

buffer was slightly modified (75 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 14 mM 2-mercaptoethanol). The data in Figure 6 show that addition of ATP at 0.2 or 0.5 mM, below the added concentration of Mg^{2+} (1.1 mM), was most beneficial. In such combinations, free [Mg^{2+}] was 0.65 or 0.44 mM, which is sufficient for an activating effect (compare Figure 4A and Figure 4B). Inspection of the equilibrium concentrations of ATP and $MgATP^{2-}$ (see table in Figure 6) again indicates that $MgATP^{2-}$ can replace ATP regarding activation of 5LO. However, the decreased 5LO activities at ATP additions of 1 and 5 mM (leading to low free Mg^{2+}) indicate that $MgATP^{2-}$ cannot replace free Mg^{2+} regarding activation of 5LO. When also Ca^{2+} (100 μM) was included in the incubations, increasing concentrations of ATP gave increased activity, without a decrease in 5LO activity at the highest ATP additions (data not shown). Also, other experiments showed that when Mg^{2+} (5 mM added) was used in combination with ATP (1 or 10 mM added), Mg^{2+} in excess of ATP gave higher 5LO activity.

Effect of Mg^{2+} on Hydrophobicity of 5LO. Ca^{2+} increases the hydrophobicity of 5LO in vitro, and promotion of membrane association is believed to be central in Ca^{2+} activation of 5LO. We investigated if Mg^{2+} has the same effect on 5LO. Figure 7 shows the results from a Triton X-114 partitioning assay. As shown previously, Ca^{2+} (200 μM) renders 5LO more hydrophobic, thus appearing in the detergent phase. Also Mg^{2+} increased the hydrophobicity of 5LO. There was slight increase in the presence of 0.8 mM free Mg^{2+}; however, about 4 mM free Mg^{2+} was required to obtain a result comparable to 200 μM Ca^{2+}. ATP (1 mM) had no effect on its own, and did not affect hydrophobicity induced by Ca^{2+}. Also, the effect of Mg^{2+} was not affected by a low concentration of ATP; however, when the addition of ATP exceeded that of Mg^{2+}, 5LO hydrophobicity was not increased. As for stimulation of 5LO activity (Figure 6), it appears that the $MgATP^{2-}$ complex cannot replace free Mg^{2+} regarding the effect on 5LO.

DISCUSSION

In this report we describe that Mg^{2+}, at concentrations which can exist in mammalian cells (0.1−1 mM), leads to dose-dependent activation of 5LO in vitro. As for activation of 5LO by Ca^{2+}, the presence of phospatidylcholine vesicles was crucial, and the resulting 5LO activity (as well as the degree of activation) was different at various combinations of arachidonate and PC (Figures 1, 2, and 4). The 5LO activity obtained with Mg^{2+} could be as high as with Ca^{2+}, provided that beneficial concentrations of arachidonate and PC were chosen for the enzyme assay. However, many aspects of Mg^{2+} activation were different as compared to the effect of Ca^{2+}. First, activation of 5LO in vitro by Ca^{2+} is apparent already at 1 μM Ca^{2+}, and maximal at 10 μM (*11*, *12*). Free Mg^{2+} concentrations of 0.4−0.8 mM gave clear activation, and the maximum response was achieved at approximately 4 mM (Figures 4 and 5). Also, a high



Added (mM)												
ATP	-	1	-	-	-	-	1	1	-	-	2	5
Ca^{2+}	-	-	0.2	-	-	-	0.2	-	0.2	0.2	-	-
Mg^{2+}	-	-	-	1	2	5	-	5	1	5	5	2
Eq. conc. (mM)												
ATP	0	1	0	0	0	0	0.53	0.03	0	0	0.07	1.63
Ca^{2+}	0	0	0.2	0	0	0	0.05	0	0.2	0.2	0	0
Mg^{2+}	0	0	0	0.76	1.50	3.73	0	2.95	0.76	3.73	2.23	0.11
$MgCl^{+}$	0	0	0	0.24	0.49	1.27	0	1.00	0.24	1.27	0.75	0.03
$CaATP^{2-}$	0	0	0	0	0	0	0.15	0	0	0	0	0
$MgATP^{2-}$	0	0	0	0	0	0	0	0.85	0	0	1.72	1.84
Mg_2ATP	0	0	0	0	0	0	0	0.10	0	0	0.15	0.01
$NaATP^{3-}$	0	0	0	0	0	0	0.32	0.02	0	0	0.05	1.32

FIGURE 7: Effect of Mg^{2+} on 5LO hydrophobicity. A mixture of 5LO and ovalbumin (150 μg/mL each) was subjected to Triton X-114 partitioning, as described under Materials and Methods. The figure shows the Coomassie-stained protein bands after SDS−PAGE of aqueous (upper) and detergent (lower) phases. The added concentrations of ATP, Ca^{2+}, and Mg^{2+} are given, as well as the calculated equilibrium concentrations of divalent cations and various complexes.

concentration of free Mg^{2+} (4 mM) was required in order to increase the hydrophobicity of 5LO to the same extent as with Ca^{2+} (Figure 7). Thus, it appears that the affinity of 5LO for Mg^{2+} is much lower than for Ca^{2+}. This is in accordance with the observation that Mg^{2+} (2 mM) did not reduce the binding of Ca^{2+} (2−100 μM) in equilibrium dialysis (*13*). Possibly, Mg^{2+} and Ca^{2+} bind to the same site on 5LO, and the low affinity for Mg^{2+} could be related to the radius for hydrated Mg^{2+}. The hydration number is larger for Mg^{2+} (36) than for Ca^{2+} (29) (*37*).

Second, it was previously shown that Ca^{2+} leads to a decreased K_m for arachidonate, and also that in the presence of Ca^{2+}, high concentrations of arachidonate lead to substrate inhibition (*8*). We confirmed those observations for Ca^{2+}, and found that Mg^{2+} did not give the same effects. For example, while a saturating dose of Ca^{2+} (1 mM) gave more product with 100 μM arachidonate than with 200 μM arachidonate, the opposite was true for 5LO stimulated with Mg^{2+} (Figure 2). Also, for Ca^{2+} the maximal initial velocity was obtained at 50 μM arachidonate, and there was a clearly reduced v_{init} at higher substrate concentrations. With Mg^{2+}, the maximal v_{init} was obtained at 100 μM arachidonate, and substrate inhibition was less pronounced (Figure 5B). It has been published that for calcium-activated 5LO, there was a correlation between the arachidonate/PC ratio and activity (*15*). We confirmed this observation (Figure 3), but no such correlation was found for 5LO stimulated with Mg^{2+}. Also for 5LO basal activity, or for the activity obtained after stimulation with ATP, such a correlation was absent. The presence of this correlation only for Ca^{2+}-activated 5LO could depend on the lowered K_m for arachidonate due to Ca^{2+}, and/or it could reflect a high efficiency of Ca^{2+} regarding promotion of 5LO membrane association.

We confirmed the finding (*10*) (Ying-Yi Zhang, personal communication) that ATP alone (without addition of divalent cations) can activate 5LO. In our hands, ATP (1 mM) was most efficient at zero or low (20 μg/mL) PC combined with high concentrations of arachidonate (Figure 2). When ATP was combined with Ca^{2+} or Mg^{2+}, activation was increased (Figures 1, 2, and 4). Since Mg^{2+} and ATP form a complex, the effects of different combinations were tested. The results (Figures 4 and 6) showed that combinations in which the concentration of Mg^{2+} exceeded that of ATP gave the best activation of 5LO enzyme activity, indicating that a fraction of free Mg^{2+} is required, and that the $MgATP^{2-}$ complex can replace ATP (but not Mg^{2+}) regarding activation of 5LO.

In a previous study, there was no effect of Mg^{2+} on 5LO activity, at concentrations up to 400 μM (*11*). However, low concentrations of arachidonate (20 μM) and PC (12.5 μg/mL) were used. Under such conditions, 5LO activation by Mg^{2+} was minute also in our hands (Figures 2 and 4). An important consideration for the possible relevance of Mg^{2+} and ATP for 5LO activity in vivo is the judgement of the concentrations of arachidonate and PC in in vitro 5LO enzyme assays, as compared to the situation in the living cell. For example, at 100 μM arachidonate together with 100 μg/mL PC, the combination of Mg^{2+} (0.5 mM) and ATP (0.2 mM) led to considerable 5LO activity, and addition also of Ca^{2+} (0.2 mM) gave only 1.5-fold activation (data not shown). On the other hand, at 20 μM arachidonate together with 20 μg/mL PC, the activity with Mg^{2+} (0.5 mM) and ATP (0.2 mM) was small, and the addition of Ca^{2+} (0.2 mM) gave a 3.3-fold activation. It is not straightforward to judge which combination of arachidonate and PC might reflect a physiological situation. However, it is obvious that Mg^{2+}

and/or ATP cannot match Ca^{2+} when it comes to activation of 5LO at low concentrations of arachidonate and PC.

Nevertheless, Mg^{2+} and ATP are present in mammalian cells, and it can be speculated that 5LO could be active in the absence of a stimulus, leading to increased intracellular calcium concentration. Activation of 5LO in the cell is complex, involving translocation of 5LO to the nuclear membrane, and it appears possible that Ca^{2+} could have several functions in this process. One well-defined function for Ca^{2+} is to bind to 5LO, thus promoting enzyme activity, and this can be observed also in 5LO enzyme assays in vitro. Our data show that Mg^{2+}, although less efficient, can substitute for Ca^{2+} regarding such 5LO activation in vitro. Putative additional Ca^{2+} effects (related to the translocation process?) could possibly be obtained also by other signal transduction pathways. Thus, it has been published that 5LO in PAF-stimulated PMNL can remain active after block of Ca^{2+} influx, and that in the presence of exogenous arachidonate 5LO activity was only partially dependent on extracellular calcium (*4*). Also, it was suggested that when leukotriene biosynthesis was stimulated by antigen, an unidentified intracellular pathway could act in conjunction with Ca^{2+} (*22*). Furthermore, recent papers describing that the 5LO inhibitor MK 886 leads to antiproliferative effects and apoptosis in HL-60 and prostate cancer cells suggest that 5LO products could be formed during cell culture without an added stimulus (*38*, *39*). At present, it seems possible that Mg^{2+} and ATP could be of relevance for basal cellular 5LO activity.

ACKNOWLEDGMENT

We thank Agneta Nordberg for expert technical assistence. We are also most grateful to Birger Lind for determinations of calcium by atomic absorption spectroscopy, and to Mats Hamberg for 17(*S*)-hydroxy-13,19(*Z*)-15(*E*)-docosatrienoic.

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BI9919246