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Arachidonic acid increases intracellular calcium in erythrocytes☆

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Abstract

Recently, we have measured in erythrocytes a voltage-modulated and dihydropyridine-inhibited calcium influx. Since arachidonic acid and other polyunsaturated fatty acids influence the activities of most ion channels, we studied their effects on the erythrocyte Ca^{2+} influx. It was measured on fresh erythrocytes, isolated from healthy donors, using the fluorescent dye Fura 2 as indicator of $[Ca^{2+}]_i$. AA (5–50 μM) and EPA (20–30 μM) stimulated a concentration-dependent increase in $[Ca^{2+}]_i$, deriving from extracellular calcium (1 mM), without affecting the intra- and extracellular pH and membrane voltage. The Ca^{2+} influx rate varied from 0.5 to 3 nM Ca^{2+}/s in the presence of AA and from 0.9 to 1.7 nM Ca^{2+}/s with EPA. The Ca^{2+} influx elicited by AA and EPA was not inhibited by dihydropyridines, while cyclooxygenase inhibitors were effective and PGE1 or PGE2 did not produce any effect. We conclude that AA could activate an erythrocyte voltage-independent Ca^{2+} transport via an intermediate product of cyclooxygenase pathway; however, a direct interaction with the membrane lipid–protein cannot be excluded.

Keywords: Erythrocytes; Fura 2; Ca^{2+} influx; Polyunsaturated fatty acids; Arachidonic acid; Cyclooxygenases; Prostaglandins

Arachidonic acid is a polyunsaturated fatty acid with four *cis* double bonds, which are the sources of its flexibility and give it the capacity to react with molecular oxygen [1]. AA is either released within the cytoplasm from cell membranes or taken by cells via the circulation. Intracellular free arachidonate has three possible destinations: diffusion outside the cell, re-incorporation into membrane phospholipids, and metabolisation. It can be metabolised by three distinct enzyme pathways, cyclooxygenase, lipoxygenase, or epoxygenase, which produce, respectively, prostaglandins and thromboxanes, leukotrienes and hydroxyeicosatetraenoic acids, and epoxides [2].

Several products of these pathways and AA itself are known to affect different kinds of ion channels, including Ca^{2+} channels that can be activated or blocked, often with concentrations as low as 1–10 μM [3,4]. Particularly, AA has been demonstrated to have an inhibitory, PKC-mediated effect on Ca^{2+} channels of hippocampal pyramidal cells and ciliary ganglion cells [5,6]. However, in the majority of studies, addition of AA to the external medium was found to increase the intracellular free calcium concentration either by mobilising Ca^{2+} from intracellular store or augmenting Ca^{2+} influx as shown in human pancreatic islets, liver microsomes, platelets, rat oligodendrocytes, and many other kinds of mammalian cells [7–11]. The Ca^{2+} entry through the plasma membrane does not seem to be due to a selective permeability of the membrane, but due to an effect of AA on the ion transport that may be direct or mediated by PKC, PLC, or by other products of AA metabolism. As the final result, the increase of cytoplasmic Ca^{2+} is able to alter the cellular state, adapting cellular functions to different stimuli.

Little is known about the effects of polyunsaturated fatty acids and their metabolites on erythrocytes. Human erythrocytes were showed to produce PGE1 and PGE2 and the production was inhibited by indometha-

☆ *Abbreviations used:* AA, arachidonic acid; $[Ca^{2+}]_i$, intracellular free calcium concentration; CO, cyclooxygenase; EPA, eicosapentaenoic acid; ETYA, eicosatetraynoic acid; ASA, acetylsalicylic acid; PGE, prostaglandin; OA, oleic acid; PKC, protein kinase C; PLC, phospholipase C.

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cin, an inhibitor of cyclooxygenase pathway [12]. Allen et al. and Rasmussen et al. [13,14] reported that PGE2 decreases the deformability, expressed as the filtration rate, of erythrocytes and hypothesised that they must contain some types of PGE2 receptors. A shrinkage of erythrocytes after PGE2 treatment was shown in a recent work, suggesting that the effect be caused by a Ca^{2+} influx, stimulating potassium efflux, through the Gardos channels [15]. Accordingly, a non-selective cation channel, permeable to Ca^{2+} and activated by PGE2, has been demonstrated in erythrocytes. [16]. Besides these in vitro studies, altered fatty acid concentrations were found in erythrocyte membranes from patients with metabolic disorders like hypercalciuria and hyperoxaluria [17–19].

To analyse the activities of fatty acids on erythrocytes, the present study examines the effects of exogenous polyunsaturated fatty acids (AA, EPA) on intracellular free calcium concentration. The effects of a monounsaturated fatty acid (OA) and an AA structural analogue not included in the CO metabolism (ETYA) were also studied. We used a technique previously described that directly allows following the Ca^{2+} fluxes and measuring the free Ca^{2+} concentration in real time [20,21]. Moreover, we have tried to explain the mechanism of measured effects by using specific inhibitors of fatty acid pathways and of Ca^{2+} channels.

Materials and methods

Materials. AA was purchased from Calbiochem, Biochemicals (San Diego, CA, USA). EPA, OA, ETYA, PGE2, PGE1, indomethacin, nifedipine, Fura 2AM, and other chemical reagents were purchased from Sigma Chemical (ST. Louis, Mo, USA). Buffers and solutions used were HBS (123 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 25 mM HEPES, pH 7.4 at 37 °C, 285 mosm/L); ATP depletion solution (140 mM NaCl, 5 mM KCl, 1 mM iodoacetate, 10 mM inosine, pH 7.4); ACG anticoagulant (2.73% citric acid, 4.48% trisodium citrate, and 2% glucose). Fura 2 AM was dissolved in dimethyl sulphoxide at 1 mM concentration and stored at −20 °C; each aliquot was diluted in HBS before each experiment to minimise the dye degradation. Fatty acids (AA, EPA, OA, and ETYA) were diluted in 100% ethanol in airtight glass vials purged with N2 and stored at −80 °C. A fresh vial was used for each experiment, to avoid the loss of activity. Lipid metabolites (PGE1 and PGE2) were dissolved in ETOH, aliquoted, and stored at −80 °C until their use. Fatty acids and AA metabolites were removed from the vial under N2 and delivered to the cuvette using a Hamilton syringe and diluted in H2O to a desired concentration immediately before use.

Preparation of erythrocytes. Blood (2 ml) from healthy human volunteers was collected in tubes containing 1/10 of ACG. Erythrocytes were prepared immediately after blood sampling and processed at room temperature. Blood was centrifuged for 5 min at 750*g* and plasma and buffy coat were discarded. Packed erythrocytes were diluted to 1% haematocrit and immediately used for experiments.

$[Ca^{2+}]_i$ measurement. To inhibit the Ca^{2+} pump activity, erythrocytes at 1% haematocrit were depleted of ATP by incubation in an ATP depletion solution for 60 min at 37 °C. After 15 min from the start of incubation, 0.1 mM Fura 2 AM (final concentration of 1 μM) was added to the cell suspension (45 min of loading). At the end of incubation, erythrocytes were washed with the ATP depletion solution to eliminate the excess Fura 2 AM and then diluted to 0.1% haematocrit in the same solution. Then, the erythrocytes were transferred into a quartz cuvette where they were incubated in the presence of fatty acid or fatty acid plus inhibitor or in their absence for 10 min. At the end, $CaCl_2$ (final concentration of 1 mM) was added in the medium and the fluorescent signal was recorded for 30 min with a luminescent spectrometer (LS 50B, Perkin–Elmer, Norwalk, CT, US). The incubation and fluorescence measurements were carried out at 37 °C under magnetic stirring. The data were analysed by the Intracellular Biochemistry Application Software Package (Perkin–Elmer, Norwalk, CT, US) that allows for a fluorescence lecture every 1.9 s. $[Ca^{2+}]_i$ was calculated as previously reported [20,21].

Experimental control and data analysis. Each experiment was performed in five separate aliquots of erythrocytes the same day and each kind of experiment was repeated on three different days. Five different concentrations of inhibitors were used in the presence of 50 μM AA or 30 μM EPA and the IC50 was calculated. The erythrocyte $[Ca^{2+}]_i$ was measured in response to experimental treatments and was also measured in the presence of diluent only (DMSO and ETOH). The eventual autofluorescence was estimated for every substance used. All experiments were performed under constant stirring and temperature (37 °C). The Ca^{2+} influx rate has been expressed as nM/s, calculated by a computerised method, as previously reported [21]. The results of the experiments have been reported in the text as a range.

Results

Effects of exogenous fatty acids on $[Ca^{2+}]_i$ in human erythrocytes

Addition of exogenous AA (5–50 μM) to ATP depleted erythrocytes, loaded with Fura 2 AM in the presence of 1 mM of external $CaCl_2$, stimulated a Ca^{2+} influx, as shown in Fig. 1a. The influx kinetic was characterised by first slow increase of calcium, followed by a phase of faster uptake, until a plateau was reached. The range of faster uptakes was 0.5–3 nM Ca^{2+}/s. The entry of calcium was dose-dependent and it reached its maximum increase at an exogenous AA concentration of 50 μM (Fig. 1b). Addition of higher concentrations of AA (⩾100 μM) and/or $CaCl_2$ (⩾20 mM) leads to lysis of erythrocytes, probably due to a detergent effect of AA, resulting in a dramatic increase of $[Ca^{2+}]_i$.

In ATP depleted erythrocytes, the presence of external 1 mM $CaCl_2$ and of exogenous EPA (range 10–30 μM), stimulated a Ca^{2+} influx similar to that stimulated by AA (Fig. 2a). The range of faster uptakes was 0.9–1.7 nM Ca^{2+}/s. The entry of calcium was dose-dependent and its maximum increase occurred with exogenous EPA at the concentration of 30 μM, as shown in Fig. 2b. Addition of higher concentrations of EPA (⩾100 μM) and/or $CaCl_2$ (⩾20 mM) leads to lysis of erythrocytes, probably due to a detergent effect.

In the absence of external $CaCl_2$, exogenous AA and EPA did not stimulate any Ca influx.

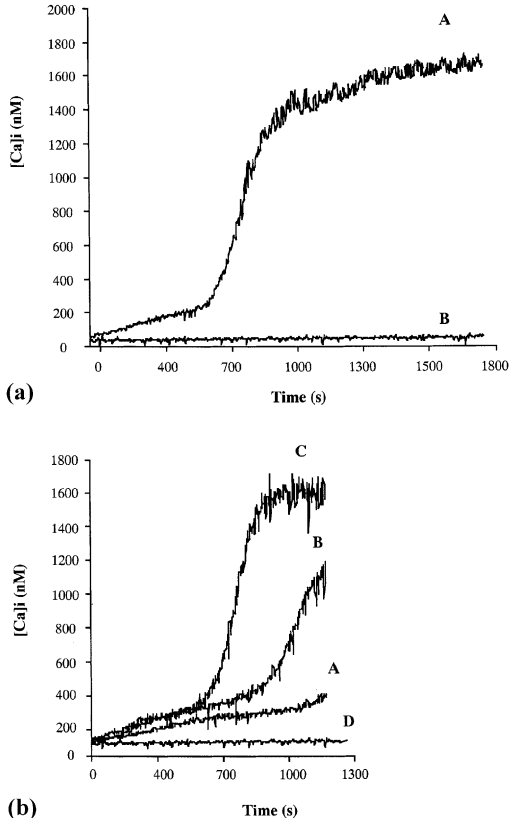

Fig. 1. (a) Ca^{2+} influx elicited by arachidonic acid in fresh erythrocytes loaded with Fura 2 and ATP depleted. The figure shows an example of the experiment. (A) The erythocytes were incubated with AA (50 μM) for 10 min. The measurement of fluorescence started when 1 mM Ca^{2+} was added into the cuvette. (B) The same experiment was performed without AA. (b) Ca^{2+} influx elicited by arachidonic acid in fresh erythrocytes loaded with Fura 2 and ATP depleted is concentration-dependent. The figure shows an example of the experiment. (A) In the presence of 5 μM AA after adding 1 mM $CaCl_2$. (B) In the presence of 25 μM AA after adding 1 mM $CaCl_2$. (C) In the presence of 50 μM AA after adding 1 mM $CaCl_2$. (D) In the presence of 50 μM AA and without external calcium.

The intra- and extracellular pH did not change during the Ca^{2+} influx stimulated by AA and EPA, so that it seems to be voltage-independent.

In contrast, addition of OA or ETYA, both in the range 1–100 μM, to ATP depleted erythrocytes in the presence of 1 mM $CaCl_2$, did not stimulate any Ca^{2+} influx.

Effect of nifedipine on Ca^{2+} influx

We investigated the effect of nifedipine, a dihydropyridine calcium antagonist, inhibitor of L-type voltage-dependent Ca^{2+} channels, on the Ca^{2+} influx stimulated by AA and EPA. Nifedipine at the concentration of 10 μM was incubated with erythrocytes during the loading with Fura 2 AM and maintained until the end of the experiments. We did not observe any inhibitory effect of nifedipine on the Ca^{2+} influx stimulated by AA and EPA.

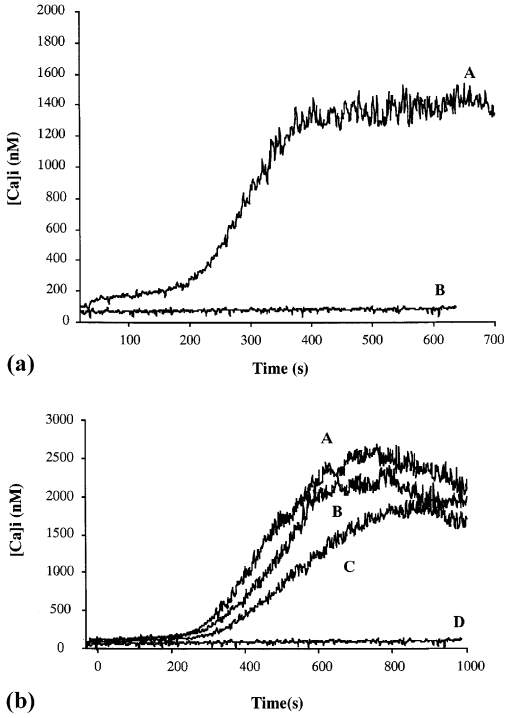

Fig. 2. (a) Ca^{2+} influx elicited by eicosapentaenoic acid in fresh erythrocytes loaded with Fura 2 and ATP depleted. The figure shows an example of the experiment. (A) The erythocytes were incubated with EPA (30 μM) for 10 min. The measurement of fluorescence started when 1 mM Ca^{2+} was added into the cuvette. (B) The same experiment was performed without EPA. (b) Ca^{2+} influx elicited by eicosapentaenoic acid in fresh erythrocytes loaded with Fura 2 and ATP depleted is concentration-dependent. The figure shows an example of the experiment. (A) In the presence of 30 μM EPA after adding 1 mM $CaCl_2$. (B) In the presence of 20 μM EPA after adding 1 mM $CaCl_2$. (C) In the presence of 10 μM EPA after adding 1 mM $CaCl_2$. (D) In the presence of 30 μM EPA and without external calcium.

Effects of cyclooxygenase inhibitors on Ca^{2+} influx

The effects of CO inhibitors, on the Ca^{2+} influx AA and EPA stimulated, were studied. ASA (1 mM) or indomethacin (1 mM) was added to a Fura 2 loaded erythrocyte suspension, 30 min before stimulation with AA or EPA. They completely inhibited the AA/EPA stimulated Ca^{2+} influx. Different concentrations of indomethacin (100–300 μM) were added in single samples to calculate the IC50 that resulted to be 160 μM for the influx stimulated by 50 μM AA (Fig. 3a) and 170 μM for the influx stimulated by 30 μM EPA (Fig. 3b).

Effects of prostaglandins on $[Ca^{2+}]_i$ in human erythrocytes

Addition of exogenous PGE1 and PGE2 (10–50 μM) to ATP depleted erythrocytes, loaded with Fura 2 AM in the presence of 1 M external $CaCl_2$, did not stimulate a Ca^{2+} influx.

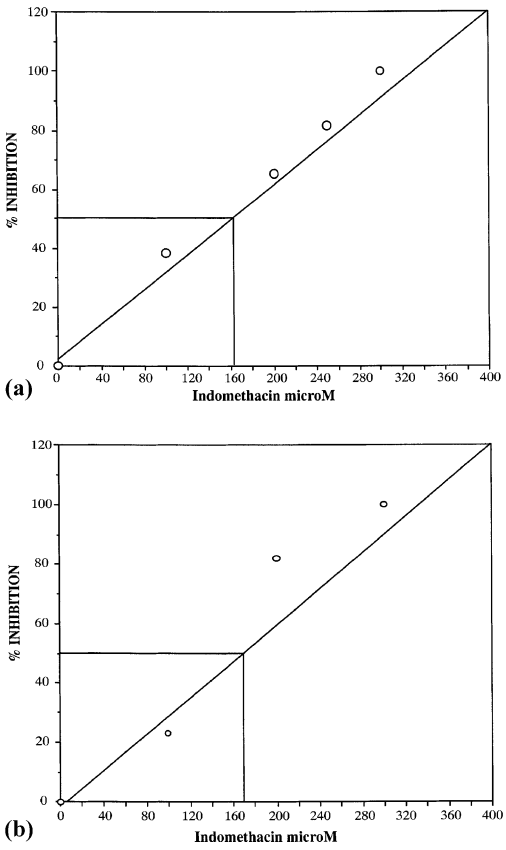

Fig. 3. (a) IC50 of indomethacin on AA induced Ca influx. (b) IC50 of indomethacin on EPA induced Ca influx.

Discussion

Our experiments, performed in human erythrocytes, showed that AA and EPA stimulated a Ca^{2+} entry, dependent on agonist concentration and deriving from extracellular calcium. Influx kinetic was characterised by an initial slow rise of calcium, followed by a phase of faster intake, until a plateau was reached. During influx intra- and extracellular pH did not change and nifedipine was ineffective as inhibitor, therefore the Ca^{2+} influx appeared to be voltage-independent. In contrast, indomethacin and ASA, both inhibitors of cyclooxygenases, inhibited the Ca^{2+} influx in a dose-dependent manner. This suggests that products of AA and EPA metabolism via CO pathway be involved in the induction of Ca^{2+} influx observed in our experiments. This inhibitory effect also provides the evidence that the Ca influx is not due to detergent activities of AA and EPA.

To have a further confirmation of this hypothesis, we studied the effects of fatty acids, OA, and ETYA, which are not substrates for the CO pathway. Particularly, ETYA is commonly used as an inhibitor of the arachidonic acid pathway [22,23]. It has also been demonstrated that ETYA inactivates cyclooxygenase, probably acting as a suicide substrate [2]. In this condition, we did not observe any Ca^{2+} influx, suggesting that the increase in $[Ca^{2+}]_i$, induced by AA and EPA, may be due to an oxidised metabolite. The absence of Ca^{2+} influx in the presence of exogenous PGE1 and PGE2 suggests that the influx was elicited by a different AA metabolite or that the experimental condition was not optimised to show their activity. In fact, Li et al. [15] estimated that only 15% of erythrocytes responded to stimulation with PGE2. If this were the case, our experiments did not allow measuring the PGE effect.

The ion flux we observed could be due to an indirect effects of polyunsaturated fatty acids on some kind of Ca^{2+} transport in human erythrocytes, similar to that observed in rabbit platelets, stimulated by a synergistic interaction of arachidonate and glucagon [24]. In addition to our findings it has also been described that arachidonic acid was able to stimulate a K^+ channel in smooth muscle by a direct effect on the membrane [3]. Alternatively, activation of PKC may be involved, since these polyunsaturated fatty acids have been shown to play a role as second messengers in the regulation of cell function, activating the phospholipid-dependent protein kinase [25].

These results indicate that polyunsaturated fatty acids, or their metabolites, may play a role as physiological second messengers in the erythrocyte metabolism, regulating some enzymatic cell function, and therefore they could play an important role in some particular disease.

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References

[1] A.R. Brash, Arachidonic acid as a bioactive molecule, J. Clin. Invest. 107 (2001) 1339–1345.
[2] P. Needleman, J. Turk, B.A. Jakschik, A.R. Morrison, J.B. Lefkowith, Arachidonic acid metabolism, Ann. Rev. Biochem. 55 (1986) 69–102.
[3] R.W. Ordway, J.J. Singer, J.V. Walsh, Direct regulation of ion channels by fatty acids, Trend Neurosci. 14 (1991) 96–100.
[4] H. Meves, Modulation of ion channels by Arachidonic acid, Prog. Neurobiol. 43 (1994) 175–186.
[5] D.O. Keyser, B.E. Alger, Arachidonic acid modulates hippocampal calcium current via protein kinase C and oxygen radicals, Neuron 5 (1990) 545–553.
[6] G. Khurana, M.R. Bennett, Nitric oxide and arachidonic acid modulation of calcium currents in postganglionic neurones of avian cultured ciliary ganglia, Br. J. Pharmacol. 109 (1993) 480–485.
[7] R.S. Rana, L.E. Hokin, Role of phosphoinositides in transmembrane signaling, Physiol. Rev. 70 (1990) 115–142.
[8] M.T. Alonso, A. Sanchez, J. Garcia-Sancho, Arachidonic acid-induced calcium influx in human platelets. Comparison with the effect of thrombin, Biochem. J. 272 (1990) 435–443.

[9] S. Ramanadham, R. Gross, J. Turk, Arachidonic acid induces an increase in the cytosolic calcium concentration in single pancreatic islet beta cells, Biochem. Biophys. Res. Commun. 184 (1992) 647–653.

[10] E.A. Dennis, S.G. Rhee, M.M. Billah, Y.A. Hannun, Role of phospholipases in generating lipid second messengers in signal transduction, FASEB J. 5 (1991) 2068–2077.

[11] B. Soliven, M. Takeda, T. Shandy, D.J. Nelson, Arachidonic acid and its metabolites increase Cai in cultured rat oligodendrocytes, Am. J. Physiol. 264 (1993) C632–C640.

[12] T. Oonishi, K. Sakashita, N. Ishioka, N. Suematsu, H. Shio, N. Uyesaka, Production of prostaglandin E1 and E2 by adult human red blood cells, Prostaglandins Lipid Mediat. 56 (1998) 89–101.

[13] J.E. Allen, H. Rasmussen, Human red blood cells: prostaglandin E2, epinephrine, and isoproterenol alter deformability, Science 174 (1971) 512–514.

[14] H. Rasmussen, W. Lake, J.E. Allen, The effect of catecholamines and prostaglandins upon human and rat erythrocytes, Biochim. Biophys. Acta. 41 (1975) 63–73.

[15] Q. Li, V. Jungmann, A. Kiyatkin, P.S. Low, Prostaglandin E2 stimulates a Ca^{2+}-dependent K+ channel in human erythrocytes and alters cell volume and filterability, J. Biol. Chem. 271 (1996) 18651–18656.

[16] L. Kaestner, I. Bernhardt, Ion channels in the human red blood cell membrane: their further investigation and physiological relevance, Bioelectrochemistry 55 (2002) 71–74.

[17] P. Messa, D. Londero, F. Massarino, L. Paganin, G. Mioni, F. Zattoni, G. Cannella, Abnormal arachidonic acid content of red blood cell membrane and main lithogenic factors in stone formers, Nephrol. Dial. Transplant 15 (2000) 1388–1393.

[18] B. Baggio, G. Gambaro, S. Zambon, F. Marchini, A. Bassi, L. Bordin, G. Clari, E. Manzato, Anomalous phospholipid *n*-6 polyunsaturated fatty acid composition in idiopathic calcium nephrolithiasis, J. Am. Soc. Nephrol. 7 (1996) 613–619.

[19] B. Baggio, G. Gambaro, Abnormal arachidonic acid content of membrane phospholipid-the-unifying hypothesis for the genesis of hypercalciuria and hyperoxaluria in idiopathic calcium, Nephrol. Dial. Transplant 14 (1999) 533–535.

[20] L. Soldati, R. Spaventa, G. Vezzoli, S. Verbi, D. Adamo, A. Caumo, R. Rivera, G. Bianchi, Characterizati on of voltage-dependent calcium influx in human erythrocytes by Fura-2, Biochem. Biophys. Res. Commun. 236 (1997) 549–554.

[21] L. Soldati, D. Adamo, S. Zerbi, A. Caumo, R. Spaventa, G. Bianchi, G. Vezzoli, Erythrocyte voltage-dependent calcium influx is reduced in hemodialyzed patients, Kidney Int. 56 (1999) 190–197.

[22] L.D. Tobias, J.G. Hamilton, The effect of 5,8,11,14-eicosatetraenoic acid on lipid metabolism, Lipids 14 (1979) 181–193.

[23] J. Capdevila, L. Gil, M. Orellana, L.J. Marnett, J.I. Mason, P. Yadagiri, J.R. Falck, Inhibitors of cytochrome P-450-dependent arachidonic acid metabolism, Arch. Biochem. Biophys. 261 (1988) 257–263.

[24] J.G. Altin, F.L. Bygrave, The influx of Ca^{2+} induced by administration of glucagon and Ca^{2+}-mobilizing agents to the perfused rat liver could involve at least two separate pathway, Biochem. J. 242 (1987) 43–50.

[25] L.C. McPhail, C.L. Claytonk, R. Snyderman, A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase, Science 224 (1984) 622–625.