The modulation of phosphatidylinositol biosynthesis in hamster hearts by methyl lidocaine

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Methyl lidocaine is an experimental anti-arrhythmic drug which has been shown to enhance the biosynthesis of phosphatidylinositol (PI) in the hamster heart. In this study, the effect of methyl lidocaine on enzymes involved in the biosynthesis of PI in the heart was examined. When the hamster heart was perfused with labelled methyl lidocaine, the majority of the compound was not metabolized after perfusion. The direct action of methyl lidocaine on an enzyme was studied by the presence of the drug in enzyme assays, whereas its indirect action was studied by assaying the enzyme activity in the heart after methyl lidocaine perfusion. CTP:phosphatidic acid cytidylyltransferase, a rate-limiting enzyme in PI biosynthesis, was stimulated by methyl lidocaine in a direct manner. Kinetic studies revealed that methyl lidocaine caused a change in the affinity between the enzyme and phosphatidic acid and resulted

in the enhancement of the reaction. Alternatively, acyl-CoA:lysophosphatidic acid acyltransferase, another key enzyme for PI biosynthesis, was not activated by the presence of methyl lidocaine. However, the enzyme activity was stimulated in hearts perfused with methyl lidocaine. The enhancement of the acyltransferase by methyl lidocaine perfusion was found to be mediated via the adenylate cyclase cascade with the elevation of the cyclic AMP level. The stimulation of protein kinase A activity by cyclic AMP resulted in the phosphorylation and activation of the acyltransferase. Interestingly, the activity of protein kinase C was not stimulated by methyl lidocaine perfusion. We conclude that the enhancement of PI biosynthesis by methyl lidocaine in the hamster heart resulted from the direct activation of the cytidylyltransferase, as well as the phosphorylation and subsequent activation of the acyltransferase.

INTRODUCTION

Phospholipids are the major structural components of the biological membrane [1]. Changes in the content and composition of the membrane phospholipids have profound effects on activities of membrane-bound proteins [2]. In addition, the conduction of electrical signals across the membrane in the heart appears to be dependent on the appropriate composition of the cardiac membrane phospholipids [3]. Beyond their structural role, membrane phospholipids participate as precursors of bioactive molecules which function as second messengers upon stimulation by receptor-mediated agonists [4]. For example, the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C is regarded as an important signal pathway for Ca^{2+} -mobilizing extracellular signal molecules [5]. In addition, recent studies have shown that the hydrolytic products generated from the breakdown of phosphatidylcholine by the action of phospholipase D may act as second messengers for cellular signalling [6,7]. Although the pathways for phospholipid biosynthesis have been elucidated, only limited information is available for the regulation of their biosynthesis.

A number of amphiphilic compounds have been shown to exhibit regulatory effects on the biosynthesis of phospholipids in mammalian tissues [8–12]. Methyl lidocaine, an experimental anti-arrhythmic drug synthesized by Astra Pharmaceuticals, is a stable amphiphilic compound with local anaesthetic properties [13]. The methyl group, which is covalently attached to the amino nitrogen of the lidocaine moiety, provides the compound with a permanent cationic charge (Figure 1). In an earlier study, the *de novo* biosyntheses of several phospholipids in hamster hearts were shown to be enhanced by methyl lidocaine perfusion [12]. Specifically, perfusion of the hamster heart with 0.5 mg/ml methyl lidocaine resulted in a 56% increase in the biosynthesis of phosphatidylinositol (PI). Part of the increase in PI biosynthesis was attributed to the increase of CTP:phosphatidic acid cytidylyltransferase activity by the direct action of methyl lidocaine [12]. However, it was not clear if the drug might also activate other biosynthetic enzymes via alternative mechanisms. In the present study, the modes for the modulation of PI biosynthesis by methyl lidocaine in the hamster heart were investigated.

Methyl lidocaine

Figure 1 The structure of methyl lidocaine

MATERIALS AND METHODS

Materials

[γ - ^{32}P]ATP (30 Ci/mmol), oleoyl-L-[^{14}C]coenzyme A (56.0 mCi/mmol) and [5- ^3H]CTP (24 Ci/mmol) were obtained from the NEN Division of Dupont Co. (Dorval, Quebec, Canada). [U- ^3H]Methyl lidocaine was custom prepared from unlabelled methyl lidocaine by the NEN Division of Dupont Co. The reagents for the determination of cyclic AMP level and protein

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; DTT, dithiothreitol; db-cAMP, $N^6,2'$ -O-dibutyryl-adenosine 3',5'-cyclic monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PKI, protein kinase A-specific inhibitor; PI, phosphatidylinositol.

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kinase C activity were obtained from Amersham Canada (Oakville, Ontario, Canada). Methyl lidocaine was a gift from Astra Pharmaceutical Products (Mississauga, Ontario, Canada). P81 papers were obtained from Whatman (Clifton, NJ, U.S.A.). Lysophosphatidic acid and phosphatidic acid were products of Sedary Research Laboratories (London, Ontario, Canada). Adenosine 5'-[γ -thio]triphosphate (ATP[S]) was purchased from Boehringer-Mannheim (Laval, Quebec, Canada). Oleoyl-CoA, ATP, ADP, CTP, PMSF, benzamidine, β -glycerol 3-phosphate, NaF, EGTA, EDTA, 3-isobutyl-1-methylxanthine (IBMX), tris(hydroxymethyl)aminomethane, dithiothreitol (DTT), Triton X-100, $N^6,2'$ -*O*-dibutyryl adenosine 3',5'-cyclic monophosphate (db-cAMP), BSA, and the catalytic subunit of protein kinase A (from bovine heart) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). SQ22536 (adenylate cyclase inhibitor) was the product of Calbiochem Co. (Windsor, Ontario, Canada). Acetic acid, $MgCl_2$ and TLC plates (Redi plate silica gel G25) were purchased from Fisher Scientific (Winnipeg, Manitoba, Canada). Kemptide and PKI (specific protein kinase A inhibitor) were obtained from Bachem Inc. (Torrance, CA, U.S.A.). All other chemicals and solvents were of reagent grade and obtained from the Canlab Division of Baxter Co. (Mississauga, Ontario, Canada).

Perfusion of isolated hamster hearts

Syrian Golden hamsters, weighing 120 ± 20 g, were used throughout the study. The isolated hamster heart was perfused at $37^\circ C$ in the Langendorff mode with Krebs-Henseleit buffer in the presence or absence of 0.5 mg/ml methyl lidocaine. After perfusion, 5 ml of Krebs-Henseleit buffer was forced through the cannula, followed by 5 ml of air. The heart was cut open, blotted dry, and homogenized in the appropriate buffer as indicated.

In order to study the fate of methyl lidocaine, isolated hamster hearts were perfused with Krebs-Henseleit buffer (pH 7.4) containing 0.5 mg/ml [3H]methyl lidocaine ($1 \mu Ci/nmol$) for a 30 min period. After perfusion, the heart was homogenized and the radioactivity in the homogenate was extracted and analysed by TLC with a solvent containing methanol/0.6 M NaCl/ NH_4OH (50:50:5, by vol.). The vast majority of the radioactivity ($92 \pm 2\%$) after perfusion was found in the methyl lidocaine fraction, indicating that there was no obvious metabolism of the compound in the heart.

Preparation of subcellular fractions

The hamster heart was homogenized in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl (pH 7.5). Subcellular fractions were prepared from the homogenate by differential centrifugation as described previously [14]. The protein content in each fraction was determined by the method of Lowry et al. [15].

CTP:phosphatidic acid cytidylyltransferase assay

The assay mixture contained 50 mM Tris/maleate (pH 6.5), 20 mM $MgCl_2$, 15 mM Triton X-100, 0.5 mM phosphatidic acid, 0.2 mg of protein and 1.0 mM [5-^3H]CTP (10000 d.p.m./nmol) [16]. The reaction was initiated by the addition of the enzyme protein into the assay mixture. The assay mixture was incubated at $37^\circ C$ for 15 min and the reaction was terminated by the addition of 2.5 ml of 0.1 M HCl in methanol. After cooling, 5 ml of chloroform was added to the mixture. The chloroform/methanol mixture was washed three times with 10 ml of 2 M $MgCl_2$ [17]. CDP-diacylglycerol in the organic phase was separated by TLC with a solvent containing chloroform/

methanol/acetic acid/water (25:14:2:4, by vol.). The radioactivity in the CDP-diacylglycerol fraction was determined by scintillation counting.

Acyl-CoA:lysophosphatidic acid acyltransferase assay

The assay mixture contained 100 mM Tris/HCl (pH 7.5), 0.2 mM L-palmitoylglycerol 3-phosphate, 90 mM sucrose, 1 mg/ml BSA, 1 mM DTT, $40 \mu M$ [$1\text{-}^{14}C$]oleoyl-CoA (4000 d.p.m./nmol) and 0.2 mg of protein in a final volume of 0.5 ml [18]. The reaction was initiated by placing the enzyme protein into the assay mixture. The mixture was incubated at $30^\circ C$ for 30 min. The reaction was terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v). Water was then added to the mixture to cause phase separation, and the labelled phosphatidic acid in the organic phase was isolated by TLC with a solvent system containing chloroform/methanol/ammonium hydroxide/water (35:15:2:1, by vol.). The phosphatidic acid fraction was visualized by exposure to iodine vapour and the radioactivity in the fraction was determined by scintillation counting.

Other enzyme assays

The activity of acyl-CoA:glycerol-3-phosphate acyltransferase was determined by the formation of the labelled lysophosphatidic acid with [$1\text{-}^{14}C$]oleoyl-CoA as substrate [18]. The activity of PI synthase was determined by the formation of labelled PI with *myo*-[3H]inositol as substrate [12].

The determination of protein kinase A and protein kinase C activities

The protein kinase A assay is adapted from the procedure of Corbin and Reimann [19]. The hamster heart after perfusion was homogenized in a buffer containing 50 mM Tris/HCl, 5 mM EDTA, 10 mM EGTA, 0.3% (w/v) 2-mercaptoethanol, 10 mM benzamidine, 50 $\mu g/ml$ PMSF, 100 mM β -glycerol 3-phosphate, 0.1 $\mu g/ml$ IBMX, 2 mM DTT and 20 mM NaF. β -Glycerol 3-phosphate was added to prevent cyclic AMP from being hydrolysed by the phosphodiesterase, whereas NaF was used as a phosphatase inhibitor and IBMX was employed as a cyclic AMP-phosphodiesterase inhibitor. Equal volumes of 50 mM potassium phosphate buffer (pH 6.8), 4.24 mM kemptide (specific substrate for protein kinase A), and $Mg[\gamma\text{-}^{32}P]ATP$ (25 $\mu Ci/\mu mol$) containing 1 mM ATP and 18 mM magnesium acetate were mixed to a total volume of 50 μl . The reaction was initiated by adding 20 μl of enzyme (5 μg) to the mixture. A reaction without enzyme was employed as the blank. The reaction mixture was incubated at $30^\circ C$ for 20 min, and the reaction was terminated by spotting the sample on to the p81 paper. The spotted paper was submerged into a 5% acetic acid solution. The paper was then washed three times and the protein kinase A activity was determined by the amount of phosphorylated kemptide bound to the paper. In the control experiment, 2 μM of PKI (a protein kinase A inhibitor) was added to determine the non-protein kinase A phosphorylation activities by other endogenous kinases.

The protein kinase C assay was a modification of the method of Hannun *et al.* [20] and was carried out with the aid of an enzyme assay kit (Amersham). The reaction mixture consisted of 50 mM Tris/HCl (pH 7.5), 12 mM calcium acetate, L- α -phosphatidyl-L-serine, 24 $\mu g/ml$ phorbol 12-myristate 13-acetate, 0.9 mM of a specific peptide for protein kinase C, 30 mM DTT and 0.05% (w/v) sodium azide in a total volume of 25 μl . Microsomal or cytosolic fraction (50 μg of protein) in a volume

of 25 μ l was added to the mixture, followed by the addition of 25 μ l of magnesium/ATP buffer containing 50 mM Tris/HCl (pH 7.5), 150 μ M [γ - 32 P]ATP (66.6 μ Ci/ μ mol), 45 mM magnesium acetate and 0.05 % (w/v) sodium azide. The blank for the assay was represented by a reaction without enzyme. The reaction was carried out at 25 °C for 15 min, and terminated with 100 μ l of a stopping solution. The reacted mixture was spotted on to the binding paper. The spotted paper was washed three times with 15 ml of a 5 % acetic acid solution. Subsequently, the paper was washed in 95 % (v/v) ethanol for 5 min, dried, and the radioactivity determined. Protein kinase C activity was calculated from the phosphorylated peptide bound to the paper.

The pool size of cyclic AMP

The pool size of cyclic AMP in hamster heart was determined with the aid of a kit from Amersham. Subsequent to perfusion, hamster hearts were immediately placed in liquid N₂. The frozen hearts were weighed and homogenized in 95 % (v/v) ethanol. The homogenate was centrifuged at 1000 g for 10 min. The supernatant, containing cyclic AMP, was dried under a stream of N₂ and assayed according to the procedure described in the cyclic AMP assay kit. Briefly, the dried sample was resuspended in 50 mM Tris/HCl (pH 7.5) and 4 mM EDTA. The assay mixture contained 4.5 nM [8- 3 H]cyclic AMP (27.8 μ Ci/nmol), cyclic AMP binding protein and protein sample in a volume ratio of 1:2:1. A standard curve ranging from 0–6 pmol of cyclic AMP was prepared. The reaction mixture was incubated at 4 °C for 2 h. Separation of the protein-bound cyclic AMP from the unbound nucleotide was achieved by adsorption of the free nucleotide on to 100 μ l of coated charcoal, followed by centrifugation at 12000 g for 2 min at 4 °C. The resulting supernatant was taken and the radioactivity determined. The amount of cyclic AMP present in the sample was calculated from the cyclic AMP standard curve.

Statistical analysis

Student's *t* test was used for all statistical analysis. The level of significance was set at *P* < 0.05.

RESULTS

The effect of methyl lidocaine perfusion on enzymes for the biosynthesis of PI

The effect of methyl lidocaine perfusion on the activities of enzymes for the *de novo* biosynthesis of PI was investigated. The hamster heart was perfused for 30 min in Krebs–Henselitt buffer containing 0.5 mg/ml methyl lidocaine [12]. After perfusion, the heart was homogenized in 0.25 M sucrose/10 mM Tris/HCl (pH 7.5), and the postnuclear fraction was obtained from the homogenate by centrifugation. The activities of the enzymes for PI biosynthesis were determined in the postnuclear fraction and the results are depicted in Table 1. Perfusion with methyl lidocaine did not change the activity of the acyl-CoA: glycerol-3-phosphate acyltransferase, CTP:phosphatidic acid cytidyltransferase or PI synthase. However, the activity of acyl-CoA:lysophosphatidic acid acyltransferase was increased by 45 % as compared with the control. The activation of acyl-CoA:lysophosphatidic acid acyltransferase by methyl lidocaine perfusion could not be reproduced by the direct addition of methyl lidocaine into the cell-free system [12]. These findings suggest that the acyltransferase was activated by methyl lidocaine through an indirect mechanism.

Table 1 Effect of methyl lidocaine perfusion on PI biosynthetic enzymes

Hamster hearts were perfused for 30 min with or without 0.5 mg/ml methyl lidocaine. Subsequently, hearts were homogenized and postmitochondrial fractions were prepared from homogenates by centrifugation. Enzyme activities were assayed as described in the Materials and methods section. Each set of values represents the mean $\pm$ S.D. of three separate experiments.

Enzymes	Enzyme activity (nmol/h per mg of protein)	
	Control	Perfusion with methyl lidocaine
Acyl-CoA: glycerol-3-phosphate acyltransferase	6.14 $\pm$ 1.20	5.84 $\pm$ 1.52
Acyl-CoA: lysophosphatidic acid acyltransferase	0.65 $\pm$ 0.14	0.95 $\pm$ 0.11*
CTP: phosphatidic acid cytidyltransferase	0.65 $\pm$ 0.09	0.61 $\pm$ 0.10
Phosphatidylinositol synthase	0.10 $\pm$ 0.03	0.11 $\pm$ 0.01

* *P* < 0.05 when compared with the control.

Mechanism for the activation of acyl-CoA:lysophosphatidic acid acyltransferase

The activation of acyl-CoA:lysophosphatidic acid acyltransferase by reversible phosphorylation was explored. In an initial set of experiments, the postnuclear fraction obtained from the hamster heart was incubated with 100 μ M ATP for 30 min in the presence of NaF and PMSF. After incubation, the acyltransferase activity in the postnuclear fraction was determined and compared with the control (without ATP). The effect of ATP[S] (a non-hydrolysable ATP analogue), ADP, or CTP on the enzyme activity was also examined. As shown in Table 2, the activity of the acyltransferase was significantly elevated (14 % as compared with control) in the presence of ATP. However, ATP[S], ADP or CTP did not produce any detectable effect on the enzyme activity. In order to demonstrate that the effect of ATP was not mediated via adenylate cyclase, SQ22536 (an

Table 2 Effect of ATP on acyl-CoA:lysophosphatidic acid acyltransferase activity

The hamster heart was homogenized in a buffer containing 10 mM Tris/HCl (pH 7.5), 0.25 M sucrose, 20 mM NaF, 2 mM DTT, 50 μ g/ml PMSF, and 4 mM MgCl₂. The postnuclear fraction was incubated with the designated nucleotides (0.1 mM) for 30 min at 37 °C prior to the assay of the acyltransferase activity. Each set of values represents the mean $\pm$ S.D. of four separate experiments.

Nucleotide	Acyl-CoA: lysophosphatidic acid acyltransferase activity (nmol/h per mg of protein)
Controls	0.65 $\pm$ 0.03
ATP	0.74 $\pm$ 0.02*
ATP + SQ22536 (1.25 mM)	0.81 $\pm$ 0.05*
ATP[S]	0.66 $\pm$ 0.04
ADP	0.60 $\pm$ 0.04
CTP	0.58 $\pm$ 0.04

* *P* < 0.05 when compared with controls.

Table 3 Effect of methyl lidocaine on protein kinase A and protein kinase C activities

Hamster hearts were perfused for 30 min with or without 0.5 mg/ml methyl lidocaine. Subsequently, hearts were homogenized, and microsomal and cytosolic fractions were prepared from homogenates by centrifugation. Protein kinase A and C activities were assayed as described in the Materials and methods section. Each set of values represents the mean $\pm$ S.D. of three separate experiments.

	Enzyme activity (pmol/min per mg of protein)	
	Control	Methyl lidocaine perfusion
Protein kinase C		
Cytosolic	5.50 $\pm$ 0.65	5.16 $\pm$ 0.24
Microsomal	4.25 $\pm$ 0.30	4.00 $\pm$ 0.60
Protein kinase A		
Cytosolic	125 $\pm$ 14	215 $\pm$ 30*

* $P < 0.05$ when compared with the control.

adenylate cyclase inhibitor) was added to the assay [21]. Co-incubation with ATP and SQ22536 did not diminish the effect of activation. Our data indicate that ATP, but not its subsequent metabolite, was an important factor for the activation of the acyltransferase, and suggest that the enzyme may be regulated via a phosphorylation-dephosphorylation mechanism.

The ability of methyl lidocaine to cause the phosphorylation of the acyl-CoA:lysophosphatidic acid acyltransferase was explored. Since the phosphorylation of the acyltransferase could arise from the activation of protein kinase A and/or protein kinase C, the effect of methyl lidocaine perfusion on both protein kinases was examined. Hamster hearts were perfused with 0.5 mg/ml methyl lidocaine for 30 min and were homogenized after perfusion. The microsomal and cytosolic fractions were obtained for the determination of protein kinase A and protein kinase C activities. Our results show that protein kinase A activity was enhanced by methyl lidocaine perfusion. The activities of protein kinase C in the cytosolic and microsomal fractions were not affected by the same treatment, indicating no translocation of the kinase took place (Table 3). In order to confirm that the activation of acyl-CoA:lysophosphatidic acid acyltransferase could be facilitated by the enhancement of protein kinase A activity, the postnuclear fraction obtained from the homogenate was incubated with 100 units of protein kinase A catalytic subunit. After incubation, the acyltransferase activity was determined. Our result shows that the incubation with protein kinase A catalytic subunit produced a pronounced increase (91%) in the acyltransferase activity. Enzyme

Table 4 Effect of methyl lidocaine on the pool size of cyclic AMP

Hamster hearts were perfused with or without 0.5 mg/ml methyl lidocaine for 5 or 30 min. Subsequent to perfusion, the cyclic AMP levels were determined. Each set of values represents the mean $\pm$ S.D. of three separate experiments.

Perfusion time (min)	Cyclic AMP levels (pmol/g wet weight)	
	Control	Methyl lidocaine
5	440 $\pm$ 15	767 $\pm$ 58*
30	406 $\pm$ 30	602 $\pm$ 14*

* $P < 0.05$ when compared with the control.

Table 5 Effect of exogenous addition of cyclic AMP on protein kinase A activity in hamster heart cytosol

Hamster hearts were perfused for 30 min with or without 0.5 mg/ml methyl lidocaine. Protein kinase A activity in the cytosolic fraction was assayed in the presence of cyclic AMP. Each set of values represents the mean of two separate experiments.

	Protein kinase A activity (pmol/min per mg of protein)				
Cyclic AMP added (pmol/ml) ...	0	20	40	80	100
Control heart	125	155	173	187	189
Perfused with methyl lidocaine	178	175	186	187	183

activity obtained after protein kinase A incubation was 1.23 ± 0.26 nmol/h per mg of protein, whereas the control was 0.65 ± 0.15 nmol/h per mg of protein.

The mechanism for the enhancement of protein kinase A activity by methyl lidocaine perfusion was examined. Since protein kinase A activity is modulated by cyclic AMP, the pool size of cyclic AMP in the perfused hamster heart was determined. Table 4 shows that the level of cyclic AMP was greatly elevated in the hamster heart after a 5 min perfusion with 0.5 mg/ml methyl lidocaine, and its level remained elevated even after 30 min of perfusion. As a further control, hamster hearts were perfused with 0.5 mg/ml methyl lidocaine and 0.25 mM SQ22536 (an adenylyl cyclase inhibitor) [22]. The presence of SQ22536 completely suppressed the elevation of the cyclic AMP level (data not shown). Our results clearly demonstrate that the level of cyclic AMP in the heart was elevated by methyl lidocaine perfusion.

The ability of elevated cyclic AMP levels to modulate protein kinase A activity in the heart was examined. The protein kinase A activity in the hamster heart cytosol was found to be significantly elevated by the exogenous addition of 20–100 pmol/ml cyclic AMP, and maximum activation of the enzyme activity was achieved at a concentration of 80 pmol/ml cyclic AMP (Table 5). Interestingly, the addition of 20–100 pmol/ml cyclic AMP to the cytosol of the hamster heart perfused with methyl lidocaine failed to elicit any significant increase in protein kinase A activity. It is plausible that the elevated level of cyclic AMP in the heart generated by methyl lidocaine perfusion had already produced maximum activation of the protein kinase A. Hence, the activity of the enzyme could not be further stimulated by the addition of exogenous cyclic AMP.

The activation of acyl-CoA:lysophosphatidic acid acyltransferase by db-cAMP

The ability of db-cAMP to activate acyl-CoA:lysophosphatidic acid acyltransferase was studied. db-cAMP mimicks the action of cyclic AMP and is transported across the membrane during perfusion [23]. Hence, the hamster heart was perfused with 1 mM db-cAMP for 30 min and the activities of protein kinase A and acyl-CoA:lysophosphatidic acid acyltransferase in the heart were determined after perfusion. As shown in Table 6, both enzyme activities in the heart were enhanced by db-cAMP. Taken together with the data obtained in preceding sections, it appears that methyl lidocaine caused the generation of cyclic AMP which stimulated the activity of protein kinase A and resulted in the phosphorylation and activation of acyl-CoA:lysophosphatidic acid acyltransferase.

Table 6 Effect of db-cAMP on the activities of acyl-CoA:lysophosphatidic acid acyltransferase and protein kinase A in hamster hearts

Hamster hearts were perfused with 1 mM db-cAMP for 30 min. After perfusion, the heart was homogenized and the postnuclear fraction was obtained for the determination of enzyme activities. Each set of values represents the mean $\pm$ S.D. of three separate experiments.

Enzyme activity	Control	db-cAMP
Protein kinase A (pmol/min per mg of protein)	101 $\pm$ 5	175 $\pm$ 30*
Acyl-CoA:lysophosphatidic acid acyltransferase (nmol/h per mg of protein)	0.61 $\pm$ 0.06	0.84 $\pm$ 0.09*

* $P < 0.05$ when compared with the control.

Figure 2 Double reciprocal plot of hamster heart CTP:phosphatidic acid cytidylyltransferase activities versus substrate concentrations

CTP:phosphatidic acid cytidylyltransferase in the microsomal fraction of the hamster heart was assayed in the presence or absence of 0.5 or 1.0 mg/ml methyl lidocaine at different substrate concentrations. (a) The assay mixture contained 0.05–0.5 mM [3 H]CTP (10 000 d.p.m./nmol), 0.5 mM phosphatidic acid, 15 mM Triton X-100 and 0.3 mg of enzyme protein. (b) The assay mixture contained 0.01–0.10 mM phosphatidic acid (PA), 1.0 mM [3 H]CTP, 15 mM Triton X-100 and 0.3 mg of enzyme protein. A double reciprocal plot of the enzyme activity versus substrate concentration was obtained from each set of results. Each point represents the mean of three separate determinations. The symbols represent: $\blacktriangledown$, incubation with 1.0 mg/ml methyl lidocaine; ∇ , incubation with 0.5 mg/ml methyl lidocaine; $\bullet$, incubation without methyl lidocaine (control).

The direct effect of methyl lidocaine on enzymes for the biosynthesis of PI

The direct effect of methyl lidocaine on enzymes in the *de novo* biosynthesis of PI was investigated. As shown in our previous study [12], the presence of methyl lidocaine in the assay mixture did not have any effect on the activities of acyl-CoA:glycerol-3-phosphate acyltransferase, acyl-CoA:lysophosphatidic acid acyltransferase and PI synthase. However, methyl lidocaine in the assay mixture caused the activation of CTP:phosphatidic acid cytidylyltransferase. These results were confirmed in the present study, and kinetic studies were carried out to examine the activation of the CTP:phosphatidic acid cytidylyltransferase. The enzyme in the microsomal fraction was assayed at different phosphatidic acid (0.1–0.10 mM) and CTP (0.05–0.50 mM) concentrations in the presence of 0.5 or 1.0 mg/ml methyl lidocaine. Subsequently, enzyme activities versus concentrations of one substrate were plotted in a double reciprocal manner. As shown in Figure 2, the K_m for phosphatidic acid was changed in the presence of methyl lidocaine, but the K_m for CTP was not affected by the same treatment. We conclude that methyl

lidocaine caused the activation of CTP:phosphatidic acid cytidylyltransferase by increasing the affinity between the enzyme and phosphatidic acid, but not the affinity between the enzyme and CTP.

DISCUSSION

Methyl lidocaine is an amphiphilic local anaesthetic which has been employed as an experimental anti-arrhythmic drug. It has a methyl group covalently attached to the amino nitrogen of the lidocaine moiety, which causes the molecule to display a permanent cationic charge [12,13]. The compound was not actively metabolized by the heart, and over 90% of the methyl lidocaine in the perfused heart was recovered in its original form.

It is clear that a positive effect on the adenylate cyclase cascade similar to that of β -adrenergic agonists [22,24] was produced by methyl lidocaine. The activation of adenylate cyclase by methyl lidocaine was first indicated by an increase in cyclic AMP level, and the notion was supported by the ability of SQ22536 (an adenylate cyclase inhibitor) to suppress the increase in cyclic AMP production. The stimulation of protein kinase A activity by the elevated cyclic AMP level was demonstrated in the methyl lidocaine perfused heart, and our result was confirmed by using db-cAMP in the perfusion. Finally, the activation of the acyl-CoA:lysophosphatidic acid acyltransferase by phosphorylation was demonstrated by the incubation of the postnuclear fraction with ATP, and more directly, by the addition of the protein kinase A catalytic subunit to the postnuclear fraction. The regulation of phospholipid biosynthetic enzymes via cyclic AMP has been well documented. For example, the modulation of acyl-CoA:glycerol-3-phosphate acyltransferase by adrenaline has been reported [25]. Acyl-CoA:lysophosphatidic acid acyltransferase has been shown to be activated by isoprenaline (isoproterenol) via protein kinase A in guinea-pig parotid gland lobules [26]. We are the first to show that the activation of acyl-CoA:lysophosphatidic acid acyltransferase by methyl lidocaine is mediated via the adenylate cyclase cascade. It is tempting for us to speculate that the binding of methyl lidocaine to a receptor is essential in order to elicit its effect on the adenylate cyclase pathway for the enhanced production of cyclic AMP and the subsequent activation of the acyl-CoA:lysophosphatidic acid acyltransferase. However, the possibility of methyl lidocaine exerting a direct effect on the activation of adenylate cyclase has not been ruled out.

The activation of acyl-CoA:lysophosphatidic acid acyltransferase via the hydrolysis of PI bisphosphate and the subsequent enhancement of protein kinase C activity was explored. Our result shows that protein kinase C activity in the heart was not activated by methyl lidocaine perfusion. It is quite likely that the action of methyl lidocaine is specific and limited to the modulation of the adenylate cyclase cascade in the hamster heart.

The direct activation of CTP:phosphatidic acid cytidylyltransferase by methyl lidocaine represents another mechanism for the modulation of PI biosynthesis. However, the exact contribution of this mechanism for the enhancement of PI biosynthesis remains undefined. This is because CTP:phosphatidic acid cytidylyltransferase is a microsomal enzyme, and the availability of the endogenous methyl lidocaine to the microsomal system is not known. Interestingly, the presence of methyl lidocaine caused a change in the affinity between the enzyme and phosphatidic acid which was not observed in the presence of lidocaine [27]. It appears that the presence of a methyl group in lidocaine would greatly alter its

biological properties and enable it to play an important role in the direct modulation of PI biosynthesis.

Beyond the modulation of PI biosynthesis, methyl lidocaine has also been shown to stimulate the biosynthesis of neutral lipids such as diacylglycerol and triacylglycerol. We have shown in a preliminary study that the acyl-CoA:diacylglycerol acyltransferase is also activated by methyl lidocaine perfusion. In a separate study, this enzyme was found to be regulated by phosphorylation [28]. It is possible that the mediation of the adenylate cyclase cascade is a common mechanism by which methyl lidocaine exerts its modulation on lipid biosynthesis in the hamster heart.

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