Human myocardial cytosolic Ca^{2+} -independent phospholipase A_2 is modulated by ATP

Concordant ATP-induced alterations in enzyme kinetics and mechanism-based inhibition

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Although phospholipases A_2 (PLsA_2) have been implicated as enzymic mediators of electrophysiological dysfunction during myocardial ischaemia and reperfusion in man, no information on the regulation of human myocardial PLsA_2 is available. We now report that human myocardial cytosolic Ca^{2+} -independent PLA_2 is modulated by ATP through mechanisms which are independent of protein phosphorylation, since: (1) ATP and its non-hydrolysable analogues reversibly augment the initial rate of crude (but not purified) human myocardial cytosolic PLA_2 activity 3–4-fold; (2) ATP and its non-hydrolysable analogues dramatically attenuate the rate of thermal denaturation of human myocardial cytosolic PLA_2 activity; (3) ATP and its non-hydrolysable analogues alter the sensitivity of human myocardial cytosolic PLA_2 to mechanism-based inhibition by (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; (4) adenine nucleotide triphosphates are more potent than guanine nucleotide triphosphates in activating and stabilizing human myocardial cytosolic PLA_2 ; (5) adenosine 5'-[β -methylene]triphosphate is more potent than its guanosine counterpart in protecting an essential thiol residue(s) in human myocardial cytosolic PLA_2 from covalent modification by 5,5'-dithiobis-(2-nitrobenzoic acid); and (6) ATP-dependent activation and stabilization of human myocardial cytosolic PLA_2 are mediated by a cytosolic protein(s) which can be functionally reconstituted with purified human myocardial cytosolic PLA_2 catalytic polypeptide. Collectively these results demonstrate that multiple physical and kinetic properties of human myocardial cytosolic Ca^{2+} -independent PLA_2 are dramatically influenced by dynamic interactions with ATP.

INTRODUCTION

Phospholipases A_2 (PLsA_2) are critical enzymic mediators of signal transduction, since their activation concomitantly initiates the formation of biologically active eicosanoids and modulates membrane molecular dynamics (c.f., Samuelsson, 1978; Dennis, 1983; Han & Gross, 1990). Although appropriate expression of activity is an integral part of homeostatic regulatory functions in mammalian cells, inappropriate expression of PLA_2 activity has been implicated in the initiation and propagation of several important disease states (see, e.g., Demediuk *et al.*, 1985; van Bilsen *et al.*, 1989; Hazen & Gross, 1991). Accordingly, elucidation of the biochemical mechanisms responsible for modulating intracellular PLsA_2 is essential for a detailed understanding of both physiological and pathophysiological processes. Furthermore, owing to their proximal location in the enzymic cascades resulting in the synthesis of a multiplicity of biologically active eicosanoids, intracellular PLsA_2 represent an attractive target for pharmacological manipulation of clinically relevant disease states.

Traditional studies have emphasized the importance of increases in intracellular Ca^{2+} as a direct modulator of Ca^{2+} -dependent PLA_2 activity. This has resulted primarily from the widespread and extensive studies of extracellular Ca^{2+} -dependent PLsA_2 over the past 25 years and the assumption that these enzymes shared physical and kinetic characteristics with intra-

cellular PLsA_2 . However, recent studies have demonstrated that the overwhelming majority of PLA_2 activity in several tissues (e.g., myocardium) is predominantly Ca^{2+} -independent (Wolf & Gross, 1985a; Hazen *et al.*, 1990) and is catalysed by polypeptides which are structurally and mechanistically distinct from the Ca^{2+} -dependent PLsA_2 (see, e.g., Roberts *et al.*, 1977; Verheij *et al.*, 1981; de Winter *et al.*, 1982; Loeb & Gross, 1986; Scott *et al.*, 1990; Thunnissen *et al.*, 1990). The physical and kinetic differences between Ca^{2+} -dependent and -independent PLsA_2 are underscored by their widely disparate molecular masses, cofactor requirements and substrate selectivities. The differences in the catalytic strategies employed by Ca^{2+} -independent and -dependent PLsA_2 have recently been exploited, resulting in the identification of a mechanism-based inhibitor which possesses > 1000-fold selectivity for inhibition of Ca^{2+} -independent in comparison with Ca^{2+} -dependent PLsA_2 (Hazen *et al.*, 1991). Furthermore, we have recently demonstrated that canine myocardial cytosolic Ca^{2+} -independent PLA_2 specifically and reversibly binds to ATP-agarose affinity matrices, whereas all Ca^{2+} -dependent PLsA_2 examined to date (both extracellular and intracellular) do not (Hazen *et al.*, 1990). We now report that multiple physical and kinetic properties of human myocardial cytosolic Ca^{2+} -independent PLA_2 are dramatically altered by interaction with ATP and that ATP potentiates mechanism-based inhibition of human myocardial cytosolic PLA_2 .

Abbreviations used: $\text{p[CH}_2\text{]}_2\text{ppA}$, adenosine 5'-[β -methylene]triphosphate; $\text{p[NH]}_2\text{ppA}$, adenosine 5'-[β -imido]triphosphate; ATP[S] , adenosine 5'-[γ -thio]triphosphate (guanosine 5'-triphosphate analogues are comparably defined); bromoenol lactone, (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; PL(s)A_2 , phospholipase(s) A_2 ; 16:0,18:1 plasmenylcholine, 1-*O*-(*Z*)-hexadec-1'-enyl-2-octadec-9'-enoyl-*sn*-glycero-3-phosphocholine; 16:0,[^3H]18:1 plasmenylcholine, 1-*O*-(*Z*)-hexadec-1'-enyl-2-[9',10'- ^3H]octadec-9'-enoyl-*sn*-glycero-3-phosphocholine.

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MATERIALS AND METHODS

Enzyme preparation

Fresh human myocardium from transplant recipients was immediately placed in ice-cold iso-osmotic saline solution. Left-ventricular tissue was rapidly isolated, trimmed of epicardial fat and visible fibrotic lesions, weighed and placed (25%, w/v) in homogenization buffer [0.25 M-sucrose (grade 1)/10 mM-imidazole/10 mM-KCl, pH 7.8, 0 °C]. Human myocardium was subsequently homogenized and fractionated by differential ultracentrifugation, yielding the cytosolic fraction as previously described (Hazen *et al.*, 1990). Myocardial cytosol was filtered through glass wool, dialysed twice against 500 vol. of homogenization buffer (8 h/dialysis) and either utilized directly or immediately frozen in individual aliquots in liquid N₂. No differences in PLA₂ kinetics, regulation by ATP or mechanism-based inhibition were observed in comparisons between fresh cytosol and cytosol stored in liquid N₂ for up to 6 months. Human myocardial cytosolic Ca²⁺-independent PLA₂ was subsequently purified 180 000-fold to near homogeneity (specific activity 67.2 μmol/min per mg) by methods similar to those utilized for canine myocardial cytosolic Ca²⁺-independent PLA₂ (Hazen *et al.*, 1990).

Preparation of synthetic phospholipids

Homogeneous 1-*O*-(*Z*)-hexadec-1'-enyl-2-[9',10'-³H]octadec-9'-enyl-*sn*-glycero-3-phosphocholine (16:0,³H]18:1 plasmenylcholine; specific radioactivity = 1.25 × 10⁶ d.p.m./nmol) was prepared by *NN*-dimethyl-4-aminopyridine-catalysed condensation of dicyclohexylcarbodi-imide-generated [³H]oleoyl anhydride with 1-*O*-(*Z*)-hexadec-1'-enyl-*sn*-glycero-3-phosphocholine as previously described (Wolf & Gross, 1985b). Polar-head-group-labelled plasmenylcholine was synthesized by condensation of [*methyl*-³H]cholinelysoplasmeylcholine (prepared by exhaustive methylation of reverse-phase-purified lysoplasmeyl ethanolamine (Creer & Gross, 1985) utilizing C³H₃I with benzyltrimethylammonium chloride as catalyst (Ayangolu *et al.*, 1984)) with unlabelled oleoyl anhydride as described above. H.p.l.c. purification and confirmation of the regiospecificity of radiolabels for each synthetic lipid were performed as described previously (Wolf & Gross, 1985b; Hazen *et al.*, 1990).

PLA₂ assays

PLA₂ activity was assessed by incubating enzyme with the indicated concentration of synthetic radiolabelled phospholipid (introduced by ethanolic injection (10 μl) in assay buffer (final concns.: 100 mM-Tris/HCl/4 mM-EGTA/4 mM-EDTA, pH 7.0) at 37 °C for 60 s in a final reaction volume of 210 μl (unless otherwise indicated). Radiolabelled products were extracted with butanol, isolated by t.l.c. and quantified by liquid-scintillation spectrometry as previously described (Hazen *et al.*, 1990). Under these conditions, reaction velocities were linear with respect to both time and enzyme concentration. Nucleotides employed in this study were initially dissolved in water (0 °C), immediately titrated to neutral pH and either utilized directly or stored in individual aliquots at -20 °C.

Thermal-denaturation, chemical-modification and suicide-inhibition studies

Thermal denaturation of human myocardial cytosolic PLA₂ was quantified by incubating cytosol (in the absence or presence of the indicated amounts of nucleotide) at 37 °C for selected periods at the end of which time an aliquot (50 μl) was removed and placed in assay buffer (containing an identical nucleotide

concentration). Remaining PLA₂ activity was quantified after injection of radiolabelled substrate as described above. Chemical modification of essential thiol residues in cytosolic PLA₂ was performed by incubating human myocardial cytosol at 0 °C for 1 min (in the presence or absence of the indicated nucleotide) with 0, 1 or 10 μM-5,5'-dithiobis-(2-nitrobenzoic acid) (Nbs₂) and subsequent quantification of remaining PLA₂ activity in assay buffer containing 1 mM-dithiothreitol (DTT) (to inactivate remaining Nbs₂) as described above. The time course of inactivation of human myocardial cytosolic PLA₂ by the suicide inhibitor (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (bromo-enol lactone) (Hazen *et al.*, 1991) was determined by incubating 10 nM-inhibitor [introduced by ethanolic injection (5 μl)] with enzyme at 20 °C for the indicated time intervals. Remaining PLA₂ activity was subsequently quantified after rapid dilution (20-fold) and addition of synthetic 16:0,³H]18:1 plasmenylcholine substrate as described above. The concentration-dependence for bromoenol lactone-mediated inhibition of human myocardial cytosolic Ca²⁺-independent PLA₂ (in the absence or presence of nucleotide) was performed similarly by preincubating selected concentrations of inhibitor with enzyme for 3 min at 20 °C before dilution (20-fold) and quantification of remaining PLA₂ activity as described above. The concentration profile of nucleotide-dependent alterations in bromoenol lactone-mediated PLA₂ inhibition was determined by direct comparisons of PLA₂ activity at various nucleotide concentrations in the presence or absence of inhibitor (3 min preincubation at 20 °C, 10 nM-bromo-enol lactone) and subsequent quantification of PLA₂ activity as described above.

Reconstitution of ATP-dependent activation and stabilization of purified human myocardial cytosolic Ca²⁺-independent PLA₂

First, endogenous PLA₂ activity in human myocardial cytosol was ablated by heat treatment at 37 °C for 20 min. This heat-treated fraction was then mixed with purified human myocardial cytosolic PLA₂ (i.e. 40 kDa catalytic polypeptide) in the presence or absence of 10 mM-ATP. PLA₂ activity was subsequently quantified either directly (to assess ATP-dependent activation) or after a 20 min 37 °C preincubation interval (to assess ATP-dependent stabilization). Similar mixing experiments were performed utilizing cytosol previously incubated at 60 °C for 45 min or cytosol previously incubated with trypsin (2%, w/w) for 24 h at 4 °C.

Source of materials and miscellaneous procedures

Protein content was determined utilizing either a Bio-Rad protein assay kit or Quantigold (Diversified Biotech) with BSA as standard. [³H]Oleic acid was purchased from duPont-New England Nuclear, and C³H₃I was obtained from Amersham International. Unlabelled oleic acid was obtained from NuChek Prep. All other lipids were obtained from Avanti Polar Lipids. Nucleotides of the highest quality (i.e. vanadate-free where possible) were obtained from either Sigma or Boehringer-Mannheim Biochemicals. Most other reagents were obtained from either Aldrich or Sigma Chemical Co.

RESULTS

ATP-dependent activation of human myocardial cytosolic Ca²⁺-independent PLA₂

Both naturally occurring and synthetic non-hydrolysable purine nucleotide triphosphates augmented the initial rate of phospholipid hydrolysis catalysed by crude human myocardial cytosol (Fig. 1). The PLA activated by adenine nucleotide triphosphates was a PLA₂, since: (1) ATP induced the release of

Fig. 1. NTP-dependent activation of human myocardial cytosolic Ca²⁺-independent PLA₂.

Initial-rate analyses of human myocardial cytosolic Ca²⁺-independent PLA₂ activity [final conditions: 100 mM-Tris, 4 mM-EGTA, 4 mM-EDTA, pH 7.0 (or for Mg·NTP, final conditions: 100 mM-Tris, 1 mM-EGTA and 10 mM Mg·NTP, pH 7.0)] were performed by incubating myocardial cytosolic protein (350 µg) with 16:0,[³H]18:1 plasmenylcholine substrate (20 µM) in the absence or presence of the indicated nucleotides (10 mM of each) for selected intervals (10–60 s). Released ³H-labelled fatty acid (the only radio-labelled product detected) was extracted with butanol, isolated by t.l.c. and quantified by liquid-scintillation spectrometry as described in the Materials and methods section. Results are means of duplicate determinations and are representative of multiple independent preparations of human ventricular myocardium. Symbols: ■, ATP; ▲, p[CH₂]ppA; ●, Mg·ATP; □, GTP; △, p[CH₂]ppG; ○, Mg·GTP; ◆, no additions.

only ³H-labelled fatty acid from *sn*-2-labelled plasmenylcholine, and (2) ATP induced the release of only [³H]lysoplasmenylcholine from polar-head-group-labelled plasmenylcholine. Adenine nucleotide triphosphates activated human myocardial cytosolic PLA₂ activity to a greater extent in direct comparisons with their guanine nucleotide counterparts (Fig. 1). Furthermore, activation of PLA₂ neither required the presence of bivalent cations nor was mediated by protein phosphorylation, since ATP, adenosine 5'-[βγ-methylene]triphosphate (p[CH₂]ppA) and Mg·ATP each activated enzymic activity. Similar results were obtained utilizing other non-hydrolyzable analogues of both ATP and GTP (e.g. adenosine 5'-[γ-thio]triphosphate (ATP[S]), adenosine 5'-[βγ-imido]triphosphate (p[NH]ppA), GTP[S], p[NH]ppG). The rank

Fig. 2. Lineweaver-Burk analysis of adenine nucleotide triphosphate-dependent activation of human myocardial cytosolic Ca²⁺-independent PLA₂.

Human myocardial cytosolic protein (350 µg) was incubated with the indicated concentrations of 16:0,[³H]18:1 plasmenylcholine substrate in assay buffer (final concns.: 100 mM-Tris, 4 mM-EGTA and 4 mM-EDTA, pH 7.0) in the presence of 0–10 mM-p[CH₂]ppA, for 60 s at 37 °C. Released ³H-labelled fatty acid was extracted with butanol, isolated by t.l.c. and quantified by liquid-scintillation spectrometry. Results are means of duplicate determinations and are representative of multiple independent preparations. Symbols: p[CH₂]ppA at: ■, 0 µM; △, 10 µM; ●, 31.6 µM; □, 100 µM; ▲, 316 µM; ○, 1 mM; ◆, 10 mM.

order of nucleotide potency for human myocardial cytosolic Ca²⁺-independent PLA₂ activation was: NTP > Mg·NTP, NDP ≫ cyclic NMP, NMP, N (where N = adenosine or guanosine).

To determine whether ATP-mediated activation of human myocardial cytosolic Ca²⁺-independent PLA₂ resulted from alterations in the affinity of enzyme for substrate, maximal reaction velocity, or both, kinetic analyses of the concentration-dependence of nucleotide-mediated PLA₂ activation were performed. The non-hydrolyzable analogue of ATP, p[CH₂]ppA, was utilized to minimize both enzyme-catalysed and non-enzyme-catalysed degradation during these incubations. Addition of p[CH₂]ppA (0–10 mM) elicited a concentration-dependent and saturable increase in the maximum rate of 16:0,[³H]18:1 plasmenylcholine hydrolysis [apparent *V*_{max} increased from 0.35 to 1.35 nmol without significant changes in the apparent affinity of enzyme for substrate (apparent *K*_m = 16 ± 1 µM)] (Fig. 2). Nucleotide-mediated activation was reversible, since dialysis or dilution attenuated activation of enzymic activity. Hill-plot analysis of the concentration-dependence of p[CH₂]ppA-

Fig. 3. Hill-plot analysis of mediated activation of human myocardial cytosolic Ca^{2+} -independent PLA_2

Human myocardial cytosolic Ca^{2+} -independent PLA_2 activity was assessed by incubating $340 \mu\text{g}$ of cytosolic protein with $20 \mu\text{M}$ $16:0$, $[^3\text{H}]18:1$ plasmenylcholine substrate in the absence or presence of the indicated concentrations of either $\text{p}[\text{CH}_2]\text{ppA}$ (○) or $\text{Mg} \cdot \text{p}[\text{CH}_2]\text{ppA}$ (●) for 60 s at 37°C . Released ^3H -labelled fatty acid was isolated and quantified as described in the Materials and methods section. Results are means of duplicate determinations.

mediated alterations in the rate of phospholipid hydrolysis suggested the presence of at least two ATP-binding sites per catalytic subunit ($n_{\text{app}} = 1.57$) and demonstrated a half-maximal concentration necessary for PLA_2 activation of $152 \mu\text{M}$ (Fig. 3). Comparisons of $\text{p}[\text{CH}_2]\text{ppA}$ -mediated activation in the absence or presence of Mg^{2+} demonstrated that inclusion of Mg^{2+} was accompanied by a 10-fold increase in the half-maximal concentration of nucleotide necessary for PLA_2 activation (i.e. $152 \mu\text{M}$ as against 1.48 mM for $\text{p}[\text{CH}_2]\text{ppA}$ and $\text{Mg} \cdot \text{p}[\text{CH}_2]\text{ppA}$ respectively) without substantial alterations in co-operativity (i.e. $n_{\text{app}} = 1.57$ as against 1.42 for $\text{p}[\text{CH}_2]\text{ppA}$ and $\text{Mg} \cdot \text{p}[\text{CH}_2]\text{ppA}$ respectively). Collectively, these results demonstrate that ATP (and its non-hydrolysable analogues) activate human myocardial cytosolic Ca^{2+} -independent PLA_2 by reversible, concentration-dependent, saturable and co-operative alterations in the observed maximum reaction velocity which do not require the participation of an intermediary kinase.

Adenine nucleotide triphosphates modulate human myocardial cytosolic Ca^{2+} -independent PLA_2 thermal denaturation

Quantification of the thermal inactivation of crude human myocardial cytosolic Ca^{2+} -independent PLA_2 activity demonstrated a first-order loss of enzyme activity during incubation at 37°C ($t_{1/2} = 2.8 \text{ min}$). Addition of either ATP or $\text{p}[\text{CH}_2]\text{ppA}$ (10 mM) to human myocardial cytosol markedly attenuated the rate of thermal inactivation of cytosolic Ca^{2+} -independent PLA_2 activity. As before, non-hydrolysable nucleotide triphosphates were employed ($\text{p}[\text{CH}_2]\text{ppA}$) to minimize both enzymic and non-enzymic degradation. Addition of $\text{p}[\text{CH}_2]\text{ppA}$ (0 – 10 mM) to human myocardial cytosol stabilized cytosolic Ca^{2+} -independent PLA_2 activity in a concentration-dependent and saturable manner (4-fold activation and 135-fold stabilization at saturating concentrations of nucleotide) (Fig. 4). Nucleotide-mediated stabilization of PLA_2 activity was reversible, since either dialysis or dilution attenuated nucleotide-dependent stabilization of thermal inactivation.

Fig. 4. $\text{p}[\text{CH}_2]\text{ppA}$ -mediated alterations in the thermal denaturation of human myocardial cytosolic Ca^{2+} -independent PLA_2

Human myocardial cytosol was heated at 37°C for the indicated time intervals in the absence or presence of selected concentrations of $\text{p}[\text{CH}_2]\text{ppA}$ (0 – 10 mM). Remaining PLA_2 activity was subsequently quantified by incubating the heated fractions ($300 \mu\text{g}$ of heat-treated cytosolic protein) with $16:0$, $[^3\text{H}]18:1$ plasmenylcholine substrate ($100 \mu\text{M}$) in assay buffer (containing an identical nucleotide concentration) for 60 s and subsequent quantification of released ^3H -labelled fatty acid as described in the Materials and methods section. The $t_{1/2}$ of thermal inactivation was calculated from linear-regression analysis of each semilogarithmic plot of remaining PLA_2 activity present at each $\text{p}[\text{CH}_2]\text{ppA}$ concentration examined. Results are means of duplicate determinations. $\text{p}[\text{CH}_2]\text{ppA}$ concns. (calculated $t_{1/2}$ in parentheses): □, $0 \mu\text{M}$ (2.8 min); ●, $10 \mu\text{M}$ (3.2 min); △, $31.6 \mu\text{M}$ (5.1 min); ■, $100 \mu\text{M}$ (9.9 min); ○, $316 \mu\text{M}$ (28.9 min); ▲, 1 mM (37.7 min); ◇, 10 mM (38.2 min).

Nucleotide-mediated alterations in the sensitivity of human myocardial cytosolic Ca^{2+} -independent PLA_2 to chemical modification of an essential thiol residue

To examine further the effects of ATP on the physical properties of human myocardial cytosolic PLA_2 , additional experiments were performed employing an independent probe of enzyme conformation. Although Ca^{2+} -independent PLA_2 activity in human myocardial cytosol was inhibited by chemical modification of an essential thiol residue in the absence of $\text{p}[\text{CH}_2]\text{ppA}$, addition of $\text{p}[\text{CH}_2]\text{ppA}$ (1 mM) markedly attenuated human myocardial cytosolic PLA_2 from group-specific chemical inactivation (Table 1). In contrast, addition of $\text{p}[\text{CH}_2]\text{ppG}$ (1 mM) only modestly attenuated chemical inactivation mediated by this thiol-group-reactive agent. Thus interaction of adenine nucleotide triphosphates with human myocardial cytosolic Ca^{2+} -independent PLA_2 substantially alters the susceptibility (i.e. accessibility) of an essential thiol residue to covalent modification by Nbs_2 .

ATP potentiates mechanism-based inhibition of human myocardial cytosolic Ca^{2+} -independent PLA_2

The effects of adenine nucleotide triphosphates on PLA_2 conformation were further examined by utilizing the Ca^{2+} -

Table 1. Nucleotide-mediated alterations in the sensitivity of human myocardial cytosolic Ca²⁺-independent PLA₂ to chemical modification of an essential thiol residue

Human myocardial cytosolic protein (200 µg) was incubated in the presence of 0, 1 or 10 µM-Nbs₂ at 0 °C for 1 min in the absence or presence of either 1 mM-p[CH₂]ppA or 1 mM-p[CH₂]ppG. PLA₂ activity was subsequently quantified by addition of 16:0,[³H]18:1 plasmenylcholine (100 µM) substrate as described in the Materials and methods section. Data are expressed as the pmol of fatty acid released. The values in parentheses are the percentages of PLA₂ activity present relative to maximum fatty acid release observed in the absence of Nbs₂ under the conditions examined. Results are means of duplicate determinations.

[Nbs ₂] (µM)	Addition...	Fatty acid released (pmol)		
		None	p[CH ₂]ppA	p[CH ₂]ppG
0		68 (100)	233 (100)	108 (100)
1		42 (61)	219 (94)	75 (69)
10		29 (43)	224 (96)	62 (57)

Fig. 5. Mechanism-based inhibition of human myocardial cytosolic Ca²⁺-independent PLA₂ in the absence or presence of saturating NTP

Human myocardial cytosolic Ca²⁺-independent PLA₂ (0.35 mg of cytosolic protein) was incubated with the indicated concentrations of bromoenol lactone in the absence (■) or presence of either 10 mM-p[CH₂]ppA (●) or 10 mM-Mg·p[CH₂]ppA (○) for 3 min at 20 °C. Aliquots from these incubations were subsequently removed, and the remaining Ca²⁺-independent PLA₂ activity was quantified by utilizing 5 µM-16:0,[³H]18:1 plasmenylcholine substrate as described in the Materials and methods section. Results are means of duplicate determinations and are expressed as the percentage of maximum PLA₂ activity relative to parallel incubations performed in the absence of inhibitor.

independent PLA₂-specific mechanism-based inhibitor bromoenol lactone) (Hazen *et al.*, 1991) as an additional probe of active-site conformation. Incubation of human myocardial cytosol with 10 nM-bromoenol lactone [in the presence of saturating concentrations of p[CH₂]ppA (10 mM)] resulted in the time-dependent irreversible inactivation of human myocardial cytosolic Ca²⁺-independent PLA₂ activity. In contrast, only diminutive amounts of PLA₂ inhibition (< 5%) were observed utilizing 10 nM-bromoenol lactone in the absence of nucleotide. Examination of the effect of nucleotides on the concentration-dependence of mechanism-based PLA₂ inhibition demonstrated that the bromoenol lactone was 50-fold more potent in

irreversibly inhibiting human myocardial cytosolic Ca²⁺-independent PLA₂ activity in the presence of saturating concentrations of nucleotide under the conditions employed (Fig. 5).

To examine the concentration-dependence of nucleotide-mediated enhancement in the potency of bromoenol lactone-mediated inhibition, comparisons of the concentration-dependence of nucleotide-mediated activation of Ca²⁺-independent PLA₂ activity with nucleotide-mediated potentiation of mechanism-based PLA₂ inhibition were performed (Fig. 6). Although 10 nM-bromoenol lactone did not significantly inhibit human myocardial cytosolic Ca²⁺-independent PLA₂ activity in the absence of nucleotide triphosphates, addition of adenine nucleotide triphosphate resulted in a concentration-dependent increase in the potency of bromoenol lactone-mediated inhibition (Fig. 6a). Inclusion of Mg²⁺ shifted the dose-response curve of nucleotide-mediated PLA₂ activation to near-physiological concentrations (Gard *et al.*, 1985) (Fig. 6a). Similarly, inclusion of Mg²⁺ shifted the dose-response profile of nucleotide-mediated alterations of mechanism-based inhibition to the right (Fig. 6b). Collectively these data demonstrate that nucleotide-enhanced inhibition and nucleotide-mediated activation and stabilization of PLA₂ activity possess similar dose-response profiles.

Reconstitution of ATP-mediated activation and stabilization of purified human myocardial cytosolic Ca²⁺-independent PLA₂ activity

To determine whether nucleotide triphosphates directly interacted with the PLA₂ catalytic polypeptide or modulated PLA₂ activity through altering interactions with an ATP-dependent regulatory protein, the effects of ATP on purified human myocardial cytosolic Ca²⁺-independent PLA₂ were examined. Incubation of purified human myocardial cytosolic Ca²⁺-independent PLA₂ with ATP did not result in the activation or stabilization of the catalytic polypeptide (Table 2). Co-incubation of human myocardial cytosol and the purified PLA₂ resulted in nearly additive amounts of PLA₂ activity in the absence of ATP. However, co-incubation of cytosol with purified human myocardial cytosolic PLA₂ in the presence of ATP resulted in greater than additive amounts of PLA₂ activity (Table 2). To characterize the nature of the cytosolic constituent(s) mediating this phenomena, endogenous Ca²⁺-independent PLA₂ activity in crude myocardial cytosol was first ablated by heat treatment at 37 °C for 20 min. Co-incubation of heat-treated cytosol (which did not contain substantial endogenous catalytic activity) with purified human myocardial cytosolic Ca²⁺-independent PLA₂ reconstituted an ATP-dependent 2.8-fold increase in purified PLA₂ activity. Similarly, preincubation of these mixtures at 37 °C for 20 min and subsequent quantification of remaining PLA₂ activity demonstrated that ATP prevented the thermal inactivation of over 80 % of the catalytic activity in the purified polypeptide (Table 2). Since heat-treated cytosol contained minimal endogenous PLA₂ activity, these results suggest that a cytosolic constituent, functionally distinct from the catalytic polypeptide, mediated reconstitution of ATP-dependent activation and stabilization of the purified PLA₂. The ATP-responsive cytosolic constituent mediating these effects was further characterized by determining its heat-lability and trypsin-sensitivity. Preincubation of human myocardial cytosol at 60 °C or with trypsin ablated the ability of the cytosolic constituent to confer ATP-dependent activation and stabilization to homogeneous human myocardial cytosolic Ca²⁺-independent PLA₂ (Table 2). Collectively these results suggest that a trypsin-sensitive cytosolic protein which is functionally distinct from the purified catalytic entity mediates the observed ATP-dependent modulation of human myocardial cytosolic Ca²⁺-independent PLA₂ activity.

Fig. 6. Dependence of nucleotide concentration on mechanism-based inhibition of human myocardial cytosolic Ca^{2+} -independent PLA_2 .

(a) Ca^{2+} -independent PLA_2 activity in human myocardial cytosol (350 μg of cytosolic protein) was assessed (utilizing 20 μM -16:0-[${}^3\text{H}$]18:1 plasmenylcholine substrate) in the absence of inhibitor at the indicated concentrations of either $\text{Mg} \cdot p[\text{CH}_2]\text{ppA}$ (●) or $p[\text{CH}_2]\text{ppA}$ (▲), or in the presence of 10 nM-bromo-enol lactone in mixtures containing the indicated concentrations of $\text{Mg} \cdot p[\text{CH}_2]\text{ppA}$ (○) or $p[\text{CH}_2]\text{ppA}$ (△) as described in the Materials and methods section. Results are means of duplicate determinations. (b) The concentration-dependence of $\text{Mg} \cdot p[\text{CH}_2]\text{ppA}$ (○)- or $p[\text{CH}_2]\text{ppA}$ (●)-mediated potentiation of mechanism-based inhibition by bromo-enol lactone was quantified by determining the percentage of PLA_2 inhibition at each nucleotide concentration examined in (a) (i.e. comparisons were made between PLA_2 activity present at each nucleotide concentration in the absence of inhibitor and that manifest in the presence of 10 nM-inhibitor). Values are expressed as the percentage of remaining PLA_2 activity in the presence of 10 nM-inhibitor after incubation (3 min at 20 °C) relative to that which is present in parallel experiments performed in the absence of inhibitor at the indicated concentration of adenine nucleotide triphosphate. Abbreviation: NA, no addition.

Table 2. Reconstitution of ATP-dependent activation and stabilization of purified human myocardial cytosolic Ca^{2+} -independent PLA_2 .

Mixing experiments were performed to characterize the cytosolic constituent mediating ATP-dependent activation and stabilization of human myocardial cytosolic PLA_2 . To determine ATP-dependent activation, protein fractions (see below) were incubated alone or in the indicated mixtures in the presence or absence of 10 mM-ATP. PLA_2 activity was quantified by incubating individual fractions or the indicated mixtures of fractions with 16:0-[${}^3\text{H}$]18:1 plasmenylcholine substrate (20 μM) for 60 s at 37 °C. Butanol extraction of released radiolabelled fatty acids and subsequent liquid-scintillation spectrometry was performed as described in the Materials and methods section. Relative activation is the ratio of PLA_2 activity in the presence of ATP to that manifest in the absence of ATP in each assay. To determine the ability of each fraction to mediate ATP-dependent stabilization, each protein fraction (or the indicated mixtures of protein fractions) were preincubated at 37 °C for 20 min in the absence or presence of 10 mM-ATP before assay. Remaining PLA_2 activity was subsequently quantified after addition of 16:0-[${}^3\text{H}$]18:1 plasmenylcholine substrate (20 μM) as described above. Relative stabilization is the percentage of PLA_2 activity remaining after preincubation at 37 °C for 20 min compared with the amount of PLA_2 activity present in the absence of preincubation at 37 °C for 20 min. Abbreviations: PLA_2 , purified PLA_2 (2 ng of the 40 kDa catalytic polypeptide); C, cytosol (300 μg of protein); C_{37} , cytosol (300 μg of protein) previously incubated at 37 °C for 20 min; C_{60} , cytosol (300 μg of protein) previously incubated at 60 °C for 45 min; C_T , cytosol (300 μg of protein) previously incubated with trypsin (2%, w/w) for 24 h at 4 °C.

Fraction	ATP (10 mM)	Zero time preincubation: 'activation' (d.p.m.)	Relative activation (fold)	20 min Preincubation: 'stabilization' (d.p.m.)	Relative stabilization (%)
PLA_2	—	66660	1.0	0	0.0
	+	65720		0	0.0
C	—	39010	3.2	0	0.0
	+	125200		130280	104.1
C_{37}	—	600	3.6	0	0.0
	+	2160		1660	76.9
C_{60}	—	0	—	0	0.0
	+	0		0	0.0
C_T	—	0	—	0	0.0
	+	0		0	0.0
$\text{PLA}_2 + \text{C}$	—	88210	2.9	2820	3.2
	+	253180		214040	84.5
$\text{PLA}_2 + \text{C}_{37}$	—	59630	2.8	0	0.0
	+	166910		134190	80.4
$\text{PLA}_2 + \text{C}_{60}$	—	63040	1.0	168	0.3
	+	65420		974	1.5
$\text{PLA}_2 + \text{C}_T$	—	56820	1.1	0	0.0
	+	60130		2720	4.5

DISCUSSION

The present results demonstrate that ATP modulates human myocardial cytosolic Ca²⁺-independent PLA₂ activity in a concentration-dependent, saturable, reversible and co-operative manner. Nucleotide-mediated alterations in human myocardial cytosolic PLA₂ activity were selective for adenine nucleotide triphosphates and did not possess an obligatory requirement for either the hydrolysis of the γ -phosphate of nucleotide triphosphate or the presence of Mg²⁺. Furthermore, the results also demonstrate that one, but not necessarily the only, component mediating nucleotide-dependent modulation of human myocardial cytosolic Ca²⁺-independent PLA₂ is a reconstitutable, trypsin-sensitive, heat-labile cytosolic protein. The similarities in the dose-response profiles of ATP-dependent activation, attenuation of thermal denaturation and potentiation of mechanism-based inhibition of human myocardial cytosolic Ca²⁺-independent PLA₂ suggest that a common nucleotide-protein interaction contributes to each of these phenomena.

Multiple independent techniques were employed to document the interaction of ATP with human myocardial cytosolic Ca²⁺-independent PLA₂. Detailed analyses of the initial rate of human-myocardial-cytosolic-PLA₂-catalysed hydrolysis of phospholipid substrate demonstrated that the 3–4-fold increase in the rate of fatty acid release mediated by ATP (and its non-hydrolysable analogues) resulted from increased maximum velocity of catalysis and was independent of alterations in apparent affinity of enzyme for substrate. Furthermore, both ATP and its non-hydrolysable analogues elicited marked alterations in the thermal-denaturation profiles of human myocardial cytosolic Ca²⁺-independent PLA₂. In addition, Nbs₂-mediated chemical inactivation of human myocardial cytosolic Ca²⁺-independent PLA₂ was selectively attenuated by p[CH₂]₃ppA. Moreover, both ATP and its non-hydrolysable analogues potentiated the inhibition of PLA₂ by the mechanism-based Ca²⁺-independent PLA₂ inhibitor bromoenol lactone (Hazen *et al.*, 1991). Since both ATP and its non-hydrolysable analogues elicited activation, stabilization, attenuation of chemical modification and potentiation of mechanism-based inhibition, the participation of an intermediary kinase in mediating these effects may be excluded. Similarly, since (1) adenine nucleotide triphosphates are significantly more potent than their guanine nucleotide counterparts, (2) Mg²⁺ ion is not an obligatory cofactor for nucleotide-mediated PLA₂ regulation and (3) both the catalytic and regulatory polypeptides are soluble entities, the participation of conventional guanine-nucleotide-binding proteins (G-proteins) in these phenomena seem unlikely (Gilman, 1987; Birnbaumer *et al.*, 1990).

Reperfusion of ischaemic myocardium through either chemical (e.g. streptokinase) or mechanical (e.g. coronary-artery bypass grafting) interventions has now become a common clinical practice (e.g., Handin & Loscalzo, 1988). During severe myocardial ischaemia, the cytosolic Mg·ATP concentration declines to levels < 1 mM (see, e.g., Opie, 1976). Upon subsequent reperfusion of reversibly injured myocardium, Mg·ATP concentrations return to near-physiological levels (i.e. 6–10 mM·Mg·ATP). Since nucleotide-dependent modulation of PLA₂ activity was titrated by physiologically and pathophysiologically

results suggest that one mechanism potentially contributing to alterations in phospholipid catabolism during reperfusion of ischaemic myocardium is ATP-mediated activation of cytosolic Ca²⁺-independent PLA₂ activity. Furthermore, since local pools of ATP can preferentially modulate the function of specific cellular proteins (see, e.g., Weiss & Lamp, 1987), it is tempting to speculate that subcellular microheterogeneity in ATP content may contribute to the local regulation of cytosolic Ca²⁺-independent PLA₂ activity. Collectively these results provide a framework for the definitive molecular characterization of the biochemical mechanisms which regulate the activity of this novel class of Ca²⁺-independent PLA₂ in man.

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