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Bimodal regulatory effect of melittin and phospholipase A_2-activating protein on human type II secretory phospholipase A_2

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Abstract

Melittin and phospholipase A_2-activating protein (PLAP) are known as efficient activators of secretory phospholipase A_2 ($sPLA_2$) types I, II, and III when phospholipid liposomes are used as substrate. The present study demonstrates that both peptides can either inhibit or activate $sPLA_2$ depending on the peptide/phospholipid ratio when erythrocyte membranes serve as a biologically relevant substrate. Low concentrations of melittin and PLAP were observed to inhibit $sPLA_2$–triggered release of fatty acids from erythrocyte membranes. The inhibition was reversed at melittin concentrations above 1 μM. PLAP-induced inhibition of $sPLA_2$ persisted steadily throughout the used concentration range (0–150 nM). The two peptides induced a dose-dependent activation of $sPLA_2$ at low concentrations, followed by inhibition when model membranes were used as substrate. This opposite modulatory effect on biological membranes and model membranes is discussed with respect to different mechanisms the interaction of the regulatory peptides with the enzyme molecules and the substrate vesicles.

Keywords: Melittin; Phospholipase A_2-activating protein (PLAP); Secretory phospholipase A_2

1. Introduction

Secretory type II phospholipase A_2 (type II $sPLA_2$) plays a key role in inflammation and signal transduction (Vadas et al., 1993) via arachidonic acid release and the eicosanoid cascade. Venom and mammalian secretory PLA_2 are comprised of enzymes with a low molecular weight (12.5 to 16 kDa). The abundance of secretory PLA_2 in various inflammatory cells or exudates as well as in plasma suggests a role of this enzyme in pathology (Pruzanski et al., 1985; Vadas et al., 1993). Accordingly, secretory PLA_2 triggers an acute inflammatory response when injected into certain tissues (Bomalaski and Clark, 1993; Vadas et al., 1989).

Most $sPLA_2$ enzymes do not exhibit any particular preference for fatty acids under optimal assay conditions, unlike cytosolic PLA_2 ($cPLA_2$) which has a high selectivity for the *sn*-2-arachidonoyl molecular species (Murakami and Kudo, 2002; Schalkwijk et al., 1990). Therefore, the pattern of fatty acids released by a recombinant type II $sPLA_2$-induced hydrolysis of the inner or outer leaflets of erythrocyte membranes has been shown to reflect the asymmetric fatty acid composition of biological membranes (Koumanov et al., 1997). In a previous paper we showed clearly that the addition of ceramides to the substrate not only activated $sPLA_2$ II but also converted this enzyme from a non-specific to a highly specific enzyme for $C_{20:4}$-containing phospholipids (Koumanov et al., 2002). In this respect, the profile of the fatty acids released in the presence of ceramide became similar to that of $cPLA_2$.

In the present paper, we extend the method of quantitative profiling of the fatty acids released by $sPLA_2$ from biomembranes in the presence of melittin and phospholipase A_2-activating protein (PLAP) because a number of activating peptides such as for example the C-reactive protein (Vadas et al., 1995) influence the effect of $sPLA_2$ on the inflammation process in vivo.

Melittin is an in vitro activator of bacterial, venom or mammalian low molecular weight $sPLA_2$ with no effect

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on $cPLA_2$ (Cajal and Jain, 1997; Clark et al., 1987; Molley et al., 1976; Rao, 1992; Steiner et al., 1993). The biological effect of melittin has usually been assessed from data obtained with vesicular phospholipid substrate. A number of studies reported that the effect of melittin on cells was a direct consequence of high PLA_2 activity (Emmerling et al., 1993; Suzuki et al., 1991; Zeitler et al., 1991).

To our knowledge, however, there has been no definite demonstration of the PLA2-activating effect of melittin on native biological membranes. Early observations were conducted with bee venom melittin contaminated with PLA_2, the latter being most probably responsible for the observed activation (Fletcher et al., 1990, 1991). The commercial availability of synthetic melittin or synthetic PLAP eliminated the problem of contamination.

The mechanism of melittin-induced activation has been described for phospholipid model membranes in an assay in which melittin was added in the form of tetramers (Hermetter and Lakowicz, 1986). It has been presumed to increase the substrate susceptibility to PLA_2 after phase transition to a non-bilayer lipid arrangement. This would activate phospholipid exchange between vesicles and enhance substrate replenishment to PLA_2 adsorbed on the interface (Cajal and Jain, 1997). Saini et al. (1997) reported that melittin binding to $sPLA_2$ inhibited its activity, an observation with great relevance to the conclusions in the present paper.

Phospholipase A_2-activating protein (PLAP) was found in mammalian tissues using the high homology in the cDNA-encoded sequence between PLAP and melittin. The presence of PLAP in mammalian tissues was confirmed using cross-reactivity with antibodies raised against the bee venom peptide (Clark et al., 1988, 1991). High concentration of PLAP was reported in inflammatory synovial fluid from patients with rheumatoid arthritis (Bomalaski et al., 1990) and in human inflammatory bowel diseases (Peterson et al., 1996). Synthesis of PLAP along with $sPLA_2$ (Bomalaski et al., 1992) has been reported to be influenced by Interleukin-1 (IL-1) and the forward induction of IL-1 synthesis by PLAP (Bomalaski et al., 1995) was considered to produce a marked amplification of the inflammatory response. PLAP was found to increase PLA_2 activity in vitro more effectively than melittin (Clark et al., 1991). Also, PLAP has been suggested to mediate the stimulation of PLA_2 through a direct interaction with the enzyme, whereas melittin has been presumed to interact mostly with the phospholipid substrate (Clark et al., 1987; Rao, 1992; Steiner et al., 1993).

The data reported so far concerning the effect of melittin and PLAP on $sPLA_2$ activity were based mostly on experiments using model phospholipid substrate and thus require additional observations with regard to their physiological relevance. In the present work an attempt was made to establish the regulatory influence of PLAP and melittin as a function of the $sPLA_2$ substrate, including physiologically relevant biomembrane phospholipids.

2. Materials and methods

2.1. Reagents

L-α-phosphatidylethanolamine (PE from egg yolk), L-α-phosphatidyl-L-serine (PS from bovine brain), sphingomyelin (SPH from egg yolk) and PLA_2 from *Crotalus adamanteus* venom were purchased from Sigma (St Louis, MO, USA). 1-Palmitoyl-2-[1-pyrenedecanoyl]-L-α-phosphatidic acid monomethyl ester was purchased from Molecular Probes (Eugene, OR, USA). Recombinant type II secretory PLA_2 was obtained from C127 mouse fibroblasts overexpressing the human enzyme (Pernas et al., 1991) and was a generous gift from Dr Olivier (Faculté de Médecine St Antoine, Paris). Synthetic melittin and PLAP were commercially available from Bachem (Basel, Switzerland).

2.2. Preparation of erythrocyte ghosts

Human erythrocytes were isolated from fresh citrated blood (Steck and Kant, 1974). All steps were performed at 4 °C. The blood was centrifuged for 10 min at 100 *g*. Red blood cells were collected and washed several times with 5 volumes of phosphate buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 8.0) and hemolyzed in 5 mM sodium phosphate, pH 8.0 (40 volumes). The ghosts were collected by centrifugation for 20 min at 22,000 *g* (Beckman J2-HS), washed (×4) with 5 mM phosphate buffer until "white ghosts" were obtained. Membrane proteins were measured using the method of Bradford (1976).

2.3. Preparation of liposomes

Liposomes were prepared by sonication of the phospholipid mixture PE/PS/SPH (50/17/33 mol/mol). The rationale for the composition of the substrate has been developed previously (Koumanov et al., 1997); PE is known to be a sensitive substrate for $sPLA_2$, PS is a negatively charged phospholipid which interacts with the basic activating peptides, and SPH is required to maintain the lamellar arrangement as determined by X-ray diffraction (Wolf et al., 2001). Typically, the phospholipids were mixed in chloroform, dried under a stream of nitrogen, hydrated in Tris–HCl buffer, pH 8.6, and sonicated (2×2 min) with a tip probe (MSE, UK) (20 kHz, approximately 100 W) until a clear dispersion was obtained.

2.4. Phospholipase A_2 assay

2.4.1. PLA_2 activity assay on erythrocyte membranes

The assay contained erythrocyte membranes representing 100 nmol of hydrolyzable phospholipids serving as substrate in 0.5 ml 100 mM Tris–HCl, pH 8.6, 5 mM $CaCl_2$, and 0.1% (w/v) fatty acid-free bovine serum albumin (BSA) to trap the products of hydrolysis. The substrate was pre-incubated for 10 min at room temperature with different concentrations of melittin or PLAP. The reaction was started with 3 mU human recombinant type II $sPLA_2$ (the activity of the enzyme preparation was calibrated by comparison with a known activity of pancreatic PLA_2). Incubations were carried out for 15 min at 37 °C with translational shaking. Released fatty acids extracted by the procedure of Dole (Tsujishita et al., 1994) and methylated by diazomethane, were quantified using gas chromatography–mass spectrometry (GC–MS). Control incubations of erythrocyte membranes incubated in the absence of $sPLA_2$ were carried out and the levels of fatty acids released by $sPLA_2$ were corrected accordingly.

2.4.2. PLA_2 activity assay on phospholipid liposomes

The incubation was carried out for 15 min at 37 °C in 0.5 ml containing 100 nmol hydrolyzable phospholipids suspended in 100 mM Tris–HCl, pH 8.6, 5 mM $CaCl_2$, and 0.1% BSA. The substrate was preincubated for 10 min at room temperature with different concentrations of melittin or PLAP. Human recombinant type II $sPLA_2$ (3 mU) was added to start the incubation. The released free fatty acids were extracted, methylated with diazomethane, and quantified as indicated above. Control incubations, in the absence of $sPLA_2$, were carried out and the levels of enzyme activity were then corrected accordingly.

2.4.3. PLA_2 activity on *1-palmitoyl-2-(1-pyrenedecanoyl)*-L-*phosphatidic acid (FPA)*

This activity was determined using a spectrofluorimeter (JY3, Jobin-Yvon, France) according to Ragvanyi et al. (1989). The incubation mixture contained 20 nmol FPA, 0.3 mU human recombinant type II $sPLA_2$ or 0.3 mU *Crotalus adamanteus* PLA_2 in 100 mM Tris–HCl, pH 8.6, 5 mM $CaCl_2$, and 0.1% BSA in a final volume of 1 ml. The substrate was preincubated with melittin or PLAP for 10 min at room temperature. The incubation was carried out in a spectrofluorimeter cuvette with constant stirring. The reaction was monitored by the emission at 397 nm, characteristic of monomeric fluorophores (excitation at 345 nm).

2.5. Gas chromatography–mass spectrometry (GC–MS) measurements

The extracted free fatty acids were methylated with diazomethane and separated with gas chromatography on a capillary column coated with Supelcowax-10 bonded phase [internal diameter 0.32 mm, length 30 m (Supelco, USA)] fitted in a gas chromatograph Hewlett Packard 5890 Series II. Fatty acids were detected with picomolar sensitivity using mass spectrometry (Nermag 10-10C, France) in the chemical ionization mode with ammonia (0.1 bar) as the reagent gas. The positive quasi-molecular ions were monitored and time-integrated. Quantification referred to an internal standard of heptadecanoic methyl ester with response factors calculated with the various fatty acid methyl ester calibrators.

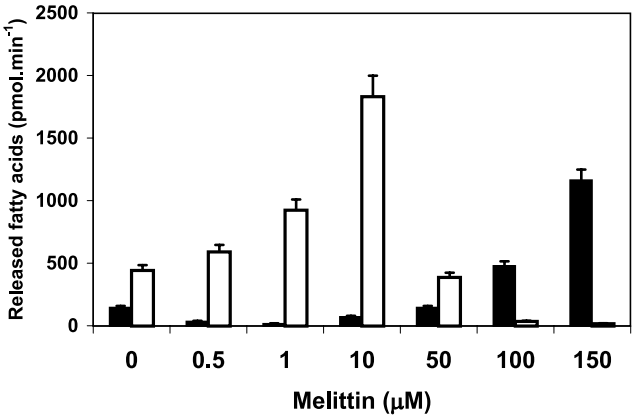

Fig. 1. Effect of melittin on secretory phospholipase A_2 $sPLA_2$ acting on erythrocyte membranes (closed bars) and phospholipid liposomes (open bars). Incubations were carried out as described under Materials and methods. Values are mean ± S.D. for triplicates in two independent experiments.

3. Results

The effect of melittin on secretory PLA_2-induced release of fatty acids was compared using two different substrates-erythrocyte membranes and phospholipid liposomes (Fig. 1). The amounts of hydrolysable phospholipids in both substrates were similar in the incubation medium (100 nmol glycerophospholipids). The activity of $sPLA_2$ on erythrocyte membranes (150 pmol of fatty acids released/min) represented only 33% of the activity observed on liposomes (450 pmol/min) in the absence of melittin. As evident from Fig. 1 (closed bars) melittin exerted an inhibitory effect on the fatty acid release from erythrocyte membranes at low concentrations (0.5–1 μM; melittin/phospholipid molar ratio=1/400–1/200). The activity of $sPLA_2$ was reported not to be specific for definite phospholipid molecular species when non-oriented human erythrocyte membranes served as substrate (Koumanov et al., 1997) but in the presence of high melittin concentrations a strong preference for C18:1 and C18:2 was observed (Table 1). The release of each fatty acid expressed relatively to its

Table 1
Effect of melittin on $sPLA_2$ activity. Experimental conditions are the same as described in Fig. 1. Released fatty acids (for 15 min incubation) were expressed as % of the amount of the corresponding fatty acids at *sn*-2 position of the substrate phospholipids determined after complete hydrolysis by *Naja naja* venom phospholipase A_2. Values are means ± SD for triplicates of two independent experiments

Melittin (μM)	Ghosts (% released fatty acids)				Liposomes (% released fatty acids)			
	C18:1	C18:2	C20:4	C22:6	C18:1	C18:2	C20:4	C22:6
0	2.26 ± 0.16	2.12 ± 0.17	2.01 ± 0.10	4.07 ± 0.24	5.26 ± 0.26	7.65 ± 0.54	11.33 ± 1.02	19.83 ± 1.59
0.5	0.24 ± 0.02	0.18 ± 0.01	0.53 ± 0.03	1.35 ± 0.08	7.55 ± 0.38	11.68 ± 0.82	18.95 ± 1.71	27.65 ± 2.21
1	0.00 ± 0.00	0.00 ± 0.00	0.36 ± 0.02	0.74 ± 0.04	9.84 ± 0.49	16.88 ± 1.18	25.94 ± 2.33	36.47 ± 2.92
10	2.54 ± 0.18	0.09 ± 0.01	0.00 ± 0.00	1.26 ± 0.08	21.17 ± 1.06	35.84 ± 2.51	36.49 ± 3.28	43.66 ± 3.49
50	4.29 ± 0.30	1.14 ± 0.09	0.37 ± 0.02	3.22 ± 0.19	4.09 ± 0.20	6.89 ± 0.48	11.21 ± 1.01	22.02 ± 1.76
100	12.44 ± 0.87	5.42 ± 0.43	2.47 ± 0.12	6.09 ± 0.37	0.42 ± 0.02	0.26 ± 0.02	1.01 ± 0.09	4.94 ± 0.40
150	29.13 ± 2.04	14.92 ± 1.19	6.06 ± 0.30	13.73 ± 0.82	0.13 ± 0.01	0.24 ± 0.02	0.96 ± 0.09	4.21 ± 0.34

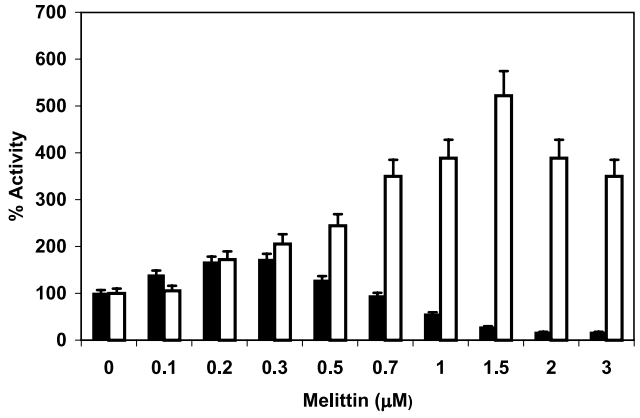

Fig. 2. Effect of melittin on human recombinant secretory PLA_2 (closed bars) in *C. adamanteus* PLA_2 (open bars), acting on fluorescent phosphatidic acid micelles. The activity was estimated from the fluorescence intensity at 397 nm relative to the control intensity in the absence of melittin. Values are mean ± S.D. (n=5).

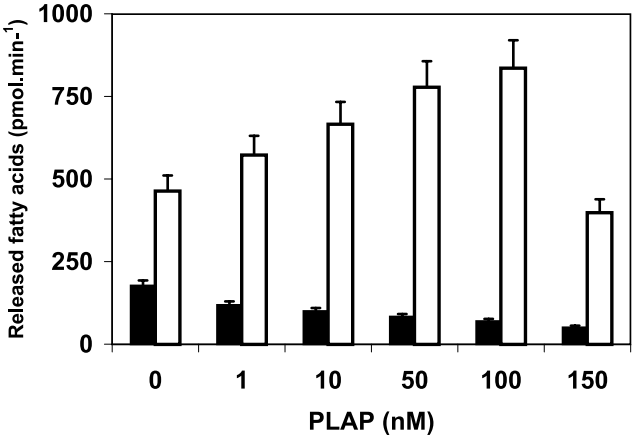

Fig. 3. Effect of phospholipase A_2 activating protein (PLAP) on secretory phospholipase A acting on erythrocyte membranes (closed bars) and phospholipi liposomes (open bars). Values are mean ± S.D. for triplicates in two independent experiments.

initial amount at *sn*-2 position of the phospholipid molecules was increased as follows: 13-fold for C18:1, 7-fold for C18:2, and 3-fold for C20:4 and C22:6 (Table 1).

The effect of melittin on $sPLA_2$ activity was just opposite when the substrate was in the form of phospholipid liposomes (Fig. 1 open bars). At concentrations from 0.5 to 10 μM (melittin/phospholipid ratio=1/400–1/20) melittin induced a strong activation of $sPLA_2$-triggered hydrolysis of the liposomal phospholipids. Here again, melittin increased predominantly the release of C18:1 and C18:2 (4-fold) as compared to that of C20:4 and C22:6 (3- and 2-fold, respectively) (Table 1). Only at high melittin concentrations (over a molar ratio of 1/4) could inhibition be detected. The changing effect of melittin (activation, followed by inhibition) as a function of the concentration was also observed using fluorescent phosphatidic acid (FPA) as substrate (Fig. 2, closed bars). Melittin activated FPA hydrolysis up to a concentration of 0.3 μM (melittin/FPA ratio=1/67) and inhibition was almost complete above 2 μM (melittin/FPA=1/10). Melittin was also tested on another type II PLA_2 present in *C. adamanteus* venom (Fig. 2, open bars). In this case a stronger activation was observed at melittin concentration of 1.5 μM (molar ratio 1/13).

The regulatory influence of PLAP on $sPLA_2$ was also found to vary as a function of the substrate. PLAP induced a dose-dependent inhibition of the $sPLA_2$-triggered fatty acid release from erythrocyte membrane phospholipids (Fig. 3, closed bars). This inhibition was gradual in the nanomolar range, whereas melittin was effective in the micromolar range. When PLAP was preincubated with liposomes composed of PE/PS/SPH, it exhibited a marked activating effect up to a concentration of about 100nM. (Fig. 3, open bars). The hydrolysis of C18:1 and C18:2 was almost double at 50 nM (molar ratio=1/4000) (Table 2). The release of C22:6 and C20:4 was augmented up to 100 nM after which a reduction was observed (Table 2). Preincubation of the fluorescent substrate FPA with PLAP for 10 min enhanced the hydrolysis by $sPLA_2$ reaching a maximum at 8 nM (ratio 1/2500) (Fig. 4). Preincubation of the enzyme, instead of the substrate with PLAP did not change $sPLA_2$ activity (data not shown).

Table 2
Effect of PLAP on $sPLA_2$ activity. Experimental conditions are the same as described in Fig. 2. Released fatty acids (for 15 min incubation) were expressed as % of the amount of the corresponding fatty acids at *sn*-2 position of the substrate phospholipids. Values are means ± SD for triplicates of two independent experiments

PLAP (nM)	Ghosts (% released fatty acids)				Liposomes (% released fatty acids)			
	C18:1	C18:2	C20:4	C22:6	C18:1	C18:2	C20:4	C22:6
0	2.26 ± 0.16	2.12 ± 0.17	2.01 ± 0.10	4.07 ± 0.24	5.26 ± 0.26	7.65 ± 0.54	11.33 ± 1.02	19.83 ± 1.59
1	1.54 ± 0.11	1.43 ± 0.09	1.48 ± 0.07	2.57 ± 0.21	7.19 ± 0.36	9.51 ± 0.57	12.81 ± 0.90	24.91 ± 2.62
10	1.43 ± 0.10	1.15 ± 0.07	1.24 ± 0.06	2.12 ± 0.17	8.63 ± 0.43	10.26 ± 0.62	14.53 ± 1.02	29.86 ± 3.14
50	1.33 ± 0.09	0.93 ± 0.06	1.07 ± 0.05	1.62 ± 0.13	9.95 ± 0.50	12.38 ± 0.74	15.87 ± 1.11	35.71 ± 3.75
100	1.19 ± 0.08	0.80 ± 0.05	0.90 ± 0.05	1.26 ± 0.10	9.41 ± 0.47	11.70 ± 0.70	16.34 ± 1.14	41.94 ± 4.40
150	1.02 ± 0.07	0.39 ± 0.02	0.43 ± 0.02	1.20 ± 0.10	8.33 ± 0.42	5.84 ± 0.35	9.69 ± 0.68	13.99 ± 1.47

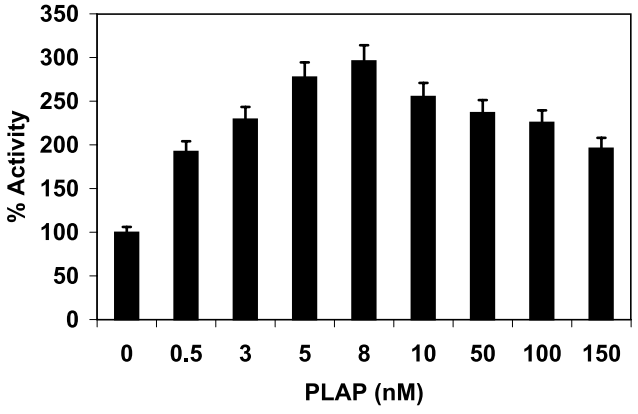

Fig. 4. Effect of PLAP on secretory phospholipase A_2 acting on fluorescent phosphatidic acid micelles. The activity was estimated from the fluorescence intensity relative to control intensity in the absence of PLAP. Values are mean ± S.D. (n=5).

4. Discussion

The present study compares the regulatory effect of two closely related peptides-melittin and PLAP, on the activity of secretory PLA_2 acting on natural or model substrates. The cellular target of this enzyme has not yet been clearly identified. Since the enzyme is abundantly secreted, it could act on the extracellular leaflet of plasma membranes or within the cell after receptor-mediated internalization (Zvaritch et al., 1996). The enzyme is assumed to take part in the inflammatory response by releasing arachidonic acid and other bioactive fatty acids and lysoderivatives from membranes of activated cells (Bomalaski et al., 1992). The rate of $sPLA_2$ synthesis is widely variable under the influence of cytokines (transcription is activated by IL-1 and TNF) but evidence for short time activation has been lacking until now. A number of reports that observed this activation, however, are based on in vitro assays using phospholipid mixtures as model substrate of $sPLA_2$ (Bomalaski et al., 1995; Rao, 1992). We believe that a more appropriate approach in investigating the modulation potency of the basic peptides is to use a biologically relevant substrate.

The influence of the so-called "activating" peptides on PLA_2 was also a priori considered invariant as a function of the concentration-a conclusion drawn with lipid mixtures used as PLA_2 substrate. This conclusion is now being reassessed.

At low concentrations melittin or PLAP were clearly activatory for $sPLA_2$ acting on pure lipid substrates. For the purpose of comparison the effect of melittin was also tested on PLA_2 from *C. adamanteus*, in order to illustrate that the observed effect was not specific mainly for human recombinant type II PLA_2. Melittin and PLAP induced activation of the hydrolysis of all fatty acids, as determined using GC–MS (Tables 1 and 2). The effect of these two peptides was tested both in the nanomolar and in the micromolar ranges. However, the data obtained showed that the effective concentration range was micromolar for melittin and nanomolar for PLAP. A possible explanation for the lower effectiveness of melittin, relative to PLAP, could be the partition equilibrium of the peptides between the lipid phase and the aqueous phase where they form aggregates (Talbot et al., 1979). It should also be noted that different activation mechanisms have been suggested for melittin and PLAP. Melittin activation has been presumed to originate from the enhanced sensitivity to PLA_2 of lipid bilayers undergoing a lamellar to non-lamellar phase transition (Batenburg et al., 1988). In model membranes containing phosphatidic acid as an anionic phospholipid which interacts with the basic peptides, the transition to hexagonal H_{II} phase has been observed at 0.3 μM melittin (Batenburg et al., 1988). Accordingly, this concentration produced strongest stimulation of $sPLA_2$ acting on fluorescent phosphatidic acid. Cajal and Jain (1997) have shown that melittin-induced activation of type I, II, or III PLA_2 is due to substrate replenishment through vesicle-vesicle contact favored by non-lamellar transition. Under the present experimental circumstances, using negatively charged phospholipids interspersed in the substrate interface, both mechanisms, phase transition and substrate replenishment, could take part in melittin-induced activation. An interesting observation was that melittin increased predominantly

the accessibility to $sPLA_2$ attack of phospholipid species enriched in C18:1 and C18:2.

Approximating a situation with physiological significance, low concentrations of melittin or PLAP were added to biological membranes serving as a substrate for $sPLA_2$. In these experiments we did not confirm the expected activation observed with low concentrations of the peptides added to purified phospholipid substrates. On the contrary, we observed that low concentrations of melittin or PLAP brought about a significant inhibition of the fatty acid release. The inhibition was reversed at concentrations over 1 μM melittin (Fig. 1) and persisted steadily for PLAP up to about 150 nM (Fig. 3). We suggest that low concentrations of the peptides added to biomembranes might not be able to partition in the phospholipid matrix and consequently modify the lipid arrangement to favour $sPLA_2$ activity. The binding of the peptides to biomembranes could only be efficient at high concentrations where activation was detected. At low concentrations, the unbound peptides are available to inhibit $sPLA_2$ as demonstrated by Saini et al. (1997).

Varying the concentrations of the so-called "activating" peptides altered their regulatory influence when biological substrate was used from inhibition of $sPLA_2$ at low concentrations to activation at relatively high concentrations. This was unexpected until the activity of $sPLA_2$ could be monitored on a complex biological substrate where the peptide binding is limited. The effect of these two peptides has generally been described only as activating when the substrate was composed of pure phospholipids to which the proteins bind with very high affinity. However, the results presented in this paper showed that this effect could be either inhibitory or activatory depending on the peptide concentration when biological membranes served as substrate. Saini et al. (1997) reported that unbound melittin inhibited $sPLA_2$ activity. The non-competitive inhibition was not followed by a stimulatory effect probably due to the limited interaction of melittin with the PE substrate used throughout the study.

These results suggest that the release of polyunsaturated fatty acids in vivo from mammalian tissues challenged by PLAP (Tsunoda and Owyang, 1994) or by PLAP enhancers, such as cholera toxin (Peterson et al., 1996) or IL-1 (Vadas et al., 1985), might not be a direct consequence of their activating effect on $sPLA_2$. It should be noted that a number of relevant biological effects of melittin or PLAP have already been differentiated from the activation of PLA_2. For instance, the lethal dose of melittin/phospholipase A_2 mixture administered in mice was lower than that of each component alone (Schmidt, 1995). This observation conducted in vivo was supported by high melittin cardiotoxicity in cultured myocytes correlating with calcium influx (Okamoto et al., 1995). Apparently, the physiological significance as well as the pathological consequences of these complex lipid-protein interactions in vivo need further clarification.

Acknowledgement

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